EFFECT OF DIETARY FATTY ACIDS AND OTHER NUTRITIONAL SUPPLEMENTS ON BIOLOGICAL PROCESSES IN DAIRY COWS

by

CLAUDIO FABIAN VARGAS-RODRIGUEZ

Lic., Universidad de Costa Rica, 2005
M.S., Kansas State University, 2013

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Abstract

The ability of nutritional supplements to generate responses in productive animals at different physiological stages, and their interaction with the particular gastrointestinal tract of ruminants have created the necessity to explore effects beyond productivity. Modulation of immune function and inflammatory processes, modifications of nutrient metabolism, and interactions with the ruminal microbial population are effects attributed to supplements that encouraged the formulation of the set of experiments described in this dissertation. The first experiment was designed to test the effects of arachidonic and docosahexaenoic acids supplemented in milk replacer for Holstein calves during the pre-weaning period on the immune system, lipid and glucose metabolism, and growth performance when animals underwent a vaccination protocol. Neither supplemental fatty acid source affected productivity, cytokine production, antibody production, or CD4 and CD8 cell proliferation. A treatment effect on glucose and NEFA plasma concentration was observed. Polyunsaturated fatty acids, mainly DHA, lowered glucose and NEFA levels compared to control; moreover, a dose effect was observed indicating that increasing amounts of PUFA decreased plasma glucose level. The second experiment tested the effects of Co carbonate (CoCarb) and Co glucoheptonate (CoGH) at different concentrations of Co on *in vitro* fermentation rate, fermentation end-products, and DM and NDF disappearance. Dry matter and NDF disappearance increased with CoGH relative to CoCarb at 1.0 ppm Co or less. CoCarb at > 3.0 ppm appeared to stimulate the biohydrogenation of long-chain fatty acids whereas CoGH had limited effects on this process. Finally, the third study evaluated responses to chromium propionate during peak lactation and interactions between chromium and rumen-protected lysine and methionine. Chromium propionate increased feed intake and tended to increase energy-corrected milk yield. Primiparous cows showed greater responses in feed intake and milk protein yield than multiparous cows. In

this study, feeding chromium propionate near peak lactation increased feed intake and tended to increase productivity but no benefits of supplementing rumen-protected lysine and methionine were observed. Overall, nutritional supplements helped to regulate different biological functions in ruminants; their utilization is not always is feasible, but the results of these experiments provide guidance about effectiveness during different physiological situations.

EFFECT OF DIETARY FATTY ACIDS AND OTHER NUTRITIONAL SUPPLEMENTS ON BIOLOGICAL PROCESSES IN DAIRY COWS

by

CLAUDIO FABIAN VARGAS-RODRIGUEZ

Lic., Universidad de Costa Rica, 2005
M.S., Kansas State University, 2013

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2016

Approved by:

Major Professor
Barry J. Bradford

Copyright

CLAUDIO FABIAN VARGAS-RODRIGUEZ

2016

Abstract

The ability of nutritional supplements to generate responses in productive animals at different physiological stages, and their interaction with the particular gastrointestinal tract of ruminants have created the necessity to explore effects beyond productivity. Modulation of immune function and inflammatory processes, modifications of nutrient metabolism, and interactions with the ruminal microbial population are effects attributed to supplements that encouraged the formulation of the set of experiments described in this dissertation. The first experiment was designed to test the effects of arachidonic and docosahexaenoic acids supplemented in milk replacer for Holstein calves during the pre-weaning period on the immune system, lipid and glucose metabolism, and growth performance when animals underwent a vaccination protocol. Neither supplemental fatty acid source affected productivity, cytokine production, antibody production, or CD4 and CD8 cell proliferation. A treatment effect on glucose and NEFA plasma concentration was observed. Polyunsaturated fatty acids, mainly DHA, lowered glucose and NEFA levels compared to control; moreover, a dose effect was observed indicating that increasing amounts of PUFA decreased plasma glucose level. The second experiment tested the effects of Co carbonate (CoCarb) and Co glucoheptonate (CoGH) at different concentrations of Co on *in vitro* fermentation rate, fermentation end-products, and DM and NDF disappearance. Dry matter and NDF disappearance increased with CoGH relative to CoCarb at 1.0 ppm Co or less. CoCarb at > 3.0 ppm appeared to stimulate the biohydrogenation of long-chain fatty acids whereas CoGH had limited effects on this process. Finally, the third study evaluated responses to chromium propionate during peak lactation and interactions between chromium and rumen-protected lysine and methionine. Chromium propionate increased feed intake and tended to increase energy-corrected milk yield. Primiparous

cows showed greater responses in feed intake and milk protein yield than multiparous cows. In this study, feeding chromium propionate near peak lactation increased feed intake and tended to increase productivity but no benefits of supplementing rumen-protected lysine and methionine were observed. Overall, nutritional supplements helped to regulate different biological functions in ruminants; their utilization is not always is feasible, but the results of these experiments provide guidance about effectiveness during different physiological situations.

Table of Contents

List of Figures

List of Tables

Acknowledgements

God, you never let me alone, even if I lost my track you returned me to your way, you always put angels to help me and my family, I couldn’t make it to the end without your divine guidance, thanks for always keep an eye on me and give me strength when I think I don't have anymore.

Dr. Bradford, I can’t express with words all the gratitude that I have toward you and your family, you have been more than my advisor, you have become an example to follow in all the aspects of life, your degree of professionalism is immeasurable and your expertise goes beyond the academic. Thank you for showing me the richness of science and passion for family.

With all the love of my heart, I want to thank to my wife Vivian, she is the love of my lifetime, she sacrificed her dreams to follow mine, she changed her family to build one with me, she pushed me to the end, and the most important, she has believed in a future with me. I love you Vi.

To my family because they represent the best give of God, day after day they support me with their prayers and they never give up on me.

I would like to thank to my committee members, to all graduate students and my lab-group partners, to the personal of KSU Dairy Unit and the crew of the ruminant nutrition lab for their constant support.

Thanks to the TiChiPuMeDoJo family, for engraving my life with thons of good memories

Dedication

To my little Ian,

for filling my existence with thousands of smiles

To Tita Guisella,

for showing me the strength in adversity

To Tito Fello,

for teaching me how to be a stalwart partner in life

Chapter 1 - Literature Review: Polyunsaturated Fatty Acids and its Role in Pre-Ruminant Diets

Introduction

Historically, functions attributed to fatty acids had been mostly energy storage and substrate contributions to cellular membranes; however, through the years the increasing research on these molecules has demonstrated that fatty acids are able to play multiple roles, including the regulation of gene expression, modulation of lipid and glucose metabolism, modification of the activity of specific enzymes and membrane channels and signaling of potent molecules (Mashek and Grummer, 2003; Poirier et al., 2006).

Despite the roles mentioned above, there is a limitation to the inclusion of lipids in rations for ruminants. The microbial population in the rumen does not have the ability to utilize the fatty acids. The presence of double bonds in the chain of the fatty acids represents a risk factor for some of the microbes, and they have to rearrange the lipid structures to reduce the degree of lipid toxicity and stabilize the ruminal environment (Maia et al., 2007; Zhang et al., 2008). This process is called biohydrogenation. Unfortunately, biohydrogenation restricts the amount of unsaturated fatty acids available for absorption in the small intestine, which limits the benefits of dietary polyunsaturates for the host animal.

Methods to protect dietary lipids against microbial activity have been proposed and utilized ((Palmquist et al., 1986; Jenkins and Bridges, 2007; Gadeyne et al., 2015), but their effectiveness is something that still is in debate and the outcomes have not been as expected when protected lipid are include in animals' rations (Gadeyne et al., 2015)**.**

In the case of calves, their rumen is poorly developed in terms of size and functionality during the first days of life; the fermentative activity is very low because the microbial population has not fully colonized this organ yet. The rumen speeds its growth when solid feed is offered to the animal and the microbial population colonizes the foregut more intensively, but before this point, calves are functionally nonruminant. Nevertheless, the rapid rate of milk intake and the clot formation in the abomasum differentiates calves from true nonruminants (Bazin and Brisson, 1976).

Young ruminants have the esophageal groove that connects the esophagus with the abomasum (the biggest stomach section before weaning), and milk is delivered directly to this organ. This feature allows the calf to consume a large amount of lipids before weaning. In fact, when rations for newborns and weaned animals are compared, the proportion of lipids differs dramatically. Normally newborns consume greater amounts of this nutrient either from whole milk or milk replacer, whereas after weaning the proportion of fat decreases ((Hill et al., 2009a).

This review will cover most of the important effects of lipids when they are included in rations for pre-weaning ruminants, and potential impacts of providing specific types and amounts of fatty acids.

Requirements for specific fatty acids for pre-ruminants

A nutritional plan aims to impact animal performance by improving the precision of balancing ingredients and specific nutrients in rations. Also, the future performance of animals for milk production is related to how animals are fed during the early stages of life, and it has been observed that more milk is produced during the first lactation when animals were fed more intensively during the pre-weaning period (Drackley et al., 2007).

However, information on the effects and requirements of specific fatty acids in calves is scarce; as a result, fatty acid profiles have been ignored when rations for newborn calves are formulated (Jenkins et al., 1986; Hill et al., 2007). A study that compared whole milk to milk replacer, formulated to have the same fat and energy content, showed superior growth and feed efficiency in animals receiving whole milk (Bascom et al., 2007). These differences were partly attributed to the fact that whole milk contained more medium-chain fatty acids, whereas in milk replacer had more long chain fatty acids. Similar responses were observed when diets formulated with tallow, coconut oil and corn oil as sources of unsaturated and saturated fatty acids were fed to newborns. Medium chain FA are not stored and they are easily hydrolyzed in tissues, whereas long chain fatty acids are accumulated in fat deposits and metabolized when they are needed; actually, higher concentrations of linoleic acid in adipose tissue was detected with corn oil diets (Jenkins et al., 1985).

The NRC (2001) has no estimates of requirements for fatty acids, but industry has enriched milk replacer with specific fatty acids and this practice has shown positive effects on growth and health status of young animals (Hill et al., 2009a; Garcia et al., 2014; Michalek et al., 2015). For example, the inclusion of increasing concentrations of linoleic acid in Holstein calf diets during the first 3 months helped to linearly increase average daily gain and feed efficiency, while also decreasing serum urea nitrogen and serum glucose concentrations (Hill et al., 2009a).

Some intensified feeding programs focus on increasing skeletal growth (Cowles et al., 2006), because by increasing the frame size it is possible to reduce the age at the first breeding (Heinrichs and Hargrove, 1987). Existing evidence associate PUFA to bone metabolism, suggesting that omega-3 fatty acids intervene in bone turnover (Kajarabille et al., 2013), a study measured alkaline phosphatase as indicators of bone formation (Watkins et al.,2001) observed how

increasing amounts of linoleic acid in the form of flax oil increased the concentrations of this parameter (Hill et al., 2009). When a commercial blend of butyric acid, coconut oil and flax seed oil was added to milk replacer and supplemented to Holstein calves (Hill et al., 2011), greater average daily gain was the outcome and the authors associated this effect with a possible increase in bone formation based on the greater concentrations of alkaline phosphatase. In addition, it was mentioned that the inclusion of butyrate in the blend could have a potential effect on pancreas development based on the increasing serum concentration of amylase observed in the groups of animals fed with the blend (Kato et al., 1989). Enriching milk replacer with coconut oil (replacing 20 % of tallow) to increase the amount of medium chain fatty acids resulted in similar growth rates compared to whole milk when they were fed to Jersey calves for 49 days (Bowen Yoho et al., 2013).

More recently**,** Garcia et al., (2015) demonstrated that increasing the amount of essential fatty acids (cannot be synthesized by the organism) in calf diets can improve animal performance. Their results showed that feeding 0.0321 and 0.036 g/kg $BW^{0.75}$ of linoleic acid and α-linolenic acid, respectively, during the first 60 d of life increased skeletal growth, observed as greater body weight gain and wither and hip heights.

When animals receive more nutrients than what they need, the body must deal with the excess in some way (storage, metabolism, or excretion), but it is possible that overfeeding fatty acids could modify some metabolic functions by reducing the synthesis of other derivatives (Hill et al., 2011). For example, the synthesis of docohexaenoic acid from linolenic acid can be decreased if excessive amounts of linoleic acid are added to diets (Buccioni et al., 2012)**.** One study supplied 1% of the dietary energy in the form of 18:2 n-6 and 18-3 n-3 fatty acids, and no treatment effects on performance of newborn calves were detected (Jenkins et al., 1986). A

previous study did not observe treatment effects on performance or immune cell response when neonatal lambs were fed milk replacer with oils rich in long chain n−3 or n−6 polyunsaturated fatty acids, although there were higher concentrations of total fatty acids and long chain fatty acids in plasma (Lewis et al., 2008).

Absorption of Fatty Acids

The concentration of lipids in whole milk and milk replacers range from 17 to 28g/100g on a dry matter basis (Hill et al., 2009b; Bateman II et al., 2012). Most milk replacers utilize animal fats because they contain similar concentrations of saturated and unsaturated fatty acids compared to whole milk. On the other hand, milk replacer has a lower concentration of medium chain fatty acids and higher content of long chain fatty acids than whole milk, thus resulting in different rates of absorption and digestibility in the small intestine of young animals (Hocquette and Bauchart, 1999).

In pre-ruminants, milk or milk replacers barely enter the rumen, because the esophageal groove delivers the milk directly into the abomasum. As the major carbohydrate in milk (lactose) and the rest of the components reach the small intestine, lactose is enzymatically hydrolyzed when stimulates insulin secretion (Saltiel and Kahn, 2001). Insulin not only regulates the metabolism of glucose but also controls lipid metabolism (Dimitriadis et al., 2011).

The formation of casein clots, catalyzed by renin and pepsin, limits the hydrolysis of fatty acids and delays the rate of absorption. Lipases can't have immediate contact with triglycerides incorporated in these clots, so the fatty acids remain attached to the glycerol backbone for longer time (Hocquette and Bauchart, 1999; Okada et al., 2010)

The coagulate moves slowly to the small intestine where it gradually releases the nutrients. The total digestion of the clot lasts between 12 and 18 h (Bauchart et al., 1996), and the fatty acids become exposed to enzymatic action during that window of time. Now in the small intestine, the fatty acids are absorbed at different rates and by different routes based on the length of their aliphatic chain. The small intestine has less difficulty to absorb the medium chain fatty acids than long chain fatty acids; in fact, in human studies, these medium chain fatty acids have been used to enhance the permeability of mucosal tissues to hydrophilic drugs (Lindmark et al., 1998). To pass the brush membrane of the enterocytes, medium and long chain fatty acids combine with biliary bile acids, which have the ability to solubilize the fatty acids due to their amphiphilic surface. The mixtures of both components result in the formation of micelles (negatively charged spherical aggregates) that facilitate fatty acid absorption (Wang et al., 2013). The micelles do not enter the enterocyte; they create a kind of channel in the apical wall and allow the fatty acid to pass across an unstirred water barrier located in the intestinal microvillus membranes (Drackley, 2000).

In addition to the micelle transport system, the presence of proteins on the surface of the enterocytes with roles as fatty acid transporters has been reported (Hayashi et al., 2013; Anderson and Stahl, 2013). These proteins directly interact with fatty acids and allow them to enter the enterocyte, possibly by simple diffusion (Wang et al., 2013). Some protein with this function, found in the intestinal cell membranes, are the fatty acid transport proteins (FATPs), fatty acid translocase (CD36), and fatty acid binding protein (FABP) (Bionaz et al., 2012; Kawano and Cohen, 2013; Tarhda and Ibrahimi, 2015). In the small intestine, two types of FABPs have been identified: the liver and intestinal-type FABPs (Poirier et al., 1996); however, the liver type particularly exhibits high binding affinity for two fatty acids, whereas the other proteins of the same family have affinity for just one fatty acid (Storch and Thumser, 2000).

Metabolism of Essential Fatty Acids

Hydrolysis

The chemical transformation of fatty acids in pre-ruminants begins when milk or milk replacer enters the mouth. Palatine glands and other tissues in the oral cavity secrete salivary lipases that hydrolyze mainly medium chain fatty acids from milk fat (Russell et al., 1980; Sun et al., 2002) ; only a small amount of long chain fatty acids can be separated from the triglycerides by this enzyme (Nelson et al., 1977). Despite the low effectiveness to release long chain fatty acids, the presence of pre-gastric esterase potentiates the action of other lipolytic enzymes present down the digestive tract (Edwards-Webb and Thompson, 1977; Sun et al., 2002). Previous studies observed how the concentration of triglycerides and phospholipids in plasma increased right after feeding, which indicates that these compounds were absorbed rapidly, and the action of the salivary enzymes could potentially contribute to this rapid hydrolysis and absorption (Bazin and Brisson, 1976). In addition, they observed that the concentration of NEFA and ketone bodies, mainly produced by the liver in pre-ruminants, did not show significant increases before feeding times, which could be due to the fact that the clot formation helped to maintain a more constant delivery of fatty acids between feeding times (Bazin and Brisson, 1976).

During neonatal life, pre-gastric esterase is charged with the hydrolysis of triaglycerides, until the pancreatic lipase increases its activity (Russell et al., 1980).

Pancreatic lipases continue the hydrolysis of fatty acids from triacylglycerides. This enzyme specifically attacks the fatty acids located in sn-1 and sn-3 positions, releasing 2 fatty acids and a monoacylglyceride (glycerol + fatty acid in sn-2 position). During the postnatal period, the enzyme activity increases 1.6 to 2.2 fold (Le Huerou-Luron et al., 1992), but it also requires the

presence of colipase, a cofactor that helps the enzyme to localize at the lipid-water surface and prevents the wash off of the enzyme by the bile salts (Parker et al., 2014).

Reassembly of Triglycerides

The re-esterification of triglycerides in mammals can occur by 2 different pathways. In most cells the process occurs predominantly by the glycerol-3-phosphate (G3P) pathway; however, the enterocytes are the exception and inside these cells, the re-assembly of triglycerides takes place by the action of 2 enzymes: monoacylglycerolacyltransferase (MAGT) and diacylglyceroltransferase (DGAT) (Alves-Bezerra and Gondim, 2012).

Chylomicron Formation and Transport

Now inside the intestinal cell, the re-formed dietary triglycerides are incorporated into chylomicrons that carry them out of the cell and distribute them to other tissues. The chylomicrons have a globular micelle-like structure with an internal core of non-polar hydrophobic molecules (triglycerides and cholesteryl esters), and the external shell composed of cholesterol, phospholipids and apolipoproteins (Randolph and Miller, 2014). Several apolipoproteins are present on the surface of chylomicrons such as apo-B48, apo-A1 and apo-AIV, but among them, apo-AIV participates more in the metabolism of lipids. For example, it participates in reverse cholesterol transport, modulates lipoprotein lipase activity, and moderates the interactions between different lipoproteins (Michalek et al., 2015). During the neonatal period when calves consume large amount of fat from milk, the expression of apo-AIV significantly increases due to the increase of lipid absorption in the small intestine (Sahl et al., 2003).

Once embedded in the chylomicrons, the triglycerides are transported from the lumen to intestinal lymph ducts or via the portal vein (Hocquette and Bauchart, 1999; Wang et al., 2013). The chylomicrons in the blood stream won't last for long because they enter into contact with the enzyme lipoprotein lipase (Mead et al., 2002). This enzyme is present in most tissues, especially adipose, muscle and heart; it plays an important role in the clearance of triglycerides from the blood stream. Lipoprotein lipase utilizes the presence of apoliproteins on the surface of chylomicrons to recognize and initiate the hydrolysis of the triglycerides. The free fatty acids can then be absorbed mainly by diffusion in most tissues, but also go to the liver as glycerol does.

Fatty Acid β-oxidation

The high pumping activity of heart demands large amounts of energy, obtained mainly from fatty acids. Heart under normal conditions utilizes very efficient fatty acid β-oxidation machinery to get energy (Grynberg and Demaison, 1996); but during challenging situations, it can switch to alternative and faster ATP-producing pathways (Sack et al., 1996; Lopaschuk et al., 2010). In the case of muscle tissue, contraction frequency and intensity dictates when and from where to get energy (glucose, ketone bodies and fatty acids); but muscle tissue also gets fuel from fatty acids by using the β-oxidation pathway (Fryan et al., 2006).

The β-oxidation pathway is a multiple phase process used by most tissues to obtain fuel from medium and long chain fatty acids (Li et al., 2012). It requires an initial activation step that occurs in the cytoplasm (Kunau et al., 1995). The enzyme acyl-CoA synthetase, in an ATP-dependent reaction, removes two inorganic phosphates and adds the coenzyme-A to the end the fatty acid, generating the activated fatty-acyl-CoA (Suzuki et al., 1990). Once the fatty acid is activated, the acyl-CoA needs to enter the mitochondria for the oxidative process. Carnitine

palmitoyl transferase-I attaches carnitine to the acyl-CoA and transports the fatty acid into the mitochondria (Holloway et al., 2006). Inside the organelle, a second enzyme called carnitine palmitoyl transferase-II detaches the fatty acyl-CoA, and returns the carnitine to the cytoplasm (Djouadi et al., 2003; Bartlett and Eaton, 2004). Now the fatty acid initiates the oxidative pathway, where the fatty acyl-CoA dehydrogenase removes 2 hydrogens and generates a double bond on the fatty acid chain. The end products of this reaction are trans-Δ^2-enoyl-CoA and FADH2, which is equivalent to 1.5 ATP (Kurtz et al., 1998; Lopaschuk et al., 2010).

In the following step, the enzyme trans-Δ^2-enoyl-CoA hydratase hydrates the double bond by adding an OH^- group to the carbon in position 3 and a H^+ to carbon in position 2; the resulting molecule is 3-L-hydroxyacyl-CoA (Guzmán and Geelen, 1993; Agnihotri and Liu, 2003).

One H^+ and one H^- are removed from 3-L-hydroxyacyl-CoA, and β-ketoacyl-CoA (with two keto-groups) is generated in a reaction where 3-L-hydroxyacyl-CoA dehydrogenase also produce 1 molecule of NADH, which yields 2.5 ATP (Schulz et al., 2011) The final step is the Claisen ester cleavage, where the action of the enzyme β-ketoacyl-CoA thiolase inserts another CoA molecule to separate the β-ketoacyl-CoA into two molecules, acetyl-CoA and a fatty acyl-CoA. This last molecule is a fatty acid chain 2 carbons shorter than the fatty acid that initiated the process (Kantor et al., 2000; Reddy and Rao, 2006). The whole oxidative process continues until no more cleavage can be done. On the other hand, the acetyl-CoA is moved to the citric acid cycle to generate additional energy.

Polyunsaturated Fatty Acid β-oxidation

The process for unsaturated and polyunsaturated fatty acids requires extra steps to remove the double bonds. The β-oxidation process occurs as it was described until a double bond is found.

In monounsaturated fatty acids, the double bond is typically located at an odd carbon. Since the cleavage steps cut two carbons at a time, the double bond will end in the 3rd carbon, so it requires isomerization that moves the double bond to the carbon in 2nd position. The enzyme charged with this task is called cis-Δ3-enoyl CoA isomerase (Mursula et al., 2001). The action of this enzyme does not requires extra energy; however, the unsaturation of a fatty acid has an energetic cost because the addition of the double bond by fatty acyl-CoA dehydrogenase is not required and no FADH is produced in this step, so less energy is generated when an unsaturated fatty acid is placed in the mitochondria for oxidation. When more than one double bond is in the fatty acid, besides the isomerase, another enzyme is involved in the process. Usually the bonds are 3 carbons apart, and after the isomerase acts on the first double bond and the cleavage happens, the following bond could be in even position. The fatty acyl-CoA dehydrogenase introduces a double bond and 2,4-dienoyl CoA is generated (Fillgrove and Anderson, 2001), which is an unusual structure that must be modified. Therefore, the 2,4-dienoyl CoA reductase consumes 1 $NADH_2$ to reduce one of the double bonds, and then the cis-Δ^3-enoyl CoA isomerase does its job (Alphey et al., 2005). Polyunsaturated fatty acids therefore generate less energy than saturated acids of the same chain length.

β-oxidation takes place not only in the mitochondria; peroxisomes can also execute this function (Cherkaoui-Malki et al., 2012). In this organelle, the process only incompletely oxidizes long and very long chain fatty acids to medium chain fatty acids (Reddy and Hashimoto, 2001). Afterwards, the resulting molecules move to the mitochondria for further oxidation. The first enzyme participating in the peroxisomal β-oxidation is the fatty acyl-CoA oxidase, which produces trans-Δ2-enoyl CoA and water. Then, enzymes similar to those in the mitochondria perform the hydration and oxidation. The final step is executed by the peroxisomal thiolase, which, in contrast

to the thiolase in the mitochondria, stops when the cleavage reaches an acyl-CoA with less than 8 carbons (Mannaerts et al., 2000).

Lipid Metabolism in the Liver

Liver plays a pivotal role in uptake, circulation and synthesis of fatty acids; several metabolic pathways, regulated by dietary lipids and hormones, have been characterized (Hocquette and Bauchart, 1999). As in other tissues, liver recognizes chylomicrons and VLDL through the apolipoproteins located on the surface of these structures. Uptake of medium and long chain fatty acids in this organ also requires their activation by the action of either acyl-CoA-synthetase or fatty acid binding protein (FABP) (Nguyen et al., 2008). Previous work demonstrated how the transcriptional expression of FABP in monogastrics increased when long chain fatty acids were supplemented in the diet (Meunier-Durmort et al., 1996), which could be significant for pre-weaned calves despite the fact that this mechanistic approach has not been proven in ruminants. Once they are activated, fatty acids follow a similar mechanism to enter either the mitochondria or peroxisomes for oxidation, or will go into the nucleus to interact with some transcription factors (Rui, 2014).

Ketone Bodies Production

In addition to β-oxidation, ketone bodies (acetoacetate, BHBA and acetone) can be produced in the liver to be used as energy currency to provide metabolic fuel to other tissues when the animal is on fasting and blood glucose levels decrease (Rui, 2014). This process is activated when the uptake of fatty acid is high and the concentration of chylomicrons and VLDL increase in the blood stream. Ketogenesis begins with 2 molecules of acetyl-CoA combined by the action

of a thiolase enzyme to produce acetoacetyl-CoA, the hydromethylglutaryl-CoA-synthase to add another acetyl-CoA, and the hydroxymethylglutaryl-CoA lyase to cut the resulting molecule in two pieces to finally produce acetoacetate (McGarry and Foster, 1980). From here, BHBA and acetone are generated, the first through the action of β-hydroxybutyrate dehydrogenase, and the second one by a non-enzymatic decarboxylation (Enjalbert et al., 2001).

Synthesis of Long Chain Fatty Acids

Importantly, in non-ruminants the liver is the major site for the formation of fatty acids, whereas in ruminants, adipose tissue does this labor (Pethick and Dunshea, 1996).

Tricarboxylate Transport System

The hepatic synthesis of fatty acids is considered the reverse of β-oxidation, and it requires acetyl-CoA produced in the mitochondria. But this molecule must be transported out to the cytosol, where the formation of the fatty acids takes place, and this action is performed by the tricarboxylate transport system (Bisaccia et al., 1989). This system involves 3 enzymes located in the cytosol, ATP-citrate lyase, malate dehydrogenase, and malic enzyme, and another enzyme found inside the mitochondria, pyruvate carboxylase (Minarik et al., 2002; Pongratz et al., 2007). The acetyl-CoA cannot cross the membrane by itself, so it is converted to citrate when combines with oxaloacetate in a reaction catalyze by citrate synthase. The citrate is pumped out, and once in the cytosol the ATP-citrate lyase detaches acetyl-CoA and oxaloacetate at the expense of 1 ATP. Malate dehydrogenase and malic enzyme reconvert the oxaloacetate to pyruvate, and this molecule is transported back into mitochondria where pyruvate carboxylase generates oxaloacetate back to start the cycle again (Attwood, 1995).

Fatty Acid Synthesis

Outside the mitochondria, acetyl-CoA carboxylase, which contains biotin, produces malonyl-CoA when it adds CO_2 to acetyl-CoA (Abu-Elheiga et al., 2001). The next step is catalyzed by fatty acid synthase; this single polypeptide chain is equipped with multiple functional sites (enzymatic roles) and an acyl-Carrier-Protein (ACP). In fact, 7 reactions occur in this protein to complete the formation of the fatty acids (von Wettstein-Knowles et al., 2006).

The malonyl CoA is anchored to the ACP by the action of the malonyl/acetyl-CoA-ACP-transacylase (MAT), releasing the CoA group and producing malonyl-ACP. This compound is the activated donor of 2 carbons and another molecule of acetyl-CoA is the receiver after being converted to acetyl-ACP by MAT in another similar enzymatic complex (Smith, 1994; Berg JM, Tymoczko JL, Stryer L, 2002).

The elongation reaction is triggered by decarboxylation of acetyl-ACP, the enzyme β-ketoacyl-ACP-synthase begins the construction of the fatty acyl chains by joining the malonyl-CoA with acetyl-ACP, and the resulting molecule is the 4-carbon acetoacetyl-ACP (Christensen et al., 2007). Then 3 enzymes, 2 reductases (β-ketoacyl-ACP-reductase and enoyl-ACP-reductase) and 1 dehydrogenase (β-hydroxyacyl-ACP-dehydrase) act alternately to catalyze the conversion to an alkyl group; this portion of the pathways repeats up to 7 times to add 2 C per cycle (von Wettstein-Knowles et al., 2006). The final product, palmitoyl-ACP, is then transformed to palmitate when a thioesterase enzyme cleaves the ACP (Svensson et al., 1995).

The main product of lipogenesis in animals is palmitate, but further elongation can be performed by different elongases present in adipose tissue, mainly to generate stearic acid. Additionallly, Δ-9-desaturases catalyze the reaction through which is possible to introduce double

bonds, converting palmitic acid to palmitoleic acid and stearic acid to oleic acid (Engler et al., 2000). Interestingly, in a study performed with rats, when animals were fed high fat diets, lipogenesis in liver was induced to elongate and desaturase the fatty acid rather than to increase de novo synthesis (Oosterveer et al., 2009) consistent with the previous statements which indicated that intracellular concentration of fatty acid synthase decreased in high fat diets (Hillgartner et al., 1995).

Omega-3 and Omega-6 Fatty Acids

Polyunsaturated fatty acids with double bonds close to the methyl end have gained a lot of attention in the last decades since an epidemiologic study demonstrated that the Eskimo population in Greenland showed low incidence of type-1 diabetes and other autoimmune and inflammatory disorders (Kromann and Green, 1980), attributed to the daily intake of fish with large content of omega-3 fatty acids.

The omega classification refers to the position of the first double bound, so omega-3s have their first double bond on carbon 3 from the methyl end, and omega-6 on carbon 6 (DeFrain et al., 2005; DeFilippis and Sperling, 2006). The presence of a double bond and the carbon where they are positioned mark significant differences in the physiological responses attributed to these fatty acids.

Some of the most important PUFA are: arachidonic acid (C20:4, n-6), α-linoleic acid (C18:2, n-6), α-linolenic acid (C18:3, n-3) eicosapentaenoic acid (C20:5, n-3) and docosahexaenoic acid (C22:6, n-3) (Schmidt and Dyerberg, 1994). The lack of mechanism to endogenously synthesize these compounds forces mammals to obtain them from dietary sources.

The omega-6 and α-linolenic acid can be found in meat, cereals, nuts and seeds; the other group is highly present in fish and seafood (Meyer et al., 2003).

Benefits for health have been attributed more to omega-3 than to omega-6 fatty acids. Eicosanoids are bioactive molecules derived from these fatty acid families (DeFilippis and Sperling, 2006); however, the position of the double bond results in a different mode of action at the cellular level. The omega-6 family has been linked to pro-inflammatory responses (Simopoulos, 2008), whereas omega-3 fatty acids are associated with anti-inflammatory roles (Kitessa et al., 2004) .

Omega-3 Fatty acids and Inflammation

Prostaglandin Metabolism

The formation of prostaglandins from omega-3 fatty acids is mediated by the action of cyclooxygenase and lipoxygenase enzymes. When arachidonic acid is release from plasma membranes by phospholipases, cyclooxygenases (COX-1 and -2) convert it to prostaglandin (PG) H_2, and the action of a series of specific isomerases produces 4 types of bioactive prostaglandins and thromboxanes. The activity of COX-2 couples more with the production of prostacyclin PGI_2, thromboxane (TX) A_2 and PGE_2 (Ricciotti and FitzGerald, 2011). On the other hand, lipoxygenase converts arachidonic acid to 5-HPETE (5-hydroperoxyeicosatetranoic acid); through a reaction catalyzed by LTA synthase, 5-HPETE is converted to leukotriene-B_4 or leukotriene-C_4, which is rapidly metabolized to leukotriene D_4 (O'Donnell, 1999).

Prostaglandine E_2 is produced during inflammation, influences dendritic cell and T-helper cell differentiation, and increases vascular permeability and sensitivity to pain (Chizzolini and Brembilla, 2009). Thromboxane A_2 is a potent platelet aggregator and vasoconstrictor, and

leukotriene-B_4 induces inflammation and leukocyte chemotaxis and adherence (Simopoulos, 2008).

When omega-3 fatty acids are included in the diet, these compounds compete with omega-6 fatty acids in the formation of phospholipids. Omega-6 fatty acids are most often placed in the n-2 position of phospholipids, but omega-3 fatty acids also use the same enzymes and position, so they are able to replace the majority of omega-6 fatty acids in the cell membrane. When phospholipases disassemble the phospholipids, a switch in the formation of prostaglandins occurs. Eicosapentaenoic acid is metabolized via the same pathway as arachidonic acid, but different end-products and different effects on the cellular function are triggered depending on the substrate entering the pathway. The cyclooxygenase produces PGE_3 from EPA instead of PGE_2 (Bagga et al., 2003), and this prostaglandin is believed to lessen the degree of inflammation; however, recent studies observed that both PGE_3 and PGE_2 increased paracellular permeability (Roche et al., 2005; Rodríguez-Lagunas et al., 2013). Omega-3 fatty acids increase the production of the platelet aggregator and vasoconstrictor thromboxane A_3, but these effects occur to a lesser degree compared to those produce by TXA_2. The LTA-synthase, using EPA as a substrate, produces a weaker inducer of inflammation and weak chemotactic leukotriene (LTB_5).

Cytokine Production

Eicosapentaenoic and docosahexaenoic acid have been proven to inhibit the production of pro-inflammatory cytokines, and the activation of peroxisome proliferator-activated receptor (PPAR) is one of the mechanism proposed. An experiment that examined if EPA or DHA generate anti-inflammatory responses by acting on these receptors observed that both fatty acids activated

PPAR-γ and reduced the concentration of lipopolysaccharides in human renal tubular cells by inhibiting the activation of nuclear factor NF-κB (Li et al., 2005).

Previous evaluations done (Mishra et al., 2004) in human umbilical vein endothelial cells showed that oxidized EPA potently decreased TNFα and IL-1ß production induced by monocyte chemoattractant protein 1 (MCP-1), but the omega-3 fatty acid had a lesser effect following IL-8 stimulation (Mishra et al., 2004). Another group (Serini et al., 2012) observed that the addition of purified DHA or EPA effectively reduced cytokine release by phytohaemagglutinin-stimulated peripheral blood mononuclear cells in vitro, and the effect was more prominent with DHA on these cells obtained from patients with Alzheimer disease. A previous study mentioned that these omega-3 fatty acids, besides causing a reduction of TNFα release, also decreased the production of IL-10 and INF-γ (Verlengia et al., 2004). They documented that the mechanism of action for these polyunsaturated fatty acids is through changes in the expression of genes clustered to cytokine production, signal transduction, transcription, cell cycle control, defense and repair, apoptosis, cell adhesion, cytoskeleton, and hormones.

Resolution of Inflammation

The first objective of the inflammatory process is to neutralize pathogenic invaders and then to initiate the repair of the damaged tissue (Maskrey et al., 2011). During acute inflammation, neutrophils initiate the defense against pathogens, and at the same time the lipid-derived pro-inflammatory prostaglandins and leukotrienes are produced. Then mononuclear cells and macrophages are recruited to clean the house and finally return the tissue to homeostasis. However, restoring to the homeostatic state does not occurs after inflammation is controlled; existing evidence proved that events such as activation of phagocytosis, apoptosis, and release and

clearance of cytokines and chemokines occur at the onset of acute inflammation (Buckley et al., 2014). This mechanism is attributed to lipid-derived substances known as specialized proresolving mediators (SPM), and these molecules have the ability to control the acute inflammation in terms of duration and the magnitude. Additionally, proresolvins also control the return to homeostasis during the decline of the disease, because they are biosynthesized right after the pro-inflammatory factors are secreted (Serhan, 2010).

Despite being anti-inflammatory, the proresolution process has significant mechanistic differences from inhibitors of inflammation. SPMs do not reduce prostaglandins or leukotrienes biosynthesis or inhibit enzymatic pathways as was observed in a study with transgenic mice that endogenously synthesized omega-3 from omega-6 (Hudert et al., 2006). These molecules stimulate the clearance of cytokines and chemokines by macrophages, stop the recruitment and infiltration of neutrophils, and also regulate the removal of apoptotic neutrophils and cellular waste (Serhan, 2010; Spite et al., 2014).

Precursors of SPM are mainly essential fatty acids in the omega-6 and omega-3 families. A group called lipoxins is derived from arachidonic acid in a process that involves the action of 5- and 15-lipoxygenases(Samuelsson et al., 1987). Another set of SPM denoted as resolvins is produced from EPA through different enzymatic mechanisms (Ogawa and Kobayashi, 2009; Allard et al., 2011; Rodriguez and Spur, 2012), but also can be synthesized when aspirin is present in a reaction that involves cyclooxygenase-2 (Serhan, 2010; Freire et al., 2011). Protectins and maresins come from DHA; protectins are synthesized by a lipoxygenase-dependent reaction (Serhan et al., 2015), whereas maresin formation requires a series of enzymatic reactions where DHA is hydroperoxidated, epoxidated and finally hydrolyzed (Sasaki et al., 2011).

The proresolvin approach provides a better understanding about how omega-3 fatty acids participate in the regulation of inflammation processes. For example, a previous report mentioned that resolvin-E1 interacted with the receptor ChemR23 to attenuate nuclear factor–κB mediated inflammation (Arita et al., 2005), and another group of researchers observed a reduction of inflammatory status in transgenic mice producing higher endogenous levels of n-3 FA, suggesting that omega-3 FA have a wide range of effects on molecular function in resolution of inflammation and organ protection (Hudert et al., 2006).

Omega 3 Fatty Acids and Energy Homeostasis

Glucose Metabolism and Lipid Metabolism

The contribution of omega-3 fatty acids in glucose metabolism has been evaluated in a wide range of experiments from clinical to cell culture models. Among the effects, improvements in insulin sensitivity in muscle tissue have been associated with the inclusion of unsaturated fatty acids in cell membranes (de Santa Olalla et al., 2009). A previous research observed a significant reduction in glucose and insulin concentrations, suggesting an increase in insulin sensitivity, when providing 1200 mg of omega-3 fatty acids to women with polycystic ovary syndrome and high risk of type 2 diabetes (Rafraf et al., 2012). However, previous studies observed adverse effects in glucose metabolism when fish oil was supplemented in rats (Borkman et al., 1989) and humans (Glauber et al., 1988). Despite the previous reports, a more recent study noted that the omega-3 index, measured as the concentration of EPA and DHA in red blood cells, was positively correlated with insulin sensitivity in overweight men (Albert et al., 2014).

Two different type of mice (wild type and GPR120 knockout) were used in an experiment to address the mechanistic effects of EPA and DHA on insulin sensitivity (Talukdar et al., 2010)

G- protein-couple receptors respond to fatty acids and are highly expressed in adipose tissue. After both groups consumed diets promoting obesity and insulin resistance, the supply of the omega-3 fatty acids enhanced insulin sensitivity and reduced inflammation in the wild type animals, but the mice without the receptor failed to respond in the same manner.

Omega-3 fatty acids have also been associated with modulation of lipid metabolism and lipoprotein concentration (Simopoulus, 1991); these compounds are associated with reduced risk of cardiovascular diseases in humans due to their ability to decrease platelet aggregation and blood viscosity by decreasing plasma concentration of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) (Illingworth et al., 1984).

A research reported the effects of DHA and EPA on glucose and lipid metabolism in bovine hepatocytes in vitro, and they found that DHA modulated the metabolism of palmitic acid toward the formation of triglycerides, phospholipids and cholesterol (Mashek et al., 2002). But in a follow up study, using radiolabeling techniques it was discovered that DHA regulates enzymes involved in the triglycerides synthesis, and suggested a more feasible effect over enzymes involved in the esterification of free fatty acids to triglycerides such as diacylglycerol acyltransferase (Mashek and Grummer, 2003).

Conclusion

Polyunsaturated fatty acids are versatile compounds with effects on a wide range of biological functions. Their different modes of action, even in animals of the same species but at different physiological age, establishes the necessity to dig more into the potential of these molecules. The impact of fatty acids on metabolic diseases and immune function gives a bonus to their supplementation in diets for livestock, not only as a source of energy but also as modifier of health.

References

Abu-Elheiga, L., M. M. Matzuk, K. A. Abo-Hashema and S. J. Wakil. 2001. Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2. Science. 291:2613-2616.

Agnihotri, G. and H. Liu. 2003. Enoyl-CoA hydratase: Reaction, mechanism, and inhibition. Bioorg. Med. Chem. 11:9-20.

Albert, B. B., J. G. Derraik, C. M. Brennan, J. B. Biggs, G. C. Smith, M. L. Garg, D. Cameron-Smith, P. L. Hofman and W. S. Cutfield. 2014. Higher omega-3 index is associated with increased insulin sensitivity and more favourable metabolic profile in middle-aged overweight men. Scientific Reports. 4:6697.

Allard, M., K. Barnes, X. Chen, Y. Cheung, B. Duffy, C. Heap, J. Inthavongsay, M. Johnson, R. Krishnamoorthy, C. Manley, S. Steffke, D. Varughese, R. Wang, Y. Wang and C. E. Schwartz. 2011. Total synthesis of resolvin E1. Tetrahedron Lett. 52:2623-2626.

Alphey, M. S., W. Yu, E. Byres, D. Li and W. N. Hunter. 2005. Structure and reactivity of human mitochondrial 2,4-dienoyl-CoA reductase: ENZYME-LIGAND INTERACTIONS IN A DISTINCTIVE SHORT-CHAIN REDUCTASE ACTIVE SITE. Journal of Biological Chemistry. 280:3068-3077.

Alves-Bezerra, M. and K. C. Gondim. 2012. Triacylglycerol biosynthesis occurs via the glycerol-3-phosphate pathway in the insect rhodnius prolixus. Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1821:1462-1471.

Anderson, C. M. and A. Stahl. 2013. SLC27 fatty acid transport proteins. Mol. Aspects Med. 34:516-528.

Arita, M., F. Bianchini, J. Aliberti, A. Sher, N. Chiang, S. Hong, R. Yang, N. A. Petasis and C. N. Serhan. 2005. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. J. Exp. Med. 201:713-722.

Attwood, P. V. 1995. The structure and the mechanism of action of pyruvate carboxylase. Int. J. Biochem. Cell Biol. 27:231-249.

Bagga, D., L. Wang, R. Farias-Eisner, J. A. Glaspy and S. T. Reddy. 2003. Differential effects of prostaglandin derived from ⊥ë-6 and ⊥ë-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. Proceedings of the National Academy of Sciences. 100:1751-1756.

Bartlett, K. and S. Eaton. 2004. Mitochondrial β-oxidation. European Journal of Biochemistry. 271:462-469.

Bascom, S., R. James, M. McGilliard and M. Van Amburgh. 2007. Influence of dietary fat and protein on body composition of jersey bull calves. J. Dairy Sci. 90:5600-5609.

Bateman II, H. G., T. M. Hill, J. M. Aldrich, R. L. Schlotterbeck and J. L. Firkins. 2012. Meta-analysis of the effect of initial serum protein concentration and empirical prediction model for growth of neonatal holstein calves through 8 weeks of age. J. Dairy Sci. 95:363-369.

Bauchart, D., D. Gruffat and D. Durand. 1996. Lipid absorption and hepatic metabolism in ruminants. Proc. Nutr. Soc. 55:39-47.

Bazin, R. C. and G. J. Brisson. 1976. Plasma lipids, ketone bodies, and glucose concentrations in calves fed high-and low-fat milk replacers. J. Dairy Sci. 59:1301-1305.

Berg JM, Tymoczko JL, Stryer L. 2002. Fatty acids are synthesized and degraded by different pathways. in Biochemistry. 5th Edition ed. Fatty acids are synthesized and degraded by different pathways. W. H. Freeman, New York.

Bionaz, M., B. J. Thering and J. J. Loor. 2012. Fine metabolic regulation in ruminants via nutrient–gene interactions: Saturated long-chain fatty acids increase expression of genes involved in lipid metabolism and immune response partly through PPAR-α activation. Br. J. Nutr. 107:179.

Bisaccia, F., A. De Palma and F. Palmieri. 1989. Identification and purification of the tricarboxylate carrier from rat liver mitochondria. Biochimica Et Biophysica Acta (BBA) - Bioenergetics. 977:171-176.

Borkman, M., D. J. Chisholm, S. M. Furler, L. H. Storlien, E. W. Kraegen, L. A. Simons and C. N. Chesterman. 1989. Effects of fish oil supplementation on glucose and lipid metabolism in NIDDM. Diabetes. 38:1314-1319.

Bowen Yoho, W. S., V. A. Swank, M. L. Eastridge, K. M. O'Diam and K. M. Daniels. 2013. Jersey calf performance in response to high-protein, high-fat liquid feeds with varied fatty acid profiles: Intake and performance1. J. Dairy Sci. 96:2494-2506.

Buccioni, A., M. Decandia, S. Minieri, G. Molle and A. Cabiddu. 2012. Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. Anim. Feed Sci. Technol. 174:1-25.

Buckley, C., D. Gilroy and C. Serhan. 2014. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. Immunity. 40:315-327.

Cherkaoui-Malki, M., S. Surapureddi, H. I. El Hajj, J. Vamecq and P. Andreoletti. 2012. Hepatic steatosis and peroxisomal fatty acid beta-oxidation. Curr. Drug Metab. 13:1412-1421.

Chizzolini, C. and N. C. Brembilla. 2009. Prostaglandin E2: Igniting the fire. Immunol. Cell Biol. 87:510-511.

Christensen, C. E., B. B. Kragelund, P. von Wettstein-Knowles and A. Henriksen. 2007. Structure of the human β-ketoacyl [ACP] synthase from the mitochondrial type II fatty acid synthase. Protein Science. 16:261-272.

Cowles, K., R. White, N. Whitehouse and P. Erickson. 2006. Growth characteristics of calves fed an intensified milk replacer regimen with additional lactoferrin. J. Dairy Sci. 89:4835-4845.

de Santa Olalla, L. M., F. Sánchez-Muniz and M. Vaquero. 2009. N-3 fatty acids in glucose metabolism and insulin sensitivity. Nutr. Hosp. 24:113-127.

DeFilippis, A. P. and L. S. Sperling. 2006. Understanding omega-3's. Am. Heart J. 151:564-570.

DeFrain, J. M., A. R. Hippen, K. F. Kalscheur and J. M. Tricarico. 2005. Effects of dietary α-amylase on metabolism and performance of transition dairy cows. J. Dairy Sci. 88:4405-4413.

Dimitriadis, G., P. Mitrou, V. Lambadiari, E. Maratou and S. A. Raptis. 2011. Insulin effects in muscle and adipose tissue. Diabetes Res. Clin. Pract. 93, Supplement 1:S52-S59.

Djouadi, F., J. Bonnefont, L. Thuillier, V. Droin, N. Khadom, A. Munnich and J. Bastin. 2003. Correction of fatty acid oxidation in carnitine palmitoyl transferase 2-deficient cultured skin fibroblasts by bezafibrate. Pediatr. Res. 54:446-451.

Drackley, J. 2000. Lipid metabolism. Farm Animal Metabolism and Nutrition.Wallingford: CAB International. 97-119.

Drackley, J., T. Overton, G. Ortiz-Gonzalez, A. Beaulieu, D. Barbano, J. Lynch and E. Perkins. 2007. Responses to increasing amounts of high-oleic sunflower fatty acids infused into the abomasum of lactating dairy cows. J. Dairy Sci. 90:5165-5175.

Edwards-Webb, J. D. and S. Y. Thompson. 1977. Studies on lipid digestion in the preruminant calf. 2. A comparison of the products of lipolysis of milk fat by salivary and pancreatic lipases in vitro. Br. J. Nutr. 37:431-440.

Engler, M. M., S. H. Bellenger-Germain, M. B. Engler, M. M. Narce and J. G. Poisson. 2000. Dietary docosahexaenoic acid affects stearic acid desaturation in spontaneously hypertensive rats. Lipids. 35:1011-1015.

Enjalbert, F., M. C. Nicot, C. Bayourthe and R. Moncoulon. 2001. Ketone bodies in milk and blood of dairy cows: Relationship between concentrations and utilization for detection of subclinical ketosis. J. Dairy Sci. 84:583-589.

Fillgrove, K. L. and V. E. Anderson. 2001. The mechanism of dienoyl-CoA reduction by 2, 4-dienoyl-CoA reductase is stepwise: Observation of a dienolate intermediate. Biochemistry (N. Y.). 40:12412-12421.

Freire, A. C., A. W. Basit, R. Choudhary, C. W. Piong and H. A. Merchant. 2011. Does sex matter? the influence of gender on gastrointestinal physiology and drug delivery. Int. J. Pharm. 415:15-28.

Gadeyne, F., G. Van Ranst, B. Vlaeminck, E. Vossen, P. Van der Meeren and V. Fievez. 2015. Protection of polyunsaturated oils against ruminal biohydrogenation and oxidation during storage using a polyphenol oxidase containing extract from red clover. Food Chem. 171:241-250.

Garcia, M., L. F. Greco, M. G. Favoreto, R. S. Marsola, D. Wang, J. H. Shin, E. Block, W. W. Thatcher, J. E. P. Santos and C. R. Staples. 2014. Effect of supplementing essential fatty acids to pregnant nonlactating holstein cows and their preweaned calves on calf performance, immune response, and health. J. Dairy Sci. 97:5045-5064.

Glauber, H., P. Wallace, K. Griver and G. Brechtel. 1988. Adverse metabolic effect of omega-3 fatty acids in non-insulin-dependent diabetes mellitus. Ann. Intern. Med. 108:663-668.

Grynberg, A. and L. Demaison. 1996. Fatty acid oxidation in the heart. J. Cardiovasc. Pharmacol. 28:.

Guzmán, M. and M. J. H. Geelen. 1993. Regulation of fatty acid oxidation in mammalian liver. Biochimica Et Biophysica Acta (BBA) - Lipids and Lipid Metabolism. 1167:227-241.

Hayashi, H., S. Maruyama, M. Fukuoka, T. Kozakai, K. Nakajima, T. Onaga and S. Kato. 2013. Fatty acid-binding protein expression in the gastrointestinal tract of calves and cows. Animal Science Journal. 84:35-41.

Heinrichs, A. J. and G. L. Hargrove. 1987. Standards of weight and height for holstein Heifers1. J. Dairy Sci. 70:653-660.

Hill, T., J. Aldrich, R. Schlotterbeck and H. Bateman. 2007. Amino acids, fatty acids, and fat sources for calf milk replacers. The Professional Animal Scientist. 23:401-408.

Hill, T., M. VandeHaar, L. Sordillo, D. Catherman, H. Bateman and R. Schlotterbeck. 2011. Fatty acid intake alters growth and immunity in milk-fed calves. J. Dairy Sci. 94:3936-3948.

Hill, T. M., H. G. Bateman II, J. M. Aldrich and R. L. Schlotterbeck. 2009a. Effects of fat concentration of a high-protein milk replacer on calf performance. J. Dairy Sci. 92:5147-5153.

Hill, T. M., H. G. Bateman II., J. M. Aldrich and R. L. Schlotterbeck. 2009b. Effect of consistency of nutrient intake from milk and milk replacer on dairy calf performance. The Professional Animal Scientist. 25:85-92.

Hillgartner, F. B., L. M. Salati and A. G. Goodridge. 1995. Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. Physiol. Rev. 75:47-76.

Hocquette, J. and D. Bauchart. 1999. Intestinal absorption, blood transport and hepatic and muscle metabolism of fatty acids in preruminant and ruminant animals. Reproduction Nutrition Development. 39:27-48.

Holloway, G. P., V. Bezaire, G. J. F. Heigenhauser, N. N. Tandon, J. F. C. Glatz, J. J. F. P. Luiken, A. Bonen and L. L. Spriet. 2006. Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. J. Physiol. (Lond.). 571:201-210.

Hudert, C. A., K. H. Weylandt, Y. Lu, J. Wang, S. Hong, A. Dignass, C. N. Serhan and J. X. Kang. 2006. Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. Proceedings of the National Academy of Sciences. 103:11276-11281.

Illingworth, D. R., W. S. Harris and W. E. Connor. 1984. Inhibition of low density lipoprotein synthesis by dietary omega-3 fatty acids in humans. Arteriosclerosis. 4:270-275.

Jenkins, K., J. Kramer, F. Sauer and D. Emmons. 1985. Influence of triglycerides and free fatty acids in milk replacers on calf performance, blood plasma, and adipose lipids. J. Dairy Sci. 68:669-680.

Jenkins, K. J., J. K. G. Kramer and D. B. Emmons. 1986. Effect of lipids in milk replacers on calf performance and lipids in blood plasma, liver, and perirenal Fat1. J. Dairy Sci. 69:447-459.

Jenkins, T. C. and W. C. Bridges. 2007. Protection of fatty acids against ruminal biohydrogenation in cattle. European Journal of Lipid Science and Technology. 109:778-789.

Kajarabille, N., J. Diaz-Castro, S. Hijano, M. Lopez-Frias, I. Lopez-Aliaga and J. J. Ochoa. 2013. A new insight to bone turnover: Role of omega-3 polyunsaturated fatty acids. Scientificworldjournal. 2013:589641.

Kantor, P. F., A. Lucien, R. Kozak and G. D. Lopaschuk. 2000. The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial long-chain 3-ketoacyl coenzyme A thiolase. Circulation Research. 86:580-588.

Kato, S., N. Asakawa, H. Mineo and J. Ushijima. 1989. Effect of short-chain fatty acids on pancreatic exocrine secretion in calves aged 2 weeks and 13 weeks. Jpn. J. Vet. Sci. 51:123-127.

Kawano, Y. and D. E. Cohen. 2013. Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. J. Gastroenterol. 48:434-441.

Kitessa, S. M., S. K. Gulati, G. C. Simos, J. R. Ashes, T. W. Scott, E. Fleck and P. C. Wynn. 2004. Supplementation of grazing dairy cows with rumen-protected tuna oil enriches milk fat with n-3 fatty acids without affecting milk production or sensory characteristics. British Journal of Nutrition. 91:271-277.

Kromann, N. and A. Green. 1980. Epidemiological studies in the upernavik district, greenland. Acta Med. Scand. 208:401-406.

Kunau, W., V. Dommes and H. Schulz. 1995. B-oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: A century of continued progress. Prog. Lipid Res. 34:267-342.

Kurtz, D. M., P. Rinaldo, W. J. Rhead, L. Tian, D. S. Millington, J. Vockley, D. A. Hamm, A. E. Brix, J. R. Lindsey, C. A. Pinkert, W. E. O'Brien and P. A. Wood. 1998. Targeted disruption of mouse long-chain acyl-CoA dehydrogenase gene reveals crucial roles for fatty acid oxidation. Proceedings of the National Academy of Sciences. 95:15592-15597.

Le Huerou-Luron, I., P. Guilloteau, C. Wicker-Planquart, J. Chayvialle, J. Burton, A. Mouats, R. Toullec and A. Puigserver. 1992. Gastric and pancreatic enzyme activities and their relationship with some gut regulatory peptides during postnatal development and weaning in calves. The Journal of Nutrition. 122:1434-1445.

Lewis, G., M. Wulster-Radcliffe and J. Herbein. 2008. Fatty acid profiles, growth, and immune responses of neonatal lambs fed milk replacer and supplemented with fish oil or safflower oil. Small Ruminant Research. 79:167-173.

Li, H., X. Z. Ruan, S. H. Powis, R. Fernando, W. Y. Mon, D. C. Wheeler, J. F. Moorhead and Z. Varghese. 2005. EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: Evidence for a PPAR-γ–dependent mechanism. Kidney Int. 67:867-874.

Li, P., X. B. Li, S. X. Fu, C. C. Wu, X. X. Wang, G. J. Yu, M. Long, Z. Wang and G. W. Liu. 2012. Alterations of fatty acid β-oxidation capability in the liver of ketotic cows. J. Dairy Sci. 95:1759-1766.

Lindmark, T., Y. Kimura and P. Artursson. 1998. Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in caco-2 cells. Journal of Pharmacology and Experimental Therapeutics. 284:362-369.

Lopaschuk, G. D., J. R. Ussher, C. D. L. Folmes, J. S. Jaswal and W. C. Stanley. 2010. Myocardial fatty acid metabolism in health and disease. Physiol. Rev. 90:207-258.

Maia, M. R., L. C. Chaudhary, L. Figueres and R. J. Wallace. 2007. Metabolism of polyunsaturated fatty acids and their toxicity to the microflora of the rumen. Antonie Van Leeuwenhoek. 91:303-314.

Mannaerts, G. P., P. P. Van Veldhoven and M. Casteels. 2000. Peroxisomal lipid degradation via beta-and alpha-oxidation in mammals. Cell Biochem. Biophys. 32:73-87.

Mashek, D. G., S. J. Bertics and R. R. Grummer. 2002. Metabolic fate of long-chain unsaturated fatty acids and their effects on palmitic acid metabolism and gluconeogenesis in bovine hepatocytes. J. Dairy Sci. 85:2283-2289.

Mashek, D. G. and R. R. Grummer. 2003. Effects of long chain fatty acids on lipid and glucose metabolism in monolayer cultures of bovine hepatocytes. J. Dairy Sci. 86:2390-2396.

Maskrey, B. H., I. L. Megson, P. D. Whitfield and A. G. Rossi. 2011. Mechanisms of resolution of inflammation: A focus on cardiovascular disease. Arteriosclerosis, Thrombosis, and Vascular Biology. 31:1001-1006.

McGarry, J. and D. Foster. 1980. Regulation of hepatic fatty acid oxidation and ketone body production. Annu. Rev. Biochem. 49:395-420.

Mead, J. R., S. A. Irvine and D. P. Ramji. 2002. Lipoprotein lipase: Structure, function, regulation, and role in disease. Journal of Molecular Medicine. 80:753-769.

Meunier-Durmort, C., H. Poirier, I. Niot, C. Forest and P. Besnard. 1996. Up-regulation of the expression of the gene for liver fatty acid-binding protein by long-chain fatty acids. Biochem. J. 319:483-487.

Meyer, B. J., N. J. Mann, J. L. Lewis, G. C. Milligan, A. J. Sinclair and P. R. Howe. 2003. Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. Lipids. 38:391-398.

Michalek, K., A. Dratwa-Chalupnik and M. Jaszczuk. 2015. Analysis of apolipoprotein A-IV expression and the levels of blood plasmamain lipid fractions of calves during the first week of life before and after theadministration of milk replacer supplemented with lactose. Turkish Journal of Veterinary and Animal Sciences. 39:423-428.

Minarik, P., N. Tomaskova, M. Kollarova and M. Antalik. 2002. Malate dehydrogenases-structure and function. Gen. Physiol. Biophys. 21:257-266.

Mishra, A., A. Chaudhary and S. Sethi. 2004. Oxidized omega-3 fatty acids inhibit NF-kappaB activation via a PPARalpha-dependent pathway. Arterioscler. Thromb. Vasc. Biol. 24:1621-1627.

Mursula, A. M., D. M. F. van Aalten, J. K. Hiltunen and R. K. Wierenga. 2001. The crystal structure of Δ3-Δ2-enoyl-CoA isomerase1. J. Mol. Biol. 309:845-853.

Nelson, J. H., R. G. Jensen and R. E. Pitas. 1977. Pregastric esterase and other oral Lipases—A review. J. Dairy Sci. 60:327-362.

Nguyen, P., V. Leray, M. Diez, S. Serisier, J. L. Bloc'h, B. Siliart and H. Dumon. 2008. Liver lipid metabolism. J. Anim. Physiol. Anim. Nutr. 92:272-283.

O'Donnell, S. 1999. Leukotrienes- biosynthesis and mechanism of action. Aust. Presc. 22:55.

Ogawa, N. and Y. Kobayashi. 2009. Total synthesis of resolvin E1. Tetrahedron Lett. 50:6079-6082.

Okada, K., K. Yajima, T. Ohba, T. Miyazaki, T. Orihashi, S. Sato and J. Yasuda. 2010. The effect of experimental inhibition of gastric acid secretion on curd formation in abomasum and weight gain of calves. Animal Science Journal. 81:89-93.

Oosterveer, M. H., T. H. Van Dijk, U. J. Tietge, T. Boer, R. Havinga, F. Stellaard, A. K. Groen, F. Kuipers and D. Reijngoud. 2009. High fat feeding induces hepatic fatty acid elongation in mice. PloS One. 4:e6066.

Palmquist, D. L., T. C. Jenkins and A. E. Joyner Jr. 1986. Effect of dietary fat and calcium source on insoluble soap formation in the rumen. J. Dairy Sci. 69:1020-1025.

Parker, R., N. M. Rigby, M. J. Ridout, A. P. Gunning and P. J. Wilde. 2014. The adsorption-desorption behaviour and structure function relationships of bile salts. Soft Matter. 10:6457-6466.

Pethick, D. and F. Dunshea. 1996. The partitioning of fat in farm animals. . Nutrition Society of Australia.

Poirier, H., P. Degrace, I. Niot, A. Bernard and P. Besnard. 1996. Localization and regulation of the putative membrane Fatty-Acid transporter (FAT) in the small intestine. European Journal of Biochemistry. 238:368-373.

Poirier, Y., V. D. Antonenkov, T. Glumoff and J. K. Hiltunen. 2006. Peroxisomal β-oxidation—A metabolic pathway with multiple functions. Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research. 1763:1413-1426.

Pongratz, R. L., R. G. Kibbey, G. I. Shulman and G. W. Cline. 2007. Cytosolic and mitochondrial malic enzyme isoforms differentially control insulin secretion. J. Biol. Chem. 282:200-207.

Rafraf, M., E. Mohammadi, M. Asghari-Jafarabadi and L. Farzadi. 2012. Omega-3 fatty acids improve glucose metabolism without effects on obesity values and serum visfatin levels in women with polycystic ovary syndrome. J. Am. Coll. Nutr. 31:361-368.

Randolph, G. J. and N. E. Miller. 2014. Lymphatic transport of high-density lipoproteins and chylomicrons. J. Clin. Invest. 124:929-935.

Reddy, J. K. and M. S. Rao. 2006. Lipid metabolism and liver inflammation. II. fatty liver disease and fatty acid oxidation. Am. J. Physiol. Gastrointest. Liver Physiol. 290:G852-8.

Reddy, J. K. and T. Hashimoto. 2001. Peroxisomal β-oxidation and Peroxisome Proliferator-activated receptor α: An adaptive metabolic system. Annu. Rev. Nutr. 21:193-230.

Ricciotti, E. and G. A. FitzGerald. 2011. Prostaglandins and inflammation. Arterioscler. Thromb. Vasc. Biol. 31:986-1000.

Roche, J. R., S. Petch and J. K. Kay. 2005. Manipulating the dietary cation-anion difference via drenching to early-lactation dairy cows grazing pasture. J. Dairy Sci. 88:264-276.

Rodriguez, A. R. and B. W. Spur. 2012. Total synthesis of the anti-inflammatory lipid mediator resolvin E2. Tetrahedron Lett. 53:1912-1915.

Rodríguez-Lagunas, M. J., R. Ferrer and J. J. Moreno. 2013. Effect of eicosapentaenoic acid-derived prostaglandin E 3 on intestinal epithelial barrier function. Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA). 88:339-345.

Rui, L. 2014. Energy metabolism in the liver. Comprehensive Physiology.

Russell, R. W., E. Caruolo and G. Wise. 1980. Effects of pregastric esterase on utilization of whole milk by preruminant calves. J. Dairy Sci. 63:1114-1122.

Sack, M. N., T. A. Rader, S. Park, J. Bastin, S. A. McCune and D. P. Kelly. 1996. Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. Circulation. 94:2837-2842.

Saltiel, A. R. and C. R. Kahn. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. Nature. 414:799-806.

Samuelsson, B., S. Dahlen, J. Lindgren, C. Rouzer and C. Serhan. 1987. Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. Science. 237:1171-1176.

Sasaki, K., D. Urabe, H. Arai, M. Arita and M. Inoue. 2011. Total synthesis and bioactivities of two proposed structures of maresin. Chemistry–An Asian Journal. 6:534-543.

Schmidt, E. B. and J. Dyerberg. 1994. Omega-3 fatty acids. Drugs. 47:405-424.

Schulz, N., H. Himmelbauer, M. Rath, M. van Weeghel, S. Houten, W. Kulik, K. Suhre, S. Scherneck, H. Vogel and R. Kluge. 2011. Role of medium-and short-chain L-3-hydroxyacyl-CoA dehydrogenase in the regulation of body weight and thermogenesis. Endocrinology. 152:4641-4651.

Serhan, C. N. 2010. Novel lipid mediators and resolution mechanisms in acute inflammation: To resolve or not? The American Journal of Pathology. 177:1576-1591.

Serhan, C. N., J. Dalli, R. A. Colas, J. W. Winkler and N. Chiang. 2015. Protectins and maresins: New pro-resolving families of mediators in acute inflammation and resolution bioactive metabolome. Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1851:397-413.

Serini, S., A. Bizzarro, E. Piccioni, E. Fasano, C. Rossi, A. Lauria, A. RM Cittadini, C. Masullo and G. Calviello. 2012. EPA and DHA differentially affect in vitro inflammatory cytokine release by peripheral blood mononuclear cells from alzheimer's patients. Current Alzheimer Research. 9:913-923.

Simopoulos, A. P. 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp. Biol. Med. (Maywood). 233:674-688.

Smith, S. 1994. The animal fatty acid synthase: One gene, one polypeptide, seven enzymes. The FASEB Journal. 8:1248-1259.

Spite, M., J. Clària and C. Serhan. 2014. Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. Cell Metabolism. 19:21-36.

Storch, J. and A. E. A. Thumser. 2000. The fatty acid transport function of fatty acid-binding proteins. Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1486:28-44.

Sun, C. Q., C. J. O'Connor and A. M. Roberton. 2002. The antimicrobial properties of milkfat after partial hydrolysis by calf pregastric lipase. Chem. Biol. Interact. 140:185-198.

Suzuki, H., Y. Kawarabayasi, J. Kondo, T. Abe, K. Nishikawa, S. Kimura, T. Hashimoto and T. Yamamoto. 1990. Structure and regulation of rat long-chain acyl-CoA synthetase. Journal of Biological Chemistry. 265:8681-8685.

Svensson, L. T., S. E. Alexson and J. K. Hiltunen. 1995. Very long chain and long chain acyl-CoA thioesterases in rat liver mitochondria Identification, Purifiction, Characterization, and Induction by Peroxisome Proliferators

Talukdar, S., E. J. Bae, T. Imamura, H. Morinaga, W. Fan, P. Li, W. J. Lu, S. M. Watkins and J. M. Olefsky. 2010. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 142:687-698.

Tarhda, Z. and A. Ibrahimi. 2015. Insight into the mechanism of lipids binding and uptake by CD36 receptor. Bioinformation. 11:302-306.

Verlengia, R., R. Gorjão, C. C. Kanunfre, S. Bordin, T. M. de Lima, E. F. Martins, P. Newsholme and R. Curi. 2004. Effects of EPA and DHA on proliferation, cytokine production, and gene expression in raji cells. Lipids. 39:857-864.

von Wettstein-Knowles, P., J. G. Olsen, K. A. McGuire and A. Henriksen. 2006. Fatty acid synthesis. FEBS Journal. 273:695-710.

Wang, T. Y., M. Liu, P. Portincasa and D. Q. Wang. 2013. New insights into the molecular mechanism of intestinal fatty acid absorption. Eur. J. Clin. Invest. 43:1203-1223.

Watkins, B. A., H. E. Lippman, L. Le Bouteiller, Y. Li and M. F. Seifert. 2001. Bioactive fatty acids: Role in bone biology and bone cell function. Prog. Lipid Res. 40:125-148.

Zhang, C., Y. Guo, Z. Yuan, Y. Wu, J. Wang, J. Liu and W. Zhu. 2008. Effect of octadeca carbon fatty acids on microbial fermentation, methanogenesis and microbial flora in vitro. Anim. Feed Sci. Technol. 146:259-269.

Chapter 2 - Effects of DHA and ARA on Performance, Nutrient Metabolism and Activation of the Immune System in Holstein Calves

C. F. Vargas-Rodriguez[*], K. Olagaray[*], R. Rusk[†], L. K. Mamedova[*], J. L. McGill[†], B. J. Bradford[*]

[*]Department of Animal Sciences and Industry

[†]Diagnostic Medicine/Pathobiology

Kansas State University Manhattan, Kansas. 66506

Abstract

Polyunsaturated FA have been linked with modulatory effects in a wide range of biological functions. The objectives for this study were: 1) to determine if docosahexaenoic (DHA) or arachidonic acid (ARA) affect pre-weaning glucose and lipid metabolism, and 2) to determine if increasing concentrations of both FA in milk replacer modulates the immune response to vaccination. Thirty Holstein newborn calves with birth weights between 31 and 45 kg were blocked by date of birth and, within block, randomly assigned to 1 of 5 treatments. Treatments were control (choice white grease), low dose of ARA (1.6 g/d), high dose of ARA (4.8 g/d), low dose of DHA (1.6 g/d) and high dose of DHA (4.8 g/d). All treatments provided a total of 1% added lipid (DM basis), with choice white grease added to ARA and DHA treatments to equalize fat content. Growth parameters, lipid and glucose metabolites, inflammation mediators (TNFα and haptoglobin), antibody titers for BRSV, BVD1 and -2, and peripheral blood mononuclear cell proliferation after vaccination were the variables measured. Data were analyzed with mixed models including the fixed effects of treatment, time, and their interaction, and the random effect block and of heifer within block. Contrasts were used to evaluate effects of fatty acid source and total essential fatty acid (EFA) supply. Dry matter intake, ADG and feed efficiency were not affected by treatments. Docosahexaenoic acid increased height at the withers relative to ARA ($P < 0.05$) but increasing EFA supply slowed the growth in pin-bone width ($P < 0.05$). Glucose concentration increased when the dose of PUFA increased ($P < 0.05$). Plasma β-hydroxybutyrate and triglyceride concentrations were not affected by treatment. Inflammatory markers, antibody titers, CD4 cells and CD8 cells were not different between treatments ($P>0.10$). The supplementation of DHA and ARA modulated glucose and lipid metabolism but had few effects on performance and immune response to vaccination in pre-weaning calves.

Keywords: inflammation, omega-3, pre-ruminants, PUFA

Introduction

Nutrient intake during the pre-weaning period affects the partitioning of nutrients among different tissues and their biological activities. Several studies have demonstrated how the improper balance of nutrients greatly affects ADG, skeletal growth and lean muscle mass of growing calves (Cowles et al., 2006; Swank et al., 2013; Garcia et al., 2015). Nevertheless, the fact that newborns have the esophageal groove, allows them to utilize alternative dietary sources whose potential is limited when the rumen is fully functional. One example is the inclusion of fat in milk replacer to increase dietary energy density. Furthermore, it is possible to deliver specific fatty acids (FA), which have been identified as modulators of different metabolic pathways (Dänicke et al., 2012; Swank et al., 2013).

Long chain FA (C22:6 specifically), tested in monolayer cultures of bovine hepatocytes, affected both glucose and lipid metabolism (Mashek and Grummer, 2003). An *in vivo* study of essential fatty acid supplementation for calves showed greater concentrations of circulating β-hydroxybutyrate when feeding milk replacer enriched with medium-chain FA in place of linoleic acid (Garcia et al., 2015). In addition, the expression of genes related to lipid metabolism increased when LCFA 20:5(*n*-3), 16:0 and 18:0 were added to Madin–Darby Bovine kidney cell culture (Bionaz et al., 2012).

Among the essential FA, special emphasis has been placed on omega-3 FA during the last decades, and multiple species have been the target of investigation. In human diets, the omega-3 FA have been evaluated for treatment of hyperlipidemia, hypertension, and rheumatoid arthritis

(Covington, 2004). The low prevalence of metabolic syndrome and type-2 diabetes in Eskimo populations in Alaska may be related to high consumption of this class of FA (Ebbesson et al., 2005). In horses, it was observed that the supplementation of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) influenced the profile of circulating FA (King et al., 2008). In rats, when as little as 6% of dietary linoleic acid was replaced with omega-3 FA (FA) from fish oil, the development of insulin resistance was prevented (Storlien et al., 1987). Arachidonic acid (ARA) with its first double bond placed in carbon 6, is another key component of cell membranes and a precursor of chemical mediators (prostaglandins, leukotrienes, and lipoxins) attributed with important actions on inflammation an resolution (Buckley et al., 2014); the responses linked to the omega-6 FA family are pro-inflammatory (Simopoulos, 2002).

Beyond their influence on metabolic activities, omega-3 FA also have been investigated for their capacity to modulate immune cell function and inflammatory responses (Greco et al., 2015). It has been reported that DHA and EPA in mice induced potent anti-inflammatory responses through their effect on G-protein receptor 120 (Talukdar et al., 2010), which is expressed primarily in adipose tissue and pro-inflammatory macrophages. In piglets during the weaning period, animals fed with 3% marine omega-3 FA had lower plasma concentration of TNFα, a response associated with a reduction of stress caused by the weaning process (Li et al., 2014). In study done with Holstein calves, omega-3 FA tended to decrease gene expression of TNFα in LPS-stimulated cells when fish oil was the source; when flax oil was used, the expression of IL-4 was decreased, (Karcher et al., 2014). This indicated the potential of the FA to regulate cytokine gene expression.

Docosahexaenoic acid has specific effects on immune function and inflammatory processes, and these are not necessarily linked to EPA as its precursor. An experiment that examined if EPA or DHA generate anti-inflammatory responses observed that both fatty acids

activated PPAR-γ and reduced the concentration of lipopolysaccharide in human renal tubular cells by inhibiting the activation of nuclear factor NF-κB (Li et al., 2005). Another group (Serini et al., 2012) observed that the addition of purified DHA or EPA effectively reduced cytokine release by phytohaemagglutinin-stimulated peripheral blood mononuclear cells *in vitro*, and the effect was more prominent with DHA.

Moreover, protectins and maresins, molecules that stimulate the clearance of cytokines and chemokines by macrophages, stop the recruitment and infiltration of neutrophils, and also regulate the removal of apoptotic neutrophils and cellular waste (Serhan, 2010; Spite et al., 2014), come from DHA; protectins are synthesized by a lipoxygenase-dependent reaction (Serhan et al., 2015), whereas maresin formation requires a series of enzymatic reactions where DHA is hydroperoxidated, epoxidated and finally hydrolyzed (Sasaki et al., 2011).

In this experiment, we aimed to determine if docosahexaenoic (DHA) or arachidonic acid (ARA) affected pre-weaning glucose and lipid metabolism. In addition, we sought to determine if increasing concentrations of DHA or ARA in milk replacer, using relatively purified sources, could modulate the immune function of calves when they undergo a vaccination protocol.

Materials and Methods

All protocols were approved by the Kansas State University Institutional Animal Care and Use Committee.

Animals and Management

Thirty Holstein heifers were enrolled within 12 h of birth. The dams gave birth in an open, cooled barn, and newborns were immediately removed from their side and their body weight was

recorded. Within the first hour of life, 1.42 L of colostrum was offered to the calves; the calves consumed almost all the colostrum offered, so drenching was not necessary. Five h later a second dose of colostrum (1.42 to 1.89 L) was offered to the newborns, and a third dose of similar amount was offered 6 h later. The aim was to supply 5.67 L of colostrum within 12 h of birth. The colostrum was collected previously and evaluated with a brix refractometer to determine total solids as an indirect measure of IgG concentration (Quigley et al., 2013). If colostrum had a brix value greater than 22%, the cut-off value for high IgG concentration (Bielmann et al., 2010; Chavatte et al., 1998), it was stored at -4°C, and when needed it was thawed in a water bath at 20°C. After the last colostrum feeding (about 12 h after birth), the calves were moved out of the maternity barn into individual hutches bedded with straw, where they remained until the end of the study.

The exclusion criteria for enrollment were: dystocia at birth (calving difficulty score ≥ 3), twins, and birth weight < 31 kg or > 45 kg. Enrolled calves were blocked by date of birth, and within block they were randomly assigned to 1 of 5 treatments (n = 6 animals per treatment). The treatments consisted of the addition of 1.0% extra fat to the basal milk replacer with the following composition:

1. Choice white grease (CWG)
2. CWG + low dose ARA (ARA = 1.6 g/d or ~ 100 mg/kg $BW^{0.75}$)
3. CWG + high dose ARA (ARA = 4.8 g/d or ~ 300 mg/kg $BW^{0.75}$)
4. CWG + low dose DHA (DHA = 1.6 g/d or ~ 100 mg/kg $BW^{0.75}$)
5. CWG + high dose DHA (DHA = 4.8 g/d or ~ 300 mg/kg $BW^{0.75}$)

The primary difference between treatments was the concentration of DHA and ARA. Two different oils were utilized as sources of extra fat: a fungal oil with a concentration of 40% ARA

(ARASCO, DSM Nutritional Products, Basel, Switzerland) and an algal oil with 40% of DHA (DHASCO, DSM Nutritional Products). Fatty acid content, energy and protein concentrations among treatments remained similar.

Color-coded treatment supplements containing the appropriate dose of each oil for 1 feeding were prepared in the Ruminant Nutrition Laboratory at Kansas State University; technicians in charge of feeding at the KSU dairy unit were blinded to the treatments.

At each feeding time (0000, 0800 and 1600 h), 0.27 kg of milk-replacer powder (Cow's Match, Land O'Lakes Animal Milk Products Co., Shoreview, MN) containing 27.59% CP, 19.85% fat, and 4.98 Mcal GE/kg DM, was weighed, mixed with 1.0 L of hot water (49°C) and put in the bottles. Pre-weighed treatment mixtures were then immediately poured into the bottles, in the following proportions: 3.0 g of CWG for the control treatment, 1.33 g of ARASCO + 2.41 g of CWG for low dose of ARA, 4.0 g of ARASCO + 1.22 g of CWG for high dose of ARA, 1.33 g of oil high in DHA + 2.41 g of CWG for low dose of DHA, 4.0 g of oil high in DHA + 1.22 g of CWG for high dose of DHA. The total fat offered to animals in each treatment was: 154 g/d (control), 156 g/d (low ARA and DHA), 161 g/d (high ARA), and 160 g/d (high DHA). The vials containing the treatments were rinsed 3 times with hot water. Extra hot water was then added to bring the mix to a final volume of 1.89 L. Milk replacer was offered at approximately 37°C, and if it was too hot, the bottles were placed in a water bath to cool before being offered to heifers. When animals weighed less than 38 kg at birth, they were fed 0.612 kg/d of milk replacer (with the full lipid treatment) for 2 weeks or until they reached 38 kg of BW (whichever occurred first), to prevent digestive problems. During the first feeding times, a few animals refused to drink the full amount of milk replacer, but after the second day all animals consumed the full ration. In addition to milk replacer, calves had *ad libitum* access to water.

Small amounts of calf starter were introduced to the calves when they were 3 d old. Initially, grain bottles were provided but when heifers consumed more than 226 g/d, grain was fed in buckets. When animals consumed more than 2 kg of starter/d, 0.453 kg/d of alfalfa was added at the bottom of the bucket to help prepare the animal to transition to a silage diet after weaning. During the entire experiment, the amounts of starter and alfalfa offered and refused were measured daily.

Weaning occurred at 63 d of age and the process lasted 7 d. Heifers were bottle fed once daily (1600 h) and treatments continued as previously assigned; therefore, during this week of weaning, milk including supplemental lipid was provided at 1/3 of the pre-weaning supply. Through the course of the study, 3 heifers (assigned to control, high dose DHA, and high dose ARA treatments, respectively) were removed from the experiment because they suffered severe diarrhea; 3 additional animals were enrolled at the end of the experiment to balance the number of animals per treatment.

Health Status

Fecal scores were recorded twice a day following the procedure described by Karcher et al. (2014). Feces was scored in 5 different categories based on consistency (1 = normal and thick, 2 = normal and less thick, 3 = thin but not watery, 4 = watery, and 5 = abnormal color) by trained personnel. When heifers displayed reduced intake, a listless appearance, or diarrhea, the personnel immediately contacted the farm management and a physical examination was performed, including measurement of body temperature (normal ~38.5ºC), respiration rate (normal ~50-75 breaths/min), and heart rate (normal ~100-150 beats/min). If these parameters were abnormal and the abdomen looked distended, heifers were medicated with 5 mL subcutaneous and 5 mL oral

clostridium antitoxin as well as 5 mL of penicillin both subcutaneous and orally; this treatment was repeated for 3 d. If animals showed respiratory distress, they were treated with tulathromycin (Draxxin®, Zoetis, Florham Park, NJ) at a dose of 1.1 mL/45 kg BW.

Performance

Body weight was recorded every 7 d. Every Friday morning after feeding, the technicians moved animals to an electronic platform scale. At the same time, height at the withers and hips of each calf were measured with a calibrated measuring stick.

A sample of milk replacer was collected every other week and samples were composited by month. A calf starter sample was collected at the end of the experiment. To determine the DM content of milk replacer and calf starter, samples were placed in a force-air oven at 105°C for 16 h. The concentration of nitrogen was determined by oxidation and detection of N_2 gas (Leco Analyzer, Leco Corp., St. Joseph, MI) and the amount of crude protein was calculated. An ether extraction procedure was utilized to determine the crude fat content in the samples (AOAC, 2000: method 920.9) and the gross energy content of milk replacer and calf starter were determined by using a bomb calorimeter.

Glucose and Lipid Metabolism

Before the morning feeding (0800 h), blood samples were collected via jugular venipuncture on days 0, 3, 21, 30, and 63. Blood was collected in one tube with potassium EDTA and another containing potassium oxalate with sodium fluoride to inhibit glycolysis (Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Immediately after collection, blood was centrifuged at 3,000 × *g* for 15 min; plasma was separated and frozen at -20°C until further analysis.

Plasma glucose concentrations were determined by enzymatic assay (kit #439-90901; Wako Chemicals USA Inc., Richmond, VA). Insulin concentrations was determined with a bovine insulin ELISA kit (#10-1201-01; Mercodia AB, Uppsala, Sweden). Samples were placed in microplates containing anti-insulin antibodies bound to the plate, and peroxidase-conjugated anti-insulin antibodies were added to the sample. The 2 monoclonal antibodies bound to insulin present in the sample and unbound antibody was washed out. To generate a colored product for measurement, 3,3'-5,5'-tetramethylbenzidine was added to the sample which reacted with the peroxidase, and the reaction was stopped by adding acid to the samples.

In addition, β-hydroxybutyrate, non-esterified FA (NEFA) and triglyceride (TG) concentrations were determined for plasma samples to monitor the effect of treatments on these lipid metabolism parameters. Concentrations of β-hydroxybutyrate were determined enzymatically (#H7587-58; Pointe Scientific Inc., Canton, MI). A 2-reagent blend containing D-3-hydroxybutyrate dehydrogenase, diaphorase enzymes, NAD and 4- iodonitrotetrazolium violet (INT) were combined and added to plasma samples. β-hydroxybutyrate reacted with NAD first and generated NADH; this product, in contact with diaphorase and INT, produced the reduced form of INT was measured by absorption at 505 nm.

Quantification of NEFA was also done using an enzymatic procedure (NEFA-HR; Wako Chemicals USA Inc.). Samples were combined with acyl-CoA synthetase to conjugate NEFA to coenzyme-A. Then acyl-CoA oxidase was added to generate hydrogen peroxide which, in presence of peroxidase and 4-aminoantipyrine, generated 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline, measured by colorimetry at 550 nm.

The concentration of triglycerides in plasma samples was estimated after a sequence of enzymatic reactions (#H7587-01; Pointe Scientific Inc., Canton, MI). In short, plasma samples

were combined in wells with a combination of reagents (mixture of lipase, glycerol kinase, glycerol phosphate oxidase, and peroxidase). Lipase separated the FA from the glycerol backbone, which was converted to glycerol-1-phosphate by glycerol kinase. The enzyme glycerol phosphate oxidase reacted with glycerol-1-phosphate to produce hydrogen peroxidase. The non-enzymatic compounds present in the reagent (4-chlorophenol, 4-aminophenazone) reacted with the hydrogen peroxide produced previously and generated a color complex that was read colorimetrically at 500 nm. A spectrophotometer (PowerWave XS; BioTek Instruments Inc., Winooski, VT) was utilized to read absorbance and with the help of Gen5 software (BioTeck Instruments Inc.).

Antibody Titers

To stimulate the development of memory B cells, heifers were vaccinated with BoviShield Gold FP5 (Zoetis) on d 49 to 56 and with BoviShield Gold Plus Ultravac-7 (Zoetis) on d 63 to 70. Vaccinations occurred on Tuesdays after the morning feeding for any heifers that fell within the age windows listed. On d 49 (prior to initial vaccination), 14 days after 1st vaccination, immediately prior to 2nd vaccination, and 10-14 days after 2nd vaccination, subsamples of blood were collected and sent to the KSU-Veterinary Diagnostic Laboratory to measure the antibody titer for 3 vaccine strains covered in BoviShield Gold FP5: bovine respiratory syncytial virus (BRSV) and bovine viral diarrhea types 1 and 2 (BVD).

The principle of the serum neutralization test is the inhibition (or neutralization) of viral replication by the presence of specific antibodies. Serum samples were serially diluted and mixed with known virus. Cultured cells were then added to this mixture and incubated to allow viral replication within the cells. If the test serum contained antibodies to the virus, the virus was neutralized, or unable to replicate, thus allowing cells to grow and monolayer normally. If the test

serum did not contain antibodies, the virus was allowed to replicate, thus damaging the cells and producing a visible cytopathic effect (CPE) in the monolayer. By making serial dilutions of test serum, the relative amount of antibody contained in the serum was determined and was given as a titer result. The highest dilution that produces neutralization was reported as the titer (Hanzlicek, 2016).

Inflammatory Mediators

Blood was sampled 1 d post-vaccination to determine the effect of the treatments on concentrations of haptoglobin and TNFα. Haptoglobin concentration was analyzed using a colorimetric method based on peroxidase activity (Cooke and Arthington, 2013). Tumor necrosis factor α was analyzed using an ELISA method (Farney et al., 2011).

Preparation of PBMC

On d 39 ± 3 (pre-vaccination), ~51 (9 ± 3 d post-vaccination), and ~70 (7 ± 3 d post-vaccination), 30 mL of blood was collected from the jugular vein into 4 ml of acid citrate dextrose. Bovine peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation from buffy coat fractions, and red blood cells were removed using hypotonic lysis. Cells were washed and re-suspended in complete RPMI (cRPMI) composed of RPMI-1640 (Gibco, Carlsbad, CA) supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 1% antibiotic-antimycotic solution, 1% non-essential amino acids, 2% essential amino acids, 1% sodium pyruvate, 50 μM 2-mercaptoethanol, and 10% fetal bovine serum.

Stimulation of PBMC

For T lymphocyte proliferation assays, PBMC were labeled with Cell Trace Violet per manufacturer's recommendations (Invitrogen, Life Technologies), plated at a concentration of 4×10^6 cells/mL in cRPMI (100 μL/well) in sterile, round-bottom, 96-well tissue-culture-treated plates (BD Biosciences), and then cultured for 6 d at 37°C in 5% CO_2. For experiments using heat-killed BRSV Strain 375 or BVD Type 2 Strain 28508-5, virus stock was inactivated for 60 min at 56°C, then 50 μL was added to the cultures (final volume of 200 μL/well). Stimulation with poke weed mitogen was used as a positive control in the proliferation experiments.

Flow Cytometry

For surface staining, cells were re-suspended at 10^7 cells/mL in fluorescence-activated cell sorting buffer and incubated for 20 min on ice with 10 μg/mL primary antibodies: mouse anti-bovine CD4 (clone ILA11A, isotype IgG2a), all mouse anti-bovine CD3 (clone MM1A, isotype IgG1), mouse anti-bovine CD8 (clone BAQ111A, isotype IgM), mouse anti-bovine γδ T cell receptor (Clone GB21A, isotype IgG2b). All antibodies were obtained from the Washington State University Monoclonal Antibody Center (Pullman, WA). Cells were washed once and re-suspended at 10^7 cells/mL with 5 μg/mL secondary antibodies: goat anti-mouse IgG1-AF647, goat anti-mouse IgG2b-PECy7 (both from Southern Biotech, Birmingham, AL), goat anti-mouse IgG2a-AF488 (from Life Technologies, Eugene, OR), and goat anti-mouse IgM-PE (from Invitrogen, Frederick, MD). Peripheral blood mononuclear cells (PBMC) were incubated for 20 min on ice, washed, and fixed in BD FACS lysis buffer. Flow cytometry data were collected on a BD LSR Fortessa X-20 flow cytometer and analyzed using FLowJo software (TreeStar, San

Carlos, CA). Background proliferation was corrected for by subtracting the number of cells that divided in mock cultures from the number of cells that divided in stimulated cultures.

Statistical Analysis

A complete randomized block design was used in this study. The data collected in this experiment were analyzed with the MIXED procedure of SAS (version 9.3.1, SAS Institute, Cary, NC) to evaluate the fixed effects of the supplemental FA, time, and treatment by time interaction. Models included the random effect of animal within block. The Kenward-Rogers method was used to estimate the denominator degrees of freedom. With an autoregressive covariance structure (AR[1]), repeated measures over time within heifer were modeled. Contrasts were used to evaluate differences between ARA and DHA (coefficients -0.6666 -0.3333 0 0.6666 0.3333) and the effect of different doses of these PUFA (coefficients 0.5 0 -1 0.5 0). Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$.

Results and Discussion

Production and Health Parameters

Samples of milk replacer, white grease choice, and the 2 oils with high content of omega-3 polyunsaturated FA were analyzed to determine the total and individual concentration of each FA; this information is detailed in Table 2.1. The oils used as source of supplemental EFA had concentrations of > 40% ARA and DHA, respectively. Additionally, the ratio of omega-6:omega-3 FA for each treatment was: 14.2:1 for control, 7.4:1 and 3.7:1 for the low and high dose of DHA, and finally 15.2:1 for low ARA and 17.4:1 for high ARA treatments.

The experiment was not designed to evaluate differences in growth of calves when they were supplemented with omega FA. Rather it was designed to test mainly differences in glucose and lipid metabolism and immune function.

On average, calves weighed 38.2 kg (SEM = 1.28 kg) at birth; this parameter was not different across treatments ($P > 0.1$). The effect of supplementing milk replacer with DHA and ARA on DMI, ADG and feed efficiency (FE) can be observed in Table 2.2. Total DMI was calculated by adding the consumption of milk replacer and calf starter (DM basis). This parameter was not affected either by the composition of EFA or the total supply of EFA; however, animals had greater consumption of DM as time passed ($P < 0.05$).

Previously**,** no changes in total DMI were observed when total fat content of milk replacer was below 23% but when the fat concentration was 30%, DMI was depressed (Tikofsky et al., 2001). In this experiment, the diets did not reach a fat content that would be expected to impair the appetite or development of the calves. The mean total DMI for this experiment was 2.0 kg/d, which was larger than that observed by Bowen Yoho et al. (2013). This group reported 0.971 kg DMI/d in an experiment designed to determine the effect of changing the FA profile in milk replacer with coconut oil as source of essential FA; however, their animals were weaned at 56 d whereas our heifers were weaned at 63 d.

Overall means for ADG and feed efficiency (ADG/DMI), were 0.75 kg/d (SEM = 0.046 kg/d) and 43.3% (SEM = 2.3%) respectively. No differences were detected for either variable when ARA or DHA were supplemented to young animals. A time effect was observed in both cases. The animals enrolled in this trial appeared to grow normally and did not exhibit any major health issues.

Similar to our results**,** (Ballou and DePeters, 2008) did not observe improvements in BW gain when they supplemented 2% fatty acid using fish oil alone or mixed with a blend of canola oil and corn oil. In contrast, greater ADG were observed in animals fed high concentration of linoleic acid (46.3 % of total FA) compared to those fed low doses (40.8% of total FA; Garcia et al., 2014).

The number of days with feces classified as abnormal did not differ with EFA supply but differences ($P < 0.05$) between ARA and DHA were observed. Calves supplemented with ARA excreted feces with less normal consistency than animals fed DHA (Table 2). None of the animals suffered severe fecal abnormality and none of the animals had any fecal score greater than 3 in the scale used in this experiment. Karcher et al. (2014) reported no differences in the number of days with abnormal fecal score when they compared the supplementation of n-3 FA from fish and flax oils on cytokine gene expression and growth of milk-fed Holstein calves.

In addition to BW, changes in body conformation were determined by measuring height at withers and hips and the width between hips and pins were reported as changes after 63 d and also weekly changes (Tables 2.3 and 2.4). For animals supplemented with DHA, changes in height at withers were greater ($P < 0.05$) than for animals supplemented with ARA. Changes in width at the pin bones were greater ($P < 0.05$) when calves received low EFA than those fed with high EFA after 63 d on treatments. Treatments did not produce any effects on BW, hip height or hip width.

When the aforementioned variables were analyzed for changes on a weekly basis (Table 2.4), the majority of them were characterized by time effects ($P < 0.05$) except for changes in pin bone width and hip height. Additionally, a significant treatment by week interaction ($P <0.05$) was detected for change in hip height and DHA tended ($P < 0.10$) to produce greater change in height at hips and withers than ARA.

Glucose and Lipid Metabolism

Variables involved in glucose and lipid metabolism are detailed in Table 2.5 and Figures 2.2 to 2.5. The treatments fed to calves during the pre-weaning period affected plasma glucose and plasma non-esterified fatty acid concentrations. The glucose levels in animals fed the control treatment averaged 116 mg/dL (SEM = 4.75). The inclusion of ARA and DHA significantly ($P < 0.05$) reduced glucose by 17% and 14%, respectively. Glucose concentration increased when the dose of PUFA increased ($P < 0.05$).

Garcia et al. (2015) observed a quadratic effect on plasma glucose concentration with increasing consumption of soybean oil, as a source of polyunsaturated FA, by pre-weaned calves. The authors attributed this effect to the activation of the peroxisome proliferator-activated receptor (PPAR-γ) in liver which stimulates β-hydroxybutyrate and glucose metabolism. Conversely, Litherland et al. (2010) performed an experiment with dairy calves to determine the effect of PPAR-γ agonists (such as omega-3 FA) on fatty acid metabolism and did not see changes in plasma glucose, NEFA, or β-hydroxybutyrate concentrations. In a study where 1,200 mg of omega-3 FA were given to women with polycystic ovary syndrome, glucose and insulin concentrations were decreased, suggesting an increase in insulin sensitivity (Rafraf et al., 2012); however, insulin concentrations were not measured in our study.

The addition of 1.6 g/d of DHA decreased ($P < 0.05$) NEFA concentration by 40% compared to control, whereas the high dose of the same fatty acid reduced NEFA concentration by 7%. The concentration of triglycerides in plasma was not affected ($P > 0.1$) by the treatments.

The concentration of glucose decreased ($P < 0.05$) during the first 30 d after peaking the first 3 d, and then increasing from d 30 to d 63. Non-esterified fatty acids and triglyceride

concentrations were high during the initial 3 d, similar to what happened with glucose, but they declined ($P < 0.05$) beyond this point. Meanwhile, β-hydroxybutyrate followed the opposite trend, with a drop in the first 3 d and then an increase toward d 63.

The reduction in plasma NEFA concentration when DHA was supplemented at a low dose was interpreted to suggest that this fatty acid has an important role in lipid metabolism. The reduction of NEFA caused by DHA was not used for the formation of circulating TG, because despite the reduction of free FA in plasma, TG concentration did not change; von Schacky et al. (1985) found that EPA and DHA supplemented to humans were incorporated into phospholipids and the concentration of plasma TG decreased over time. Based on this observation, plasma NEFA may have been utilized for the formation of phospholipids in the current study. Conversely, Mashek et al. (2002) observed that DHA and EPA have poor rates of incorporation into cellular phospholipids when they evaluated the effect of different FA on glucose and lipid metabolism of bovine hepatocytes.

Inflammatory and Immune Response

Omega-3 and omega-6 FA are compounds associated with inflammatory responses; the group with the double bond in *n*-3 position tends to have anti-inflammatory effects, whereas the *n*-6 group stimulates inflammation (Karcher et al., 2014).

Adding DHA and ARA at different concentrations in this study did not impact ($P > 0.10$) the plasma concentrations of TNFα or haptoglobin (Table and Figure 2.6), both mediators of inflammation (Li et al., 2014). A significant time effect ($P < 0.05$) was observed for haptoglobin but not for TNFα. Hill et al. (2011) observed that the mRNA abundance of TNFα, measured in whole blood samples, decreased after vaccination when linoleic acid was supplied to Holstein

calves. The inclusion of omega-3 FA in pig diets reduced the concentration of TNFα compared to animals fed a vegetable oil supplement (Li et al., 2014)**;** authors attributed this effect the ability of these FA to inhibit NF-κB, an activator of inflammatory gene transcription in the nucleus.

Figures 2.7 and 2.8 report the effect of treatments on antibody titer production when animals underwent vaccination for prevention of BVD types 1 and 2 and BRSV. In this case, only a time effect ($P < 0.05$) was observed. The antibody production for d 30 was greater compared to d 63 and 82, possibly because the animals at that point still had large amounts of maternal antibodies. Studies where the effect of FA on antibody titers were measured in calves after being vaccinated are scarce; some of the existing references (Hill et al., 2011; Esselburn et al., 2013) observed increased titers to bovine BVD and PI3 in response to a lipid supplement but it contained animal fat, butyrate, medium chain FA and linolenic acid, and not omega-3, so comparisons with this study are complicated.

The suppression of T-helper type 1 cytokine production is one of the benefits attributed to the *n*-3 PUFA (Lin et al., 2007). Proliferation assays for CD4 and CD8 lymphocytes challenged with BRSV and BVD were performed in samples collected on d 39 (before the vaccination) and d 7 to 9 after vaccinations. The overall treatment effect and interaction between treatments and sampling times were not significant ($P > 0.1$) but a time effect was observed ($P < 0.05$; Figure 2.9). The low percentage of CD4 lymphocytes indicated that immune function was not fully activated yet. The vaccination and booster activated a CD4 response to BRSV followed by the activation of CD8 cells; however, EFA treatments were not different than control, indicating that these compounds had no effect on activation of this process. Similar results were observed when diets enriched with DHA were fed to rats with gut-derived sepsis (Lin et al., 2007); the percentage of CD4 and CD8 lymphocytes were not different when treatments were compared to the control.

In another study, the effect of a fish oil therapy evaluated in HIV positive patients with hyperlipidemia did not cause any variation in CD4 cell count (Ranieri, 2007).

Conclusions

In summary, the utilization of highly concentrated sources of ARA and DHA did not provide enough evidence to clarify the modulatory effect of these compounds on inflammatory and immune response. Polyunsaturated fatty acid lowered blood glucose and NEFA indicating a possible regulatory effect on glucose and lipid metabolism.

Acknowledgments

We thank Land O'Lakes for providing the milk replacer used as the basal diet in this experiment. We also want to show our gratitude toward the staff and undergraduate student and employees at the Kansas State University Dairy Teaching and Research Center (Manhattan) for animal care and technical assistance.

References

AOAC. 2000. Official Methods of Analysis. 17th ed. Assoc. Off. Anal. Chem, Arlington, VA.

Ballou, M. A., and E. J. DePeters. 2008. Supplementing milk replacer with omega-3 fatty acids from fish oil on immunocompetence and health of jersey calves. J. Dairy Sci. 91:3488-3500.

Bionaz, M., B. J. Thering, and J. J. Loor. 2012. Fine metabolic regulation in ruminants via nutrient–gene interactions: Saturated long-chain fatty acids increase expression of genes involved in

lipid metabolism and immune response partly through PPAR-α activation. Br. J. Nutr. 107:179-191

Bowen Yoho, W. S., V. A. Swank, M. L. Eastridge, K. M. O'Diam, and K. M. Daniels. 2013. Jersey calf performance in response to high-protein, high-fat liquid feeds with varied fatty acid profiles: intake and performance. J. Dairy Sci. 96:2494-2506.

Cooke, R., and J. Arthington. 2013. Concentrations of haptoglobin in bovine plasma determined by ELISA or a colorimetric method based on peroxidase activity. J. Anim. Physiol. Anim. Nutr. 97:531-536.

Covington, M. B. 2004. Omega-3 fatty acids. American Family Physician. 70 (1):133-140.

Cowles, K., R. White, N. Whitehouse, and P. Erickson. 2006. Growth characteristics of calves fed an intensified milk replacer regimen with additional lactoferrin. J. Dairy Sci. 89:4835-4845.

Dänicke, S., J. Kowalczyk, L. Renner, J. Pappritz, U. Meyer, R. Kramer, E. Weber, S. Döll, J. Rehage and G. Jahreis. 2012. Effects of conjugated linoleic acids fed to dairy cows during early gestation on hematological, immunological, and metabolic characteristics of cows and their calves. J. Dairy Sci. 95:3938-3953.

Ebbesson, S. O., P. M. Risica, L. O. Ebbesson, J. M. Kennish and M. E. Tejero. 2005. Omega-3 fatty acids improve glucose tolerance and components of the metabolic syndrome in Alaskan Eskimos: the Alaska Siberia project. Int. J. Circumpolar Health. 64:.

Esselburn, K. M., K. M. O'Diam, T. M. Hill, H. G. Bateman II, J. M. Aldrich, R. L. Schlotterbeck and K. M. Daniels. 2013. Intake of specific fatty acids and fat alters growth, health, and titers following vaccination in dairy calves. J. Dairy Sci. 96:5826-5835.

Farney, J. K., L. K. Mamedova, B. H. Godsey and B. J. Bradford. 2011. Technical note: validation of an ELISA for measurement of tumor necrosis factor alpha in bovine plasma. J. Dairy Sci. 94:3504–3509.

Garcia, M., L. F. Greco, M. G. Favoreto, R. S. Marsola, D. Wang, J. H. Shin, E. Block, W. W. Thatcher, J.E.P. Santos, and C. R. Staples. 2014. Effect of supplementing essential fatty acids to pregnant nonlactating holstein cows and their preweaned calves on calf performance, immune response, and health. J. Dairy Sci. 97:5045-5064.

Garcia, M., J. H. Shin, A. Schlaefli, L. F. Greco, F. P. Maunsell, J.E.P. Santos, C. R. Staples, and W. W. Thatcher. 2015. Increasing intake of essential fatty acids from milk replacer benefits performance, immune responses, and health of preweaned holstein calves. J. Dairy Sci. 98:458-477.

Greco, L. F., J. T. N. Neto, A. Pedrico, R. A. Ferrazza, F. S. Lima, R. S. Bisinotto, N. Martinez, M. Garcia, E. S. Ribeiro, G. C. Gomes, J. H. Shin, M. A. Ballou, W. W. Thatcher, C. R. Staples, and J. E. P. Santos. 2015. Effects of altering the ratio of dietary n-6 to n-3 fatty acids on performance and inflammatory responses to a lipopolysaccharide challenge in lactating holstein cows. J. Dairy Sci. 98:602-617.

Hanzlicek, G. A. 2016. KSU-veterinary diagnostic laboratory principle of the procedure to measure the antibody titers. Personal comunication.

Hill, T., M. VandeHaar, L. Sordillo, D. Catherman, H. Bateman and R. Schlotterbeck. 2011. Fatty acid intake alters growth and immunity in milk-fed calves. J. Dairy Sci. 94:3936-3948.

Karcher, E., T. Hill, H. Bateman, R. Schlotterbeck, N. Vito, L. Sordillo and M. VandeHaar. 2014. Comparison of supplementation of n-3 fatty acids from fish and flax oil on cytokine gene expression and growth of milk-fed holstein calves. J. Dairy Sci. 97:2329-2337.

King, S., A. AbuGhazaleh, S. Webel and K. Jones. 2008. Circulating fatty acid profiles in response to three levels of dietary omega-3 fatty acid supplementation in horses. J. Anim. Sci. 86:1114-1123.

Li, H., X. Z. Ruan, S. H. Powis, R. Fernando, W. Y. Mon, D. C. Wheeler, J. F. Moorhead and Z. Varghese. 2005. EPA and DHA reduce LPS-induced inflammation responses in HK-2 cells: Evidence for a PPAR-γ–dependent mechanism. Kidney Int. 67:867-874.

Li, Q., J. H. Brendemuhl, K. C. Jeong and L. Badinga. 2014. Effects of dietary omega-3 polyunsaturated fatty acids on growth and immune response of weanling pigs. Journal of Animal Science and Technology. 56:1 - 7.

Lin, M., C. Hsu, S. Yeh, C. Yeh, K. Chang, P. Lee and W. Chen. 2007. Effects of ω-3 fatty acids on leukocyte Th1/Th2 cytokine and integrin expression in rats with gut-derived sepsis. Nutrition. 23:179-186.

Litherland, N. B., M. Bionaz, R. L. Wallace, J. J. Loor and J. K. Drackley. 2010. Effects of the peroxisome proliferator-activated receptor-α agonists clofibrate and fish oil on hepatic fatty acid metabolism in weaned dairy calves1. J. Dairy Sci. 93:2404-2418.

Mashek, D. G., S. J. Bertics and R. R. Grummer. 2002. Metabolic fate of long-chain unsaturated fatty acids and their effects on palmitic acid metabolism and gluconeogenesis in bovine hepatocytes. J. Dairy Sci. 85:2283-2289.

Mashek, D. G. and R. R. Grummer. 2003. Effects of long chain fatty acids on lipid and glucose metabolism in monolayer cultures of bovine hepatocytes. J. Dairy Sci. 86:2390-2396.

Quigley, J. D., A. Lago, C. Chapman, P. Erickson and J. Polo. 2013. Evaluation of the brix refractometer to estimate immunoglobulin G concentration in bovine colostrum. J. Dairy Sci. 96:1148-1155.

Rafraf, M., E. Mohammadi, M. Asghari-Jafarabadi and L. Farzadi. 2012. Omega-3 fatty acids improve glucose metabolism without effects on obesity values and serum visfatin levels in women with polycystic ovary syndrome. J. Am. Coll. Nutr. 31:361-368.

Ranieri, R. 2007. Effect of omega-3 fatty acids on the lipid structure of HIV-positive patients on antiretroviral therapy: personal observation of 20 subjects. Infez Med. 15:237-241.

Sasaki, K., D. Urabe, H. Arai, M. Arita and M. Inoue. 2011. Total synthesis and bioactivities of two proposed structures of maresin. Chemistry–An Asian Journal. 6:534-543.

Serhan, C. N. 2010. Novel lipid mediators and resolution mechanisms in acute inflammation: To resolve or not? The American Journal of Pathology. 177:1576-1591.

Serhan, C. N., J. Dalli, R. A. Colas, J. W. Winkler and N. Chiang. 2015. Protectins and maresins: New pro-resolving families of mediators in acute inflammation and resolution bioactive metabolome. Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1851:397-413.

Serini, S., A. Bizzarro, E. Piccioni, E. Fasano, C. Rossi, A. Lauria, A. RM Cittadini, C. Masullo and G. Calviello. 2012. EPA and DHA differentially affect in vitro inflammatory cytokine release by peripheral blood mononuclear cells from alzheimer's patients. Current Alzheimer Research. 9:913-923.

Simopoulos, A. P. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomedicine & Pharmacotherapy. 56:365-379.

Spite, M., J. Clària and C. Serhan. 2014. Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. Cell Metabolism. 19:21-36.

Storlien, L. H., E. W. Kraegen, D. J. Chisholm, G. L. Ford, D. G. Bruce, and W. S. Pascoe. 1987. Fish oil prevents insulin resistance induced by high-fat feeding in rats. Science. 237:885 - 888.

Swank, V. A., W. S. B. Yoho, K. M. O'Diam, M. L. Eastridge, A. J. Niehaus, and K. M. Daniels. 2013. Jersey calf performance in response to high-protein, high-fat liquid feeds with varied fatty acid profiles: blood metabolites and liver gene expression. J. Dairy Sci. 96:3845-3856.

Talukdar, S., E. J. Bae, T. Imamura, H. Morinaga, W. Fan, P. Li, W. J. Lu, S. M. Watkins, and J. M. Olefsky. 2010. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. Cell. 142:687-698.

Tikofsky, J. N., M. Van Amburgh, and D. Ross. 2001. Effect of varying carbohydrate and fat content of milk replacer on body composition of Holstein bull calves. J. Anim. Sci. 79:2260-2267.

Von Schacky, C., S. Fischer, and P. C. Weber. 1985. Long-term effects of dietary marine omega-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. J. Clin. Invest. 76:1626-1631.

Tables and Figures

Table 2.1. Fatty acid profile of basal milk replacers and essential fatty acid supplements.

Fatty acid (g/100g total FA)	DHA-rich oil	ARA-rich oil	White grease choice	Milk replacer
C6:0	0.0	0.0	0.0	0.2
C8:0	0.3	0.0	0.0	0.4
C10:0	1.2	0.0	0.1	0.6
C12:0	4.8	0.0	0.1	0.6
C14:0	12.2	0.3	1.6	2.3
C14:1	0.2	0.0	0.0	0.1
C15:0	0.0	0.1	0.1	0.2
C16:0	11.3	8.7	22.1	23.1
C16:1	2.0	0.1	2.8	2.0
C17:0	0.0	0.3	0.4	0.5
C18:0	0.8	7.8	8.6	11.8
C18:1n9t	0.0	0.1	0.2	0.2
C18:1n10t	0.0	0.0	0.0	0.2
C18:1n11t	0.0	0.0	0.3	0.2
C18:1n9c	18.3	20.1	41.7	33.7
C18:1n11c	0.1	0.4	3.2	2.3
C18:2n6c	**0.7**	**6.8**	**14.9**	**17.7**
C20:0	0.1	0.7	0.2	0.2
C18:3n6	0.0	2.8	0.0	0.0
C20:1	0.1	0.3	0.9	0.6
C18:3n3	0.0	0.1	0.6	1.1
CLA 9c,11t	0.0	0.0	0.1	0.1
CLA 9t,11t	0.0	0.0	0.1	0.1
C20:2	0.0	0.6	0.8	0.6
C22:0	0.2	1.3	0.0	0.1
C20:3n6	0.0	3.8	0.2	0.2

C20:3n3	0.0	0.0	0.1	0.1
C20:4n6	**0.0**	**44.2**	**0.4**	**0.3**
C23:0	0.0	0.1	0.0	0.1
C24:0	0.1	1.0	0.0	0.1
C20:5n3	0.0	0.1	0.0	0.0
C24:1	0.0	0.1	0.0	0.0
C22:5n3	0.7	0.0	0.1	0.1
C22:6n3	**46.7**	**0.0**	**0.0**	**0.0**

Table 2.2. Effect of supplemental DHA and ARA on performance of weaned Holstein calves

	Treatment						Effect	
Parameter	**Control**	**DHA-Low**	**DHA-High**	**ARA-Low**	**ARA-High**	**SEM**	**Treatment**	**[1]ARA vs DHA**
Dry matter intake, kg/d	2.03	2.06	2.10	1.87	1.95	0.154	NS	NS
Average daily gain, kg/d	0.756	0.783	0.757	0.747	0.707	0.0459	NS	NS
Feed efficiency	0.430	0.451	0.417	0.467	0.402	0.0292	NS	NS
Abnormal fecal days, n	7.83	9.67	9.83	9.67	13.5	1.689	NS	$P < 0.05$

[1]ARA = arachidonic acid, DHA = docosahexaenoic acid

Table 2.3. Effect of supplemental DHA and ARA on growth parameters of weaned Holstein calves after 63 d on treatments

	Treatment						Effect	
Parameter	**Control**	**DHA-Low**	**DHA-High**	**ARA-Low**	**ARA-High**	**SEM**	**Treatment**	**[1]EFA dose**
Δ-Body Weight, kg	48.42	48.73	47.69	46.39	44.38	2.85	NS	NS
Δ-Withers height, cm	15.01	17.25	17.75	15.04	15.29	1.09	NS	NS
Δ-Hip height, cm	17.98	16.44	18.16	15.88	16.12	1.34	NS	NS
Δ-Hip width, cm	7.10	7.28	7.47	7.55	6.93	0.67	NS	NS
Δ-Pins width, cm	6.57	8.73	8.40	9.04	8.74	0.75	NS	$P < 0.05$

Δ = changes

[1]Essential fatty acid

Table 2.4. Effect of supplemental DHA and ARA on growth parameters of weaned Holstein calves (average per week)

	Treatment						Effect		
Parameter	**Control**	**DHA-Low**	**DHA-High**	**ARA-Low**	**ARA-High**	**SEM**	**Treatment**	**Treat × week**	**[1]ARA vs DHA**
Body weight gain, kg/week	5.29	5.48	5.30	5.23	4.95	0.320	NS	NS	NS
Δ-Withers height, cm/week	1.88	1.89	1.92	1.73	1.61	0.14	NS	$P < 0.10$	NS
Δ- Hip height, cm/week	2.10	1.93	2.03	1.79	1.69	0.15	NS	NS	$P < 0.05$
Δ- Hip width, cm/week	0.91	0.86	0.88	0.78	0.77	0.07	NS	NS	NS
Δ- Pins width, cm/week	1.16	0.92	1.02	0.88	0.89	0.10	NS	NS	NS

[1]ARA = arachidonic acid, DHA = docosahexaenoic acid

Table 2.5. Effect of supplemental DHA and ARA on glucose and lipid metabolites

	Treatment						Effect	
Parameter	**Control**	**DHA-Low**	**DHA-High**	**ARA-Low**	**ARA-High**	**SEM**	**Treatment**	**[1]EFA dose**
Glucose, mg/dL	115.51	97.65	99.75	93.11	98.38	4.75	$P < 0.05$	$P < 0.05$
NEFA[1], µM	258.1	153.6	239.5	254.2	245.4	29.4	$P < 0.05$	NS
β-hydroxybutyrate, µM	106.89	103.26	114.62	101.64	106.56	9.94	NS	NS
Triglycerides, mg/dL	18.56	17.87	18.92	15.97	17.41	3.04	NS	NS

NEFA = non-esterified fatty acid, TG = triglycerides

Table 2.6. Effect of supplemental DHA and ARA on inflammatory mediators

	Treatment						Effect
Parameter	**Control**	**DHA-Low**	**DHA-High**	**ARA-Low**	**ARA-High**	**SEM**	**Treatment**
TNFα[1] ,pg/mL	3.46	3.73	2.91	3.59	4.18	0.73	NS
Haptoglobin, µg/mL	165	183	153	103	149	27	NS

[1]TNFα= Tumor necropsies factor

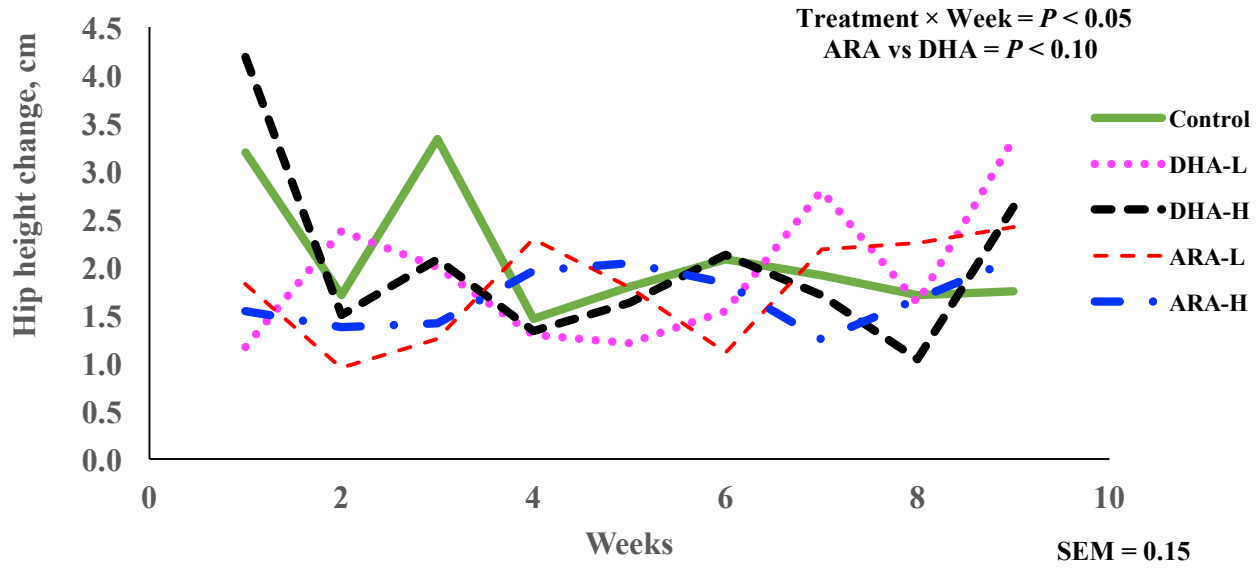


Figure 2.1. Change in hip height of Holstein weaning calves supplemented with docosahexaenoic and arachidonic acid. The X-axis represent number of weeks and Y-axis represent the changes in hip height measure in cm. Each treatment was supplemented during 63 d to 6 different animals. Measures were recorded weekly.

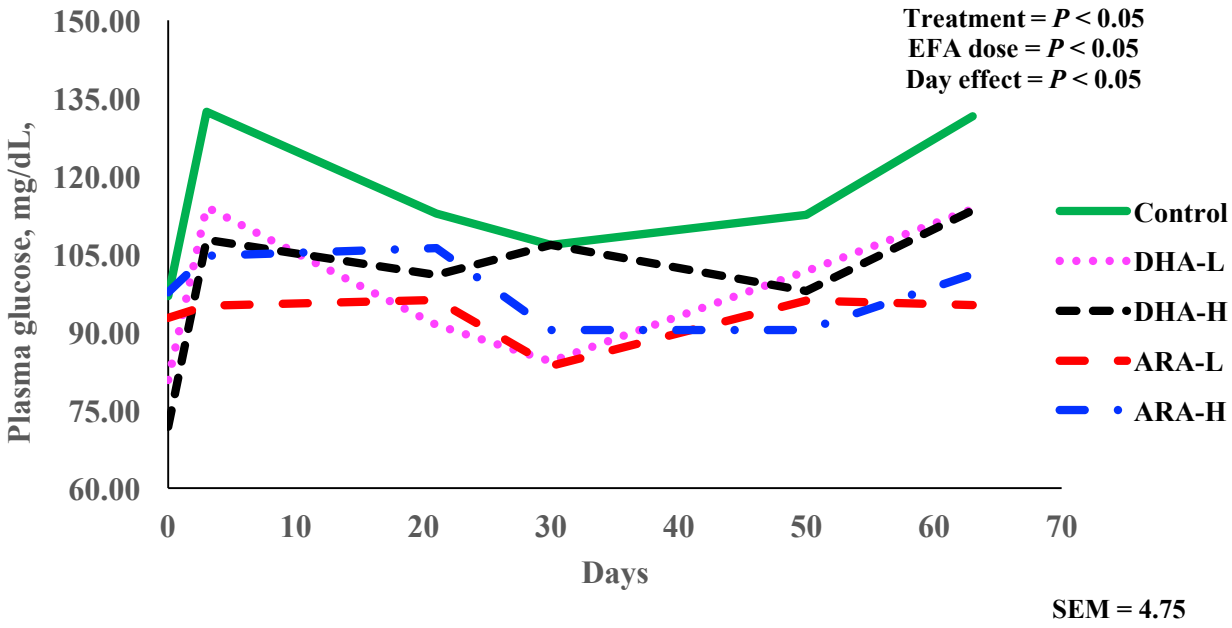


Figure 2.2. Effect of supplemental docosahexaenoic and arachidonic acid on plasma glucose concentration. The X-axis represent number of days on the experiment and Y-axis represent plasma glucose concentration measured in mg/dL. Each treatment was supplemented during 63 d to 6 different animals. Plasma samples for glucose were collected on d 0, 3, 21, 30, 50 and 63.

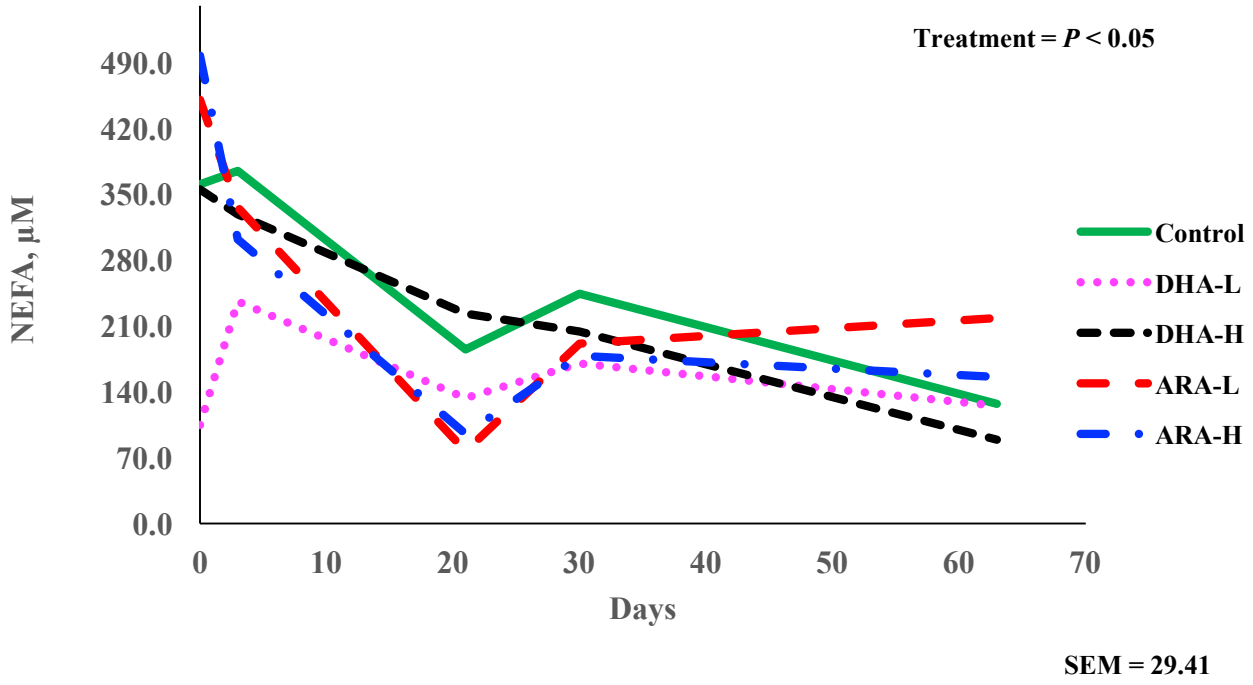


Figure 2.3. Effect of supplemental docosahexaenoic and arachidonic acid on plasma non-esterified fatty acid concentration. The X-axis represent number of days on the experiment and Y-axis represent plasma NEFA concentration measured in µM. Each treatment was supplemented during 63 d to 6 different animals. Plasma samples for NEFA were collected on d 0, 3, 21, 30 and 63.

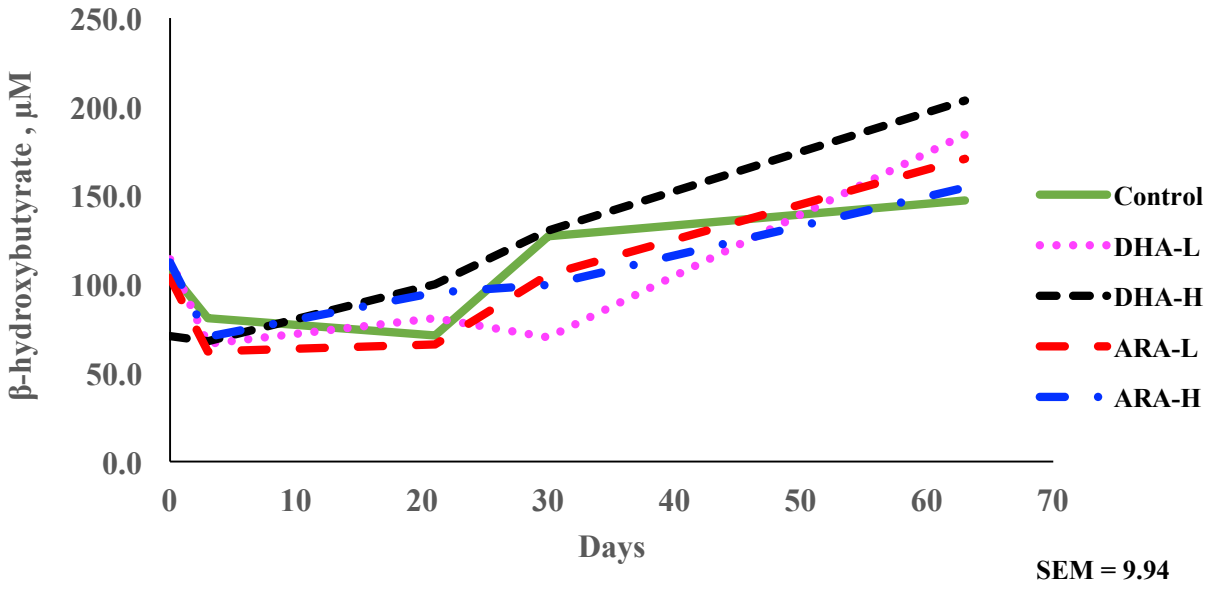


Figure 2.4. Effect of supplemental docosahexaenoic and arachidonic acid on plasma β-hydroxybutyrate concentration. The X-axis represent number of days on the experiment and Y-axis represent plasma β-hydroxybutyrate concentration measured in µM. Each treatment was supplemented during 63 d to 6 different animals. Plasma samples for β-hydroxybutyrate were collected on d 0, 3, 21, 30 and 63.

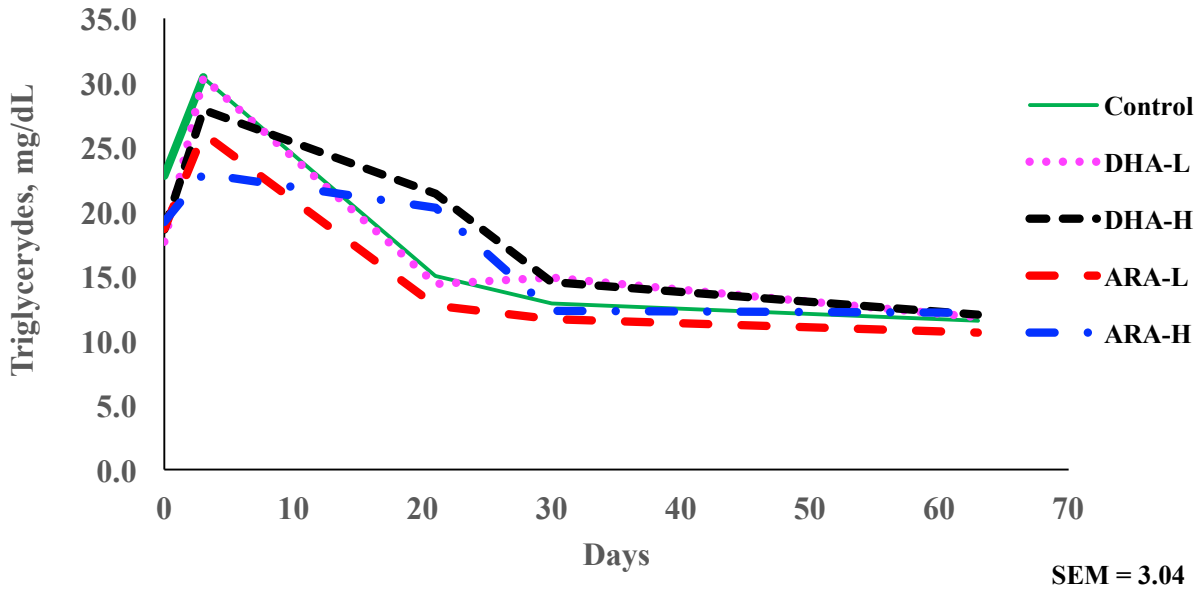


Figure 2.5. Effect of supplemental docosahexaenoic and arachidonic acid on plasma triglycerides concentration. The X-axis represent number of days on the experiment and Y-axis represent plasma triglycerides concentration measured in mg/dL. Each treatment was supplemented during 63 d to 6 different animals. Plasma samples for triglycerides were collected on d 0, 3, 21, 30 and 63.

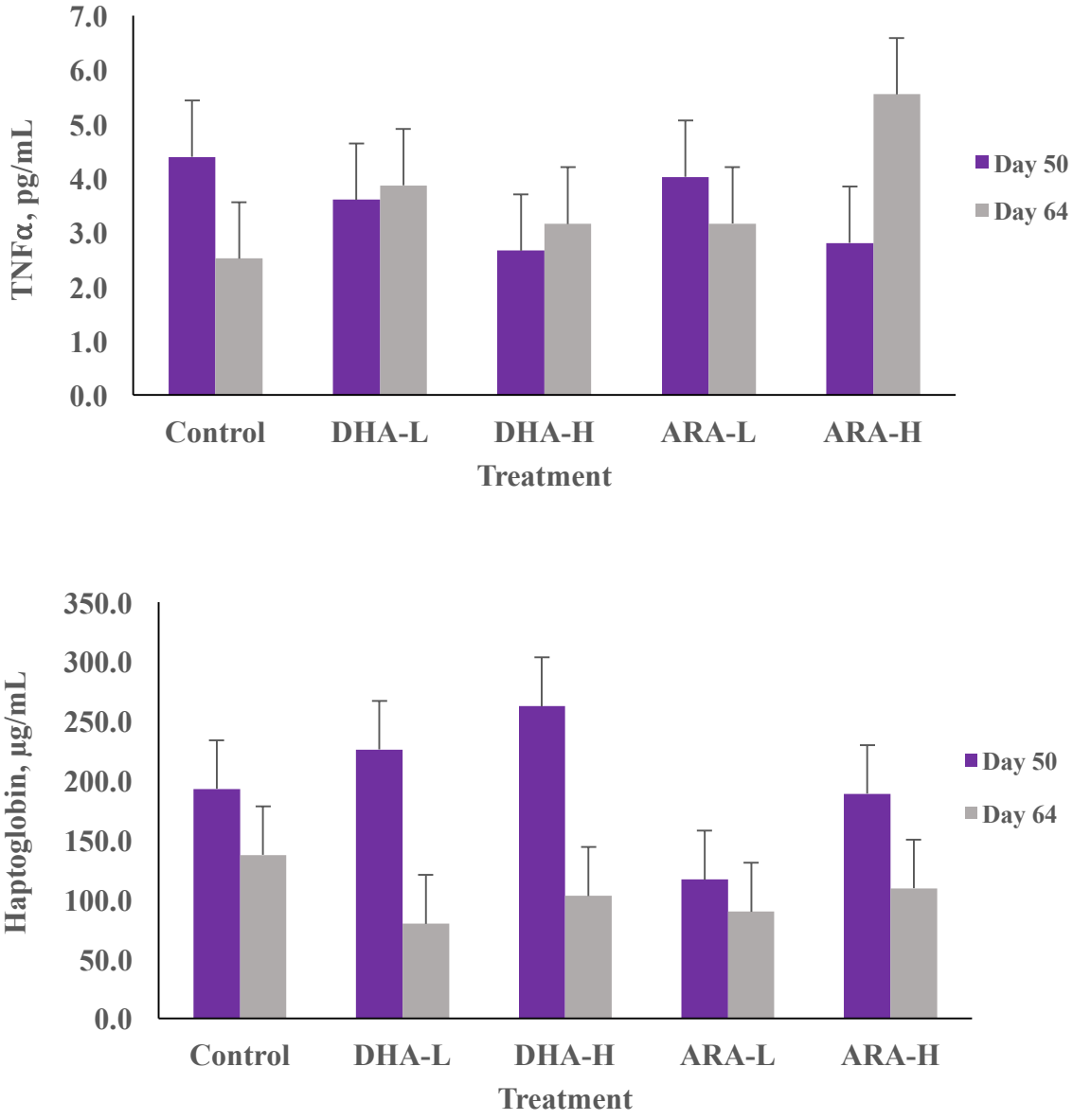


Figure 2.6. Effect of supplemental docosahexaenoic and arachidonic acid on tumor necrosis factor-α and haptoglobin concentration. The X-axis indicates the treatments used during the experiment, the Y-axis represent plasma haptoglobin concentration measured in µg/mL. Each treatment was supplemented during 63 d to 6 different animals. Plasma samples for haptoglobin were collected on d 50 and 64, one day after vaccination.

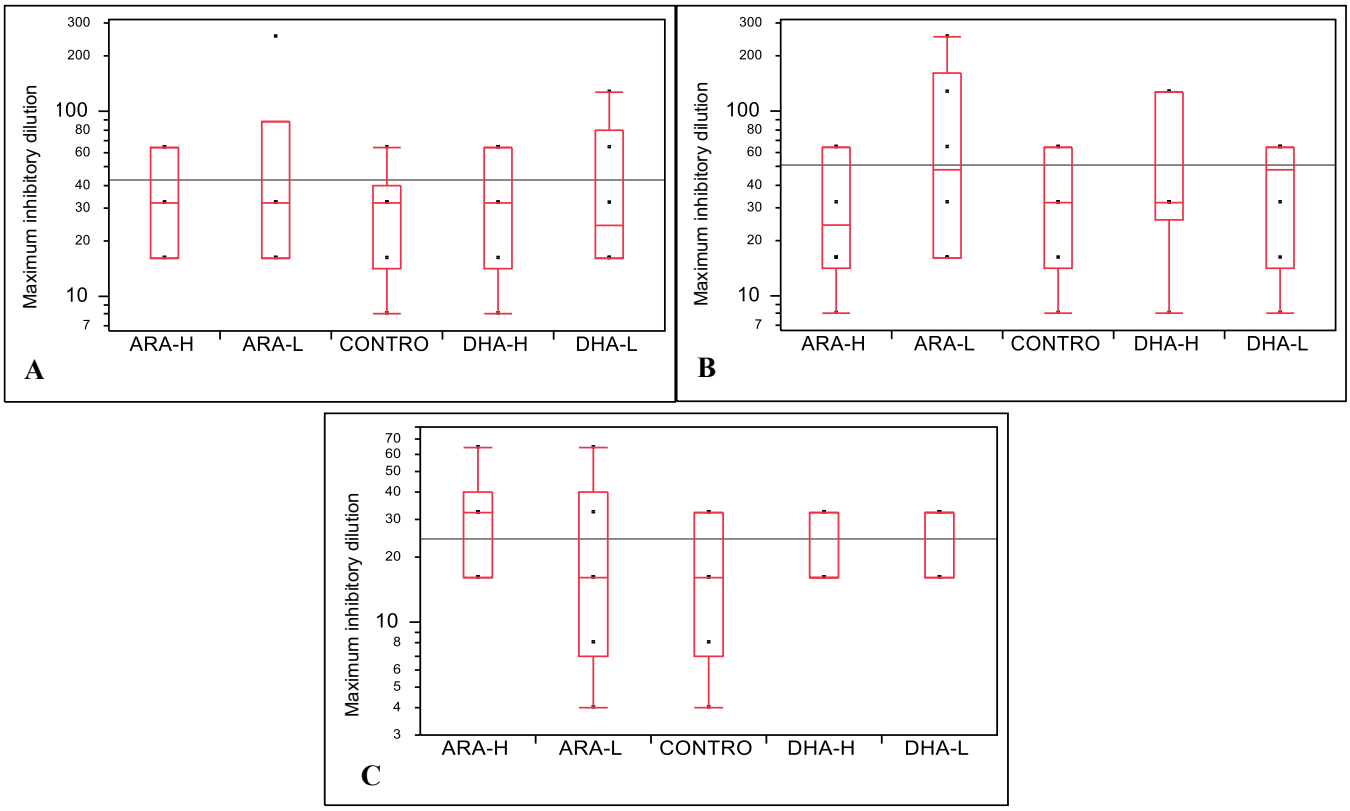


Figure 2.7. A) Effect of supplemental docosahexaenoic and arachidonic acid on antibody titer for bovine viral diarrhea type 1 (BVD1). B) Effect of supplemental docosahexaenoic and arachidonic acid on antibody titer for bovine viral diarrhea type 2 (BVD1). C) Effect of supplemental docosahexaenoic and arachidonic acid on antibody titer for bovine respiratory syncytial virus. The X-axis indicates the treatments used during the experiment, the Y-axis represent the maximum inhibitory dilution of serum for antibody reactivity for the mentioned strains of virus. Each treatment was supplemented during 63 d to 6 different animals. Plasma samples for antibody titer were collected on d 49, 63 and 82.

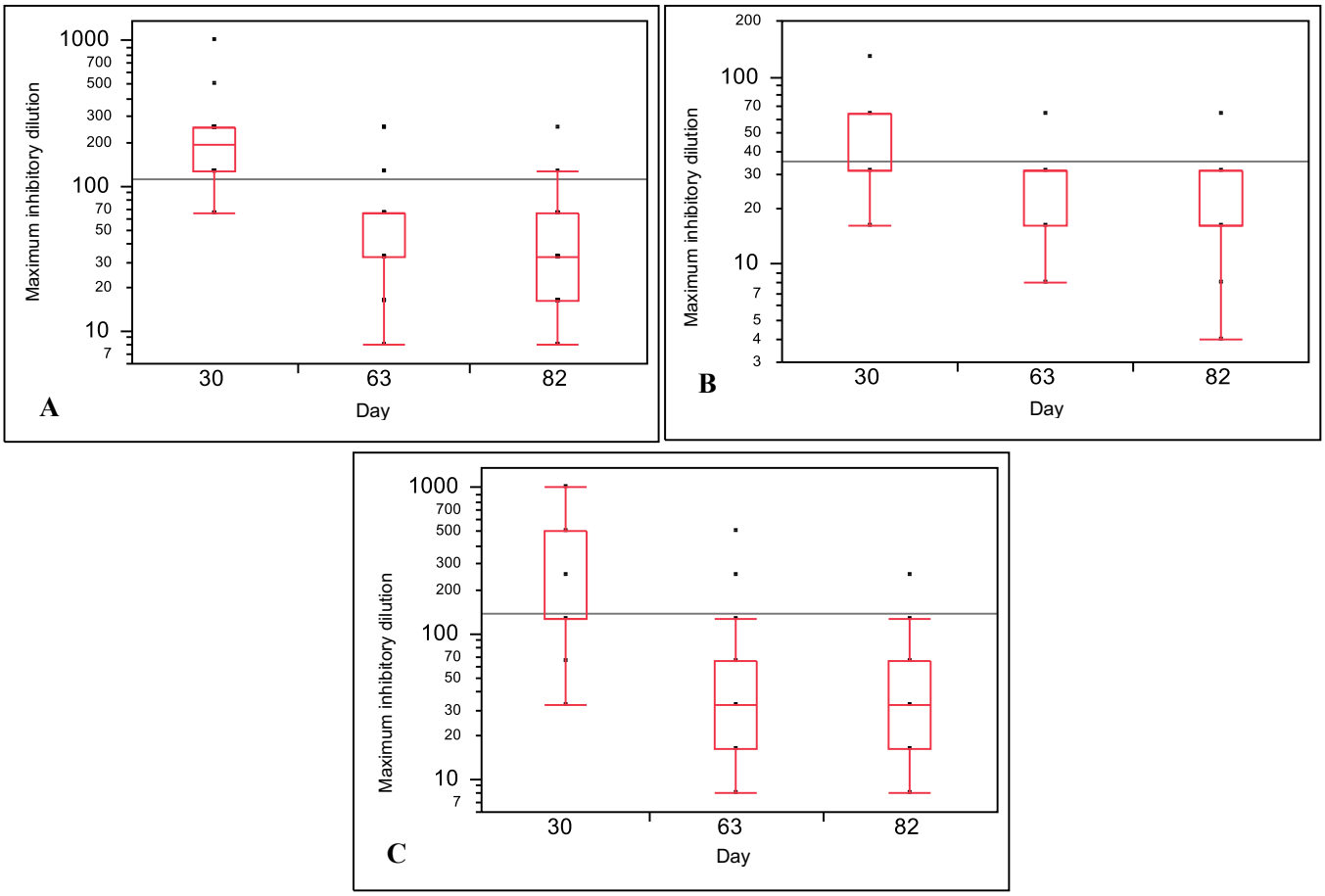


Figure 2.8. A) Effect of supplemental docosahexaenoic and arachidonic acid on antibody titer for bovine viral diarrhea type 1 over time. B) Effect of supplemental docosahexaenoic and arachidonic acid on antibody titer for bovine viral diarrhea type 2 over time. C) Effect of supplemental docosahexaenoic and arachidonic acid on antibody titer for bovine respiratory syncytial virus over time. The X-axis indicate sampling dates; the Y-axis represent the maximum inhibitory dilution of serum for antibody reactivity for the mentioned strains of virus. Each treatment was supplemented during 63 d to 6 different animals. Plasma samples for antibody titer were collected on d 49, 63 and 82.

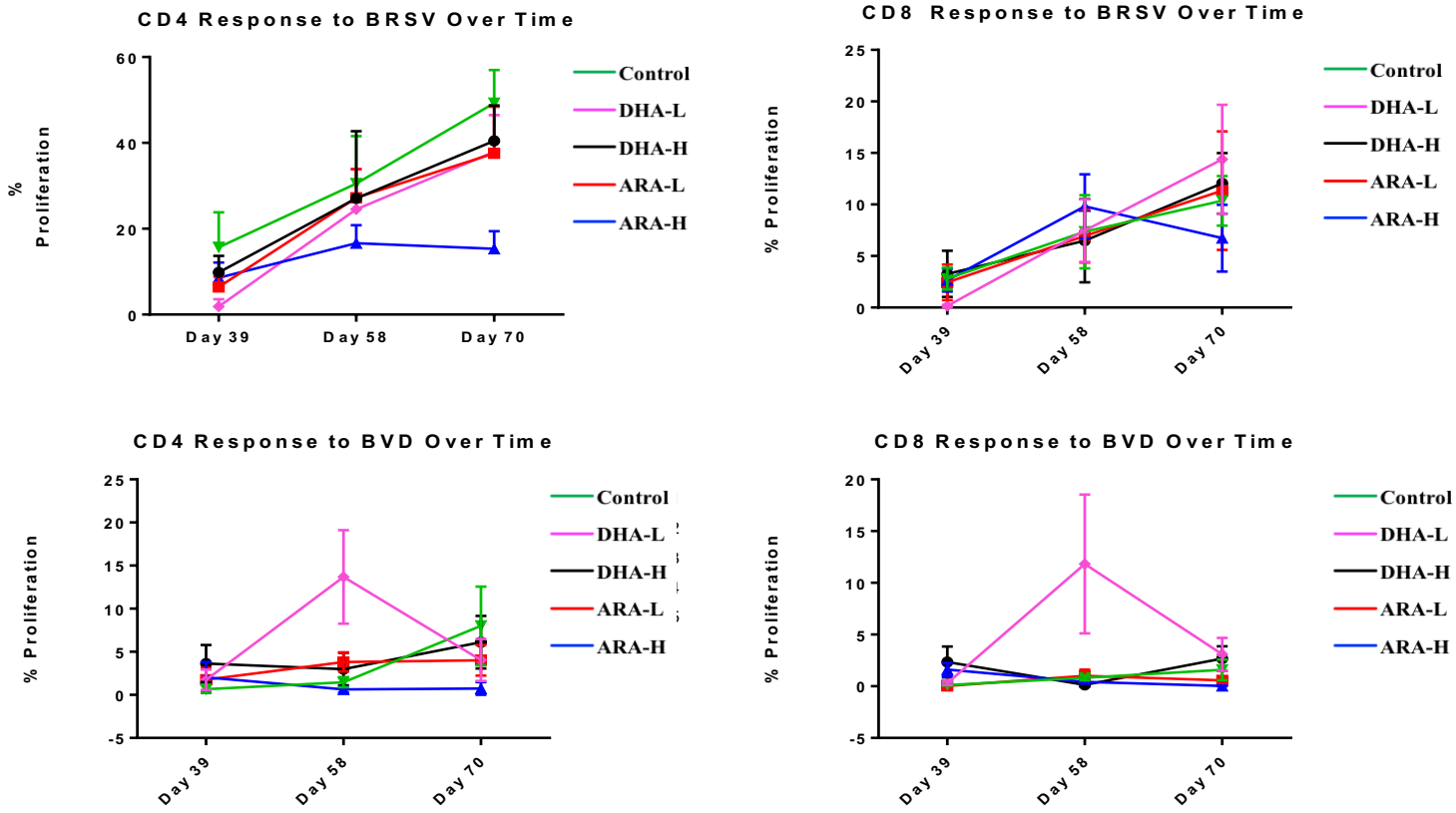


Figure 2.9. Effect of supplemental docosahexaenoic and arachidonic acid on CD 4 cell proliferation in response to bovine viral diarrhea and bovine respiratory syncytial virus. The X-axis indicates the sampling dates used for this analysis, the Y-axis

represents the percentage of cells proliferating when treatments were supplemented to Holstein calves during pre-weaning period. Each treatment was supplemented during 63 d to 6 different animals.

Chapter 3 - Effects of Cobalt Source on Rate and Extent of Dry Matter and Fiber Degradation *In Vitro*

C. F. Vargas-Rodriguez[*], A. J. Carpenter[*], J. DeFrain[†], and B. J. Bradford[*]

[*]Department of Animal Sciences and Industry, Kansas State University, Manhattan, 66506;
[†]ZINPRO Corporation, Eden Prairie, MN 55344

Abstract

Positive effects on fiber degradation have been observed when supplemental cobalt (Co) was added to diets for ruminants but dose-dependent effects of different Co sources on ruminal fermentation have received little attention. Our objective was to determine the effects of different sources and concentrations of Co on *in vitro* fermentation rate, fermentation end-products, and DM and NDF disappearance (DMD and NDFD, respectively). Ruminal fluid was collected from heifers fed a high-forage diet with < 0.1 ppm supplemental Co and fermentation substrate contained no measurable Co. Different inclusion levels (0.0, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, and 15.0 ppm Co) of Co glucoheptonate (CoGH) and Co carbonate (CoCarb) were tested *in vitro* during Experiment 1. Gas production was recorded every 15 min and, after 24 h, pH was measured and contents of each flask were used to determine DMD and NDFD. Experiment 2 evaluated the effects of Co (CoGH and CoCarb at 0, 0.33, 1, 3, and 9 ppm Co) on gas production, VFA concentration and NH_3 concentrations. In both studies, each treatment combination had 4 replicates and samples were incubated for 24 h. Asymptotic gas production curves were modeled with the NLIN procedure of SAS using the Gauss-Newton fit method. Gas production kinetic values and all other data were modeled to assess the effects of Co concentration, source, and their interaction. Regardless of source, Co significantly decreased ($P < 0.05$) the rate of gas production in Experiment 1. Gas production tended to decrease ($P < 0.10$) more rapidly with increasing CoGH relative to similar levels of CoCarb. Dry matter disappearance was greater for CoGH compared to CoCarb across levels of Co tested. Neutral detergent fiber disappearance was increased by 21% for CoGH at concentrations between 0.1 and 1.0 ppm but a pronounced drop in NDFD occurred at 15 ppm Co for this treatment. The effects of Co source on gas production kinetics and pH change were inconsistent between experiments 1 and 2, largely because of dramatic effects at 15 ppm.

Relative to no Co supplementation, concentrations of branched chain VFA decreased ($P < 0.05$) with Co supplementation. CoCarb dose-dependently and linearly decreased ($P < 0.05$) the amount of unsaturated fatty acids and also linearly increased ($P < 0.05$) saturated fatty acids after incubation, presumably reflecting an increased extent of biohydrogenation. In the case of CoGH, the effect of concentration was minimal, resulting in substantial differences in FA profiles between the Co source at concentrations of 3 and 9 ppm. In summary, CoGH increased DMD and NDFD at 1 ppm Co or less relative to CoCarb but decreased NDFD at 15 ppm Co. Furthermore, CoGH had limited effects on the biohydrogenation of LCFA, whereas CoCarb ≥ 3.0 ppm appeared to stimulate this process.

Key words: Minerals, Digestibility, Fiber, Gas production

Introduction

Microbial populations in the rumen are capable of using cobalt (Co) to produce cobalamin and its biologically-active analogs; these compounds act as enzymatic cofactors in several metabolic processes such as gluconeogenesis and methionine synthesis (Kadim et al., 2003; Girard et al., 2009). Vitamin B_{12} synthesis from dietary Co occurs through a complex process that requires 30 enzymatically-catalyzed steps to form a corrin ring, where Co is placed in the center of the structure and attached to 4 pyrol-derived nitrogen molecules (Raux et al., 2000).

Despite the ability of ruminal microbes to synthesize cobalamin from Co, the process is inefficient and makes ruminants susceptible to Co deficiency. In the presence of adequate Co, the rate of conversion does not exceed 15% whereas, when the amount is low, as little as 3% is converted to cobalamin (Costigan and Gerdes, 1991). In addition, vitamin B_{12} is degraded in the

rumen and its absorption is reduced as a result (Costigan and Gerdes, 1991). In addition to its pro-vitamin role, major benefits have been observed on fiber degradation when extra Co has been added to the diet (Kišidayová et al., 2001), perhaps in part because the divalent cation character of Co allows it to serve as a linkage between the negatively-charged outer plant cell wall and negatively-charged bacterial membranes. Inadequate levels of this mineral in rations for ruminants caused unstable fermentation patterns and lower apparent nutrient digestibility associated with shifts in microbial populations (Kadim et al., 2003).

Some studies have tested the impact of the form of minerals on animal performance. Some references indicate more beneficial effects when Co is included in a chelated form (Formigoni et al., 2011), whereas others observed 10% higher bioavailability when an inorganic source of Co was supplied (Kawashima et al., 1997). More recently, a study reported that when fed at 10 times NRC (2001) recommendations, both Co forms seem to have similar effects compared to a control diet containing the recommended concentration of Co (Akins et al., 2013).

The objective of this study was to determine the effects of Co carbonate (CoCarb) and Co glucoheptonate (CoGH) levels on in vitro fermentation rate, DM disappearance, fiber degradation, fermentation end-products, and fatty acid biohydrogenation.

Materials and Methods

All the experimental procedures were approved by Kansas State University Institutional Animal Care and Use Committee.

Experiment 1: Evaluation of Fermentation Response to Varying Inclusion Levels of Cobalt Glucoheptonate and Cobalt Carbonate *In Vitro*

For this experiment, 3 cannulated Holstein heifers were used as ruminal fluid donors. These animals were housed in tie-stalls at the Kansas State University Dairy Cattle Teaching and Research Unit and fed a high-forage diet (Table 3.1) with minimal supplemental Co (< 0.1 ppm as Co carbonate). Before any collection of rumen fluid, heifers were fed this diet for a 14-d adaptation period to reduce the possible carryover of Co from the previous diet. The animals were fed once daily.

Ruminal liquor was collected from the 3 animals, composited and placed into a preheated thermal recipient to maintain temperature during transportation. In the laboratory, ruminal fluid was strained through 8 layers of cheesecloth and placed into a large separatory funnel, which was maintained at 37°C for 30-40 min to allow for stratification of the mat, fluid, and protozoal fractions. The protozoa-rich fraction was voided from the funnel and the clarified liquid layer was mixed 1:2 with McDougall's buffer.

The basal substrate was composed of corn silage (22%), alfalfa hay (21%), corn grain (25%), cottonseed (4%), dried distiller grains (14%) and soybean meal (14%). These materials were dried and ground through a 1-mm screen and subsequently blended into the appropriate proportions. Standard proximate chemical analyses and mineral analyses were conducted to characterize the substrate composition via wet chemistry. This mixture (2.5 g) was added to each fermentation flask, which was equipped with pressure sensitive membranes and RF transmitters that recorded the volume of fermentative gasses produced at 15-min intervals (RFS Gas Production System, Ankom Technologies, Macedon, NY).

The experimental treatments consisted of 2 different sources of Co: CoGH (chelated;

COPRO® 25, Zinpro Corporation, Eden Prairie, MN; CoGH) and feed grade CoCarb (inorganic; Akins et al., 2013). Each source was evaluated at 8 different inclusion levels: 0, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, and 15.0 ppm Co (w/v based on 150 mL of ruminal inoculum). Glucose was also added to the CoCarb treatments such that the total mass added was uniform across sources of Co for each concentration (0, 0.8, 4.1, 8.2, 16.3, 40.8, 81.6, and 122.4 mg glucose / flask, respectively). Each treatment combination was added to 4 different flasks (4 repetitions / treatment; 64 treatment flasks in total). Sources and levels were randomly distributed within the incubator to avoid any effect of location on overall treatment estimates.

Finally, 150 mL of the ruminal inoculum was added to each flask. Four flasks contained inoculum only and were treated as blanks. Gas (CO_2) was used to remove oxygen from the flasks and then the flasks were placed in an incubator at 39°C for 24 h. The tubes were gently swirled every 3 to 4 h during the incubation.

After 24 h of fermentation, the pH of the solution in each flask was measured and contents of the flasks were chilled in an ice bath to cease microbial activity. The solution was weighed and put in aluminum pans. The pans were placed in a 55°C oven for 48 h to determine DM content. The remaining material was used to determine the concentration of NDF in the presence of sodium sulfite and amylase (Van Soest et al., 1991) with an Ankom Fiber Analyzer (Ankom Technology, Fairport, NY).

Experiment 2: Effects of Cobalt Glucoheptonate and cobalt carbonate on VFA Yield and Long Chain Fatty Acids Biohydrogenation During *In Vitro* Fermentation

The extraction of rumen fluid and its preparation were identical to the procedure followed during Experiment 1. The treatments for this experiment were chosen based on gas production,

pH, and DMD obtained from the previous study. Treatments, which were applied as in Experiment 1, provided 0, 0.33, 1, 3, and 9 ppm Co (w/v based on 150 mL of ruminal inoculum), with glucose again added to CoCarb to equalize the total mass added across treatments. Four replicates were included for each treatment (40 treatment flasks total).

At the end of fermentation (24 h), pH was measured, and the contents of the flasks were chilled in an ice bath to cease microbial activity. A 10-mL sample was centrifuged at 30,000 × g for 20 min and a 4 mL sample of supernatant was combined with 1 mL of metaphosphoric acid for storage at -20ºC. These samples were used to characterize concentrations of VFA (HACH. 2000) and NH_3 (Broderick and Kang, 1980). Another 10 mL was utilized to characterize concentrations of long-chain fatty acids (LCFA) by GC analysis (Sukhija and Palmquist, 1988).

Data Analysis

Asymptotic gas production curves were modeled with the NLIN procedure of SAS (version 9.3, SAS Institute, Cary, NC) using the Gauss-Newton fit method (Schofield et al, 1994). Individual flask data were modeled to determine the lag phase, gas production rate, and asymptote; these values were subsequently evaluated as dependent variables. Gas production kinetic values and all other data from each flask were evaluated using the MIXED procedure of SAS to evaluate the effects of Co concentration, source, and their interaction (SAS Institute Inc., 2011). Contrasts were then used to evaluate the overall effect of Co supplementation compared with control, the overall effect of source, the linear and quadratic responses to concentration, and the linear and quadratic interactions of concentration and source. Source and concentration effects are shown independently unless there was evidence of an interaction.

Results

Experiment 1

The basal substrate utilized in these experiments contained 21.5% CP, 3.97% ether extract, 24.8% NDF, and 18.9% ADF (DM basis). The Co concentration in the basal substrate was less than the detection limit of 1 ppm (Method EPA 6010) and therefore provided little background Co (less than 0.017 ppm in final solution).

Effects of treatments on gas production are shown in Table 3.2. Cobalt supplementation decreased the rate of gas production compared to control and tended to increase the lag time between inoculation and commencement of gas production. The asymptotic total 24-h gas production tended to decrease more rapidly for CoGH as concentrations increased (linear dose by source interaction, $P < 0.10$) and CoGH also decreased gas production rate compared to CoCarb ($P < 0.05$). Level of Co did not affect pH change; however, CoGH caused less change ($P < 0.05$) in pH relative to CoCarb (Table 3.2).

In vitro DMD and NDFD of basal substrate, as affected by Co treatments, is presented in Figure 3.1. There was a trend for a linear decrease ($P < 0.10$) in DMD as Co levels increased. In addition, CoGH ($P < 0.05$) increased DMD compared to CoCarb (63.8 ± 1.23% vs. 61.3 ± 1.24% across all levels); however, no interaction ($P > 0.10$) between concentration and source was observed.

A significant interaction between source and level was observed for NDFD, at Co concentrations of 0.1 to 1.0 ppm, CoGH increased ($P < 0.05$) NDFD by 21% (60.2 vs. 49.8%, SE = 2.54%) compared to similar levels of CoCarb but CoGH levels >1.0 ppm led to a decrease in NDFD. In fact, when CoGH was supplemented at 15 ppm Co, only 15% of NDF was degraded.

Experiment 2

Relative to no Co supplementation, the rate of gas production during Experiment 2 decreased ($P < 0.05$) with the addition of CoCarb and CoGH (Table 3.3) and a similar effect was observed for the asymptotic volume of gas produced. There was no evidence that different concentrations of Co affected the lag component of the cumulative gas production curve; however, lag time was increased ($P < 0.05$) by CoGH compared to CoCarb (219.8 vs. 199.3 min, SE = 6.15).

Regardless of Co source, increasing Co concentrations linearly reduced pH change (Table 3.4). In this experiment, a source effect on pH was not observed as in Experiment 1. Supplementing Co decreased *in vitro* ammonia concentrations compared to control (25.5 vs. 24.7 mM, SE = 0.55mM; $P < 0.05$; Table 3.4). Ammonia concentrations were approximately 25 mM with the addition of 0.33 to 3.0 ppm but decreased to approximately 23 mM at 9.0 ppm Co (quadratic, $P < 0.05$). Individual and total concentrations of VFA are also shown in Table 3.4. Relative to the non-supplemented control, concentrations of valerate and branched chain VFA (isobutyrate, and isovalerate) decreased with Co supplementation ($P < 0.05$). Concentrations of acetate, isobutyrate, and isovalerate decreased in a quadratic manner as supplemental Co increased ($P < 0.05$).

Results of LCFA profile analysis following 24 h of in vitro incubation revealed numerous interactions between source and level of Co ($P < 0.05$; Supplemental Table 1). To better understand the effect of added Co source and levels on the biohydrogenation of fatty acids, the LCFA were grouped into 5 different categories: polyunsaturated fatty acid (PUFA), unsaturated fatty acid (UNS), saturated fatty acids (SAT), trans-fatty acids (TRANS), and conjugated linoleic fatty acids (CLA). The effects of Co on these categories of LCFA are shown in Figure 3.3. These graphs show how the interaction between levels and sources of Co modified the relative proportion of UNS and SFA. CoCarb linearly decreased ($P < 0.05$) PUFA and UNS concentrations and also linearly

increased SAT concentrations, presumably reflecting an increased extent of biohydrogenation. In the case of CoGH, the effect of concentration was minimal, resulting in substantial differences in FA profiles between Co sources at concentrations of 3 and 9 ppm. Compared to profiles in the control treatment (Fig. 3.3, Supplemental Table 1), levels of CoCarb > 1.0 ppm seemed to promote biohydrogenation.

Discussion

The animals used as rumen fluid donors in these experiments were fed a high-forage diet. Existing evidence indicates that *in vitro* digestibilities of DM, OM and NDF were improved when substrate was incubated with inoculum obtained from animals fed only with alfalfa compared to a higher concentrate diet (Hussein et al., 1994).

The NRC (2001) estimated 20 ng/mL (ppb) as the critical concentration of Co in ruminal fluid for adequate vitamin B_{12} production (maintaining tissue concentrations of vitamin B_{12} > 0.3 μg/L). Results in this study clearly demonstrate responses to Co supplementation at higher levels, likely independent of vitamin B_{12} synthesis. In general, ruminal Co concentrations are approximately 80% of dietary Co concentrations (Kirsten et al., 2008; Suttle, 2010). Therefore, the treatments in this study are relevant for predicting responses to dietary Co ranging from 0 to 18.75 ppm.

Across both experiments, supplemental Co lowered the rate of gas production by almost 6% in comparison with the control; however, the effects of Co source on the kinetics of gas production were inconsistent across experiments. In Experiment 1, CoGH decreased the rate of gas production but in Experiment 2 this outcome was not observed. On the other hand, the lag time was affected by source in Experiment 2 but not during Experiment 1. Another in vitro study,

performed with rumen fluid from goats, showed a greater rate of gas production when 0.35 ppm of Co (in the form of $CoSO_4 \bullet 7H_2O$) was added to the substrate (Nasser, 2013). These results are not in agreement with the kinetics of gas production obtained in our study and may be due to differences in the form of Co used.

In contrast to the gas production results, the *in vitro* DMD and NDFD responses suggested that moderate levels of supplemental Co improved ruminal degradation of substrate. In an experiment with cannulated lambs (Bishehsari et al., 2010), increasing concentrations of Co (0, 0.25, 0.50, 0.75 and 1.0 ppm as $CoSO_4 \bullet 7H_2O$) improved the degradation of DM. In a separate study using similar concentrations, DM disappearance was greater at the intermediate supplementation levels, whereas low levels of Co (0.1 and 0.12 ppm as $CoSO_4 \bullet 7H_2O$) decreased the apparent nutrient digestibility (Wang et al., 2007). Results of DMD and NDFD analyses in Experiment 1 were similar to the results described in these references, although the effect of dose for DMD was marginal ($P = 0.06$). Maximal digestibility of both DM and NDF was achieved at 0.1 to 0.5 ppm Co.

CoGH significantly increased DMD relative to CoCarb in Experiment 1, seemingly in direct contrast to the decrease in gas production rate by CoGH. The results for NDFD suggest that the rumen availability of Co was higher in the CoGH because at levels between 0.1 and 1.0 ppm, NDFD was greater for CoGH but declined sharply at 15 ppm Co with this source. These responses align with the results of Kawashima et al. (1997), who indicated that Co glucoheptonate had higher solubility than Co carbonate.

Several authors have concluded that Co augments the digestion of the fiber fraction by increasing the activity of fibrolytic bacteria (Scott and Dehority, 1965; Bishehsari et al., 2010; Nasser, 2013). Two different studies measured effects on microbial activity; one (Nasser, 2013)

observed higher protozoal populations at 1.0 ppm Co ($CoSO_4 \bullet 7H_2O$) and the other (Kišidayová et al., 2001) found a significant 10% increase in cellulose digestibility with supplemental Co ($CoSO_4$). These findings also agree with an early publication (Scott and Dehority, 1965) which claimed that cellulolytic bacteria such as *Ruminococcus flavefaciens* need vitamin B_{12} for maximal growth.

Vitamin B_{12} is an essential cofactor of methylmalonyl CoA mutase, which is required in the fermentative conversion of succinate to propionate (Takahashi-Iñiguez et al., 2012). There is evidence that supplemental Co can augment the activity of microbial methylmalonyl CoA mutase (Tiffany et al., 2006). This is supported by Costigan and Gerdes (1991), who reported that *Selenomonas ruminantium*, a ruminal species that has propionate as its major end-product, showed greater uptake of Co when ionic Co-60 was added to the medium. In another study with *Prevotella ruminicola* 23, concentrations of succinate and acetate were higher when vitamin B_{12} was absent but, when the vitamin was included, a higher concentration of propionate was observed (Strobel, 1992). It is important to consider that the methylmalonyl CoA mutase reaction is not the only mechanism in the rumen to generate propionate (Tiffany et al., 2006).

Previous research demonstrated greater production of propionate with CoGH compared to Co propionate at the same levels of supplemental Co (Tiffany et al., 2003). We found no differences in VFA profiles between Co sources in the current experiments, nor did Co concentration impact propionate concentrations. Our findings do not support proposed impacts of Co on propionate production, perhaps in part due to low basal concentrations of Co in the substrate, which may have been adequate to support sufficient vitamin B_{12} concentrations.

Two of the three studies that have evaluated the effects Co on branched chain fatty acid and ammonia concentrations *in vitro* have found no changes in these parameters (Stemme et al.,

2008; Nasser, 2013), although they added less than 1 ppm Co ($CoSO_4$) to the inoculum in these studies. Another report found increasing amounts of isobutyrate when Co was supplemented at 4 ppm (Kawashima et al., 1997). The decrease in isobutyrate and isovalerate concentrations in response to Co supplementation in Experiment 2 did not agree with these previous findings. In contrast, the response we detected was driven primarily by inhibitory effects observed only at 3 ppm Co and greater. These results, accompanied by the decrease in NDFD at high concentrations of CoGH, may indicate that excessive amounts of Co can be detrimental for ruminal microbes, especially with highly soluble sources.

The results obtained in this study in regard to the concentration of LCFA differ from a previous report that did not find differences in LCFA molar proportions when Co ($CoCO_3$) was added between 0.05 and 1.0 ppm (Tiffany et al., 2006). Another study, conducted to test whether different oral doses of Co acetate (3, 6 and 9 ppm) affected production parameters and ruminal function in lactating sheep (Frutos et al., 2014), revealed no changes in the ruminal concentrations of LCFA or bacterial populations. By looking at the categories of the LCFA, our outcomes suggest an impact of added CoCarb on the microbes involved in the biohydrogenation of fatty acids. At high levels of CoCarb inclusion, the shift in LCFA profile from UNS to SAT fatty acids was consistent with an increase in the extent of biohydrogenation after 24 h of incubation; furthermore, the biohydrogenation pathway that produces *trans*-10, *cis*-12 CLA as an intermediate is associated with milk fat depression (Griinari et al., 1998). The concentration of this FA linearly decreased with increasing CoCarb supplementation. Although these effects occurred at concentrations that showed alterations in ammonia and branched-chain fatty acid concentrations, these measures did not differ by source as LCFA did, making it difficult to propose a mechanism underlying this response.

Conclusions

Results of Experiments 1 (NDFD) and Experiment 2 (branched-chain VFA) appear to indicate that Co concentrations in the 9 to 15 ppm range have negative effects on *in vitro* fermentation. For NDFD in particular, CoGH had a stimulatory effect at moderate concentrations (0.1 to 1.0 ppm) but clearly inhibited NDFD at a concentration of 15 ppm. Overall, CoGH increased DMD relative to CoCarb. Source effects on gas production kinetics and pH change during fermentation were inconsistent between Experiments 1 and 2 because of dramatic effects of 15 ppm CoGH in the first study, though source by concentration effects were not significant for these variables. Finally, CoCarb enhanced FA biohydrogenation at Co concentrations of 3 ppm and greater.

Overall, our results suggest that Co does impact ruminal fermentation. Considering all of the outcomes we measured, the optimal response for ruminal microbes appears to be achieved with as little as 0.1 to 0.5 ppm Co. In that range of concentrations, CoGH enhanced DMD by 6% units and NDFD by 18% units whereas CoCarb increased DMD by 5% units and NDFD by 7% units relative to no Co supplementation.

Acknowledgments

We thank Zinpro for providing funding to support this study. We thank the staff and student employees at the Kansas State University Dairy Teaching and Research Center (Manhattan) for animal care and technical assistance.

References

Akins, M. S., S. J. Bertics, M. T. Socha, and R. D. Shaver. 2013. Effects of cobalt supplementation and vitamin B_{12} injections on lactation performance and metabolism of Holstein dairy cows. J. Dairy Sci. 96:1755-1768.

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

Bishehsari, S., M. M. Tabatabaei, H. Aliarabi, D. Alipour, P. Zamani and A. Ahmadi. 2010. Effect of dietary cobalt supplementation on plasma and rumen metabolites in Mehraban lambs. Small Ruminant Research. 90:170-173.

Broderick, G. A. and, J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. J. Dairy Sci. 63: 64-75.

Costigan, P., and R. Gerdes. 1991. Assimilation of inorganic cobalt into the rumen microorganism. Page 54 in Recent advances in animal nutrition, Australia. RAAN, Australia.

Formigoni, A., M. Fustini, L. Archetti, S. Emanuele, C. Sniffen, and G. Biagi. 2011. Effects of an organic source of copper, manganese and zinc on dairy cattle productive performance, health status and fertility. Anim. Feed Sci. Technol. 164:191-198.

Girard, C. L., D. E. Santschi, S. P. Stabler, and R. H. Allen. 2009. Apparent ruminal synthesis and intestinal disappearance of vitamin B12 and its analogs in dairy cows. J. Dairy Sci. 92:4524-4529.

Griinari, J. M., D. A. Dwyer, M. A. McGuire, D. E. Bauman, D. L. Palmquist and K.V. Nurmela. 1998. Trans-octadecenoic acids and milk fat depression in lactating dairy cows. J. Dairy Sci. 81:1251-1261.

HACH. (2000). Volatile acids, Esterification method. Method 8196.

Hussein, H. S., G. C. Fahey Jr., B. W. Wolf, and L. L. Berger. 1994. Effects of cobalt on *in vitro* fiber digestion of forages and by-products containing fiber. J. Dairy Sci. 77:3432-3440.

Kadim, I. T., E. H. Johnson, O. Mahgoub, A. Srikandakumar, D. Al-Ajmi, A. Ritchie, K. Annamalai, and A. S. Al-Halhali. 2003. Effect of low levels of dietary cobalt on apparent nutrient digestibility in Omani goats. Anim. Feed Sci. Technol. 109:209-216.

Kawashima, T., P. R. Henry, D. G. Bates, C. B. Ammerman, R. C. Littell and J. Price. 1997. Bioavailability of cobalt sources for ruminants. 3. *In vitro* ruminal production of vitamin B_{12} and total corrinoids in response to different cobalt sources and concentrations. Nutr. Res. 17:975-987.

Kišidayová, S., P. Sviatko, P. Siroka, and D. Jalč. 2001. Effect of elevated cobalt intake on fermentative parameters and protozoan population in RUSITEC. Anim. Feed Sci. Technol. 91:223-232.

Nasser, M. 2013. Influence of dietary cobalt on performance, nutrient digestibility and rumen activity in lambs. Lucrari Stiintifice. 53:678-683.

Raux, E., H. L. Schubert and M. Warren. 2000. Biosynthesis of cobalamin (vitamin B_{12}): a bacterial conundrum. Cell. Mol. Life Sci. 57:1880-1893.

SAS Institute Inc. 2011. SAS/STAT 9.3 User's Guide. Version 9.3 ed. SAS Institute Inc., Cary, NC.

Scott, H. W., and B. A. Dehority. 1965. Vitamin requirements of several cellulolytic rumen bacteria. J. Bacteriol. 89:1169-1175.

Stemme, K., P. Lebzien, G. Flachowsky and H. Scholz. 2008. The influence of an increased cobalt supply on ruminal parameters and microbial vitamin B12 synthesis in the rumen of dairy cows. Arch. Anim. Nutr. 62:207-218.

Strobel, H. J. 1992. Vitamin B_{12}-dependent propionate production by the ruminal bacterium prevotella ruminicola. Appl. Environ. Microbiol. 58:2331-2333.

Sukhija, P. S., and D. L. Palmquist. 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J. Agric. and Food Chem. 36: 1202-1206.

Suttle, N. F. 2010. Minerals in nutrition of livestock. 4th ed. Pages 223-254. Midlothian, UK.

Takahashi-Iñiguez, T., E. García-Hernandez, R. Arreguán-Espinosa and M. E. Flores. 2012. Role of vitamin B(12) on methylmalonyl-CoA mutase activity. J. Zhejiang Univ. Sci. B. 13:423-437.

Tiffany, M. E., V. Fellner, and J. W. Spears. 2006. Influence of cobalt concentration on vitamin B12 production and fermentation of mixed ruminal microorganisms grown in continuous culture flow-through fermentors. J. Anim. Sci. 84:635-40.

Tiffany, M. E., J. W. Spears, L Xi and J. Horton. 2003. Influence of dietary cobalt source and concentration on performance, vitamin B_{12} status, and ruminal and plasma metabolites in growing and finishing steers. J. Anim. Sci. 81:3151-3159.

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

Wang, R. L., X. H. Kong, Y. Z. Zhang, X. P. Zhu, Narenbatu, and Z. H. Jia. 2007. Influence of dietary cobalt on performance, nutrient digestibility and plasma metabolites in lambs. Anim. Feed Sci. Technol. 135:346-352.

Tables and Figures

Table 3.1. Ingredient and nutrient composition of the diet fed to the ruminally-cannulated heifers during Experiment 1 and 2.

Item	%, DM basis
Ingredient	
Wheat straw	36.91
Alfalfa hay	23.05
Corn grain, ground	21.73
Corn distillers grain, dried	17.93
Soybean meal	0.87
Trace mineral salt[1]	0.13
Vitamin A premix	0.0091
Vitamin D premix	0.0297
Vitamin E premix	0.0800
Nutrient	
DM, % as fed	60.6
CP	11.9
NDF	48.5
ADF	32.3
EE	2.5
NFC	31.8

[1]Provided 50 ppm Co, 300 ppm Cu, 2000 ppm Fe, 70 ppm I, 2000 ppm Mn, 3500 ppm Zn

Table 3.2. Effect of different sources and concentrations of cobalt on *in vitro* asymptotic volume (V), rate (K), lag phase (L) of cumulative gas production curves and pH changes during Experiment 1.

Treatment	V, mL	K, mL/min	L, min	pH change
No supplemental Co	222.7	0.0031	192.4	-0.710
CoCarb (Co, ppm)				
0.1	222.8	0.0030	208.9	-0.713
0.5	222.8	0.0030	187.7	-0.695
1.0	216.1	0.0031	199.4	-0.763
2.0	213.5	0.0030	198.8	-0.753
5.0	221.0	0.0028	196.3	-0.655
10.0	222.5	0.0030	201.8	-0.735
15.0	209.1	0.0031	204.7	-0.745
CoGH (Co, ppm)				
0.1	219.8	0.0030	200.2	-0.725
0.5	224.4	0.0029	206.2	-0.680
1.0	220.6	0.0028	203.6	-0.680
2.0	218.9	0.0030	191.2	-0.693
5.0	218.6	0.0028	201.7	-0.675
10.0	213.9	0.0028	194.9	-0.658
15.0	210.5	0.0028	196.1	-0.703
SE	6.66	0.0001	6.15	0.029
Contrast[1]	e	AB	a	B

[1]A = No supplemental Co vs. all other treatments; B = source effect; C = linear dose; D = quadratic dose; E = linear dose × source; F = quadratic dose × source; Upper case = $P < 0.05$; lower case = $P < 0.10$.

Table 3.3. Effect of different sources and concentrations of cobalt on *in vitro* asymptotic volume (V), rate (K), and lag phase (L) of cumulative gas production curves and pH changes during Experiment 2

Treatment	V, mL	K, mL/min	L, min
No supplemental Co	230.80	0.0035	205.11
CoCarb (Co, ppm)			
0.33	220.23	0.0032	183.75
1.0	222.61	0.0034	210.39
3.0	227.93	0.0034	200.43
9.0	222.96	0.0033	202.72
CoGH (Co, ppm)			
0.33	227.53	0.0033	216.20
1.0	214.2	0.0032	215.39
3.0	224.07	0.0033	213.97
9.0	216.38	0.0032	233.54
SE	5.62	0.0001	13.44
Contrast[1]	A	A	B

[1]A = No supplemental Co vs. all other treatments; B = source effect; C = linear dose; D = quadratic dose; E = linear dose × source; F = quadratic dose × source; Upper case = $P < 0.05$; lower case = $P < 0.10$.

Table 3.4. *In vitro* volatile fatty acid concentrations (m*M*), ammonia concentration (m*M*) and pH change with different sources and concentrations of cobalt in Experiment 2

Treatment	Acetate	Propionate	Butyrate	Valerate	Isobutyrate	Isovalerate	Total VFA	NH_3	pH change
No supplemental Co	62.68	38.04	13.72	2.99	1.45	1.73	114.4	25.50	-0.624
CoCarb (Co, ppm)									
0.33	61.27	38.02	13.35	2.87	1.43	1.66	112.6	25.81	-0.642
1.0	62.04	37.85	13.43	2.91	1.46	1.69	113.3	25.26	-0.617
3.0	62.96	38.95	13.61	2.90	1.40	1.65	115.5	25.53	-0.653
9.0	62.07	38.40	13.54	2.86	1.30	1.53	114.0	22.60	-0.648
CoGH (Co, ppm)									
0.33	61.22	37.42	13.43	2.87	1.41	1.66	112.1	25.49	-0.593
1.0	64.10	39.18	14.01	2.93	1.44	1.68	115.8	25.81	-0.631
3.0	61.63	37.31	13.64	2.89	1.40	1.62	112.5	24.20	-0.622
9.0	60.10	37.46	13.39	2.86	1.34	1.51	110.8	23.11	-0.643
SE	1.37	0.53	0.23	0.05	0.03	0.03	1.9	0.55	0.039
Contrast[1]	D			A	ACD	ACD		ACD	C

[1]A = No supplemental Co vs. all other treatments; B = source effect; C = linear dose; D = quadratic dose; E = linear dose × source; F = quadratic dose × source; Upper case = $P < 0.05$; lower case = $P < 0.10$.

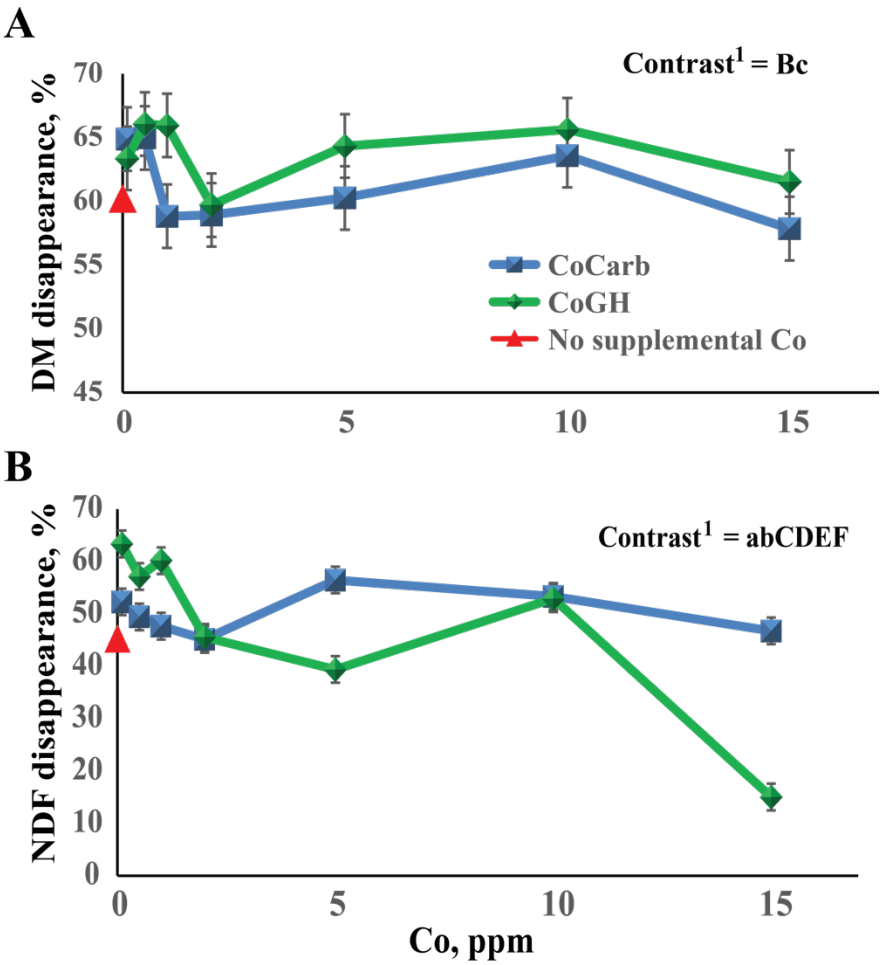


Figure 3.1. ***In vitro*** **DM and NDF disappearance with different sources and concentrations of cobalt. The X-axis represent the concentration of Co expressed in ppm, and the Y-axis indicate the in vitro disappearance of NDF expressed as a percentage. [1]A = No supplemental Co vs. all other treatments; B = source effect; C = linear dose; D = quadratic dose; E = linear dose × source; F = quadratic dose × source; Upper case = *P* < 0.05; lower case = *P* < 0.10.**

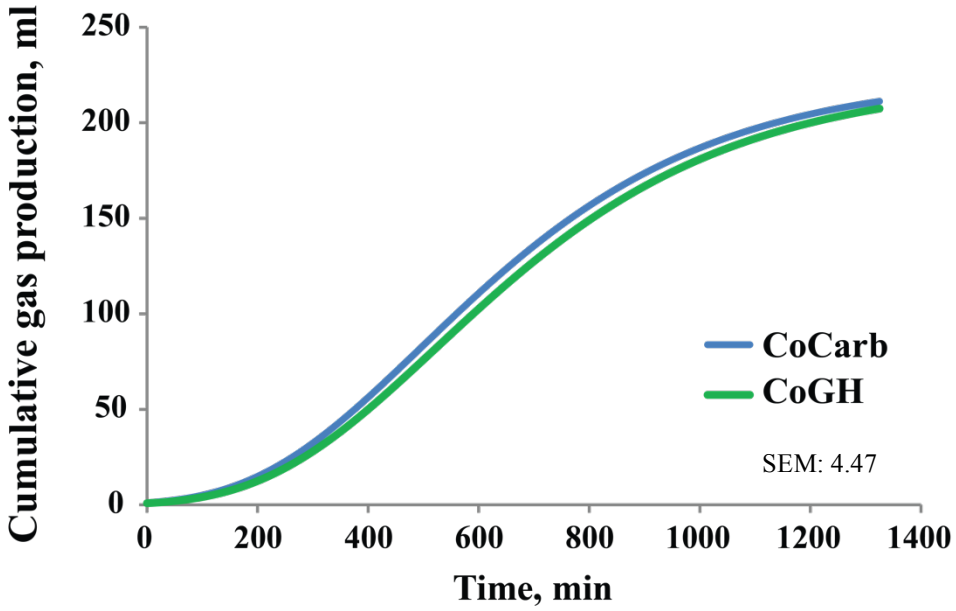


Figure 3.2. In vitro gas production with two different sources of cobalt during Experiment 2. The X-axis represents incubation time expressed in minutes, and the Y-axis indicate the quantity of gas produced for each source of Cobalt, measured in ml.

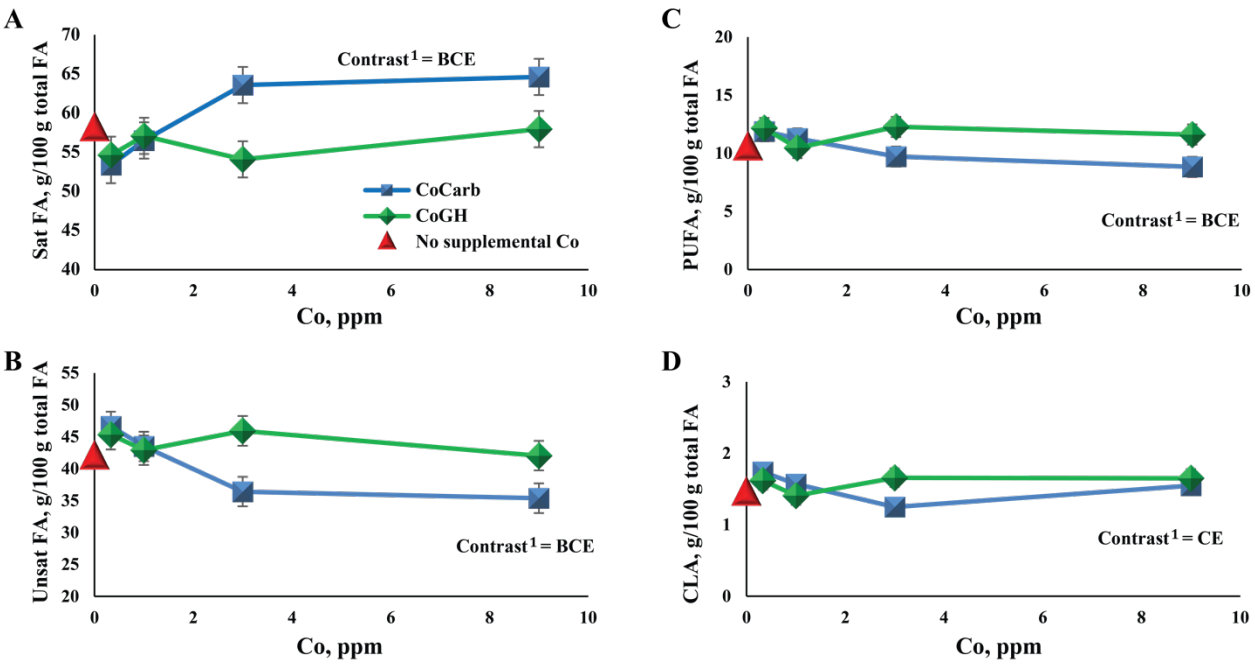


Figure 3.3. In vitro A) saturated fatty acid concentration (Sat), B) unsaturated fatty acid concentration (Unsat), C) polyunsaturated fatty acid concentration (PUFA), and D) conjugated linoleic acid concentration (CLA) with different sources and concentrations of cobalt. The X-axis represent the concentration of Co expressed in ppm, and the Y-axis indicate the grams of fatty acid in 100 g of total fat. [1]A = No supplemental Co vs. all other treatments; B = source effect; C = linear dose; D = quadratic dose; E = linear dose × source; F = quadratic dose × source; Upper case = $P < 0.05$; lower case = $P < 0.10$.

Chapter 4 - Effects of supplemental chromium propionate and rumen-protected amino acids on productivity, diet digestibility, and energy balance of peak lactation dairy cattle

C. F. Vargas-Rodriguez[*], K. Yuan[*], E. C. Titgemeyer[*], L. K. Mamedova[*], K. E. Griswold[†], and B. J. Bradford[*1]

[*]Department of Animal Sciences and Industry, Kansas State University, Manhattan 66506

[†]Kemin Industries, Des Moines, IA 50317

Correspondence Author: bbradfor@ksu.edu

This manuscript has been published in: J. Dairy Sci. (2014) 97:3815–3821

Abstract

Chromium (Cr) feeding in early lactation increased peak milk production in some studies, but responses to dietary Cr during peak lactation have not been evaluated. Furthermore, interactions of essential amino acids (AA) and Cr have not been explored. Our objective was to evaluate responses to CrPr (KemTRACE brand Chromium Propionate 0.04%, Kemin Industries, Inc.) and rumen-protected Lys (LysiPEARL, Kemin Industries, Inc.) and Met (MetiPEARL, Kemin Industries, Inc.) and their interaction in peak lactation cows. Forty-eight individually fed Holstein cows (21 primiparous, 27 multiparous, 38 ± 15 days in milk) were stratified by calving date in 12 blocks and randomly assigned to 1 of 4 treatments within block. Treatments were control, CrPr (8 mg/d Cr), RPLM (10 g/d Lys and 5 g/d Met, intestinally available), or CrPr plus RPLM. Treatments were premixed with ground corn and top-dressed at 200 g/d for 35 d. Diets consisted of corn silage, alfalfa hay, and concentrates, providing approximately 17% CP, 31% NDF, and 40% NFC. Dry matter intake (DMI) significantly increased ($P > 0.05$) with the inclusion of CrPr (22.2 vs. 20.8 ± 0.67 kg/d), and energy-corrected milk (ECM) yield tended to increase ($P = 0.09$). In addition, CrPr increased milk protein yield and tended to increase DMI in primiparous cows but not in multiparous cows. A CrPr by week interaction ($P < 0.05$) was detected for milk lactose content, which was increased by CrPr during week 1 only (4.99 vs. 4.88 ± 0.036%). As a proportion of plasma AA, lysine increased and methionine tended to increase in response to RPLM, but the inclusion of RPLM decreased ($P < 0.05$) N efficiency (milk protein N / N intake). Digestible energy intake, gross energy digestibility, and energy balance were not affected ($P > 0.10$) by treatments. There were no treatment effects on feed efficiency or changes in body weight or body condition score ($P > 0.10$). In summary, feeding CrPr increased DMI and tended to increase ECM in cows fed for 5 wk near peak lactation, with primiparous cows showing greater

responses in DMI and milk protein yield than multiparous cows.

Key words: lysine, methionine, chromium, essential amino acid

Introduction

After parturition, cows must adapt to milk secretion, but their daily DMI rarely matches the nutrient demands for that activity (Dalbach et al., 2011). Because of these extremely high nutrient requirements, cows near peak lactation are most likely to experience AA deficiencies, which can limit peak milk and, in turn, decrease whole-lactation productivity.

Chromium (**Cr**) is involved in many metabolic functions (Mertz, 1993; Bryan et al., 2004); it activates certain enzymes and stabilizes AA and nucleic acids (National Research Council, 1997; Khalili et al., 2012). Some studies utilized supplemental Cr in diets for lactating cattle and reported increases in milk production (Hayirli et al., 2001; McNamara and Valdez, 2005), whereas others detected enhanced immune responsiveness and disease resistance, particularly in animals under stress conditions (Spears et al., 2012).

It is also known that Cr can potentiate the action of insulin by binding to intracellular insulin receptor sites and promoting signal transduction (Kegley et al., 2000), thereby enhancing carbohydrate metabolism. In addition, Cr can alter protein synthesis (Gentry et al., 1999); though the mechanisms underlying this effect are not completely understood, the impact of Cr on insulin sensitivity has been clearly demonstrated in cattle (Sumner et al., 2007), and insulin signaling promotes protein synthesis. However, there is currently no information about interactions between AA nutrition and Cr supplementation in dairy cattle. Therefore, a critical need exists to further explore responses to Cr in the presence and absence of supplemental AA near peak lactation.

Materials and Methods

The Kansas State University Institutional Animal Care and Use Committee approved all experimental procedures.

Design and Treatments

Forty-eight lactating Holstein cows (21 primiparous and 27 multiparous, 38 ± 15 DIM) were used in a randomized complete block design with 4 treatments. The cows were stratified by calving date in 12 blocks, and assigned randomly to treatments within block.

All cows were housed in tie-stalls and individually fed a common diet (Table 4.1). Analysis by the Cornell Net Carbohydrate and Protein System version 6.1 (NDS version 3, Ruminant Management & Nutrition, Reggio Emilia, Italy) estimated metabolizable Lys supply at 148 g/d (6.38% of MP) and metabolizable Met supply at 47 g/d (2.03% of MP) with 22 kg/d DMI in the control diet. Treatments were premixed with ground corn and offered as a top-dress at a rate of 200 g/cow daily for 35 d. Treatments were control, Cr propionate (**CrPr**; 8 mg/d Cr in the form of 20 g/d KemTRACE Chromium Propionate 0.04%, Kemin Industries, Inc., Des Moines, IA), rumen-protected lysine and methionine (**RPLM**; 10 g/d lysine and 5 g/d methionine, intestinally available), or both (CrPr+RPLM). The RPLM supplement was composed of 48.8 g/d of LysiPEARL and 15.3 g/d of MetiPEARL (Kemin Industries, Inc.), and was predicted to provide Lys and Met supplies of 6.77% and 2.23% of MP, respectively. Cows were milked 3 times daily (0300, 1100, and 1900 h) and fed once daily (1600 h) for ad libitum intake, targeting 10% daily refusals.

Sample and Data Collection

Feed offered and feed refused were measured for each cow daily to determine DMI. Milk yield was recorded for each cow daily. Body weights and BCS (Wildman et al., 1982) were measured on d 1 and 35. Milk samples were collected 3 d per week for milk composition analysis.

Samples of feed ingredients were collected weekly and frozen for analysis. On d 19 to 21 and 33 to 35, samples of TMR and feed refusals were collected daily and fecal samples were collected at 9 h intervals, representing every 3 h of a 24-h period.

Sample Analysis

Samples of diet ingredients, TMR, and feed refusals were dried in a 55°C forced air oven for 48 h, composited by collection period (d 21 vs. 35), ground through a 1-mm screen (Wiley mill, Arthur H. Thomas, Swedesboro, NJ), and analyzed for DM, OM, CP, NDF, and ether extract (**EE**). The DM content was determined by drying at 105°C in a forced-air oven for 16 h. Ash concentration was determined after 4 h of oxidation at 500°C in a muffle furnace. Nitrogen content was determined by oxidation and detection of N_2 (Leco Analyzer, Leco Corp., St. Joseph, MI). Concentration of NDF was determined using an Ankom Fiber Analyzer (Ankom Technology, Fairport, NY) including amylase and sodium sulfite (Van Soest et al., 1991). Crude fat was determined by ether extraction (AOAC, 2000; method 920.9).

Milk samples were analyzed for concentration of fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments, Chaska, MN), MUN (MUN spectrophotometer, Bentley Instruments), and somatic cells (SCC 500, Bentley Instruments) by Heart of America DHIA (Manhattan, KS).

Energy Balance

The concentration of acid detergent insoluble ash (ADIA) was determined (Van Soest et al., 1991) in dried samples of feed ingredients, refusals, and feces. This parameter was used as an endogenous indigestible marker to estimate fecal output (Cochran et al., 1986). The gross energy

(GE) content of feed, top-dressed treatments, refusals, and dried fecal samples were determined by bomb calorimetry. Intake of GE was calculated as GE in feed offered (calculated from analysis of feed ingredients) minus GE in refusals; fecal GE was subtracted to determine DE intake. After calculating DE concentration of the diets by cow (DE intake / DMI), ME and NE_L concentrations were calculated using the following formulas (National Research Council, 2001): ME (Mcal/kg DM) = [1.01 × (DE, Mcal/kg) - .045] + 0.0046 × (EE, % - 3), and NE_L (Mcal/kg) = 0.703 × ME (Mcal/kg) – 0.19 + [(0.097 × ME, Mcal/kg + 0.19)/97] × (EE, % - 3). Intakes of ME and NE_L were then determined by multiplying these concentrations by DMI. Milk energy output (Mcal) was calculated as 9.29 × kg of milk fat + 5.47 × kg of milk protein + 3.095 × kg of milk lactose, and maintenance energy requirements (Mcal/d) estimated as 0.08 × kg of $BW^{0.75}$ (National Research Council, 2001). To determine NE_L balance, milk and maintenance energy were subtracted from NE_L intake.

Statistical Analysis

One cow on CrPr+RPLM developed severe mastitis on d 20 of treatment and was subsequently removed from the study. No data were collected or analyzed for this cow. Milk and DMI data were averaged by week prior to analysis. Data were analyzed using the Mixed Procedure of SAS (version 9.2; SAS Institute Inc., Cary, NC) to assess the fixed effects of parity (primiparous vs. multiparous), time, CrPr, RPLM, and 2-, 3-, and 4-way interactions, and the random effect of block. With the exception of CrPr × RPLM, interactions were removed from models when $P > 0.30$. Repeated measures over time within cow were modeled with an autoregressive (AR[1]) covariance structure. Denominator degrees of freedom were estimated using the Kenward-Rogers

method. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P < 0.10$. Treatment means were separated with pair-wise *t*-tests when interactions were significant.

Results and Discussion

Dry matter intake was significantly increased by CrPr ($P < 0.05$), but was not significantly affected by RPLM when fed for 5 wk near peak lactation (Table 2). Although neither RPLM nor CrPr significantly altered yields of milk or milk components, CrPr tended to increase ECM ($P = 0.09$) by 6% (Table 4.2). In addition, there was evidence of parity × CrPr interactions for both DMI ($P = 0.06$) and milk protein yield ($P = 0.04$), in both cases indicating positive responses to CrPr in primiparous cows but not in multiparous cows (Figures 4.1A and 4.1B).

Several lines of evidence indicate that Cr supplementation during the periparturient period improves DMI and milk production (Hayirli et al., 2001; McNamara and Valdez, 2005). Hayirli et al. (2001) reported that supplementation of 0, 3.9, 8.3, and 16.5 mg Cr/d from Cr-methionine resulted in a linear increase in prepartum DMI. Besong et al. (1996) observed increased milk yield in the first 8 wk of lactation in cows supplemented with 0.8 mg Cr/kg DMI as Cr-picolinate. Smith et al. (2005) found that supplementation of 0.03 or 0.06 mg of Cr/kg $BW^{0.75}$ increased DMI and milk yield in early lactation. In heat stress conditions, Cr supplementation from 3 wk prepartum through 12 wk postpartum improved postpartum DMI and increased milk yield by 6.7%, 12.3%, and 16.5% at 4, 8, and 12 wk postpartum, respectively (Soltan, 2010). In these studies, feed efficiency was essentially unaffected, because the increases in milk yield and DMI in response to Cr supplementation paralleled each other.

The interaction of RPLM and CrPr affected milk protein content ($P = 0.04$, Table 4.2). Somewhat counter-intuitively, in the absence of CrPr, RPLM decreased milk protein content ($P <$

0.01), but no effect of RPLM was detected in the presence of CrPr ($P = 0.77$). Rumen-protected lysine and methionine also decreased the efficiency of N utilization for milk protein ($P = 0.05$). Rumen-protected methionine (RPM) supplementation has been reported to increase milk protein content in many studies (Doreau and Chilliard, 1997; Leonardi et al., 2003; Pacheco et al., 2006), but not in others (Papas et al., 1984; Davidson et al., 2008). Fewer studies (Armentano et al., 1997; Rulquin and Delaby, 1997) observed increased milk protein yield. Dietary supplementation of methionine or both methionine and lysine can significantly increase N utilization efficiency (Wang et al., 2010), but Noftsger and St-Pierre (2003) did not observe an improvement in N efficiency when they evaluated supplemental methionine in a digestibility trial. Likewise, varying results have been reported for milk and milk fat yields. In light of the inconsistent data, a meta-analysis (Patton, 2010) was conducted to investigate the effect of RPM supplementation on production. The results indicated that RPM addition to diets increased milk protein, both as percentage (0.07%) and yield (27 g/d), and slightly increased milk yield. However, DMI and milk fat percentage were slightly decreased. Therefore, although there is a lack of research on the simultaneous addition of both RPL and RPM on milk components, the decreased milk protein content by RPLM in our study was unexpected.

There was a CrPr × week interaction ($P = 0.04$) for lactose content, reflecting significantly greater lactose content (4.99 vs. 4.86 ± 0.036%) in response to CrPr during wk 1, with no differences observed from wk 2 on. Previous work has demonstrated enhanced glucose utilization in cattle supplemented with Cr (Sumner et al., 2007), consistent with the view that Cr acts primarily as an insulin sensitizing agent. However, we are not aware of other studies in lactating cows reporting increased milk lactose content in response to supplemental Cr, so it is somewhat unclear what caused this response or why it was transient.

Intake of GE and DE and digestibility of GE and DM were similar across treatments ($P > 0.10$), and no treatment effects were detected for NE_L balance, BW change, or BCS change (Table 4.3). The negative values for BW and BCS changes, which suggested that cows were in a catabolic state, appear to conflict with the positive calculated NE_L balances. However, BW and BCS change data covered the entire 35-d experiment, whereas NE_L balance was only determined on d 21 and 35. It is likely that many cows (mean 38 DIM at the start and 73 DIM at the end of the study) moved from a negative energy balance to a positive energy balance during the course of the study. Nevertheless, the accuracy of calculated NE_L balance may have been limited due to biased estimations of fecal output with the ADIA marker. Dietary ADIA concentrations were relatively low in this experiment, potentially increasing variability in ADIA intake estimates, which could result in underestimated fecal mass and overestimated supply of energy. The magnitude of this potential bias was estimated by comparing NE_L values calculated from measured energy digestibility vs. model-derived NE_L values based on ingredient characterization alone (Table 4.1). This comparison suggested that marker-based digestibility analysis may have overestimated ME supply by approximately 17%. Regardless, relative treatment differences were valid because of the common basal diet, and the lack of an effect of Cr on energy balance is consistent with previous findings in early lactation (Smith et al., 2005; Sadri et al., 2009).

Plasma AA profiles are presented in Table 4.4. The proportion of lysine significantly increased ($P = 0.05$) and that of methionine tended to increase ($P = 0.07$) in response to RPLM. On the other hand, the proportion of threonine was significantly decreased by RPLM ($P < 0.01$). A tendency for a CrPr × RPLM interaction ($P = 0.06$) was observed for tryptophan, reflecting a decreased proportion of tryptophan by CrPr in the presence of RPLM ($P = 0.03$), but not in the absence of RPLM ($P = 0.64$). The plasma lysine and methionine responses to RPLM were less

than might have been expected, given the lack of increased milk protein yield. We observed approximately a 10% increase in lysine and 6% increase in methionine as a proportion of AA in response to estimated supplementation of 10 and 5 g/d, respectively. Similar supplementation rates have increased plasma concentrations by more than 30% (Rogers et al., 1987), though without any change in milk protein yield in mid-lactation cows producing approximately 30 kg milk/d. Compared to our work, the cows evaluated by Rogers et al. (1987) may have been more responsive to AA supplementation (in terms of plasma concentrations) because of lower microbial supply of AA and because of stable AA demands for protein synthesis. Few studies have evaluated responses to supplemental AA in cows during the transition from the catabolic state in early lactation to an anabolic state. Previous work has demonstrated that muscle repletion occurs during this stage of lactation and has suggested that increases in intestinally available methionine may enhance this process (Phillips et al., 2003). Whether or not such a response interferes with potential milk protein responses to bypass essential AA is unknown and may be a fruitful area of investigation, especially considering the complex interactions of AA supply and insulin signaling to influence both muscle deposition and milk protein synthesis (Bequette et al., 2001).

Conclusions

The supplementation of CrPr increased DMI and tended to increase ECM yield of peak-lactation cows when fed for a 5-wk period, and DMI as well as milk protein yield was particularly enhanced in primiparous cows. The inclusion of RPLM increased lysine and tended to increase methionine as a proportion of plasma AA, but decreased the efficiency of N utilization for milk protein. These findings indicate that responses to dietary Cr in the dairy cow are not limited to early lactation.

Acknowledgments

We thank Kemin Industries (Des Moines, IA) for providing funding to support this study. We thank the staff and student employees at the Kansas State University Dairy Teaching and Research Center for animal care and technical assistance.

References

AOAC. 2000. Official Methods of Analysis. 17th ed. Association of Official Analytical Chemists, Arlington, VA.

Armentano, L. E., S. J. Bertics, and G. A. Ducharme. 1997. Response of lactating cows to methionine or methionine plus lysine added to high protein diets based on alfalfa and heated soybeans. J. Dairy Sci. 80:1194-1199.

Bequette, B. J., C. E. Kyle, L. A. Crompton, V. Buchan, and M. D. Hanigan. 2001. Insulin regulates milk production and mammary gland and hind-leg amino acid fluxes and blood flow in lactating goats. J. Dairy Sci. 84:241-255.

Besong, S., J. A. Jackson, C. L. Hicks, and R. W. Hemken. 1996. Effects of a supplemental liquid yeast product on feed intake, ruminal profiles, and yield, composition, and organoleptic characteristics of milk from lactating holstein cows. J. Dairy Sci. 79:1654-1658.

Bryan, M. A., M. T. Socha, and D. J. Tomlinson. 2004. Supplementing intensively grazed late-gestation and early-lactation dairy cattle with chromium. J. Dairy Sci. 87:4269-4277.

Cochran, R. C., D. C. Adams, J. D. Wallace, and M. L. Galyean. 1986. Predicting digestibility of different diets with internal markers: Evaluation of four potential markers. J. Anim. Sci. 63:1476-1487.

Dalbach, K. F., M. Larsen, B. M. L. Raun, and N. B. Kristensen. 2011. Effects of supplementation with 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester on splanchnic amino acid metabolism and essential amino acid mobilization in postpartum transition holstein cows. J. Dairy Sci. 94:3913-3927.

Dairy Records Management Systems. 2013. DHI glossary. Accessed June 17, 2013. http://www.drms.org/PDF/materials/glossary.pdf.

Davidson, S., B. A. Hopkins, J. Odle, C. Brownie, V. Fellner, and L. W. Whitlow. 2008. Supplementing limited methionine diets with rumen-protected methionine, betaine, and choline in early lactation holstein cows. J. Dairy Sci. 91:1552-1559.

Doreau, M., and Y. Chilliard. 1997. Digestion and metabolism of dietary fat in farm animals. Br. J. Nutr. 78(Suppl. 1): S15-S35.

Gentry, L. R., J. M. Fernandez, T. L. Ward, T. W. White, L. L. Southern, T. D. Binder, D. L. Thompson, D. V. Horohov, A. M. Chapa, and T. Sahlu. 1999. Dietary protein and chromium tripicolinate in suffolk wether lambs: Effects on production characteristics, metabolic and hormonal responses, and immune status. J. Anim. Sci. 77:1284-1294.

Hayirli, A., D. R. Bremmer, S. J. Bertics, M. T. Socha, and R. R. Grummer. 2001. Effect of chromium supplementation on production and metabolic parameters in periparturient dairy cows. J. Dairy Sci. 84:1218-1230.

Kegley, E. B., D. L. Galloway, and T. M. Fakler. 2000. Effect of dietary chromium-L-methionine on glucose metabolism of beer steers. J. Anim. Sci. 78:3177-3183.

Khalili, H., A. D. Foroozandeh, M. Toghyani, and G. Ghalamkari. 2012. Reproductive performance by dairy cows fed supplemental chromium-methionine in transition period. Afr. J. Biotechnol. 11(93):16029-16033.

Leonardi, C., M. Stevenson, and L. E. Armentano. 2003. Effect of two levels of crude protein and methionine supplementation on performance of dairy cows. J. Dairy Sci. 86:4033-4042.

McNamara, J. P., and F. Valdez. 2005. Adipose tissue metabolism and production responses to calcium propionate and chromium propionate. J. Dairy Sci. 88:2498-2507.

Mertz, W. 1993. Chromium in human nutrition: A review. J. Nutr. 123:626.

National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

National Research Council. 1997. The Role of Chromium in Animal Nutrition. Natl. Acad. Press, Washington, DC.

Noftsger, S., and N. R. St-Pierre. 2003. Supplementation of methionine and selection of highly digestible rumen undegradable protein to improve nitrogen efficiency for milk production. J. Dairy Sci. 86:958-969.

Pacheco, D., C. G. Schwab, R. Berthiaume, G. Raggio, and H. Lapierre. 2006. Comparison of net portal absorption with predicted flow of digestible amino acids: Scope for improving current models? J. Dairy Sci. 89:4747-4757.

Papas, A. M., C. J. Sniffen, and T. V. Muscato. 1984. Effectiveness of rumen-protected methionine for delivering methionine postruminally in dairy cows. J. Dairy Sci. 67:545-552.

Patton, R. A. 2010. Effect of rumen-protected methionine on feed intake, milk production, true milk protein concentration, and true milk protein yield, and the factors that influence these effects: A meta-analysis. J. Dairy Sci. 93:2105-2118.

Phillips, G. J., T. L. Citron, J. S. Sage, K. A. Cummins, M. J. Cecava, and J. P. McNamara. 2003. Adaptations in body muscle and fat in transition dairy cattle fed differing amounts of protein and methionine hydroxy analog. J. Dairy Sci. 86: 3634-3647.

Rogers, J. A., U. Krishnamoorthy, and C. J. Sniffen. 1987. Plasma amino acids and milk protein production by cows fed rumen-protected methionine and lysine. J. Dairy. Sci. 70:789-798.

Rulquin, H., and L. Delaby. 1997. Effects of the energy balance of dairy cows on lactational responses to rumen-protected methionine. J. Dairy Sci. 80:2513-2522.

Sadri, H., G. R. Ghorbani, H. R. Rahmani, A. H. Samie, M. Khorvash and R. M. Bruckmaier. 2009. Chromium supplementation and substitution of barley grain with corn: Effects on performance and lactation in periparturient dairy cows. J. Dairy Sci. 92:5411-5418.

SAS Institute Inc. 2011. SAS/STAT 9.3 User's Guide. Version 9.3 ed. SAS Institute Inc., Cary, NC.

Smith, K. L., M. R. Waldron, J. K. Drackley, M. T. Socha, and T. R. Overton. 2005. Performance of dairy cows as affected by prepartum dietary carbohydrate source and supplementation with chromium throughout the transition period. J. Dairy Sci. 88:255-263.

Soltan, M. A. 2010. Effect of dietary chromium supplementation on productive and reproductive performance of early lactating dairy cows under heat stress. J. Anim. Physiol. Anim. Nutr. (Berl). 94(2):264-272.

Spears, J. W., C. S. Whisnant, G. B. Huntington, K. E. Lloyd, R. S. Fry, K. Krafka, A. Lamptey, and J. Hyda. 2012. Chromium propionate enhances insulin sensitivity in growing cattle. J. Dairy Sci. 95:2037-2045.

Sumner, J. M., F. Valdez, and J. P. McNamara. 2007. Effects of chromium propionate on response to an intravenous glucose tolerance test in growing Holstein heifers. J. Dairy Sci. 90:3467-3474.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci.

74:3583-3597.

Wang, C., H. Y. Liu, Y. M. Wang, Z. Q. Yang, J. X. Liu, Y. M. Wu, T. Yan, and H. W. Ye. 2010. Effects of dietary supplementation of methionine and lysine on milk production and nitrogen utilization in dairy cows. J. Dairy Sci. 93:3661-3670.

Wildman, E. E., G. M. Jones, P. E. Wagner, R. L. Boman, H. F. Troutt Jr., and T. N. Lesch. 1982. A dairy cow body condition scoring system and its relationship to selected production characteristics. J. Dairy Sci. 65:495-501.

Tables and Figures

Table 4.1. Ingredient and nutritional composition of the basal diet

Ingredient	**% of DM**
Corn silage	31.5
Alfalfa hay	23.4
Wet corn gluten feed[1]	6.8
Ground corn	23.1
Whole cottonseed	4.6
Mechanically extracted soybean meal[2]	2.1
Solvent extracted soybean meal	5.1
Ca salts of long-chain fatty acids[3]	0.8
Micronutrient premix[4]	2.6
Nutrient	
DM, % as-fed	57.9
OM	91.3
CP	16.7
NDF	31.7
ADF	20.1
fNDF[5]	22.1
NFC	39.8
Ether extract	3.1

GE, Mcal/kg	4.11
DE[6], Mcal/kg	3.34
ME[7], Mcal/kg	2.92
NE_L[8], Mcal/kg	1.87
Model-predicted ME[9], Mcal/kg	2.50

[1]SweetBran, Cargill Inc., Blair, NE

[2]Soy Best, Grain States Soya, West Point, NE

[3]Megalac-R, Church & Dwight Co, Princeton, NJ

[4]Premix consist of 45.1% limestone, 32.2% of sodium bicarbonate, 6.4% magnesium oxide, 5.2% sodium chloride, 5.2% vitamin E premix (44 IU/g), 0.45% vitamin A premix (30 IU/g), 0.19% vitamin D premix (30 IU/g), 2.1% 4-Plex (Zinpro Corp., Eden Prairie, MN; contains 2.58% Zn, 1.48% Mn, 0.90% Cu, 0.18% Co, 8.21% Met, and 3.80% Lys), 0.96% selenium premix (600 ppm Se), 0.45% Zinpro 100 (Zinpro Corp.; contains 10% Zn and 20% Met), 0.03% ethylenediamine dihydriodide premix (3.65% I), 0.88% Kallsil (Kemin Industries), and 0.88% Myco CURB (Kemin Industries).

[5]forage NDF

[6]DE = (Gross energy intake – gross energy in feces)/DMI.

[7]ME = [1.01 × (DE, Mcal/kg) - 0.045] + 0.0046 × (EE, % -3).

[8]NE_L = 0.703 × ME (Mcal/kg) – 0.19 + [(0.097 × ME, Mcal/kg + 0.19)/97] × [EE, % -3].

[9]ME predicted by CNCPS 6.1 (NDS version 3, Ruminant Management & Nutrition, Reggio Emilia, Italy).

Table 4.2 . Chromium propionate (CrPr) and rumen-protected lysine and methionine (RPLM) effects on intake, productivity, and milk composition of lactating dairy cows

	Control		RPLM			*P*-value		
Item	Control	CrPr	Control	CrPr	SEM	CrPr	RPLM	Interaction
DMI, kg/d	19.9	22.2	21.7	22.3	1.10	< 0.05	0.18	0.23
Milk yield, kg/d	40.5	43.7	42.4	43.3	1.44	0.14	0.61	0.39
Milk fat, %	4.20	4.13	3.95	3.97	0.15	0.88	0.19	0.74
Milk protein, %	2.75	2.67	2.62	2.68	0.04	0.66	0.09	0.04
Milk lactose, %	4.90	4.99	4.89	4.90	0.04	0.26	0.24	0.37
MUN, mg/dL	13.2	14.0	13.8	13.1	0.56	0.89	0.70	0.09
SCC linear score	1.59	1.10	1.58	1.65	0.50	0.66	0.56	0.56
Fat yield, kg/d	1.68	1.81	1.66	1.70	0.07	0.27	0.37	0.58
Protein yield, kg/d	1.12	1.16	1.12	1.17	0.03	0.22	0.93	0.86
Lactose yield, kg/d	2.01	2.17	2.09	2.15	0.07	0.15	0.74	0.54
Milk N efficiency[2], %	30.2	27.6	27.0	27.1	1.17	0.19	< 0.05	0.15
ECM[1], kg/d	43.1	46.3	43.1	44.9	1.47	0.09	0.62	0.65

ECM/DMI	2.18	2.08	2.02	2.02	0.08	0.53	0.16	0.56

[1]ECM = (0.327 × milk yield) + (12.95 × fat yield) + (7.65 × protein yield); (Dairy Record Management Systems, 2013)

[2] N efficiency = milk protein N / N intake.

Table 4.3. Chromium propionate (CrPr) and rumen-protected lysine and methionine (RPLM) effects on energy balance of lactating dairy cows

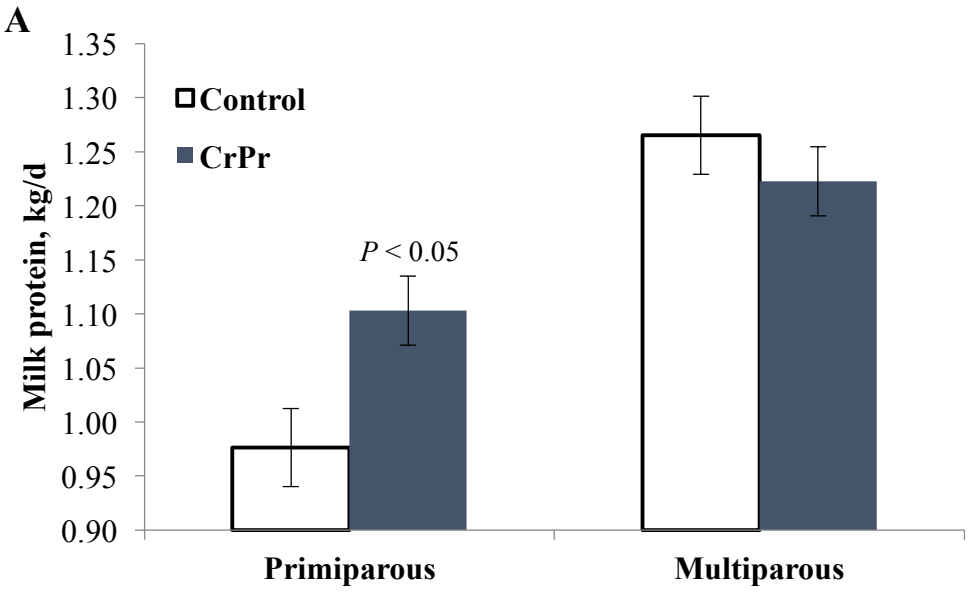
	Control		RPLM			*P*-value		
Item	Control	CrPr	Control	CrPr	SEM	CrPr	RPLM	Interaction
GE_{intake}, Mcal/d	92.8	100.8	98.1	101.1	4.18	0.10	0.42	0.46
DE_{intake}, Mcal/d	76.1	82.5	80.8	81.2	3.31	0.20	0.52	0.25
$GE_{digested}$, %	81.4	82.0	82.2	81.1	1.43	0.86	0.97	0.51
DM digestibility, %	81.0	81.7	82.6	80.9	1.35	0.67	0.72	0.33
NE_L balance, Mcal/d	3.3	6.3	7.7	6.7	1.89	0.56	0.15	0.25
BW change, kg / 28 d	-14.6	-9.9	-13.7	-7.9	4.15	0.21	0.74	0.89
BCS change, / 28 d	-0.31	-0.32	-0.33	-0.27	0.09	0.06	0.86	0.32

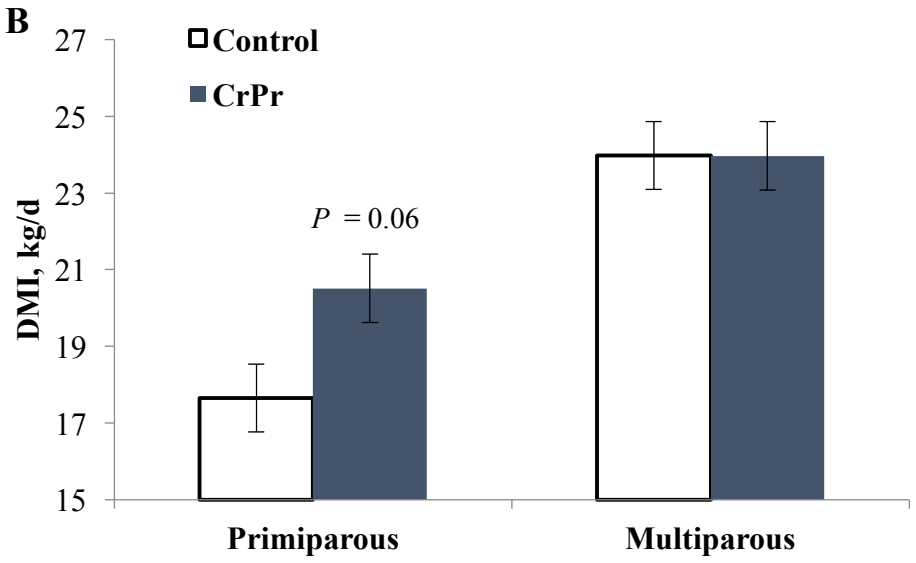
Table 4.4. Chromium propionate (CrPr) and rumen-protected lysine and methionine (RPLM) effects on plasma amino acids on lactating dairy cows

Amino Acid	Control		RPLM			*P*-value		
(Molar % of total AA)	Control	CrPr	Control	CrPr	SEM	CrPr	RPLM	Interaction
Glycine	15.57	15.07	14.98	13.92	1.10	0.48	0.42	0.80
Valine	12.19	12.19	11.90	11.07	0.65	0.47	0.28	0.53
Alanine	10.88	11.84	11.72	11.53	0.43	0.37	0.52	0.18
Glutamine	8.70	7.97	8.95	9.44	0.58	0.81	0.09	0.22
Leucine	8.20	8.08	8.11	7.89	0.45	0.66	0.71	0.91
Isoleucine	6.82	6.63	6.77	6.71	0.40	0.72	0.96	0.86
Threonine	4.52	4.72	4.17	3.97	0.24	0.99	0.02	0.25
Citruline	4.40	4.65	4.08	4.18	0.34	0.32	0.23	0.81
Serine	3.95	3.96	3.99	3.81	0.17	0.58	0.73	0.55
Arginine	3.61	4.07	4.01	3.94	0.21	0.30	0.45	0.16
Lysine	3.34	3.37	3.80	3.59	0.17	0.55	0.05	0.47

Glutamate	3.02	2.91	2.78	2.85	0.28	0.91	0.47	0.67
Tyrosine	2.14	2.15	2.30	2.23	0.10	0.75	0.22	0.65
Histidine	2.10	2.09	1.89	2.05	0.07	0.28	0.09	0.25
Asparagine	2.06	1.85	2.20	2.22	0.24	0.63	0.18	0.55
Phenylalanine	1.97	1.96	2.13	2.03	0.08	0.43	0.11	0.52
Taurine	1.75	1.90	1.58	1.80	0.17	0.27	0.41	0.83
Ornithine	1.73	1.68	1.77	1.73	0.10	0.56	0.64	0.97
Tryptophan	1.63	1.68	1.73	1.53	0.07	0.18	0.69	0.06
Methionine	0.95	0.99	1.06	1.01	0.04	0.93	0.07	0.12
Aspartate	0.32	0.38	0.33	0.34	0.02	0.13	0.51	0.28
Total amino acids, m*M*	2.47	2.39	2.14	2.39	0.10	0.39	0.11	0.11

Figure 4.1. Interactions of chromium propionate (CrPr) and parity for milk protein yield (A) and dry matter intake (B). Supplements were fed for 35 d near peak lactation, and DMI and milk production responses were analyzed by week throughout the study. Values are LSM ± SEM, n = 10 to 13.





Appendix A - Supplemental Table and Figure

Supplemental Table 1. In vitro long chain fatty acid concentrations with different sources and concentrations of cobalt in Experiment 2

g/100g of FA	0	CoCarb 0.33	CoCarb 1	CoCarb 3	CoCarb 9	CoGH 0.33	CoGH 1	CoGH 3	CoGH 9	SEM	Contrast[1]
C6:0	3.92	2.76	3.36	5.39	5.45	3.14	4.09	2.89	4.17	0.67	bCE
C8:0	0.06	0.04	0.05	0.07	0.08	0.04	0.05	0.04	0.06	0.01	bCE
C10:0	0.52	0.46	0.51	0.81	1.21	0.48	0.98	0.58	0.95	0.15	ACe
C11:0	0.18	0.14	0.17	0.24	0.26	0.17	0.21	0.14	0.19	0.03	bCE
C12:0	0.60	0.51	0.57	0.76	0.78	0.54	0.66	0.51	0.65	0.07	bCE
C14:0	1.83	1.57	1.74	2.22	2.23	1.67	1.95	1.59	1.91	0.16	bCE
C15:0	1.72	1.50	1.62	2.15	2.22	1.55	1.86	1.50	1.80	0.17	BCE
C16:0	21.78	21.28	21.64	22.60	22.18	21.48	21.99	21.24	21.47	0.45	bE
C16:1	0.40	0.38	0.41	0.47	0.46	0.39	0.41	0.39	0.43	0.02	C
C17:0	0.88	0.74	0.81	1.10	1.17	0.78	0.97	0.73	0.89	0.10	BCE
C18:0	25.42	23.69	24.34	26.74	27.50	23.09	24.17	23.23	24.04	0.82	BCE
C18:1n11c	1.89	1.74	1.84	2.10	2.17	1.82	2.02	1.79	2.04	0.09	CE
C18:1n11t	18.46	20.27	19.28	14.29	13.44	20.05	16.57	20.60	17.71	1.35	BCE
C18:1n9c	7.75	8.98	8.20	6.77	6.55	8.62	8.26	8.59	7.69	0.53	BCE
C18:1n9t	2.36	2.06	2.24	2.78	2.89	2.11	2.55	2.03	2.40	0.22	bCE
C18:3n3	0.73	0.82	0.76	0.63	0.64	0.79	0.68	0.84	0.85	0.08	bC
C18:2n6c	7.33	8.57	7.86	6.30	5.88	8.71	7.09	8.81	7.77	0.91	BCE
C18:2n6t	0.14	0.11	0.13	0.19	0.19	0.13	0.17	0.11	0.16	0.02	CE
C20:0	0.53	0.56	0.54	0.50	0.51	0.54	0.50	0.57	0.54	0.02	E
C20:1	0.15	0.14	0.13	0.13	0.12	0.14	0.13	0.15	0.15	0.01	Abe
C20:2	0.03	0.04	0.04	0.02	0.01	0.03	0.03	0.03	0.02	0.01	Ce
C21:0	0.07	0.07	0.07	0.07	0.07	0.07	0.08	0.06	0.07	0.004	

C22:0	0.42	0.45	0.44	0.37	0.39	0.44	0.39	0.46	0.44	0.03	E
C22:5n3	1.06	0.82	0.93	1.33	1.43	0.87	1.18	0.79	1.10	0.15	bCE
C23:0	0.15	0.14	0.14	0.13	0.12	0.14	0.14	0.14	0.15	0.01	BE
C24:0	0.49	0.53	0.51	0.46	0.48	0.52	0.46	0.54	0.53	0.06	dE
C24:1	0.12	0.10	0.11	0.17	0.21	0.10	0.15	0.08	0.14	0.02	BCE
CLA10t12c	0.35	0.38	0.37	0.32	0.31	0.37	0.35	0.38	0.37	0.02	bcE
CLA9c11c	0.05	0.06	0.05	0.03	0.03	0.06	0.04	0.06	0.07	0.01	BE
CLA9c11t	0.56	0.71	0.62	0.47	0.55	0.63	0.52	0.73	0.73	0.07	dE
CLA9t11t	0.50	0.60	0.53	0.44	0.43	0.56	0.51	0.47	0.50	0.04	C

[1]A = No supplemental Co vs. all other treatments;

B = source effect;

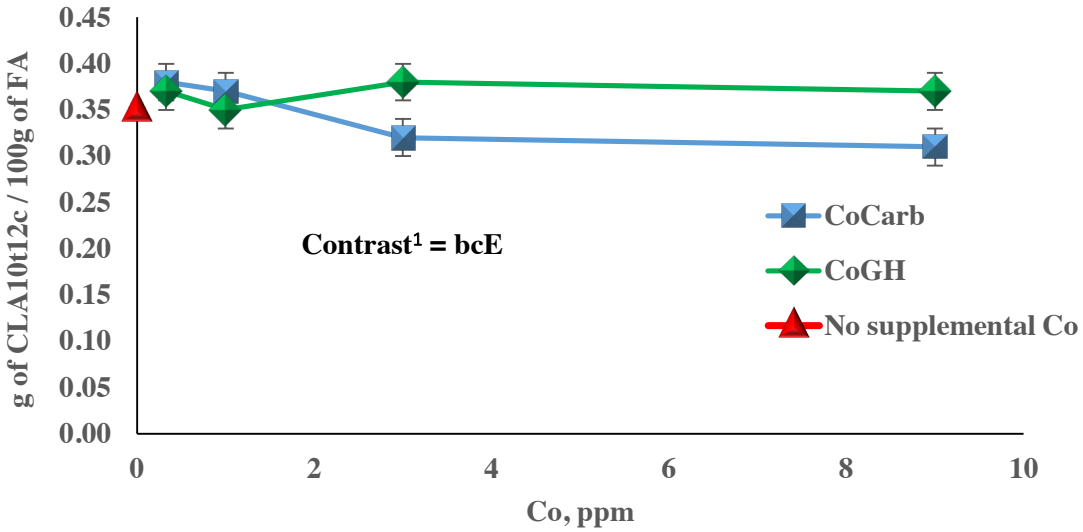
C = linear dose;

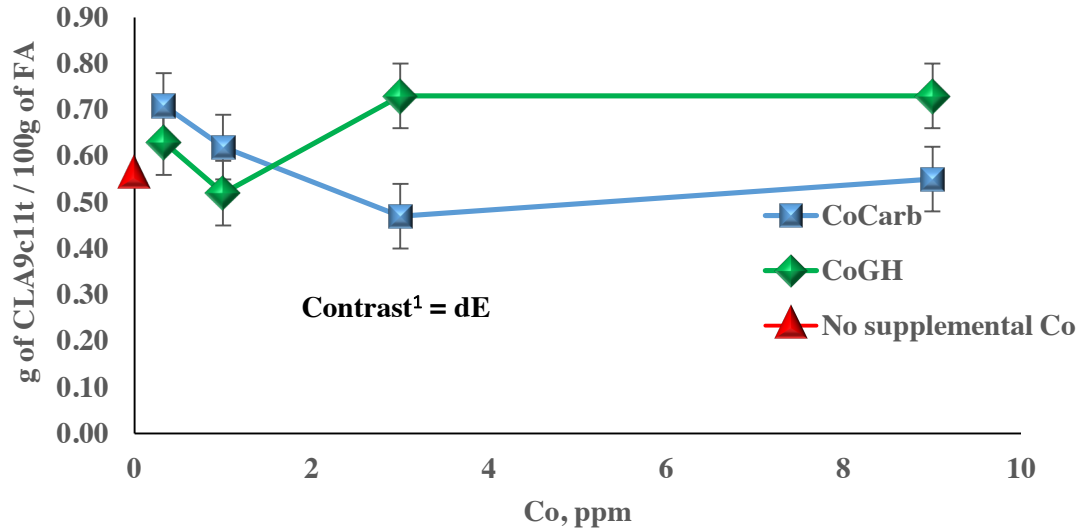
D = quadratic dose;

E = linear dose × source;

F = quadratic dose × source;

Upper case = $P < 0.05$; lower case = $P < 0.10$.





[1]A = No supplemental Co vs. all other treatments;

B = source effect;

C = linear dose;

D = quadratic dose;

E = linear dose × source;

F = quadratic dose × source;

Upper case = $P < 0.05$; lower case = $P < 0.10$.

Supplemental Figure 1. In vitro *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA concentrations with different sources and concentrations of cobalt. The X-axis represent the concentration

of Co expressed in ppm, and the Y-axis indicate the grams of fatty acid in 100 g of total fat.

[1]A = No supplemental Co vs. all other treatments; B = source effect; C = linear dose; D = quadratic dose; E = linear dose × source; F = quadratic dose × source; Upper case = $P < 0.05$; lower case = $P < 0.10$.

Appendix B - Copyright Permission

Chapter 4: Effects of supplemental chromium propionate and rumen-protected amino acids on productivity, diet digestibility, and energy balance of peak lactation dairy cattle

C. F. Vargas-Rodriguez[*], K. Yuan[*], E. C. Titgemeyer[*], L. K. Mamedova[*], K. E. Griswold[†], and B. J. Bradford[*1]

[*]Department of Animal Sciences and Industry, Kansas State University, Manhattan 66506

[†]Kemin Industries, Des Moines, IA 50317

This manuscript has been published in *Journal of Dairy Science*. J. Dairy Sci. 97:3815–3821

Permission for Authors

Authors can use their articles for a wide range of scholarly, non-commercial purposes as outlined below. These rights apply for all Elsevier authors who publish their article as either a subscription article or an open access article.

Authors can use either their accepted author manuscript or final published article for: Use at a conference, meeting or for teaching purposes; Internal training by their company; Sharing individual articles with colleagues for their research use (also known as 'scholarly sharing'). **Use in a subsequent compilation of the author's works**; Inclusion in a thesis or dissertation; reuse of portions or extracts from the article in other works; preparation of derivative works (other than for commercial purposes).