Digestibility of diets containing Enertia s/f® and soybean oil in equines and effect of cobalt chloride on fermentation of alfalfa and smooth bromegrass hay by equine cecal microorganisms

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Laura Kay Fehlberg

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Major Professor
Teresa Douthit

Abstract

Supplementation of lipids in the form of triglycerides is a safe method for increasing energy density in equine diets; however the effects of calcium salts of fatty acids (CSFA) on nutrient digestibilities in the horse are unknown. Eight cecally cannulated Quarter Horses were utilized in a crossover design in which horses consumed 1.5% BW smooth bromegrass hay and 0.5% BW concentrate supplemented with 4.1% soybean oil (SB) or 4.9% Enertia s/f® (E), a proprietary CSFA, for 28 d. Feces were collected for determination of apparent total tract digestibility of nutrients, and cecal digesta was evaluated for pH and concentrations of VFA and LCFA. Serum was collected following a 16-h fast and analyzed for triglycerides and cholesterol. Apparent total tract digestibilities of DM, NDF, ADF, CP, ether extract, and GE were unaffected by lipid source ($P > 0.10$). Serum triglycerides tended to be greater in horses consuming E compared to SB ($P = 0.10$); but, serum cholesterol concentrations were not different ($P = 0.45$). Cecal pH was unaffected by lipid source. Cecal concentration of total VFA tended to be greater in horses consuming SB compared to E at 2-h post feeding ($P = 0.07$). Cecal propionate concentrations were greater at 2-h post feeding for horses fed SB compared to horses fed E ($P = 0.03$). A treatment × time interaction was detected for total cecal LCFA concentration in which total cecal LCFA were greater in horses consuming E compared to SB at 2-h following consumption of a meal but became more similar as time progressed ($P < 0.01$). Secondly, an *in vitro* study was conducted to determine the effect of Co chloride on fermentation parameters using an equine cecal fluid inoculum in a 2 × 5 factorial arrangement of treatments. Alfalfa or smooth bromegrass hay (5 g DM) were utilized as substrates and were supplemented with 0.0, 0.5, 5.0, 25.0, or 50.0 mg Co/kg substrate DM. Cultures containing alfalfa had greater IVDMD compared to those with smooth bromegrass hay ($P < 0.01$). There was a forage × Co interaction

in which gas production increased with time and was greater in cultures containing alfalfa ($P < 0.01$). Production of acetate, propionate, butyrate, isobutyrate, isovalerate, and total VFA, as well as acetate:propionate, were increased in cultures containing alfalfa compared to those containing brome hay ($P < 0.05$).

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Chapter 1 - Literature Review

Introduction

The equine gastrointestinal tract (GIT) consists of a foregut for enzymatic digestion and a hindgut that contains an enlarged cecum for microbial fermentation. Its native diet is comprised of a long-stem forages consumed in small, frequent meals throughout the day (Janis, 1976). As performance and energy demands increase, the diet is altered to contain supplemental concentrates, and the horse is fed larger meals with less frequency (Santos et al., 2010). Concentrates utilize non-structural carbohydrates (NSC) to increase energy density of diets; however, starch may exceed the digestive and absorptive capacity of the small intestine and flow into the hindgut resulting in gastrointestinal disturbance (Julliand et al., 2006). To decrease the likelihood of gastrointestinal disturbances, vegetable oils are used to decrease NSC of the concentrate while maintaining its energy density (Delobel et al., 2008).

Equine diets typically do not include large amounts of lipid, but the GIT of horses adapts to accommodate increased intakes as demonstrated by an increase in lipase activity and increased fat digestibility (Kronfeld et al., 2004; Lorenzo-Figueras et al., 2007). Though the equine GIT appears to utilize greater amounts of lipid than that of ruminants, an overload of lipid may exceed small intestinal capacity and flow into the cecum in a manner similar to that of starch (Palmquist and Jenkins, 1980; Meyer et al., 1997). If lipid enters the cecum, it may have adverse effects on digestibilities of other nutrients, particularly fiber (Jansen et al., 2000; Kronfeld et al., 2004). Even so, fat supplemented diets are common in the equine industry, particularly with performance horses.

A higher fat diet increases energy density without necessitating additional DM consumption (McCann et al., 1987). Supplemental fat also may increase oxidation of fatty acids

which could provide the extra energy needed by performance horses (Scott et al., 1992; Hughes et al., 1995). Supplementing fat also may attenuate symptoms of metabolic disorders, especially those associated with increased NSC consumption, such as polysaccharide storage myopathy (PSSM; Oldham et al., 1990; Julen et al., 1995).

In ruminants, addition of fat to diets often is limited to no more than 6% to avoid negative effects of lipids on fibrolytic activity in the rumen (Palmquist and Jenkins, 1980). In an attempt to ameliorate decreased fiber fermentation, ruminally inert fats were developed. These "by-pass" fats, such as calcium salts of fatty acids (CSFA), are designed to pass through the rumen undigested and become available for digestion and absorption in the small intestine (Jenkins and Palmquist, 1984). The use of CSFA in equine diets is undocumented.

Cobalt salt supplementation is suspected to increase fiber digestibility. Because it is a divalent cation, Co has the potential to facilitate microbial attachment to fiber particles (Waterman et al., 2017). Cobalt also has potential to increase oxygen carrying capacity of the blood by stimulating erythropoiesis. It adapts tissues to decreased oxygen supply by inducing hypoxia (Ho et al., 2015). Adapting tissues to decreased oxygen supply and increasing the oxygen carrying capacity of blood enables Co to function as a performance enhancing drug in aerobic sports.

The purpose of this review is to analyze fat digestion and absorption in horses and determine associative effects of feeding fat. Emphasis will be on the effect of fat on digestibility of fiber and physiological changes that occur when fat is increased in the equine diet. Additionally, efficacy and mode of action of CSFA will be addressed. Lastly, it will discuss effects of Co on fiber digestion and potential for use of Co as a performance enhancing drug.

Introduction to lipids

Lipids typically are found in equine diets as triglycerides, phospholipids, glycolipids, or sterols. Triglycerides (hydrolyzed to diglycerides, monoglycerides, and FFA), are the major storage lipid in plants and therefore the predominant source of fat in the diet (Hallebeek and Beynen, 2002). Triglycerides and diglycerides are polar, water insoluble, non-swelling lipids that will form a layer above an aqueous solution. Monoglycerides and phospholipids are polar, water insoluble, swelling lipids. Because of their swelling capability, these molecules have some ability to interact with water and readily solubilize in micelles (Shiau, 1981).

Triglycerides consist of a glycerol backbone with 3 fatty acid attachments. Each fatty acid is an unbranched carbon chain that can vary in length from 2 to 28 carbons; however, 12- to 22-carbon chains are most common in typical feedstuffs. Fatty acids are classified as either saturated or unsaturated. Saturated fatty acids contain only single carbon to carbon bonds. These fatty acids have relatively high melting points, making them solid at room temperature. Unsaturated fatty acids have one or more double bonds and have decreased melting points compared to saturated fatty acids, thus they are likely to be liquid at room temperature. Fatty acids contain a carboxylic acid group (delta carbon) on one end and a methyl group (omega carbon) on the opposite end. The omega nomenclature of unsaturated fatty acids is based on distance of the double bond from the terminal methyl group. For example, an omega-3 (n-3) fatty acid would have a double bond connecting the third and fourth carbon from the methyl end (Warren and Vineyard, 2013).

Lipid Metabolism

Mechanical digestion

Digestion of lipids in horses begins in the mouth with chewing and grinding of feedstuffs (Merritt and Julliand, 2013). Salivary secretions are initiated by mastication and originate predominately from the parotid salivary glands. Equine saliva consists of mostly water and is essentially devoid of digestive enzymes; therefore, only mechanical size reduction occurs in the oral cavity (Alexander, 1966). Mechanical digestion continues in the stomach, where the churning mechanism created by peristalsis and the closure of the pyloric sphincter generates small lipid droplets from the original fat particles. In humans it is recognized that gastric mechanical digestion cleaves triglycerides into diglycerides and FFA; however, this has not been verified in the horse (Niot et al., 2009). Cleavage enhances emulsification, providing a larger surface area for digestive enzymes to affix (Merritt and Julliand, 2013).

Enzymatic digestion

As lipid droplets decrease in size and emulsify into chyme in the stomach, the enzyme gastric lipase begins digestion. Gastric lipase is released from zymogen cells that reside in the fundic region of the stomach. This region, known for its secretory activity, is located in the glandular portion of the stomach. The extent of fat digestion that occurs within the stomach is unknown, but it is likely limited because of a rapid rate of passage in horses. In other species, hydrolysis of triglycerides begins with gastric lipase and yields diglycerides and FFA (Moreau et al., 1988). These fatty acids, in addition to partially digested protein, stimulate the release of cholecystokinin (CCK), a gastrointestinal hormone. Cholecystokinin is released from mucosal cells in the small intestine and prompts pancreatic and hepatic secretions (Merritt and Julliand, 2013). The pancreas secretes a variety of enzymes, including lipase. Contrary to other species,

lipase is the most abundant enzyme secreted by the equine pancreas (Lorenzo-Figueras et al., 2007). In addition to digestive enzymes, bicarbonate also is secreted by the pancreas to neutralize chyme, which also assists in lipid emulsification. Diglycerides combine with phospholipids and bile salts to form emulsion particles. Because horses lack a gallbladder to store bile, bile is continuously secreted into the duodenum from the liver to provide bile salts for formation of emulsion particles (Merritt and Julliand, 2013). Bile salts are necessary to shift the pH optimum of pancreatic lipase from 8 to 6, which is closer to the average pH in the duodenal lumen, but they also prevent lipase from accessing triglycerides (Shiau, 1981). Co-lipase is needed as an "anchor" to allow lipase attachment to the triglycerides and lipolysis to occur (Carey et al., 1983). Lipase is then able to breakdown triglycerides within the core of the emulsion particle, producing 2 monoacylglycerides and 2 FFA's (Warren and Vineyard, 2013).

Core components of emulsion particles are hydrolyzed and migrate to the surface because they are less hydrophobic than triglycerides, therefore increasing surface area of the emulsion particle. As the surface coat components increase and core components decrease, surface pressure increases and a bilayer of the surface components detaches. Bile salts, though poor emulsifiers, assist in lipid hydrolysis by adsorbing to the lipid-water interface in the lumen of the small intestine and solubilizing products of lipolysis: monoglycerides, FFA, and lysophospholipids (Carey et al., 1983). The solubilized surface products, in conjunction with phospholipids, lead to mixed micelle formation. The micelle contains a FFA core, surrounded by a slightly water soluble outer layer of bile salts, monoglycerides, and lysophospholipids (Warren and Vineyard, 2013). Solubility and size of micelles enables the digestive products of lipolysis to diffuse across the aqueous barrier of the small intestinal lumen and reach the brush border membrane (Niot et al., 2009). The environment surrounding the brush border membrane has an

acidic pH because of the H^+/Na^+ antiport that resides on the luminal membrane of enterocytes. The acidic pH causes protonation of FFA's, resulting in long chain fatty acids (LCFA) that are not contained within a micelle formation (Shiau, 1981). The antiport mechanism facilitates interaction between products of lipolysis and enterocytes for absorption by providing an acidified environment. This concept was validated in rats given a pharmacological agent that inhibited the H^+/Na^+ antiport; therefore, an acidic pH was not developed and LCFA did not interact with enterocytes for absorption (Niot et al., 2009).

Absorption

The major anatomical site of fatty acid absorption is the duodeno-jejunum, with very limited absorption in the ileum (Niot et al., 2009). Previously it was believed that passive diffusion through the lipid bilayer of enterocytes was the only mechanism for absorption of monoglycerides, LCFA, and lysophospholipids (Shiau, 1981; Niot et al., 2009). It has since been documented in humans that absorption can occur by passive diffusion, also known as protein-independent diffusion, or facilitated diffusion, in which proteins are needed to transport lipolytic products across the apical membrane of enterocytes (Mansbach and Gorelick, 2007). The contribution ratio of these 2 absorption processes in horses is unknown (Warren and Vineyard, 2013). Three transport proteins have been identified in humans: plasma membrane-associated fatty acid-binding protein ($FABP_{pm}$; Stremmel et al., 1985), fatty acid transport protein 4 (FATP4; Schaffer and Lodish, 1994), and CD36 (Abumrad et al., 1993). It is currently assumed that these transport proteins also exist in the equine small intestine (Warren and Vineyard, 2013). Fatty acid transport protein 4 is expressed primarily in the jejunum of humans, with minimal expression in the duodenum and no expression in the colon (Stahl et al., 1999). When FATP4 expression is blocked in humans, LCFA uptake is reduced by 40 to 50% confirming that this

transport protein is utlized for LCFA uptake by enterocytes (Mansbach and Gorelick, 2007). Also expressed in the duodeno-jejunum of humans is the glycoprotein CD36, also known as fatty acid translocase (Lobo et al., 2001). In rats consuming a high fat diet, this transmembrane glycoprotein appears to be vital, demonstrated by an upregulation of expression of this protein in response to increased fat (Poirier et al., 1996). Plasma membrane-associated fatty acid-binding protein also may play a role in LCFA transportation into cells, as it can bind LCFA for transport (Stremmel et al., 1985) with a greater affinity for unsaturated fatty acids comparted to saturated fatty acids (Shiau, 1981). This protein is found in the jejunum, and, to a minor extent, in the ileum; however, it also is found in the crypt cells of the colon. Because LCFA absorption does not occur in the colon, the purpose of $FABP_{pm}$ in the colon is unknown (Isola et al., 1995).

Following absorption into cells, monoglycerides and LCFA are transported via intestinal-fatty acid binding protein (I-FABP; Niot et al., 2009) to the endoplasmic reticulum (ER) and resynthesized into triglycerides (Mansbach and Gorelick, 2007; Warren and Vineyard, 2013). Peptide YY, a paracrine agent that is released when dietary fat reaches the ileum, upregulates the protein expression of I-FABP allowing for more efficient fatty acid assimilation into cells (Hallden and Aponte, 1997). Once transported to the membrane of the ER, triglycerides are resynthesized by either the monoglyceride acylation pathway (MAP) or the phosphatidic acid pathway (PAP). The MAP is predominant during the absorptive state and occurs in the smooth ER (Shiau, 1981; Niot et al., 2009). This pathway works through progressive acylation, initially using the enzyme acyl CoA synthetase to yield a fatty acid with an acyl CoA attached (FA-CoA) which acts as an acyl donor. Fatty acid CoA is used for the acylation of a monoglyceride via the enzyme monoglyceride acyl transferase to form a diglyceride. Fatty acid CoA is used again as an

acyl donor, acylating a diglyceride into a triglyceride by employing the enzyme diglyceride acyl transferase (Shiau, 1981).

In the fasted state in humans, the PAP is predominant and takes place on the surface of the rough ER. This pathway utilizes α-glycerophosphate as the acyl receptor, but the acylation process is similar to the MAP. Unlike MAP, end products of the PAP can be a triglyceride or a phospholipid (Shiau, 1981; Niot et al., 2009). In most mammals, following the resynthesis of triglycerides, cholesterol, and phospholipids, a lipoprotein, identified as a chylomicron, is formed. In humans, chylomicron synthesis requires apolipid protein (apo) B48. A second apolipid protein, apo AIV, also is added to the particle and is believed to be a stabilizer (Niot et al., 2009). Apolipid protein B48 combines with microsomal triglyceride transfer protein (MTP), phospholipids, Apo AIV, and cholesterol to form a "dense particle" that then fuses with a triglyceride mass. This particle, known as the prechylomicron, is transported to the Golgi via prechylomicron transport vesicle (PCTV; Mansbach and Gorelick, 2007). Once adhered to the Golgi, glycosylation and addition of minor apolipid proteins to the prechylomicron occurs (Niot et al., 2009). After this final stage of maturation, chylomicrons are secreted into the lymphatic system by exocytosis. Introduction into circulation occurs through the thoracic duct. It is unclear whether movement of chylomicrons into circulation is the same in the horse as in other species (Warren and Vineyard, 2013). Marchello et al. (2000) identified chylomicrons in plasma of horses consuming high fat diets. In ruminants, formation of chylomicrons is dependent on amount of dietary fat (Byers and Schelling, 1988). Because the digestive system of horses is adapted to a forage-based diet that often does not include large amounts of lipid, it is likely that triglycerides are transported as very low-density lipoproteins (VLDL) or that horses must first be adapted to a diet with a greater fat inclusion level before chylomicrons can be identified. Very

low-density lipoproteins would be formed in the same manner as chylomicrons in enterocytes and behave very similarly (Warren and Vineyard, 2013).

Long-chain fatty-acid metabolism

If fat is fed at a level that surpasses the digestive and absorptive capabilities of the small intestine, it will proceed into the cecum. Meyer et al. (1997) observed 60 mg fat/kg BW entered the ileum in horses consuming 0.93 kg fat/BW. It was suspected that most of this subsequently entered the cecum. Fat metabolism in the cecum is not well researched; therefore, the extensive work conducted in ruminants is summarized. In the rumen, fat must first undergo lipolysis. The glycerol backbone of triglycerides is hydrolyzed by lipolytic microbes from the 3 fatty acids (Jenkins, 1993). Glycerol can be metabolized by microbes to produce acetate, propionate, and butyrate (Wright, 1969). Free fatty acids undergo some extent of biohydrogenation (Hess et al., 2008). This process hydrogenates unsaturated fatty acids into saturated fatty acids by hydrolyzing a double bond to form a single bond. Biohydrogenation appears to be a protective mechanism for microbes because unsaturated fatty acids have antimicrobial properties. Unsaturated 18-carbon FFA most commonly produce stearic acid (C18:0), the product of complete biohydrogenation (Jenkins, 1993). Biohydrogenation also was demonstrated in young pigs consuming a fat supplemented diet. The concentration of unsaturated fatty acids in the feces decreased while stearic acid increased as compared to concentrations in the ileum. This was not observed in germ free pigs and was therefore attributed to microflora in the hindgut (Carlson and Bayley, 1968). Absorption of LCFA does not occur in the fermentative compartment of the cow. Instead, LCFA flow into the small intestine and are absorbed (Jenkins, 1993). It is therefore assumed that if LCFA arrive in the hindgut of the horse, absorption will not occur and LCFA will be excreted in the feces.

Fat digestibility

In the horse, fat is digested and absorbed in the small intestine and fat that escapes digestion will enter the cecum (Meyer et al., 1997). The extent of digestion depends on a multitude of variables, including adaptation time to a fat supplemented diet, fat source, and meal size. Lipids can be derived from forages, grains, and added fats, including vegetable and animal sources, and are generally measured by ether extract (EE) content (Kronfeld et al., 2004). Unfortunately, EE content varies significantly between ingredients. The majority of lipids in plants are nonsaponifiable substances, which contribute to the EE content and do not provide energy to the animal (Palmquist and Jenkins, 2003). Of all feedstuffs, forages have the least available fat (Warren and Vineyard, 2013). Digestibility of EE in a forage-only diet consumed by horses ranged from 42 to 49%. Lipid digestibility is limited in forages because structural components encompass lipids, yielding them indigestible (Fonnesbeck et al., 1967). In horses consuming a grain-only diet, digestibility of EE ranged from 55 to 76% (Hintz and Schryver, 1989). Diets with added fats elicited the greatest digestibility of EE in horses at 88 to 94% because added fats and oils contain primarily readily digestible triglycerides (Kane et al., 1979).

Kronfeld et al. (2004) asserted that a diet consisting of forage and grains would have an apparent digestibility of EE of 55% but would approach 100% for added fats. When the amount of fat in the diet increases, apparent digestibility of EE increases due to the adaptation of lipolytic enzymes by increasing activity. Adaptation takes 4 to 14 d, depending on breed and age of horse (Julen et al., 1995; Bush et al., 2001; Kronfeld et al., 2004). Julen et al. (1995) supplemented diets of horses with fat and observed EE digestibility increasing from 40% to 74% from d 0 to d 7 and continued at this level to the conclusion of the study. Bush et al. (2001) noted that digestibility of EE increased as lipid consumption increased.

The extent of fat digestion is largely determined by the amount of time feedstuffs spend proximal to the hindgut. In many species, fat slows gastric emptying (Meyer et al., 1986). It is postulated that this occurs because lipids in the small intestine provide feedback inhibition to the stomach, thereby slowing emptying. In the horse, this assumption has not been validated. When horses were fed a bolus of carbohydrates or fat, gastric emptying time was not different (Lorenzo-Figueras et al., 2005). Meals provided by Lorenzo-Figueras et al. (2005) were isocaloric and nearly isovolumetric; however, molarity and particle size were not addressed, both of which can affect rate of gastric emptying (Meyer et al., 1986). Geor et al. (2001) observed faster gastric emptying time in horses consuming a corn oil-supplemented diet; however, the diets were not isovolumetric. Increases in volume could increase rate of passage because of increased DM intake; therefore, faster emptying time could not be attributed to corn oil alone. Wyse et al. (2001) noted decreased gastric emptying time with soya oil and an indigestible fat. These conflicting reports make it difficult to determine if fat elicits the same response in horses as it does in animals that routinely consume greater amounts of fat.

Fat in equine diets

Equine diets containing forages and cereal grains typically do not contain large amounts of lipids, as they normally comprise of 4% or less of the diet (Warren and Vineyard, 2013). Grass hay generally contains 20 to 24 g crude fat/kg DM, with legume hay containing only slightly more at approximately 25 g crude fat/kg DM (Bonfaied et al., 2003). Concentrates have a wider range of lipid contents. For example, oat grain, barley grain, and rice bran contain 54, 23, and 208 g crude fat/kg DM, respectively. Because of the low crude fat content in grains and forages, oils are used by nutritionists to increase energy density of diets. Common oils, such as canola oil, corn oil, or soybean oil, contain 1000 g crude fat/kg DM, only differing in their fatty

acid composition (Palmquist, 1991; Warren and Vineyard, 2013). A commercial concentrate is considered to have added fat if the crude fat content is > 5% (Beynen and Hallebeek, 2002). Varying concentrations of essential fatty acids (EFA) can make some oils nutritionally superior to others. Fat can be top dressed in the form of oil; however, this may lead to nutrient imbalances that are expensive to correct. Therefore, a fat-added concentrate is a practical alternative for horse owners (Warren and Vineyard, 2013). Fat often is supplemented in equine diets to increase energy density, enhance performance, and for metabolic modulations. Detrimental effects of fat supplementation have not been well-documented.

Essential fatty acids

Linoleic acid (LA; C18:2n-6) and α-linolenic acid (ALA; C18:3n-3) are polyunsaturated fatty acids that are considered EFA for equines, meaning they are required in the diet (Harris et al., 2005); however, there has not been a deficiency of either identified in horses (Warren and Vineyard, 2013). Mammals cannot synthesize LA or ALA because they lack the enzymes Δ12- and Δ15-desaturase that are utilized to inter-convert 18-carbon fatty acids (O'Neill et al., 2002; Warren and Vineyard, 2013). Plants and algae readily synthesize LA and ALA, and therefore are sources of EFA for equine diets (Calder and Grimble, 2002; Warren and Vineyard, 2013). The NRC (2007) recommends a minimum intake level of 0.5% DM for LA and there are no current guidelines for ALA. These recommendations were based on other mammalian species and justification for the horse was not identified. A diet consisting solely of grass or legume hay may provide the recommended daily intake, but with the addition of concentrates, such as flaxseed or rice bran that contains large amounts of LA (59 g/kg DM and 71 g/kg DM, respectively) the daily recommendation is easily met. Flaxseed also contains an abundance of ALA at 228 g/kg DM. Fats and oils can be concentrated sources of both LA and ALA. Corn oil and soybean oil

tend to contain more LA compared to most fats and oils in equine diets, while flaxseed oil contains the greatest concentration of ALA (Warren and Vineyard, 2013).

Fat supplementation for performance horses

The desire to increase energy density of diets for performance horses leads to fat supplementation. Fat contains 9.5 Mcal GE/kg, which equates to 2.25 times the amount of energy provided by carbohydrates (Warren and Vineyard, 2013). Kane et al. (1979) reported that increasing the amount of fat in the diet of ponies increased DE and ME in proportion to the amount of fat added. Because of increased energy density of fat, calories can be added to diets without increasing DM intake. This is desirable in performance horses, especially racehorses, where increased gut fill could slow race speeds (McCann et al., 1987). Unfortunately, adding lipids in place of starch and sugar to diets decreases the availability of substrates utilized in anaerobic metabolism. Horses rely predominately on anaerobic metabolism during high speed events and decreasing dietary carbohydrates may lead to decreased race speeds (Lacombe et al., 2001).

The addition of fat to equine diets also may decrease reactivity to stimuli. Holland et al. (1996) observed a decrease in spontaneous activity in horses consuming 10% added fat. This was accompanied by less total travel time because horses spent fewer seconds reacting to novel stimuli. Additionally, cortisol concentration was decreased at rest and in response to stimuli in fat-supplemented horses, especially when compared to horses consuming a diet with more starch and sugar. Decreased stress also was demonstrated by decreased heart rates in horses consuming the diet with increased fat, but only in response to a stimuli (Redondo et al., 2009). While horses appear to be less excitable when consuming more fat, this usually is accompanied by concomitant decreases in starch and sugar in the diet, thus these observations may be confounded

by fewer calories coming from nonstructural carbohydrates (NSC). Recurrent exertional rhabdomyolysis is a stress-induced myopathy characterized by abnormal intracellular Ca regulation that affects 5% of Thoroughbred racehorses (McKenzie et al., 2003). A Thoroughbred exhibiting symptoms of RER would not be able to race, and, therefore, there are serious economic repercussions to this syndrome. Increased fat in diets of horses with RER decreased stress, excitability, and muscle tightness, allowing the horses to race (Macleay et al., 1999).

Horses consuming diets with increased fat exhibit physiological changes beneficial to performance. For muscles to contract, they must have substrate available to utilize as energy. The primary substrates utilized are glycogen and triglycerides, with protein only contributing 10 to 15% (Lacombe et al., 2003). During submaximal work, aerobic metabolism is the primary method of generating energy and substrate utilization includes FFA and muscular glycogen. The advantage of a fat-supplemented diet is that it is associated with glycogen sparing. This means there is increased oxidation of FFA for energy during work, thus conserving glycogen for later use (Lacombe et al., 2003). Anaerobic metabolism, utilized during maximal work, employs muscular glycogen as its primary energy source (Lacombe et al., 2001). This increased glycogen availability then enhances the substrate pool available for anaerobic metabolism and may lead to increased performance. Conversely, adding dietary carbohydrates enhances glycogen deposition in humans (Williams, 1993), but carbohydrate loading has not been shown to be an effective means of increasing muscular glycogen stores in horses (Clark et al., 1990).

Respiratory quotient (RQ) is a measure of substrate utilization, with a greater RQ indicating carbohydrate utilization and a lesser RQ indicating increased FFA utilization. In horses adapted to a fat-supplemented diet for 10 wk, their RQ when exercised at the same workload was less than that of horses consuming a control diet with a greater concentration of

NSC and a lesser amount of fat (Dunnett et al., 2002). When horses were fed a diet containing 18% added fat, RQ at rest and during exercise was less than that of horses consuming the control diet. Together these indicate an increase in oxidation of FFA when horses consume supplemental fat (Pagan et al., 2002). In this same study, onset of blood lactate accumulation (OBLA), a measurement of anaerobic threshold, was utilized during a high intensity exercise test. This is often used as an indicator of fatigue and fitness level, therefore increasing the time to OBLA is desirable in performance horses, especially those competing in high intensity events. In horses consuming increased fat, horses took longer to reach OBLA (Pagan et al., 2002). A decrease in RQ accompanied by an increase in OBLA in horses consuming diets with increased fat indicates that substrate utilization shifts to FFA oxidation and time to fatigue is delayed. This does not directly relate to performance and race times, and, therefore, one must be cautious remarking on changes in performance.

Metabolic modulation with added fat

In horses consuming diets with added fat, several metabolic changes occur that may affect performance and health of the animal. Calories derived from fat often replace calories from another source, typically NSC, or the lipid is applied as a topdress and simply provides additional calories (Kronfeld and Harris, 2003). If an iso-caloric exchange between fat and NSC occurs, starch and sugar concentrations in the diet decrease and metabolic changes ensue to better utilize increased dietary triglycerides. Perhaps counter-intuitively, plasma triglycerides decease while cholesterol and lipoprotein lipase (LPL) increase (Orme et al., 1997; Geelen et al., 1999). When LPL increases, more triglycerides in VLDL are hydrolyzed and surface material of VLDL, including cholesterol, are transferred to high-density lipoproteins (HDL). This explains why increased cholesterol and decreased triglycerides are observed in horses consuming a fat-

supplemented diet (Stanley et al., 1986). It was proposed by Geelen et al. (1999) that increased LPL, in conjunction with decreased plasma triglycerides, indicates that horses consuming a diet with added fat adapt metabolically to the diet change. However, it is also possible that these changes may be the result of decreased dietary NSC (Orme et al., 1997; Dunnett et al., 2002).

Fat-supplemented diets are beneficial to horses with the exertional rhabdomyolysis polysaccharide storage myopathy (PSSM). Horses with this syndrome store excessive glycogen in skeletal muscle, demonstrate increased metabolism of glycogen during exercise, and have increased insulin sensitivity. Though the etiology of how supplemental fat decreases symptom severity in horses with PSSM is not entirely known, decreased serum creatine kinase (CK) at rest and during exercise was observed when PSSM-affected horses consumed a fat-supplemented diet when compared to control horses (Ribeiro et al., 2004). Adding fat to the diet has the potential to minimize postprandial spikes in glucose, allows for decreased intake of NSC, and is associated with decreased serum CK concentrations. Together these could reduce glycogen deposition and attenuate symptoms of PSSM.

Fat effects on fiber digestibility

The effect of fat on fiber digestibility in horses has been of particular interest, but research findings are conflicting. Fiber digestibility in ruminants is depressed when they are fed diets exceeding 6% fat (Palmquist and Jenkins, 1980). It has been suggested that if fat were to escape absorption in the small intestine and enter the equine cecum, it may hinder fibrolytic microorganisms (Jansen et al., 2000). Delobel et al. (2008) concluded that because fiber digestibility increased, fat did not arrive in the cecum when horses consumed a diet with 8% added linseed oil. Increased fiber digestibility as opposed to no change in fiber digestibility was explained by decreased NSC in the fat-added diet. A meta-analysis on the effects of fat on fiber

digestibility in horses detected a decrease in ADF digestibility with supplemental fat but no change in DM or NDF digestibilities. It was proposed that fiber digestibility was not adversely affected by fat because the small intestine precedes the fermentative compartment in the horse, and most fat would be absorbed prececally (Sales and Homolka, 2011).

Introduction to ruminally inert fats

Fat intake exceeding 6% of the diet in ruminants elicits antimicrobial action in the rumen (Palmquist and Jenkins, 1980). It was suggested that this may occur through a direct effect, in which the membrane of the microbe is altered, or by physical coating of feed particles, disabling microbial enzyme attachment (Palmquist and Jenkins, 1980). An antimicrobial effect would result in decreased cellulolytic activity, potentially decreasing the acetate:propionate ratio in the rumen (Jenkins, 1993). Ruminally inert fats may be utilized to increase energy density of feed without inhibiting fiber degradation. Because fat is protected from microbial degradation in the rumen, it passes into the small intestine (Palmquist and Jenkins, 1980). The degree of inertness varies greatly, depending on the process utilized to produce the ruminally inert fat (Gulati et al., 1997).

Ruminal inertness can be achieved through a variety of chemical processes including encapsulation of a triglyceride with formaldehyde-treated protein (Scott et al., 1970), extruding vegetable oil seeds (Deacon et al., 1988), pelleting fat with starch to form prilled fat (Grummer, 1988), and formation of soaps of saturated or unsaturated fatty acid with Ca (Jenkins and Palmquist, 1984). Gulati et al. (1997) conducted an *in vitro* experiment comparing extruded fat, prilled fat, Ca salts of fatty acids (CSFA), and formaldehyde-treated fat. They detected less biohydrogenation in CSFA compared to other fat supplements and concluded that CSFA would be the most ruminally inert if it reacted similarly *in vivo*.

Calcium salts of fatty acids

Because Ca is a divalent cation, attachment to LCFA results in an insoluble soap (Jenkins and Palmquist, 1984). This can be achieved with saturated or unsaturated LCFA. In the rumen, about 50% biohydrogenation occurs in CSFA when predominantly unsaturated LCFA are utilized, whereas a CSFA derived from an animal-vegetable fat blend is 87% biohydrogenated (Wu et al., 1991). Biohydrogenation converts unsaturated fatty acids to saturated fatty acids, and unsaturated fatty acids have a greater digestibility compared to saturated fatty acids. Calcium salts of fatty acids are more inert if they contain predominately saturated LCFA; however, CSFA that are composed primarily of unsaturated fatty acids are of interest in the beef cattle industry. Increased flow of unsaturated fatty acid into the small intestine causes tissues to contain greater unsaturated fatty acids, yielding a healthier beef product (Wood et al., 2004). Unfortunately, as fatty acids become more unsaturated, ruminal inertness decreases (Jenkins and Bridges, 2007).

Reports of effects CSFA on digestibility of nutrients and fermentation parameters tend to vary. Hill and West (1990) and Alexander et al. (2002) observed greater EE digestibilities when cattle were fed CSFA-supplemented diets, while Ngidi et al. (1986) reported reduced digestibility of EE. Because the ruminant's small intestine has a limited capacity for fat digestion and absorption, it was proposed that increased fat may lead to decreased digestibility of EE (Ngidi et al., 1986). There is also evidence that digestibility of fiber components does not change in ruminants consuming a CSFA-supplemented diet, therefore indicating that CSFA are inert in the rumen and do not affect fiber digestibility (West and Hill, 1990; Alexander et al., 2002).

For a CSFA to be inert, dissociation of the Ca must not occur in the rumen but should readily occur in the abomasum and small intestine. Dissociation of Ca from the fatty acid depends on pH, with a lesser pH resulting in greater dissociation (Jenkins and Palmquist, 1984).

Differences in pH between the rumen and small intestine enable fat to be inert in the rumen because Ca is attached. Sukhija and Palmquist, (1990) noted that Ca salt of soybean fatty acids dissociated at the greatest pH (6.0) and less saturated fatty acids led to a decrease in the pH at which dissociation occurred. Conversely, Ca soap formation occurs at a pH of 6.5 (Zangenberg et al., 2001). Thus, it appears that CSFA inertness depends on the type of fat used and the pH of the rumen at time of consumption.

Enertia s/f® is a proprietary CSFA supplement provided by ADM Alliance Nutrition (Quincy, IL). It contains 84% crude fat, provided by palmitic (44%), oleic (40%), and linoleic (9.5%) fatty acids. This supplement is primarily marketed for use in dairy cattle rations to increase energy density of diets without eliciting adverse effects on fiber digestibility that typically occur with fat supplementation greater than 6%.

Markers for digestibility of nutrients

In ruminants and non-ruminants, total collection of feces allows the most accurate determination of total tract digestibility of nutrients (Sales, 2012). Because total fecal collection methods are often cumbersome or not feasible with larger sample sizes, internal and external markers have been proposed as alternative methods for measurement of digestibilities (Kanani et al., 2015). Retention time for external markers tends to be less than that of the digesta, leading to premature excretion and inaccurate calculations of digestibility coefficients (Faichney et al., 1989). Internal markers are an indigestible portion of the diet that will pass through the GIT at a rate similar to that of digesta. Internal markers can present bias if there is not full recovery in the feces or if representative samples are not obtained (Kanani et al., 2015). In horses, common internal markers include acid insoluble ash (AIA) and acid detergent lignin (ADL). Other indigestible markers also have been utilized, such as acid detergent insoluble ash (ADIA) and

indigestible ADF (Sales, 2012). External markers are less favorable because of potential health concerns and varying rate of passage (Myers et al., 2006) and therefore the focus of this review will be on internal markers.

In horses, variation in diet influences the efficacy of internal markers. Kanani et al. (2014) fed horses bermudagrass hay of varying quality to compare ADIA, ADL, and alkaline-peroxide lignin as internal markers and found that ADIA was nearest to 100% recovery. Concentration of ADIA in hay and feces varied for different dietary ingredients, but because of the low cost and rapid analysis it was considered the most suitable internal marker for the complete forage diet provided. Miraglia et al. (1999) fed mixtures of forage and concentrate, collected total feces, and utilized AIA and ADL as internal markers. Calculations of digestibility coefficients utilizing total fecal collection or AIA were determined to be similar, but ADL severely underestimated DM digestibility and was therefore found unsuitable as a marker. In contrast Bergero et al. (2005) reported ADL did not underestimate digestibility coefficients and therefore was determined to be an adequate marker for an all-forage diet.

Introduction to cobalt

Cobalt is a trace element utilized in multiple biochemical pathways, notably the formation of cobalamin, also known as vitamin B_{12} (Kobayashi and Shimizu, 1999). Ruminal microorganisms synthesize vitamin B_{12} from dietary Co and a deficiency is characterized by unthriftiness (Suttle and Jones, 1989; Kinobe, 2016). A deficiency has not been observed in horses, thus it is likely that most equine dietary components contain adequate Co (Coenen, 2013; Vervuert and Kienzle, 2013). Even so, the interest in Co supplementation to horses is increasing, particularly in the race horse industry. Cobalt induces hypoxia-like responses, allowing tissues to adapt to low oxygen conditions which could theoretically decrease time to fatigue in race horses

(Ho et al., 2015; Kinobe, 2016). It has been proposed that Co can enhance fiber digestibility, but research has yielded mixed results (Hubbert et al., 1956; Lopez-Guisa and Satter, 1992).

Cobalt requirement for microorganims

Ruminal and cecal microorganisms require Co for vitamin B_{12} synthesis; therefore, a diet deficient in Co may lead to vitamin B_{12} deficiency in the animal (Davies, 1971; McDonald and Suttle, 1986). Cobalt is at the center of a corrin ring formed in the production of vitamin B_{12} (Martens et al., 2002). Vitamin B_{12} is a growth factor for ruminal microorganisms and is utilized to produce and metabolize propionate (Strobel, 1992). Vitamin B_{12} is required by methylmalonyl mutase, which catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA. This conversion is an intermediate step in the production of propionate and metabolism of propionate to glucose (Nagaraja et al., 1997). In a vitamin B_{12}-deficient batch culture, succinate accumulates, verifying that vitamin B_{12} is necessary in the conversion of succinate to propionate (Strobel, 1992). Propionate is an important VFA because it is gluconeogenic. Ruminants can produce up to 40% of their glucose requirement in the liver via gluconeogenesis (Kennedy et al., 1994). If Co is limiting, gluconeogenesis will be reduced resulting in a less efficient animal. Additionally, Kennedy et al. (1994) observed reduced appetite resulting in reduced gain in lambs consuming a Co-deficient diet.

There is evidence that vitamin B_{12} is absorbed in the cecum of the horse. When horses were dosed with labeled Co, subsequently labeled vitamin B_{12} was detected in urine (Stillions et al., 1971). Because serum levels of vitamin B_{12} remained constant in horses consuming a diet with supplemental Co and no dietary vitamin B_{12}, it was concluded that horses do not have a dietary vitamin B_{12} requirement, that it is synthesized via dietary Co in sufficient quantities and it is absorbed in the lower GIT (Stillions et al., 1971).

Role of cobalt in fiber digestibility

Cobalt supplementation has been proposed to increase fiber digestion by ruminal microbes. Cobalt is a divalent cation and therefore has the potential to increase the affinity between negatively-charged plant cell walls and negatively-charged bacterial membranes. If microbes can more readily attach to fiber particles, digestibility of these particles will increase (Lopez-Guisa and Satter, 1992; Waterman et al., 2017). Hussein et al. (1994) observed that Co did not have an effect on IVDMD, OM disappearance, nor fiber disappearance in ruminal contents. Conversely, Lopez-Guisa and Satter (1992) observed increased DM digestibility with supplemental Co in heifers consuming a forage-only diet. Thus, they suggested that Co supplementation is effective in increasing digestibility of a diet consisting of predominately of forage, and it is most effective with low-quality forage (Lopez-Guisa and Satter, 1992). Results from this study were confounded by use of Co and Cu supplementation concurrently, and, therefore, it cannot be distinguished if Co supplementation was the cause of increased DM digestibility.

Cobalt deficiency *in vivo* results in reduced appetite and thereby decreased DM consumption which results in decreased apparent fiber digestibility. Thus it is difficult to decipher whether Co deficiency or decreased DM consumption leads to the subsequent decrease in apparent fiber digestibility. In Co-supplemented animals, increased fiber digestion simply may be a result of increased fiber intake because appetite was no longer suppressed (Salsbury et al., 1953); therefore, an *in vitro* experiment was conducted to determine if Co supplementation increases cellulose digestibility. Digestibility of cellulose was decreased at 12 ppm added Co and the greatest depression occurred at 200 ppm. Researchers also observed no effect on cellulose

digestion with supplemental vitamin B_{12}. They concluded Co had a negative effect on fiber digestion *in vitro*.

In horses, very little research has been conducted regarding Co's effect on fiber digestion. In a recent study by LeCompte (2015), horses consumed diets with varying levels of supplemental Co. No differences were observed in digestibilities of DM, NDF, ADF, or lignin; therefore, they concluded that fiber digestion was not affected by Co supplementation.

Cobalt as a performance enhancing drug

Cobalt alters the hypoxia inducible factor (HIF) pathway, which enables cells to adapt to an environment with decreased oxygen supply (Ho et al., 2015). Gene modulation of HIF also leads to increased numbers of red blood cells (RBC), induced by increased expression of erythropoietin. Increased RBC increase the oxygen carrying capacity of the blood (Déry et al., 2005). Though Co misuse has not been documented in equine sports, its misuse has been observed in humans. A threshold for equine urinary and plasma Co levels has been set at 75 ng/mL and 2 ng/mL, respectively, to identify Co intake exceeding normal consumption (Ho et al., 2015). Not only would Co supplementation provide an unfair advantage to racehorses, it also may have detrimental side effects caused by long term use. Though toxicity has not been documented in horses, humans that utilize Co as a performance enhancing drug or to combat anemia may suffer a variety of maladies (Simonsen et al., 2012; Ho et al., 2015). Cobalt toxicity has the potential to affect the thyroid, and to cause Co induced asthma, allergic dermatitis, or cardiomyopathy (Underwood, 1975). It can also have a negative impact on the immune system and may have carcinogenic potential (Ho et al., 2015). Cobalt toxicity may occur due to inhibition of critical enzymes by attaching to sulfhydryl groups and competing with Ca in Ca channels (Simonsen et al., 2012). Because of potential toxicity and drug testing in performance

horses, dietary Co supplementation for nutritional benefits must be at levels that do not lead to values that exceed urinary and serum thresholds.

Summary

Supplemental fat can be added to equine diets to increase energy density and produce physiological changes that may result in increased performance. The effect of fat on digestibility of nutrients remains unclear due to variation between studies. Some of this variation may be attributed to different methods used to calculate digestibility coefficients. These methods include total fecal collections or the use of external or internal markers. Internal markers, particularly ADIA, appear to be an accurate and cost effective way to determine digestibility coefficients for equine diets.

Additionally, CSFA-supplemented diets provide a way to increase caloric density without sacrificing microbial activity in ruminants. The extent to which ruminants can utilize fat once it enters the small intestine appears to be limited compared to non-ruminants, and, therefore, may surpass digestive capacity at greater inclusion rates leading to decreased digestibility of EE.

Lastly, the effects of Co on fiber digestion are unclear because of varying results provided by different researchers. Cobalt may have the ability to increase aerobic performance in racehorses; however, toxicity and misuse must be addressed in the industry. Because of the potential for toxicity, if Co is supplemented to horses in an attempt to enhance fiber digestion, the amount of Co added must be carefully calculated to maintain serum and urine Co levels below published threshold values.

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Chapter 2 - Effect of cobalt chloride on fermentation of alfalfa and smooth bromegrass hay by equine cecal microorganisms

L. K. Fehlberg, J. M. Lattimer, J. S. Drouillard, and T. L. Douthit

Department of Animal Sciences and Industry, Kansas State University, Manhattan, KS 66506

Abstract

Cobalt is required by microorganisms to synthesize vitamin B_{12}, which is an important coenzyme for energy metabolism. It has been suggested that increasing Co above requirements can increase fiber digestibility in bovines; however, effects of Co on equine cecal microorganisms are unknown. Our objective was to evaluate effects of Co chloride on *in vitro* gas production, VFA production, and IVDMD using strained cecal fluid from 4 cannulated Quarter Horses as inoculum. Alfalfa or smooth bromegrass hay was provided as substrate (5 g DM) with Co added at 0.0, 0.5, 5.0, 25.0, or 50.0 mg/kg substrate DM. Fermentation bottles containing 10 mL of equine cecal fluid and 140 mL of McDougall's buffer were incubated in duplicate, and pH, VFA concentrations, and IVDMD were measured after 48 h of fermentation. Gas production was measured every 15 min during the fermentation period. Terminal pH was not affected by Co nor forage type ($P \geq 0.19$). There was greater IVDMD for cultures containing alfalfa (29.2%) compared to smooth bromegrass hay (19.4%; $P < 0.01$). There was a forage × Co interaction in which gas production was greater in cultures containing alfalfa compared to smooth bromegrass hay, becoming more different as time progressed ($P < 0.01$). There was a linear forage × cobalt interaction ($P = 0.05$) in acetate:propionate (A:P), in which increasing Co resulted in increased A:P in cultures containing alfalfa while increasing Co concentration resulted in decreased A:P in cultures containing smooth bromegrass hay. Concentrations of acetate, propionate, butyrate, isobutyrate, isovalerate, and total VFA were increased in cultures containing alfalfa compared to those containing brome hay ($P < 0.05$). Cobalt had minimal effects on fermentation parameters measured in *in vitro* cultures; however, fermentation was greater in cultures containing alfalfa compared to those containing smooth bromegrass hay.

Introduction

The effect of Co on fiber digestion in ruminants has been of particular interest because it is a divalent cation. It has been suggested that divalent cations may enhance attraction between the negatively charged plant cell wall and negatively charged bacterial cell wall. Increased affinity between bacteria and plant particles could increase fermentation (Waterman et al., 2017). Analysis of data collected from *in vitro* cultures do not support this claim, as increased Co concentrations suppress microbial fermentation, as indicated by decreased cellulose digestion (Salsbury et al., 1953; Hubbert et al., 1956). Volatile fatty acid concentrations also may be modified by Co supplementation. Tiffany et al. (2006) observed decreased ruminal succinate concentrations and increased valerate, isovalerate, and butyrate concentrations with Co supplementation. Microorganisms require Co to synthesize vitamin B_{12}, and vitamin B_{12} is a cofactor for multiple reactions, including intermediate steps in propionate production and metabolism and gluconeogenesis (Strobel, 1992; Girard et al., 2009). Symptoms of Co deficiency are thought to be a product of vitamin B_{12} deficiency, and include decreased appetite and growth rate in cattle and sheep (Kennedy et al., 1994; Nasser, 2010).

In horses, a Co deficiency has not been identified; however, absorption of vitamin B_{12} from the hindgut has been verified (Stillions et al., 1971). The NRC (2007) recommends including Co in equine diets at 0.05 mg/kg DM, but this is based on deficiency symptoms observed in ruminants. This study was conducted to determine if varying levels of Co chloride in a culture containing equine cecal fluid inoculum and alfalfa or smooth bromegrass hay would alter fermentative activity, as measured by gas production, pH, VFA concentrations, and IVDMD.

Materials and methods

Animals

Four cecally cannulated Quarter Horses were used as cecal fluid donors. All horses were fitted with a permanent cecal cannula (flexible rumen cannula, #7c; 3.8 cm center diameter and 8.9 cm wall thickness: Bar Diamond, Parma, ID; Beard et al., 2011). All horses were consuming a common diet consisting of *ad libitum* prairie grass hay prior to collection. *Ad libitum* access to water and a salt block was provided.

Experimental design

The experimental design consisted of a randomized complete block design with a 2 × 5 factorial treatment arrangement consisting of 2 forage substrates (alfalfa and smooth bromegrass hay; Table 2.1) and 5 concentrations of supplemental Co (0, 0.5, 5, 25, or 50 mg/kg substrate DM) as Co chloride. There were 8 blocks (horse) with duplicate cultures of each Co-forage combination for each donor animal.

Inoculum and fermentation

Alfalfa and smooth bromegrass hay were ground using a Wiley mill (1 mm screen: Thomas Scientific, Philadelphia, PA) and Co chloride treatments were solubilized in 1 mL of distilled water. Five g of substrate DM were added to 250-mL screw top fermentation flasks (#7056; Ankom Technology, Macedon, NY) and placed in a 39°C incubator approximately 12 h before cecal inoculum was added. One-hundred forty milliliters of McDougall's buffer (McDougall, 1948) at pH 7 were added to each flask 7 h before cecal inoculum was applied and flasks remained in a 39°C incubator. There was 1 blank for each replicate that contained only cecal fluid and McDougall's buffer to serve as a baseline for gas and VFA production.

Cecal contents were obtained via gravity flow through cecal fistula, strained through 4 layers of cheesecloth, and collected into prewarmed insulated thermoses and immediately transported back to the laboratory. Cecal fluid was strained through 4 layers of cheesecloth into 1,000-mL separatory funnels, gassed with N_2, and incubated at 39°C for approximately 30 min until cecal fluid stratified. Once stratified, the bottom sediment layer was discarded and the aqueous, microbe-rich middle layer was used as inoculum. Ten milliliters of cecal fluid and 1 mL of Co chloride were added to fermentation flasks containing substrate and stirred. Each flask was flushed using N_2 and pH was recorded using a portable pH meter (Thermo Scientific Orion 3 Star Portable pH Meter, Waltham, MA). A pressure monitoring module (RF Gas Production System; Ankom Technology, Macedon, NY) was screwed onto flasks which were placed into a shaking incubator (New Brunswick Scientific Inc., New Brunswick, NJ) at 39°C with moderate agitation for 48 h. Cumulative gas production was measured from each flask every 15 min for 48 h. Upon termination, flasks were removed from the incubator and exposed to oxygen.

After the 48-h fermentation period, cumulative gas pressures were converted into moles of gas using the 'ideal' gas law (Ankom Technology Corp., 2014): $n = p (V/RT)$. In this formula, n is gas produced (mol), p is pressure (kPa), V is head-space volume in flasks (L), T is temperature (K), and R is the gas constant (8.314472 $L.kPa.K^{-1}.mol^{-1}$). Moles of gas were converted into volume of gas (mL) using Avogadro's law (Ankom Technology Corp.): Gas produced (mL) = $n \times 22.4 \times 1000$, where 22.4 is the volume occupied by 1 mole of gas at 39°C.

Final pH was recorded using a portable pH meter (Thermo Scientific Orion 3 Star Portable pH Meter, Waltham, MA). Four milliliters of fluid were obtained from each flask, combined with 1 mL of 25% (wt/vol) meta-phosphoric acid solution, and stored at -18°C until

VFA analysis. Remaining contents from each flask were poured into 19.0 × 12.7-cm aluminum pans and dried at 55ºC for 72 h to determine IVDMD.

Volatile fatty acid analyses

Samples for VFA analysis were frozen for at least 24 h, later thawed, homogenized, and centrifuged at 17,000 × g for 15 min. Supernatant was transferred into 12 mm × 32 mm gas chromatography (GC) vials, vortexed (Scientific Industries Vortex-Genie 2, Houston, TX), and VFA were measured using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a flame ionization detector and a 15 m × 0.53 mm × 0.5 µm Supelco 58812-011 Nukol column (Supelco columns; Sigma-Aldrich, St. Louis, MO). Hydrogen gas was the carrier at a flow rate of 40 mL/min. Initial oven temperature was 70ºC and increased 15ºC/min to 130ºC then increased by 60ºC/min until a final temperature of 220ºC was achieved and held for 2 min. Inlet and detector temperatures were 300ºC. Volatile fatty acids were quantified by comparing to known standards (Supelco Volatile Fatty Acid Standard Mix; Sigma-Aldrich, St. Louis, MO) containing acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate.

Statistical analyses

Data were analyzed using the MIXED procedure of SAS (Version 9.4). The model for *in vitro* VFA production, final pH, IVDMD, and gas production included fixed effect of Co, forage type, this interaction, and random effect of block (horse). The experimental unit was fermentation flask. Gas production analyses used the MIXED procedure of SAS and included a repeated measures statement for time of interaction. The model included fixed effects of Co concentration, forage type, time, and 2- and 3-way interactions between Co, time, and forage; and block (horse) as the random effect. The repeated statement included fermentation flask as the

subject, time, and compound symmetry was used as the covariance structure. The 101 Kenward-Rogers correction was applied for degrees of freedom estimation for all analyses. Significance was declared at $P < 0.05$, and a tendency was considered to be present when $0.05 < P < 0.10$. Differences among LSMEANS were determined using the PDiff option of SAS.

Results

There was no forage × Co interaction for terminal pH ($P = 0.86$), nor was it affected by forage type or Co concentration (Table 2.2; $P \geq 0.19$). There was no forage × Co interaction ($P = 0.17$) nor effect of Co on IVDMD ($P = 0.57$). *In vitro* DM disappearance was greater ($P < 0.0001$) in cultures containing alfalfa (29.2%) compared to those containing smooth bromegrass hay (19.4%). There was a forage × Co interaction in which gas production was greater in cultures containing alfalfa compared to smooth bromegrass hay, becoming more different as time progressed (Figure 2.1; $P < 0.01$). Gas production in cultures containing smooth bromegrass hay was greater in those containing 5 mg Co/kg substrate DM than 50 mg Co/kg substrate DM from h 41 to 48 ($P < 0.05$), however Co concentration did not have an effect on cultures containing alfalfa at any time point ($P > 0.10$). There were no Co × time nor forage × Co × time interactions for gas production ($P = 1.00$).

There was no forage × Co interaction ($P > 0.10$) and no effect of Co on total VFA, propionate, butyrate, valerate, isobutyrate, or isovalerate (Table 2.3; $P \geq 0.32$). There was an effect of forage in which cultures containing alfalfa had greater concentrations of total VFA, acetate, propionate, butyrate, isobutyrate, and isovalerate ($P < 0.05$). There also was a linear forage × cobalt interaction ($P = 0.05$) in acetate:propionate (A:P), in which increasing Co resulted in increased A:P in cultures containing alfalfa while increasing Co concentration resulted in decreased A:P in cultures containing smooth bromegrass hay. Acetate:propionate was

greater in cultures containing alfalfa and 50 mg Co/kg substrate DM compared to 5 mg Co/kg substrate DM ($P < 0.05$), however there was no effect of Co on cultures containing smooth bromegrass hay. Molar percentage of acetate was greater in cultures containing alfalfa and 0.5 or 50 mg Co/kg substrate DM compared to 5 mg Co/kg substrate DM ($P < 0.05$). Molar percentage of propionate was greater in cultures containing smooth bromegrass hay and 50 mg Co/kg substrate DM compared to 5 mg Co/kg substrate DM ($P < 0.05$). There were no differences observed in molar percentages of butyrate, valerate, isobutyrate, and isovalerate ($P > 0.10$).

Discussion

This is the first published experiment to utilize equine cecal fluid as an inoculum to determine effects of Co chloride on IVDMD, gas production, pH, and VFA. Cobalt supplementation did not affect IVDMD or pH in equine cecal cultures containing alfalfa nor smooth bromegrass hay. This is in contrast with observations of Lopez-Guisa and Satter (1992), who observed increased DM disappearance in heifers consuming a low quality forage diet supplemented with Co and Cu above NRC requirements. Because both minerals were supplemented together, this creates confounded results and therefore it is not possible to determine if Co alone would have a similar effect on DM disappearance. In a similar study, cows consuming low quality forage experienced greater *in situ* NDF and OM disappearance after 96 h in response to Co supplementation (Waterman et al., 2017). The current experiment concluded after 48 h, which may have been inadequate time to observe effects on IVDMD. Aside from the fact that higher quality hay was used in the current experiment (greater CP, and lesser NDF and ADF), the alfalfa hay used as a substrate contained 0.33 mg Co/kg DM and the smooth bromegrass hay contained 0.12 mg Co/kg substrate DM. Consequently, the hay utilized likely provided adequate Co for microorganisms; thus, supplemental Co beyond this did not enhance

fermentation. In studies conducted by Hubbert et al. (1956) and Salsbury et al. (1953), cellulose digestion decreased in cultures using a ruminant inoculum containing supplemental Co. They suggested that if Co is supplied in sufficient amounts within substrates, then excess Co likely would be detrimental. We were unable to verify this conclusion, as supplemental Co did not appear to be detrimental, as demonstrated by no changes in IVDMD or total VFA concentrations in response to supplementation. Because cumulative gas production was greater in cultures containing smooth bromegrass hay with 5 mg Co/kg substrate DM compared to 50 mg Co/kg substrate DM, it appears that in this forage type, 5 mg Co/kg substrate DM was most favorable for fermentation. It is probable that because alfalfa provided more Co, supplemental Co was unnecessary and therefore did not confer any detectible benefits to fermentation.

Vitamin B_{12} plays an important role in the production of propionate. When *Prevotella ruminicola* 23, a bacteria found in the rumen, was grown in the absence of vitamin B_{12}, acetate and succinate were the predominant VFA produced; however, in the presence of vitamin B_{12}, propionate concentration increased (Strobel, 1992). Thus, it has been suggested that vitamin B_{12} is required by bacteria to convert succinate to propionate. In the current study, molar proportion of propionate was greater in cultures containing smooth bromegrass hay and 50 mg Co/kg substrate DM compared to 5 mg Co/kg substrate DM. Because no values were different than baseline (0 mg Co/kg substrate DM), propionate was most likely unaffected by Co in these cultures. Differences detected in molar proportions, because they are expressed as a percentage of total VFA, were likely due to shifts in total VFA rather than propionate concentrations. Tiffany et al. (2006) added Co to a continuous culture of bovine ruminal contents at 0, 0.05, 0.10, and 1.0 mg/kg DM and noted no change in molar proportions of acetate, propionate, or A:P; however, molar proportions of butyrate, valerate, and isovalerate increased with

supplemental Co. Though these results differ from the current study, it may be due to their utilization of concentrate instead of forage substrate. It appears that Co was most likely provided in adequate amounts by the substrates utilized and therefore supplemental Co did not affect fermentation by equine cecal microorganisms, as measured by gas production, pH, VFA concentration, and IVDMD.

Summary

Cobalt is required by microorganisms to synthesize vitamin B_{12}. Vitamin B_{12} is important in many biochemical pathways. Pertinent to this experiment, it is involved in intermediate steps in the production of propionate (Strobel, 1992). It is also involved in energy metabolism; therefore a decrease in Co, resulting in a vitamin B_{12} deficient animal, leads to decreased growth and general unthriftiness (Nasser, 2010). It has been suggested that Co may increase fiber digestibility because of divalent cation activity (Nasser, 2010; Waterman et al., 2017); however, this has not been established in horses. We evaluated effects of Co concentration on IVDMD, gas production, and VFA concentrations in cultures of mixed cecal microorganisms containing alfalfa or smooth bromegrass hay. Fiber digestibility was not enhanced by Co supplementation *in vitro*. It is likely that the amount of Co provided by the substrates was sufficient for microbial function, and, therefore, supplemental Co did not have an effect. Greater IVDMD, gas production, and VFA concentrations in cultures containing alfalfa compared to smooth bromegrass hay provides evidence that alfalfa has greater digestibility *in vitro*. Supplemental Co provided only limited differences in the response variables measured, thus it was not possible to identify any clear trends in the effect of Co on equine cecal cultures fed alfalfa or smooth bromegrass hay.

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Table 2.1. Proximate analysis of alfalfa and smooth bromegrass hays fed to equine cecal cultures

DM basis	Alfalfa hay	Smooth bromegrass hay
DM, %	90.4	92.2
NDF, %	39.7	63.3
ADF, %	31.4	35.6
CP, %	22.4	9.6
EE, %	2.2	2.7
Non-structural carbohydrates, %	25.5	17.7
Water-soluble carbohydrates, %	5.3	11.3
Ether soluble carbohydrates, %	2.9	5.9
Ash, %	10.24	6.7
Co, mg/kg	0.33	0.12
DE, Mcal/kg	2.43	2.12

Table 2.2. Terminal pH and IVDMD in equine cecal cultures fed alfalfa or smooth bromegrass hay, supplemented with 0.0, 0.5, 5.0, 25.0, or 50.0 mg Co/kg substrate DM

	Alfalfa hay					Smooth bromegrass hay								
	Cobalt, mg/kg substrate DM											P-value		
Item	**0**	**0.5**	**5**	**25**	**50**	**0**	**0.5**	**5**	**25**	**50**	**SEM**	**Forage**	**Cobalt**	**F×C[1]**
Terminal pH	6.39	6.32	6.35	6.36	6.35	6.29	6.31	6.28	6.27	6.35	0.06	0.19	0.95	0.86
IVDMD	27.75	30.53	29.62	29.88	28.38	21.15	17.60	20.15	20.12	17.95	0.01	< 0.01	0.57	0.17

[1]Forage × Co interaction

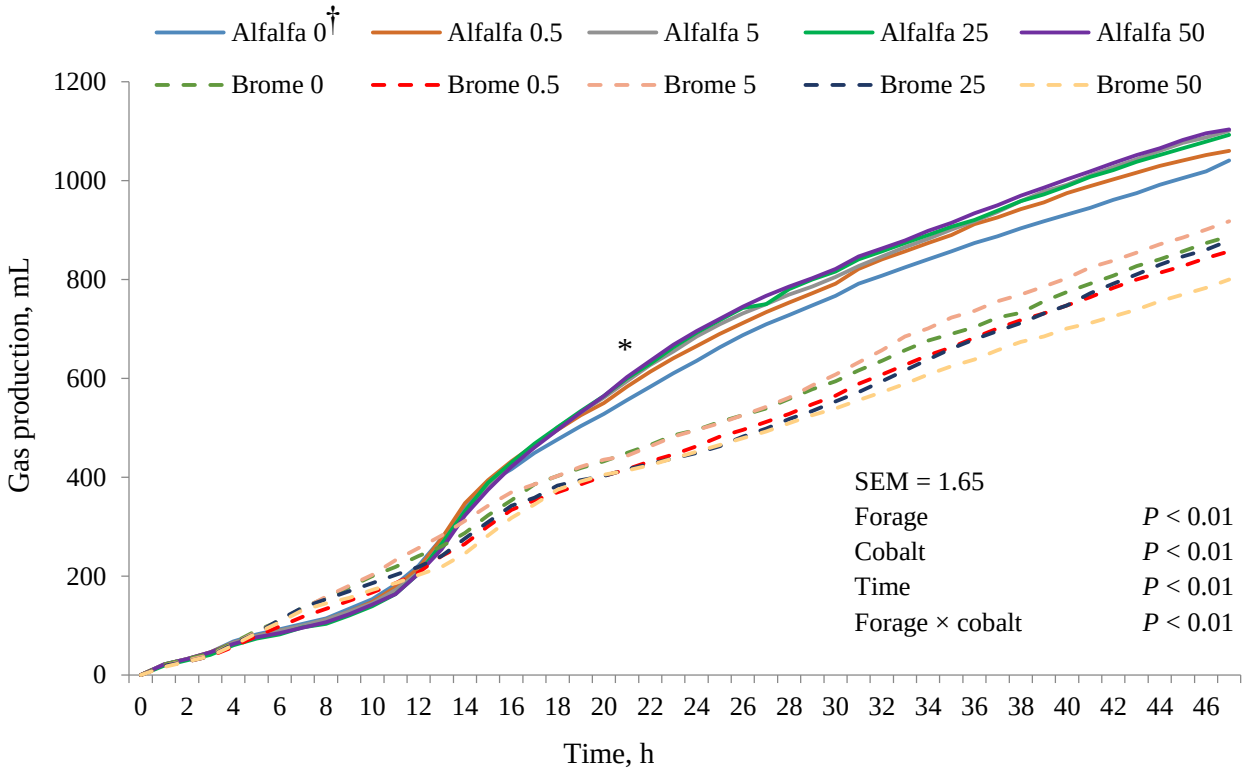

Figure 2.1. Gas production in fermentation flasks containing 0.0, 0.5, 5.0, 25.0, or 50.0 mg Co/kg substrate DM as Co chloride, 5 g substrate DM as alfalfa or smooth bromegrass hay, 10 mL strained cecal fluid, 140 mL McDougall's buffer and incubated at 39°C for 48 h. Gas production was not affected by Co over time ($P = 1.00$) and there was no forage × Co × time interaction ($P = 1.00$).

*Indicates a difference in gas production in cultures containing alfalfa compared to smooth bromegrass hay from h 21 to 48 ($P < 0.01$)

†Legend indicates alfalfa or smooth bromegrass hay (brome) with cobalt concentration (0.0, 0.5, 5.0, 25.0, or 50.0 mg Co/kg substrate DM)

Table 2.3. Volatile fatty acid (VFA) concentrations in equine cecal cultures fed alfalfa and smooth bromegrass hay, supplemented with 0.0, 0.5, 5.0, 25.0, or 50.0 mg Co/kg substrate DM

	Alfalfa					Smooth bromegrass hay						P-value		
Item	**Cobalt, mg/kg substrate DM**													
VFA, mmol	**0**	**0.5**	**5**	**25**	**50**	**0**	**0.5**	**5**	**25**	**50**	**SEM**	**Forage**	**Cobalt**	**F×C[1]**
Total	20.23	19.83	21.30	21.55	22.04	15.80	15.12	16.15	15.02	14.98	1.14	< 0.01	0.77	0.63
Acetate	1.87	1.86	1.94	1.99	2.08	1.39	1.35	1.45	1.33	1.31	0.11	< 0.01	0.83	0.47
Propionate	0.77	0.74	0.82	0.82	0.80	0.66	0.61	0.64	0.62	0.64	0.05	< 0.01	0.76	0.84
Acetate:propionate	2.49^{ab}	2.57^{ab}	2.40^{b}	2.43^{ab}	2.62^{a}	2.16	2.22	2.25	2.14	2.07	0.15	< 0.01	0.70	0.11*
Butyrate	0.27	0.24	0.28	0.27	0.28	0.25	0.24	0.26	0.23	0.22	0.03	0.05	0.75	0.67
Valerate	0.03	0.03	0.04	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.007	0.29	0.74	0.65
Isobutyrate	0.04	0.04	0.04	0.04	0.04	0.02	0.02	0.02	0.02	0.02	0.002	< 0.01	0.62	0.88
Isovalerate	0.04	0.04	0.05	0.04	0.04	0.02	0.02	0.02	0.02	0.02	0.003	< 0.01	0.84	0.85
VFA, mmol/total mmol														
Acetate	61.49^{ab}	62.86^{a}	60.90^{b}	61.49^{ab}	62.79^{a}	58.84	59.40	59.33	58.95	58.50	0.97	< 0.01	0.32	0.17
Propionate	25.10	24.92	25.75	25.54	24.23	27.78^{ab}	27.01^{ab}	26.70^{a}	27.97^{ab}	28.56^{b}	1.21	< 0.01	0.77	0.09*
Butyrate	8.85	7.90	8.74	8.39	8.66	10.44	10.36	10.83	9.94	9.58	0.85	< 0.01	0.53	0.62
Valerate	1.20	1.13	1.21	1.21	1.15	1.25	1.48	1.41	1.30	1.47	0.30	0.13	0.99	0.94
Isobutyrate	1.39	1.33	1.39	1.38	1.30	0.81	0.81	0.76	0.86	0.89	0.05	< 0.01	0.74	0.12
Isovalerate	1.41	1.38	1.43	1.43	1.31	0.88	0.89	0.93	0.89	0.87	0.09	< 0.01	0.69	0.96

[1]Forage × Co interaction
[*]Linear Co × forage interaction ($P < 0.05$)
[ab]Means without a common superscript letter are different ($P < 0.05$)

Chapter 3 - Digestibility of diets containing Enertia s/f® and soybean oil in equines

L. K. Fehlberg, J. M. Lattimer, C. I. Vahl, J. S. Drouillard, and T. L. Douthit

Kansas State University, Manhattan, KS 66506

Abstract

Fat supplementation, particularly in the form of vegetable oil, is a safe way to increase energy density of equine diets. Calcium salts of fatty acids (CSFA) are frequently fed to ruminants, but their efficacy in equine diets is unknown. The purpose of this study was to compare Enertia s/f®, a proprietary CSFA, and soybean oil with respect to impact on apparent total tract nutrient digestion and cecal fermentation parameters in horses. Eight cecally cannulated Quarter Horses were used in a crossover design in which horses consumed a diet for 28 d consisting of 1.5% BW (as-fed) smooth bromegrass hay and 0.5% BW (as-fed) of pelleted concentrate containing 4.9% Enertia s/f®(E) or 4.1% soybean oil (SB). Fecal samples were collected over a 3-d period and analyzed for apparent total tract digestibilities of DM, NDF, ADF, CP, ether extract (EE), and GE using acid-detergent insoluble ash as an internal marker. Cecal digesta samples were obtained 0, 2, 4, 6, 8, 10, and 12 h following the morning meal and analyzed for pH, and concentrations of VFA and long chain fatty acid (LCFA). Serum was collected following fecal and cecal collections and a 16-h fast and analyzed for triglycerides and cholesterol. Apparent total tract digestibilities of DM, NDF, ADF, CP, EE, and GE were unaffected by lipid source ($P > 0.10$). Serum triglycerides tended to be greater in horses consuming E compared to SB ($P = 0.10$); however, serum cholesterol was not different ($P = 0.45$). Mean cecal pH in all horses regardless of treatment decreased below baseline during h 2, 4, 6, and 8 ($P < 0.01$) then increased to a level similar to baseline. Cecal concentrations of total VFA tended to be greater in horses consuming SB compared to E at h 2 ($P = 0.07$). Cecal concentrations of acetate, propionate, butyrate, and acetate:propionate increased above baseline during h 4, 6, and 8 ($P < 0.001$) and returned to baseline values at h 10 and 12 ($P > 0.10$). Cecal propionate concentrations were less at h 2 for horses fed E compared to horses fed SB (P = 0.03).

A treatment by time interaction was detected for total cecal LCFA concentration ($P < 0.01$); LCFA concentration was greater at h 2 for horses consuming E compared to horses fed SB ($P = 0.02$). Soybean oil and the Enertia s/f® can be added to diets of horses to increase energy density and have similar effects on digestion of DM, NDF, ADF, GE, EE, and CP.

Introduction

The gastrointestinal tract (GIT) of horses evolved to accommodate continuous grazing. A complex hindgut allows for degradation of structural carbohydrates, including cellulose, hemicellulose, and soluble fiber through microbial fermentation. The end products of fermentation are predominately acetate, propionate, and butyrate which can contribute up to 80% of the horse's energy requirement (Al Jassim and Andrews, 2009). However, many performance horses have a greater energy requirement than what can be met by structural carbohydrates alone; therefore, grain often is added to the diet to increase energy density. Grains contain nonstructural carbohydrates (NSC) that can be digested enzymatically in the small intestine, providing energy directly to the horse in the form of glucose (Julliand et al., 2006). If the amount of grain consumed surpasses the digestive capacity of the small intestine (3.4 g/kg BW; Potter et al., 1992), starch and other rapidly fermentable carbohydrates may arrive in the cecum and colon and can cause digestive upset via disruption of the microflora (Julliand et al., 2006).

Fat can be used as a replacement for NSC as a means to safely increase energy density in equine diets (Kane et al., 1979) while potentially decreasing DM consumption (Hintz et al., 1978). In ruminants, greater than 6% ether extract (EE) in the diet depresses fiber fermentation (Palmquist and Jenkins, 1980; Doreau and Chilliard, 1997; Alexander et al., 2002). Lipids have a direct antimicrobial effect by binding to microbial lipid bilayers and negatively altering plasma membrane potential, thereby inhibiting growth (Jenkins, 1993). It also has been proposed that

lipids physically coat feed particles, inhibiting microbial attachment that is necessary for cell wall digestion (Cheng et al., 1991). Like NSC, fat can be fed at a level that exceeds small intestinal absorptive capacity and results in passage into the hindgut, although the level at which this occurs is disputed and relatively undefined (Jansen et al., 2000; Jansen et al., 2002; Sales and Homolka, 2011). Assuming a similar microbial environment in the cecum and the rumen (Al Jassim and Andrews, 2009), it is probable that if enough fat reaches the cecum, fiber digestion is inhibited. Although others have not reported decreased nutrient digestibilities with fat supplementation in horses (Kane et al., 1979; Julen et al., 1995; Bush et al., 2001), amount and type of fat consumed would have a large impact on findings.

In dairy cattle, feeding fats and oils can have adverse effects on fiber digestibility, thus efforts have been made to combat this issue (Palmquist and Jenkins, 1980). Deleterious effects of fat on fiber digestibility in the rumen require the presence of a free carboxyl group on fatty acids; therefore, it was proposed that addition of a different functional group may decrease lipid attachment to microflora and feed particles (Czerkawski et al., 1966). Divalent minerals, such as Ca, react with FFA to form insoluble salts that are mostly inert in the rumen and therefore do not affect fiber digestibility (Jenkins and Palmquist, 1984). Most fatty acids dissociate from a Ca attachment at pH < 5.5, and therefore will become FFA in the abomasum and small intestine (Sukhija and Palmquist, 1990). Once in the small intestine, FFA are readily absorbed and utilized by the host animal at a rate comparable to other fat sources (Baumann et al., 2003). In monogastrics, any ingested calcium salts of fatty acids (CSFA) would directly enter the stomach and small intestine. Theoretically CSFA would dissociate in the small intestine and be absorbed, similarly to what is observed in the bovine after CSFA exit the rumen; however, this has yet to be validated in the horse.

While the amount of liquid fat added to diets often is limited by pellet durability and palatability, CSFA may be a viable option to increase fat inclusion because they are a dry ingredient and can be added pre-pelleting (Casals et al., 2006). In concentrates, an inclusion rate greater than 2 to 3% added fat may result in a friable pellet (Partridge et al., 1986). Post-pellet liquid application (PPLA) of fat is a feasible alternative, but no more than 15% fat may be added to the diet because of decreased palatability in horses (Hallebeek and Beynen, 2002). Also, PPLA is not available in many commercial feed mills. There have been claims from commercial feed manufacturers that inclusion of CSFA can increase pellet durability compared to traditional fat supplements and that they do not result in decreased palatability.

Digestibility of CSFA in horses is unknown, so the purpose of this study was to compare the influence of Enertia s/f® (Archers Daniel Midland Alliance Nutrition, Quincy, IL), a proprietary CSFA, and soybean oil, a common source of supplemental fat in equine diets, on digestibility of DM, OM, NDF, ADF, CP, EE, and GE in horses.

Materials and methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University. Eight Quarter Horses (4 mares, 4 geldings) with a mean BW of 535 kg were used. All horses were fitted with a permanent cecal cannula (flexible rumen cannula, #7c; 3.8 cm center diameter and 8.9 cm wall thickness; Bar Diamond, Parma, ID; Beard et al., 2011). Horses were housed in an enclosed facility in randomly assigned individual stalls (3.05 m × 3.66 m) bedded with pine shavings and had *ad libitum* access to water. All horses received turnout from 5 to 6 h/d in a dry lot. There were 4 horses per dry lot to accommodate socialization and exercise.

Experimental design

Horses were blocked by weight and gender and assigned to 1 of 2 initial treatments in a crossover design. Treatments were formulated to be isocaloric and isonitrogenous and consisted of either 4.1% soybean oil (SB) or 4.9% Enertia s/f® (E), which were provided in a pelleted concentrate. Enertia s/f®, which had less crude fat (84% EE), was added at a level to bring crude fat in both concentrates to 4.1%. Diets consisted of 1.5% BW smooth bromegrass hay and 0.5% BW pelleted concentrate (as-fed basis), divided equally between 2 meals fed at 0700 and 1900 h (Tables 3.1, 3.2, and 3.3). Horses were maintained on their assigned treatment diets for a 28-d acclimation period followed by a 5-d collection period. Horses then were crossed over to the opposite treatment and the same feeding regimen was repeated.

Sample collection

Cecal samples from all horses were collected 0, 2, 4, 6, 8, 10, and 12 h following the morning feeding on d 29 and 62. Approximately 50 mL of cecal contents were obtained through cannulae via gravity flow, strained through 4 layers of cheesecloth and collected into a container (Specimen Storage Containers, #14955117A, Fisher Scientific, Pittsburg, PA). Immediately after collection, pH of strained cecal fluid was measured using a portable pH meter (Accumet Portable pH Meter AP62, Fisher Scientific, Pittsburg, PA). A 1-mL aliquot of strained cecal fluid from each horse for each time period was transferred in duplicate into microcentrifuge tubes. Deproteinization was achieved with 250 µL of 25% (wt/vol) meta-phosphoric acid solution and samples were stored at -18°C until VFA analysis. Twenty-five milliliters of remaining strained cecal fluid were transferred to a 25 mm × 150 mm glass screw top tube with a Teflon lined cap and stored at -18°C until analysis of long chain fatty acids (LCFA; Fisher Scientific, Pittsburg, PA). Following final cecal collection on a given day, there was a 12-h buffer period where no

collections were obtained. On d 30 and 63, shavings were removed from all stalls. For the next 3 d, feces were collected directly off the stall floors every 6 h. Feces contaminated with cecal fluid or urine were discarded. Each day, feces from each horse were homogenized and a sample was transferred to a 1-gallon Ziplock bag. Samples from each day from each horse were homogenized together into 1 sample (approximately 2.3 kg) and stored at -18°C for further analysis of DM, OM, NDF, ADF, CP, EE, and GE.

Following fecal collections, horses were fasted for 16 h and approximately 15 to 20 mL of whole blood were obtained via jugular venipuncture using a 38.1-mm 20-gauge needle. Blood was collected into a 10 mL non-heparinized red top Vacutainer serum tube (#366430, Becton, Dickson and Company, Franklin Lakes, NJ), left at room temperature until a clot was identified, and centrifuged at 1,400 × g at room temperature for 20 min. Serum was removed using a transfer pipette and approximately 1 mL was deposited into 1.5-mL microcentrifuge tubes in quadruplicate. Tubes were stored at -18°C for future analysis of serum triglycerides (TG) and cholesterol.

Laboratory analyses

Fecal samples were dried at 55°C in a forced air oven for 48 h and ground using a Wiley mill (1 mm screen; Thomas Scientific, Philadelphia, PA). Samples from the hay and concentrates also were ground through a 1-mm screen using a Wiley mill. Subsamples (0.5 g) of feedstuffs and feces were dried at 105°C in a forced air oven to determine DM. Separate 3-g subsamples of hay, concentrate, and feces were combusted in a muffle oven at 450°C for 8 h for quantification of ash and OM (Undersander et al., 1993). Dried feces, hay, and concentrate were analyzed for NDF and ADF using a batch processor while following the procedure outlined by Goering and van Soest (1970; Ankom Technology Corp., Fairport, NY). Following ADF analysis, bags were

secured in tins, weighed, and combusted in a muffle oven at 450°C for 8 h to determine acid detergent insoluble ash (ADIA; Kanani et al., 2014). Acid detergent insoluble ash was calculated using the following equation:

$$ADIA\ (\%) = 100 * \left[\frac{Sample\ weight\ after\ combustion\ (g)}{Initial\ weight\ of\ sample\ (g)}\right]$$

Adiabatic bomb calorimetry was used to determine gross energy in dried feed and feces (AOAC, 1990). The crude fat content of feed and feces was determined using the EE technique with an acid hydrolysis where hydrochloric acid was utilized to liberate fat from the calcium (AOAC, 2012a). Crude protein in feed and feces was determined using the combustion method to determine N content (g) and multiplying N by 6.25 (AOAC, 2012b). Acid detergent insoluble ash was used as an internal marker for the calculation of the digestibility of DM, NDF, ADF, CP, EE, and GE. Digestibilities were calculated using the following equation:

$$Digestibility\ (\%) = 100 * [1 - \left(\frac{nutrient\ in\ feces\ (\%) * ADIA\ in\ feed\ (\%)}{nutrient\ in\ feed\ (\%) * ADIA\ in\ feces\ (\%)}\right)]$$

Total dietary compositions were determined using this equation:

$$Nutrient\ in\ diet\ (\%) = [nutrient\ in\ hay\ (\%) * 0.75] + [nutrient\ in\ grain\ (\%) * 0.25]$$

Following deproteinization of VFA, strained cecal samples were frozen for at least 24 h, later thawed, homogenized, and centrifuged at 17,000 × g for 15 min. The aqueous supernatant was transferred into 12 mm × 32 mm gas chromatography (GC) vials, vortexed (Scientific Industries Vortex-Genie 2, Houston, TX), and analyses were completed using a packed column (6’ × ¼”, 4 mm ID glass, packed with GP 10% SP-1200, 1% H_3PO_4; Supelco #1-1965; Agilent Technologies, Santa Clara, CA) with a flame ionization detector (compressed air set at 200 mL/min and H_2 set at 20 mL/min). Nitrogen was used as the carrier gas with a flow rate of 60 mL/min. The detector and injector were set at 250°C and the column was at a constant

temperature of 130°C. Volatile fatty acids were quantified by comparing to known standards (Supelco Volatile Fatty Acid Standard Mix; Sigma-Aldrich, St. Louis, MO) containing acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate.

For LCFA analysis (C8 to C24) cecal samples were removed from the -18°C freezer and transferred directly to a precooled drying chamber of a lyophilizer (SP Scientific, Warminster, PA; Genesis Model 35EL) programmed with vacuum of 100 mTorr, shelf temperature of -10°C, and condenser chamber temperature of -70°C. After 48 h, samples were removed and interesterified to create methyl-esters (Sulchija et al., 1988). Interesterified samples were centrifuged at 500 × g for 10 min and the upper organic solvent layer was transferred to screw top 12 mm × 32 mm GC vials for gas chromatography. A SP-2560 capillary column (100 m × 0.25 mm with a 0.2 μm film; Agilent and J&W columns, Santa Clara, CA) with a flame ionization detector was used. Hydrogen was used as the carrier gas with a flow rate of 1 mL/min and a split ratio of 1:100. The injection and detector temperatures were set at 250°C. The initial oven temperature was 140°C with a temperature increase rate of 2°C/min until 200°C was reached, followed by an increase of 4°C/min until a final oven temperature of 245°C was achieved. Volatile fatty acids were quantified by comparing to known standards (Supelco 37 FAME mix #47885-U; Sigma-Aldrich, St. Louis, MO) containing C6:0, C8:0, C10:0, C11:0, C12:0, C14:0, C14:1, C15:0, C15:1, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1n9t, C18:1n10t, C18:1n11t, C18:1n9c, C18:1n11c, C18:2n6t, C18:2n6c, C20:0, C18:3n6, C20:1, C18:3n3, CLA 9c11t, C21:0, CLA 10t12c, CLA 9c11c, CLA 9t11t, C20:2, C22:0, C20:3n6, C22:1n9, C20:3n3, C20:4n6, C23:0, C22:2, C24:0, C20:5n3, C24:1, C22:5n3, and C22:6n3.

Serum triglyceride (TG) and cholesterol were measured at the Animal Health and Diagnostic Center at Cornell University using the triglyceride GPO-PAP method and cholesterol

CHOD-PAP method, respectively (Roche ModP, Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim; Roche Diagnostics, Indianapolis, IN).

Statistical analyses

Data were analyzed using the MIXED procedure of SAS (Version 9.4). For digestibility variables (DM, NDF, ADF, CP, EE and GE) and serum concentrations of TG and cholesterol, the model included fixed effect of treatment and the random effect of replicate (horse) and period to determine the least-squares means (LSMEANS) and experimental unit was horse. For pH, VFA, and LCFA, a repeated measures statement was utilized and the model included fixed effect of treatment and treatment by hour interaction and random effect of replicate (horse) and period to determine the LSMEANS. Linear and quadratic contrasts averaged over treatments for 7 equally spaced time points were included to determine hour trends. Repeated statement included horse as the subject, time, and autoregressive(1) as the covariance structure. The 101 Kenward-Rogers correction was applied for degrees of freedom estimation for all analyses. Significance was declared at $P < 0.05$, and a tendency was considered to be present when $0.05 < P < 0.10$. Differences among LSMEANS were determined using the PDiff option of SAS. A Bonferonni adjustment was used to correct for multiplicity.

Results

Body weights were not different at initiation nor conclusion of the project, and they were not affected by treatment ($P > 0.10$). Dry matter intake was not different between horses on either treatment ($P > 0.10$; Table 3.4). Apparent total tract digestibilities of DM, NDF, ADF, CP, EE, and GE were unaffected by lipid source ($P > 0.10$). Concentrations of cholesterol in the serum were not different between horses fed either diet ($P > 0.10$). In contrast, concentrations of

TG in the serum tended to be greater in horses fed the E diet (22.13 mg/dL) compared to those on the SB diet (19.00 mg/dL; $P = 0.09$; Table 3.5).

There was an effect of time on cecal pH ($P < 0.01$; Figure 3.1). Mean cecal pH, regardless of lipid source, decreased below baseline (h 0, 7.14) at h 2, 4, 6, and 8 (6.88, 6.83, 6.86, 6.95, respectively; $P < 0.01$) then increased to a level similar to baseline pH at h 10 and 12 (7.04, 7.18, respectively; $P > 0.10$). In horses consuming the SB diet, cecal pH decreased below baseline (h 0, 7.13) at h 2, 4, and 6 (6.80, 6.85, 6.83, respectively; $P < 0.01$) and then increased to a level similar to baseline at h 8, 10, and 12 (6.90, 7.07, 7.14, respectively; $P > 0.10$). Cecal pH of horses consuming the E diet was below baseline (h 0, 7.15) at h 4 and 6 (6.80, 6.89, respectively; $P < 0.01$) and increased to a level similar to baseline at h 8, 10, and 12 (7.00, 7.02, 7.21, respectively; $P > 0.10$). There were no treatment differences in cecal pH ($P > 0.10$).

Total cecal VFA concentrations were quadratically affected by time ($P < 0.001$; Table 3.6), with mean cecal VFA concentrations increased above baseline (h 0, 41.17 mM) during h 4, 6, and 8 (56.79 mM, 56.75 mM, and 53.43 mM, respectively; $P < 0.001$). Mean total cecal VFA concentration returned to baseline values at h 12 (40.75 mM; $P > 0.10$). Total cecal VFA concentrations tended to be greater in horses consuming the SB diet (51.25 mM) compared to horses consuming the E diet (37.02) at h 2 ($P = 0.07$). By h 4, total cecal VFA concentrations were not different in horses consuming the 2 diets and continued to be similar through h 12 ($P > 0.10$).

Cecal concentrations of acetate, propionate, butyrate, valerate ($P < 0.01$) and isobutyrate ($P = 0.02$) across all horses regardless of lipid source demonstrated a quadratic effect of time. Mean cecal acetate, propionate, and butyrate were increased from baseline (h 0) at h 4, 6, and 8 ($P < 0.05$). Cecal acetate concentration tended to be greater in horses consuming the SB diet

(38.05 mM) compared to the E diet (27.97 mM) at h 2 ($P = 0.08$). Horses consuming the E diet had less cecal propionate (6.53 mM) compared to horses consuming the SB diet (9.76 mM) at h 2 ($P = 0.04$). In all horses regardless of diet, mean cecal acetate:proprionate (A:P) was affected by time linearly ($P < 0.0001$), with the greatest ratio at h 2 (4.12) and the A:P at h 12 (3.68) was less than at h 2 and 4 (4.12 and 3.95, respectively; $P < 0.05$). In horses consuming the E diet, A:P increased above baseline (3.91) at h 2 (4.32, $P < 0.0001$), however horses consuming the 2 diets were not different at h 2 ($P > 0.10$).

A treatment × time interaction was observed in total cecal concentration of LCFA ($P = 0.0082$; Table 3.7), in which total cecal concentrations of LCFA were greater at h 2 in horses consuming the E diet (183.23 µg/mL) compared to horses consuming the SB diet (120.57 µg/mL; $P = 0.02$). There also was a treatment × time interaction identified in total cecal concentrations of saturated LCFA ($P = 0.02$). A tendency for increased cecal concentrations of saturated LCFA were detected in horses consuming the E diet compared to horses consuming the SB diet at h 2 (101.68 µg/mL and 70.95 µg/mL, respectively; $P = 0.07$) and at h 8 (68.19 µg/mL and 43.54 µg/mL respectively; $P = 0.07$). Cecal concentrations of unsaturated LCFA were also affected by a treatment × time interaction ($P < 0.0079$). In horses consuming the E diet, cecal unsaturated LCFA concentration (81.55 µg/mL) was greater than in horses consuming the SB diet (42.62 µg/mL) at h 2 ($P = 0.006$). There was a treatment × time interaction in concentrations of cecal palmitic acid (C16:0) in which concentrations were greater in horses consuming the E diet (65.49 µg/mL) compared to those consuming the SB diet (43.65 µg/mL; $P < 0.003$). A treatment × time interaction was also detected in oleic methyl ester (C18:1n9c; $P = 0.02$). Mean cecal C18:1n9c concentration was greatest at h 2 compared to all other time points (24.93 µg/mL; $P < 0.001$) in both treatment groups. Even so, at the same time point (h 2), horses

ingesting the E diet had increased cecal C18:1n9c concentration (32.10 µg/mL) above that of horses consuming the SB diet (17.76 µg/mL; $P = 0.004$). A treatment × time interaction was also observed in cecal linoleic methyl ester (C18:2n6c; $P = 0.03$), demonstrated by a greater mean concentration in horses ingesting the E diet (21.18 µg/mL) compared to horses ingesting the SB diet (10.19 µg/mL; $P = 0.02$) at h 2. A treatment × time interaction was also observed in eicosapentoenoic acid (C22:5n3; $P = 0.006$), with increased cecal C22:5n3 concentration at h 12 in horses consuming the SB diet (0.83 µg/mL) compared to the E diet (0.34 µg/mL; $P = 0.01$).

Discussion

Proximate analysis of the experimental pelleted diets revealed that diets were not equal in EE content. Despite the greater inclusion level calculated to provide an isolipid diet, E concentrate had decreased EE content compared to SB concentrate. Decreased EE content in E concentrate could be attributed to improper mixing at the feed mill or incomplete dissociation of Ca from the fatty acids when using the acidified ether method during laboratory analysis (Cruywagen et al., 2000). If the differences in EE concentration were a product of incomplete recovery in the laboratory, then the values provided in the proximate analysis would have underestimated EE content and thus horses may have received the intended amount of fat.

This experiment was the first of its kind to evaluate the effect of CSFA on nutrient digestibility in the horse. In dairy cows supplemented with 4.5% added soy fatty acids or soy calcium soaps, no differences in DM, NDF, ADF, CP, nor crude fat digestibilities were detected (Jenkins and Palmquist, 1984). Furthermore, digestibilities of DM, ADF, and NDF in dairy cows fed varying fat sources added at 6.8%, including an animal-vegetable blend and a palm oil calcium soap, were not different (Palmquist, 1991). This experiment verifies that

supplementation with CSFA does not alter digestion of most basic nutrients in the horse either, despite differences in gastointestinal anatomy.

On d 17 of the experiment during period 1, a horse consuming the E diet presented with signs of colic. A veterinarian from the Veterinary Health Center at Kansas State University was consulted and a physical exam was completed. Large colon displacement was suspected, however vital signs were within normal limits (temperature: 98.7°F, heart rate: 24 beats per minute, respiratory rate: 16 breaths per minute). Mineral oil and electrolytes were mixed with 18.9 L of water and administered via nasogastric tube. Flunixin (700 mg), xylazine (200 mg) and butorphonal (5 mg) were administered intravenously and feed was withheld for 12 h. Hay was slowly reintroduced starting 12 h post colic and grain was reintroduced 24 h post colic. Because of hindgut disturbances that may have occurred, the acclimation period was extended by 1 wk to allow normal motility and fermentation to be restored. The acclimation period of period 2 was also extended 1 wk, to ensure both acclimation periods were 28 d. No other signs of colic were noted throughout the duration of the experiment. Following the final collection of blood and termination of the experiment, the same horse was found dead the following morning (12 h following the conclusion of the trial), taken to Veterinary Health Center at Kansas State University, and necropsied to determine cause of death. The horse had no previous history of colic and had been cannulated nearly 7 yr earlier. Necropsy technicians were unable to determine cause of death nor the cause of colic. Data obtained from this horse were not outliers compared to other horses on study; therefore, data collected from this horse were included in the statistical analyses.

Even though digestibilities of nutrients were not different between horses on the 2 diets in this study, the coefficients differ from what has been reported in other studies. While DM

digestibility may be slightly decreased in a cecally cannulated horse compared to an intact horse (Jansen et al., 2002), this most likely does not fully account for the large difference in DM digestibility noted in the current study compared with other studies. Delobel et al. (2008) included fat in diets of horses at a comparable level and observed a 20% greater DM digestibility than the current study; however, their forage:concentrate ratio was 50:50 whereas it was 75:25 in the current study. Increased bulk in the diet, such as from the long stemmed hay provided in the current experiment, increases rate of passage and decreases time available to digest feedstuffs (Stevens and Hume, 1996), which may help explain the decreased digestibility observed in the current experiment. In this study, OM digestibility was similar to DM digestibility, which is in accordance with what others have observed (Cuddeford and Hughes, 1990; Lindberg and Karlsson, 2001; Gatta et al., 2005). In the current study, NDF and ADF digestibilities were 20 to 30% less than what has been reported in the literature with similar fat supplementation levels in horses, although dietary NDF and ADF provided in previous work was less than that of the current study (Lindberg and Karlsson, 2001; Gatta et al., 2005; Delobel et al., 2008). The poor quality hay used in this experiment likely contributed to the decreased digestibility coefficients calculated.

In contrast to what was noted for DM, NDF, and ADF, digestibility of CP in the present study was greater than what has been reported in other studies (West and Hill, 1990; Miraglia et al., 1999; De Marco et al., 2012). However, Delobel et al. (2008) supplemented with 8% fat in a concentrate fed to horses at a 50:50 forage:concentrate ratio and noted a CP digestibility similar to that reported for the current study. It is difficult to compare digestibility of CP between studies because of the variability in protein sources and the differing amounts and forms of fat added. Digestibility of EE in the current study was similar to observations in previous experiments when

fat was supplemented to horses (Jansen et al., 2002; Gatta et al., 2005; Delobel et al., 2008). Initially fecal crude fat was analyzed in the current study using the EE method without acid hydrolysis. On second analysis, the crude fat content was determined using the EE method with an acid hydrolysis to liberate any Ca attachments that may still exist. Because both laboratory analyses yielded the same fecal EE content, it was determined that fats in the feces were not in the form of Ca soaps.

Serum was collected for the measurement of TG and cholesterol after a 16-h fast in order to obtain true basal values and decrease variability due to differences in nutrient absorption rates between animals (Davinder and Naugler, 2013). Others have reported that horses consuming soybean oil have decreased serum TG, and they have suggested this is due to increased activity of lipoprotein lipase (LPL; Orme et al., 1997; Geelen et al., 1999). Lipoprotein lipase activity increases with increasing lipid in the diet, thus TG are cleared from the blood at a quicker rate. If more SB was absorbed than E and stimulated more LPL activity, this may explain the tendency for horses consuming the SB diet to have decreased serum TG compared to those consuming the E diet. This hypothesis is supported by the fact that E contains a saturated palm sourced fat, which has decreased digestibility compared to soybean oil (Doreau and Chilliard, 1997). Alternatively, if less SB was absorbed, that too could provide a simple explanation for decreased TG. Concentrations of TG in the serum of horses fed either diet were comparable to those reported in an equine study using corn oil in exercising horses and are within the normal reference range for horses (O'Connor et al., 2007). Serum cholesterol concentrations were greater in the current study than those reported in horses in light to medium work supplemented with vegetable oils (Geelen et al., 1999; O'Connor et al., 2007), but similar to horses at maintenance (Siciliano and Wood, 1993).

Kronfeld et al. (2004) reported that fat digestibility in the horse should be near 100%, with the assumption that fat is completely digested in the small intestine. Certainly, many factors could impact fat digestibility, including DMI, total fat intake, and fat source. Meyer et al. (1997) found that preileal fat digestibility in horses fed a diet with 1 g added fat/ kg BW was between 73 and 86%, concluding that any undigested fat would enter the cecum. There was evidence of fat entering the cecum in the current study, as there were LCFA in cecal fluid of horses from both treatment groups, thus indicating digestion and absorption of fat in the small intestine is not complete when total dietary fat is approximately 4%. Increased concentration of LCFA in the cecum of horses consuming E compared to SB diet at h 2 is most likely due to decreased absorption in the small intestine. Perhaps there was insufficient dissociation of the Ca attachment from the LCFA that hindered absorption (Palmquist and Jenkins, 1980) or the greater concentration of palmitic acid in Enertia s/f® compared to soybean oil led to decreased prececal digestion (Doreau and Chilliard, 1997).

In ruminants, unsaturated LCFA depress fiber fermentation moreso than saturated LCFA (Jenkins, 1993). Because cecal unsaturated LCFA were elevated in horses consuming the E diet compared to horses consuming the SB diet at h 2, it is possible that fiber fermentation was depressed. Depressed fermentation when the first dietary fractions were reaching the hindgut is further validated by decreased total cecal VFA concentration in horses consuming the E diet compared to horses consuming the SB diet at h 2. However, because differences between treatments in cecal unsaturated LCFA and VFA concentrations were only noted at h 2, it is unlikely that any deleterious effects on fermentation were sustained. At h 4, dilution of LCFA by other substrates entering the cecum may have negated any depressive effects on fermentation.

This is further demonstrated by the lack of differences in NDF and ADF digestibilities between horses consuming either diet.

Summary

As horses are asked to perform as athletes, there is interest in increasing the energy density of their diets. Increasing starch intake has the potential to elicit hindgut disturbances and increase excitability, both of which are undesirable in performance horses. Recently, interest has shifted to increasing dietary fat as it does not cause cecal acidosis and has been shown to decrease reactivity; however, the amount total fat in a concentrate is limited by palatability and pellet quality. Enertia s/f® can potentially provide a means to add more fat pre-pelleting; but the effects of Enertia s/f® in horses were formerly unknown. Enertia s/f® does not appear to be absorbed in the foregut to the same extent as soybean oil. Additionally, inclusion of Enertia s/f® at this level may depress fermentative activity at 2 h following a morning meal, but it is insufficient to elicit deleterious effects on total tract digestibility of fiber in the diet. Total tract digestibilities reported in the current study demonstrate that Enertia s/f® can be included at 4% added fat in the concentrate of horses with similar effects on nutrient digestibility to those of soybean oil.

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Table 3.1. Proximate analysis (DM basis) of smooth bromegrass hay and pelleted concentrates containing soybean oil (SB) or Enertia s/f® (E)

Item	Smooth bromegrass hay[a]	SB concentrate[b]	E concentrate[c]
DM, %	92.2	89.6	90.1
NDF, %	63.3	37.9	39.8
ADF, %	35.6	22.3	23.0
CP, %	9.6	15.4	15.3
EE, %	2.7	7.9	6.8
Non-structural carbohydrates, %	17.7	--	--
Water-soluble carbohydrates, %	11.3	--	--
Ether soluble carbohydrates, %	5.9	--	--
Ash, %	6.7	--	--
DE (Mcal/kg)	2.12	3.04	2.93

[a]Fed 1.5 % BW (as-fed) to all horses
[b]Fed 0.5% of BW (as-fed) to horses in the SB treatment group
[c]Fed 0.5% of BW (as-fed) to horses in the E treatment group

Table 3.2. Composition (DM basis) of pelleted concentrates containing either soybean oil (SB) or Enertia s/f®(E)

Ingredient, %	SB concentrate	E concentrate
Corn	9.09	9.84
Soybean hulls	30.56	30.61
Alfalfa meal	10.50	10.50
Enertia s/f®	0.00	4.94
Soybean oil	4.17	0.00
Wheat middlings	36.73	36.25
Molasses	4.00	4.00
Soybean meal, 48%	2.13	2.15
Limestone	1.76	0.63
Sodium chloride	1.00	1.00
Copper sulfate	0.02	0.02
Zinc oxide	0.02	0.02
Vitamin A 30,000, IU/g	0.02	0.02

Table 3.3. Long chain fatty acid (LCFA) composition of smooth bromegrass hay and pelleted concentrates containing soybean oil (SB) or Enertia s/f® (E)

LCFA[1,2]	Smooth bromegrass hay	SB concentrate	E concentrate
C6:0	0.00	0.00	0.00
C8:0	0.12	0.01	0.05
C10:0	0.07	0.06	0.10
C11:0	0.00	0.00	0.00
C12:0	3.07	0.09	0.21
C14:0	1.23	0.12	0.81
C14:1	0.00	0.00	0.00
C15:0	0.25	0.07	0.11
C16:0	22.59	13.07	34.58
C16:1	0.75	0.18	0.25
C17:0	0.45	0.18	0.21
C18:0	2.10	3.34	4.01
C18:1n11t	0.13	0.00	0.00
C18:1n9c	3.79	19.93	28.78
C18:1n11c	0.53	1.41	0.98
C18:2n6t	0.44	0.03	0.05
C18:2n6c	19.18	52.24	24.83
C20:0	2.06	0.37	0.44
C18:3n3	35.88	7.32	2.92
CLA 9c11t	0.07	0.00	0.01
C21:0	0.19	0.05	0.04
C18:2n10t12c	0.00	0.00	0.01
C18:2n9c11c	0.00	0.05	0.00
C18:2n9t11t	0.18	0.11	0.22
C20:2	0.07	0.10	0.15
C22:0	2.44	0.37	0.24
C20:3n6	0.00	0.02	0.02
C22:1n9	0.26	0.07	0.10
C20:3n3	0.29	0.02	0.04
C20:4n6	0.00	0.00	0.00
C23:0	0.43	0.09	0.10
C22:2	0.16	0.01	0.02
C24:0	2.94	0.24	0.25
C24:1	0.00	0.05	0.07
C22:5n3	0.14	0.00	0.00
Total LCFA	100	100	100

[1]LCFA presented as a % of total LCFA

[2]Nomenclature of LCFA defined as number of carbons:number of double bonds and orientation of carbon atom on either side of the double bond described as t = *trans* or c = *cis*.

Table 3.4. Total tract digestibility coefficients and DMI of horses consuming diets supplemented with soybean oil (SB) or Enertia s/f® (E) for 29 d†

Item[1]	SB diet	E diet	SEM
DMI, %	94.83	94.49	1.80
DM, %	41.42	42.44	5.85
OM, %	41.13	42.11	3.95
NDF, %	19.64	21.12	3.47
ADF, %	18.48	19.54	2.18
CP, %	86.56	86.41	0.98
Ether extract, %	79.11	77.37	1.90
GE, %	39.29	39.90	5.22

[1]Digestibility coefficients presented on a percentage basis calculated using acid detergent insoluble ash as an internal marker

†Diet consisted of 1.5% BW smooth bromegrass hay (as-fed) and 0.5% BW pelleted concentrate (as-fed) containing either 4.1% SB or 4.9% E

Table 3.5. Serum[1] triglycerides and cholesterol in horses consuming diets supplemented with soybean oil (SB) or Enertia s/f® (E) for 32 d†

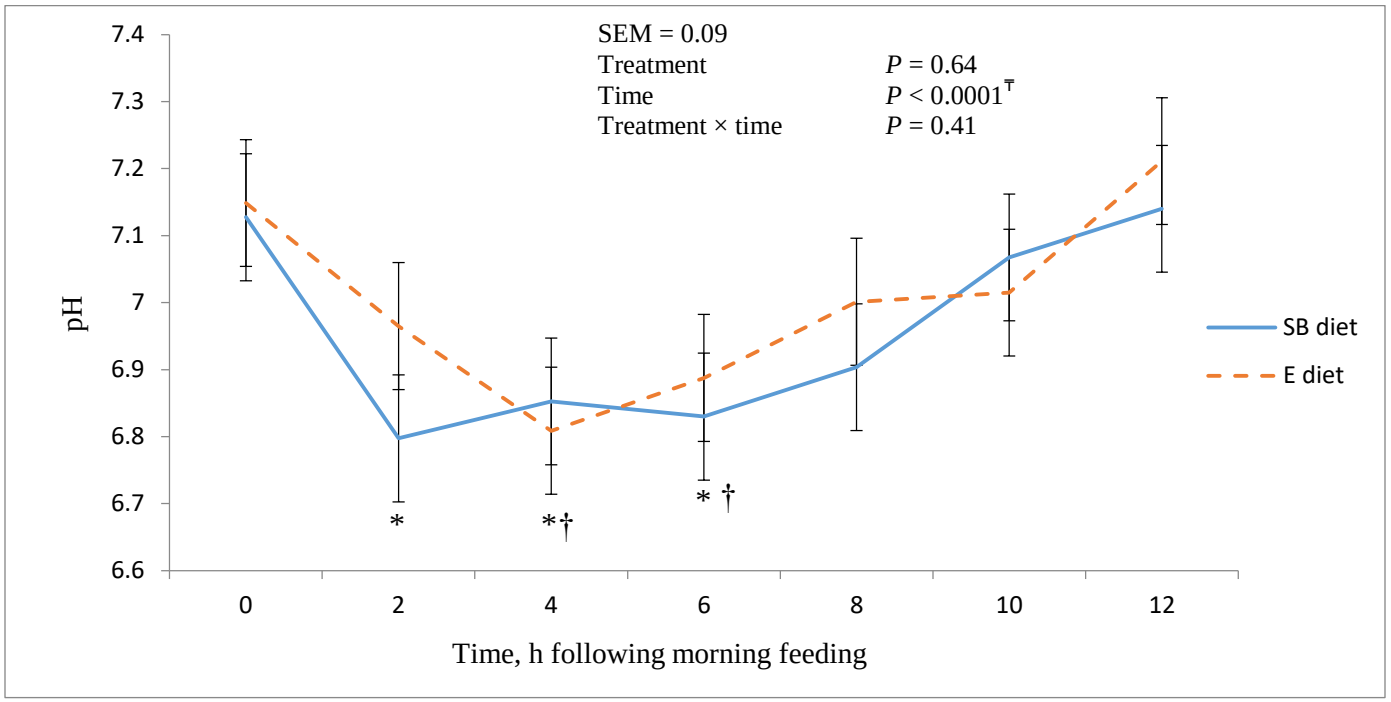
Item	SB diet	E diet	SEM
Serum triglycerides, mg/dL	19.0[A]	22.1[B]	1.91
Serum cholesterol, mg/dL	104.9	103.1	4.14

[1]Serum was collected following a 16-h fast

†Diet consisted of 1.5% BW smooth bromegrass hay (as-fed) and 0.5% BW pelleted concentrate (as-fed) containing 4.1% SB or 4.9% E

[A, B]Indicates a difference between treatments at $0.05 < P < 0.10$

Figure 3.1. Cecal pH[1] in horses consuming diets supplemented with soybean oil (SB) or Enertia s/f® (E) for 28 d‡



[1]Cecal pH recorded every 2 h for 12 h
‡Diet consisted of 1.5% BW smooth bromegrass hay (as-fed) and 0.5% BW pelleted concentrate (as-fed) containing 4.1% SB or 4.9% E
*Indicates cecal pH below baseline (h 0) value in horses consuming SB ($P < 0.05$)
†Indicates cecal pH below baseline (h 0) value in horses consuming E ($P < 0.05$)
Ŧ Indicates a quadratic effect of time averaged across treatments ($P < 0.05$)

Table 3.6. Cecal VFA concentration in horses consuming diets supplemented with soybean oil (SB) or Enertia s/f® (E) for 28 d†

VFA, mM	Time	SB diet	E diet	Overall means	SEM	Fixed effects[1] ($P < 0.05$)
Total VFA	0	43.06^{a}	39.28^{acd}	41.17^{ad}	3.86	T*
	2	51.25^{C,ab}	37.02^{D,acd}	44.14^{ac}		
	4	56.20^{b}	57.38^{b}	56.79^{b}		
	6	57.77^{b}	55.73^{be}	56.75^{b}		
	8	55.13^{bc}	51.72^{be}	53.43^{bc}		
	10	52.39^{ab}	45.53^{cde}	48.96^{c}		
	12	43.50^{ac}	38.05^{d}	40.78^{d}		
Acetate	0	31.70^{a}	29.19^{a}	30.45^{ad}	2.77	T*
	2	38.05^{C,a}	27.97^{D,a}	33.01^{ac}		
	4	41.64^{b}	43.10^{b}	42.37^{b}		
	6	42.71^{b}	41.11^{bc}	41.91^{b}		
	8	40.07^{a}	38.27^{bc}	39.17^{bc}		
	10	38.19^{a}	34.03^{acd}	36.11^{c}		
	12	31.35^{a}	28.21^{ad}	29.78^{d}		
Propionate	0	8.37^{a}	7.54^{a}	7.96^{ad}	0.86	T*
	2	9.76^{A,ab}	6.53^{B,a}	8.14^{a}		
	4	10.85^{ab}	10.80^{b}	10.82^{b}		
	6	11.37^{b}	11.18^{b}	11.27^{b}		
	8	11.17^{b}	10.23^{b}	10.70^{bc}		
	10	10.53^{ab}	8.67^{ab}	9.60^{c}		
	12	8.95^{ab}	7.45^{a}	8.20^{d}		
Acetate:propionate	0	3.78^{ab}	3.91^{ac}	3.84^{ac}	0.18	T‡
	2	3.93^{b}	4.32^{b}	4.12^{b}		
	4	3.87^{bc}	4.04^{c}	3.95^{a}		
	6	3.77^{ab}	3.72^{a}	3.75^{c}		
	8	3.57^{a}	3.76^{ac}	3.67^{c}		
	10	3.68^{ab}	3.96^{ac}	3.82^{ac}		
	12	3.56^{ac}	3.81^{ac}	3.68^{ac}		
Butyrate	0	2.48^{a}	2.14^{ac}	2.31^{a}	0.37	T*
	2	2.85^{ab}	2.02^{a}	2.43^{ac}		
	4	3.04^{ab}	2.97^{b}	3.00^{bc}		
	6	3.18^{ab}	3.03^{b}	3.11^{bc}		
	8	3.37^{b}	2.85^{c}	3.11^{bc}		
	10	3.13^{ab}	2.49^{a}	2.81^{c}		
	12	2.70^{ab}	2.05^{a}	2.37^{d}		

VFA, mM	Time	SB diet	E diet	Overall means	SEM	Fixed effects
Isobutyrate	0	0.21	0.17[ab]	0.19[a]	0.05	T*
	2	0.24	0.24[a]	0.24[b]		
	4	0.30	0.21[ab]	0.25[b]		
	6	0.21	0.17[ab]	0.19[a]		
	8	0.22	0.16[ab]	0.19[a]		
	10	0.22	0.13[b]	0.18[a]		
	12	0.21	0.16[ab]	0.18[a]		
Valerate	0	0.17[a]	0.13[ab]	0.15[a]	0.04	T[*]
	2	0.20[ab]	0.16[ab]	0.18[bc]		
	4	0.22[b]	0.18[a]	0.20[c]		
	6	0.18[ab]	0.16[ab]	0.17[bd]		
	8	0.18[ab]	0.15[ab]	0.16[bd]		
	10	0.19[ab]	0.13[ab]	0.16[bd]		
	12	0.17[ab]	0.11[b]	0.14[d]		
Isovalerate	0	0.13	0.11		0.03	
	2	0.15	0.09			
	4	0.15	0.11			
	6	0.13	0.09			
	8	0.13	0.07			
	10	1.14	0.08			
	12	0.14	0.08			

[†]Diet consisted of 1.5% BW smooth bromegrass hay (as-fed) and 0.5% BW pelleted concentrate (as-fed) containing 4.1% SB or 4.9% E

[1]T = time effect, X = treatment effect, and I = treatment × time effect

[A,B]Indicates a difference between treatments at $P < 0.05$

[C,D]Indicates a difference between treatments at $0.05 < P < 0.10$

[a,b,c,d,e]Indicates a difference within treatments at $P < 0.05$

*Indicates a quadratic effect of time averaged across treatments ($P < 0.05$)

[‡]Indicates a linear effect of time averaged across treatments ($P < 0.05$)

Table 3.7. Cecal long chain fatty acid (LCFA) concentration in horses consuming diets supplemented with soybean oil (SB) or Enertia s/f® (E) for 28 d†

LCFA, µg/mL[1]	Time	SB diet	E diet	SEM	Fixed effects[2] ($P < 0.05$)
Total LCFA	0	99.79^{ab}	96.94^{a}	17.56	T, I
	2	$120.57^{A,a}$	$183.23^{B,b}$		
	4	81.56^{b}	110.22^{a}		
	6	76.47^{b}	107.73^{a}		
	8	76.47^{b}	112.57^{a}		
	10	81.10^{b}	99.55^{a}		
	12	104.10^{ab}	90.36^{a}		
Palmitic acid (C16:0)	0	34.88^{abcd}	33.04^{a}	7.86	T, I
	2	$43.65^{A,acd}$	$65.49^{B,b}$		
	4	30.49^{b}	41.73^{a}		
	6	27.70^{b}	45.18^{a}		
	8	27.19^{b}	44.71^{a}		
	10	31.19^{c}	37.49^{a}		
	12	42.69^{d}	29.76^{a}		
Palmitoleic acid (C16:1)	0	0.47	0.49	0.07	
	2	0.43	0.44		
	4	0.38	0.44		
	6	0.44	0.47		
	8	0.42	0.50		
	10	0.42	0.44		
	12	0.46	0.44		
Margaric acid (C17:0)	0	1.97	2.00	0.31	
	2	2.19	2.13		
	4	1.53	1.92		
	6	1.91	2.30		
	8	1.65	1.95		
	10	1.87	1.98		
	12	2.36	1.92		
Stearic acid (C18:0)	0	17.87^{a}	18.63^{a}	1.91	T
	2	17.99^{a}	22.81^{b}		
	4	10.55^{b}	13.90^{c}		
	6	9.72^{b}	13.81^{c}		
	8	9.45^{b}	13.88^{c}		
	10	11.04^{b}	14.09^{c}		
	12	15.34^{a}	14.40^{c}		

LCFA, µg/mL	Time	SB diet	E diet	SEM	Fixed effects
Trans-vaccenic acid (C18:1n11t)	0	8.14	7.17[a]	2.08	
	2	9.90	10.91[bc]		
	4	5.91	10.26[ac]		
	6	6.13	8.59[ac]		
	8	7.74	11.93[b]		
	10	6.34	10.60[ac]		
	12	7.19	9.87[ac]		
Oleic acid (C18:1n9c)	0	10.99[a]	9.97[a]	2.84	T, I
	2	17.76[A, b]	32.10[B, b]		
	4	10.53[a]	15.26[a]		
	6	9.36[a]	13.76[a]		
	8	9.42[a]	12.67[a]		
	10	9.58[a]	10.47[a]		
	12	11.61[ab]	8.86[a]		
Vaccenic acid (C18:1n11c)	0	2.23[a]	2.55[abc]	0.62	T
	2	1.99[ab]	3.11[a]		
	4	1.27[b]	1.93[b]		
	6	1.32[ab]	1.85[b]		
	8	1.86[ab]	3.03[ac]		
	10	1.96[ab]	2.77[abc]		
	12	2.29[ab]	2.65[abc]		
Trans-linoleic acid (C18:2n6t)	0	1.00	0.78[a]	0.12	T
	2	1.04	0.81[a]		
	4	1.19	1.01[ab]		
	6	1.10	0.99[ab]		
	8	1.23	1.17[b]		
	10	1.13	0.93[a]		
	12	1.03	0.88[a]		
Linoleic acid (C18:2n6c)	0	7.82	7.47[a]	2.09	T, I
	2	10.19[A]	21.18[B, b]		
	4	7.43	9.56[a]		
	6	7.19	9.47[a]		
	8	7.34	8.88[a]		
	10	6.26	7.74[a]		
	12	7.48	7.37[a]		

LCFA, µg/mL	Time	SB diet	E diet	SEM	Fixed effects
Arachidic acid	0	2.18	2.30[a]	0.37	T
(C20:0)	2	1.98	3.08[b]		
	4	1.81	2.04[a]		
	6	1.40	1.84[a]		
	8	1.43	2.13[a]		
	10	1.59	2.12[a]		
	12	1.95	2.17[a]		
Gamoleic acid	0	2.92	3.23[a]	0.60	T
(C18:3n3)	2	2.71	4.35[b]		
	4	2.59	2.85[a]		
	6	2.40	2.98[a]		
	8	2.50	3.12[a]		
	10	2.40	2.93[a]		
	12	2.87	3.11[a]		
Conjugated	0	1.04	0.82[a]	0.72	T
linoleic acid	2	2.72	4.88[b]		
(C18:2n9c11t)	4	0.67	1.00[a]		
	6	0.49	0.48[a]		
	8	0.48	0.61[a]		
	10	1.07	0.61[a]		
	12	1.22	0.98[a]		
Heneicosylic	0	0.18	0.18[a]	0.03	
acid	2	0.17	0.28[b]		
(C21:0)	4	0.20	0.18[a]		
	6	0.12	0.16[a]		
	8	0.13	0.17[a]		
	10	0.16	0.17[a]		
	12	0.17	0.19[a]		
Conjugated	0	0.09[ab]	0.06[a]	0.06	T
linoleic acid	2	0.25[a]	0.40[b]		
(C18:2n10t12c)	4	0.08[b]	0.05[a]		
	6	0.03[b]	0.06[a]		
	8	0.02[b]	0.10[a]		
	10	0.10[ab]	0.00[a]		
	12	0.09[ab]	0.02[a]		
Conjugated	0	0.00	0.00	0.12	
linoleic acid	2	0.00	0.00		
(C18:2n9c11c)	4	0.00	0.00		
	6	0.00	0.00		
	8	0.00	0.59		
	10	0.00	0.00		
	12	0.00	0.00		

LCFA, µg/mL	Time	SB diet	E diet	SEM	Fixed effects
Conjugated linoleic acid (C18:2n9t11t)	0	0.71[ab]	0.58[a]	0.23	T
	2	1.23[a]	1.80[b]		
	4	0.33[b]	0.45[a]		
	6	0.28[b]	0.28[a]		
	8	0.30[b]	0.34[a]		
	10	0.46[ab]	0.30[a]		
	12	0.64[ab]	0.33[a]		
Behenic acid (C22:0)	0	2.30	2.46[a]	0.45	T
	2	2.09	3.56[b]		
	4	2.18	2.36[a]		
	6	1.56	1.97[a]		
	8	1.53	2.25[a]		
	10	1.73	2.23[a]		
	12	2.05	2.40[a]		
Dihomogamma linolenic acid (C20:3n6)	0	0.30[abc]	0.23	0.10	T
	2	0.09[bd]	0.00		
	4	0.14[bd]	0.00		
	6	0.41[ac]	0.00		
	8	0.07[bd]	0.00		
	10	0.02[d]	0.02		
	12	0.00[d]	0.00		
Erucic acid (C22:1n9)	0	0.09[a]	0.09[ab]	0.02	T
	2	0.15[b]	0.18[a]		
	4	0.07[a]	0.12[ab]		
	6	0.08[a]	0.14[ab]		
	8	0.06[a]	0.14[ab]		
	10	0.07[a]	0.11[b]		
	12	0.09[a]	0.11[b]		
Alpha-linolenic acid (C20:3n3)	0	0.47[a]	0.43[ac]	0.08	T
	2	0.53[ab]	0.50[ac]		
	4	0.66[b]	0.61[b]		
	6	0.63[b]	0.64[b]		
	8	0.53[ab]	0.57[bc]		
	10	0.49[a]	0.44[c]		
	12	0.45[a]	0.44[c]		

LCFA, µg/mL	Time	SB diet	E diet	SEM	Fixed effects
Arachidonic acid	0	0.04	0.06	0.02	
(C20:4n6)	2	0.05	0.00		
	4	0.00	0.03		
	6	0.03	0.03		
	8	0.03	0.00		
	10	0.04	0.01		
	12	0.05	0.02		
Tricosylic acid	0	0.54	0.51	0.09	T
(C23:0)	2	0.54	0.81		
	4	0.51	0.56		
	6	0.38	0.44		
	8	0.36	0.47		
	10	0.37	0.45		
	12	0.43	0.49		
Docosadienoic	0	0.10	0.14	0.03	T
(C22:2)	2	0.04	0.11		
	4	0.03	0.04		
	6	0.01	0.05		
	8	0.04	0.07		
	10	0.08	0.12		
	12	0.08	0.14		
Lignoceric acid	0	2.71	2.96	0.52	
(C24:0)	2	2.33	3.53		
	4	2.46	2.67		
	6	1.79	2.30		
	8	1.80	2.63		
	10	2.05	2.65		
	12	2.52	2.78		
Nervonic acid	0	0.20	0.21	0.04	T
(C24:1)	2	0.18	0.34		
	4	0.18	0.20		
	6	0.10	0.14		
	8	0.08	0.13		
	10	0.13	0.15		
	12	0.20	0.19		
Eicosapentaenoic	0	0.51^{a}	0.54^{a}	0.12	T, I
acid	2	0.35^{ab}	0.38^{ab}		
C22:5n3	4	0.15^{b}	0.31^{ab}		
	6	0.42^{ab}	0.26^{b}		
	8	0.33^{ab}	0.43^{a}		
	10	0.47^{ab}	0.41^{a}		
	12	$0.83^{A,c}$	$0.34^{B,a}$		

LCFA, µg/mL	Time	SB diet	E diet	SEM	Fixed effects
Total saturated	0	62.63^{abc}	62.09^{a}	10.95	T, I
LCFA	2	$70.95^{C,ac}$	$101.68^{D,b}$		
	4	49.72^{bc}	65.36^{a}		
	6	44.85^{b}	67.80^{a}		
	8	$43.54^{C,b}$	$68.19^{D,a}$		
	10	50.02^{ab}	61.18^{a}		
	12	67.50^{c}	53.95^{a}		
Total	0	37.17^{a}	34.85^{a}	7.26	T, I
unsaturated	2	$49.62^{A,b}$	$81.55^{B,b}$		
LCFA	4	31.83^{a}	44.86^{a}		
	6	31.35^{a}	40.59^{a}		
	8	32.76^{a}	44.38^{a}		
	10	31.08^{a}	38.41^{a}		
	12	36.60^{a}	35.72^{a}		

†Diet consisted of 1.5% BW smooth bromegrass hay (as-fed) and 0.5% BW pelleted concentrate (as-fed) containing 4.1% SB or 4.9% E

[1]Nomenclature of LCFA defined as number of carbons:number of double bonds and orientation of carbon atom on either side of the double bond described as t = trans or c = cis.

[2]T = time effect, X = treatment effect, and I = treatment × time effect

^{A,B}Indicates a difference between treatments at $P < 0.05$

^{C,D}Indicates a difference between treatments at $0.05 < P < 0.10$

^{a,b,c,d,e}Indicates a difference within a treatment at $P < 0.05$