THESIS

TRACE MINERAL SOURCE IMPACTS RUMEN TRACE MINERAL DISTRIBUTION AND FIBER DIGESTION IN STEERS FED A LOW-QUALITY FORAGE-BASED DIET

Submitted by

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ABSTRACT

TRACE MINERAL SOURCE IMPACTS RUMEN TRACE MINERAL DISTRIBUTION AND FIBER DIGESTION IN STEERS FED A LOW-QUALITY FORAGE-BASED DIET

Twelve Angus steers (BW 452.8 ± 21.8 kg) fitted with ruminal cannulae were used to determine the impact of trace mineral (TM) source on neutral detergent fiber (NDF) digestibility, short chain fatty acid (SCFA) production, ruminal solubility of Cu, Zn, and Mn, and relative binding strength of trace minerals located in the rumen insoluble digesta fraction. Steers were fed a low-quality grass hay diet (DM basis: 10.8% CP, 63.1% NDF, 6.9 mg Cu/kg, 65.5 mg Mn/kg, and 39.4 mg Zn/kg) supplemented with protein for 21 d. Treatments consisted of 20, 40, and 60 mg supplemental Cu, Mn, and Zn/kg DM, respectively, from either sulfate (STM) or hydroxy (HTM) sources (n=6 steers/treatment). Following a 21-d adaptation period, total fecal output was collected for 5 d. Dry matter digestibility tended ($P < 0.07$) to be reduced (51.9 vs. 53.4 ± 0.52%) and NDF digestibility was reduced ($P < 0.04$; 40.4 vs. 42.7 ± 0.67%) in STM vs. HTM supplemented steers. On d-6, rumen fluid was collected at 0, 2, and 4 h post feeding and analyzed for SCFA. There were no treatment x time interactions for any response variables measured. However, treatment was a significant ($P < 0.05$) source of variation for butyric acid and total SCFA production. Steers receiving HTM had less ($P < 0.02$) butyric acid and greater ($P < 0.05$) total SCFA than STM supplemented steers. Steers were then fed the same low-quality grass hay diet without supplemental Cu, Zn, or Mn for 14 d. On d-15, steers received a pulse dose of 20 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either STM or HTM sources (n=6 steers/treatment). Ruminal samples were obtained at 2-h intervals starting at -4 h and ending at

24 h relative to dosing. There was a treatment x time interaction for ruminal soluble Cu, Mn and Zn concentrations. Ruminal soluble mineral concentrations were greater ($P < 0.05$) for Cu at 4, 6, 8, 10, 12, and 14 h; for Mn at 4 and 6 h; and for Zn at 4, 6, and 8 h post dosing in STM compared to HTM supplemented steers. Concentrations of Cu and Zn in ruminal solid digesta were also affected by treatment, time, and treatment x time. At 12 h post dosing, Cu and Zn concentrations were greater ($P < 0.05$) in HTM supplemented steers when compared to STM supplemented steers. Upon dialysis against Tris-EDTA the % Zn released was greater at 12 h ($P < 0.03$) and 24 h ($P < 0.05$) and the % Cu released was greater ($P < 0.02$) at 24 h post dosing when compared to STM supplemented steers. Results indicate that Cu and Zn from HTM have low solubility in the rumen, may improve fiber digestibility and appear to be less tightly bound to ruminal solid digesta than Cu and Zn from STM.

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CHAPTER 1 – REVIEW OF LITERATURE

INTRODUCTION

Micro or "trace" elements differ from macro elements based strictly on the amount provided in the diet (NRC, 2016). Trace elements are required at concentrations less than 100 mg/kg diet dry matter, while macro elements are required at concentrations above 100 mg/kg diet dry matter (McDowell, 1992). According to NRC (2016), 15 trace elements are considered essential for mammals. Ten of these trace elements are considered essential for beef cattle. These include chromium (Cr), cobalt (Co), copper (Cu), iodine (I), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and Zinc (Zn). Additional trace elements, including boron (B), fluorine (F), lithium (Li), silicon (Si), tin (Sn), and vanadium (V), have been previously identified as essential for certain animal species (NRC, 2016; McDowell, 1992; Underwood and Suttle, 1999). Although elements such as Cu, Mn, and Zn are required in many biochemical reactions within the animal, pure elemental sources of Cu, Mn, Zn, etc. are not fed to the animal. Instead, elements are typically consumed by animals associated with salts, amino acids, proteins, carbohydrates, halogens, etc. (e.g. Cu-sulfate, Cu-lysine, Cu-chloride) and are referred to as minerals. Therefore, for the purposes of this literature review, trace elements will be referred to as trace minerals with the understanding that the element is biologically active within the animal but is ingested in mineral form.

Although uncertainty exists about which trace minerals are considered essential, trace minerals can be easily categorized into a subset that has been shown to be extremely important in livestock nutrition; Co, Cu, Fe, I, Mn, Se, and Zn. An additional 20 to 30 trace minerals occur naturally in feed, plant, and animal tissue, and it is unknown whether they have a biological function in mammals.

It has been well documented that deficiencies of various trace minerals can result in metabolic diseases. However, the interactions between trace minerals and metabolic processes are tremendously complex. As an example, Cu, Zn, Mn, Fe, Se, Co, and I have been identified as crucial components for carbohydrate, lipid, protein, and vitamin metabolism and absorption. In addition, these trace minerals are found to be heavily involved in hormone production, immunity, and cellular homeostasis. Appropriate trace mineral supplementation can also improve health, immune response, and at much higher rates of supplementation in swine and poultry, can alter microbial colonization of the gut, resulting in improved gut health (Faulkner and Weiss, 2017).

These trace minerals function primarily as catalysts in enzyme systems within cells. These requiring metal enzymes can be divided into two categories: 1) metal-activated enzymes and 2) metalloenzymes. Metal-activated enzymes may or may not have an absolute requirement for a metal; however, the presence of a metal is typically required for optimizing enzyme activity. Metalloenzymes have a very specific characteristic, and that is to hold a tightly bound metal ion at or near the active site of the enzyme. Metal ions bound to metalloenzymes are actively involved in catalysis. Removal of the metal ion will cause the enzyme to become non-functional. Enzymes involved in electron transport, bone metabolism, immune response, gene expression, nutrient metabolism, and protection of cells from oxidative stressors all have been shown to require certain trace elements for proper function.

When targeting optimum animal performance, carbohydrates, protein, and lipids are the primarily focus of ruminant nutritionists. Supplements containing trace minerals are formulated after the basal diet is balanced. Very little value/consideration is given to the trace minerals contained in the primary basal dietary ingredients. Though the minimum concentrations of

essential trace minerals needed to avoid deficiencies have been well researched, there is still a need for further research demonstrating optimal levels and sources of trace minerals for proper immune function and growth (Cohen, 2014; Lineman, 2013). However, identifying optimal concentration levels and sources of trace minerals can be challenging because of trace mineral complexity within the body interactions. Various studies have targeted the impact of trace mineral supplementation on beef cattle performance and immunity during the feedlot phase of beef production. However, results have been highly variable (Malcolm-Callis et al., 2000; Rhoads et al., 2003). A comprehensive understanding of the animal's mineral requirements as well as an understanding of the mechanisms of each trace mineral with respect to digestion, absorption, and utilization is needed.

Trace mineral absorption

Zinc

The activities of over 200 enzymes depend on Zn, of which the metalloenzyme carbonic anhydrase was the first to be identified (McDowell, 1992). In addition to its importance with respect to enzyme activity, Zn is also heavily involved in protein synthesis, carbohydrate metabolism, glycolysis, and transcription and translation (McDowell, 1992). Nonetheless, along with enzyme activity, Zn has been associated with an increase in feed intake and nucleic acid biosynthesis, resulting in normal growth (Hambidge et al., 1986). Throughout the absorption process, certain dietary factors can alter the absorption of Zn but will differ between non-ruminants and ruminants. Certain trace minerals such as Cu and Fe can influence Zn absorption. Other main factors involved in Zn absorption include the levels and sources of Zn. The overall understanding of Zn absorption will aid in determining the optimal levels of dietary Zn, and its source provided to beef cattle.

Most studies focused on examining the mechanisms of Zn absorption have been conducted with mice and rats, whereas very little research has been conducted in ruminants. Zinc absorption takes place primarily in the first meter of the duodenum in ruminants and in the first portion of the jejunum in monogastric animals. These locations, respectively, are also the main site of Zn re-excretion (endogenous Zn) from the animal (Miller, 1970). Zinc absorption can be divided into four phases according to Cousins (1982). Figure 1.1 demonstrates the processes involved in Zn absorption across the enterocyte. The first phase involves the solubilization of Zn in the lumen of the intestine. Zinc typically becomes soluble at a low pH (2-4) and therefore, the majority of Zn is absorbed prior to the increase of duodenal pH. Once Zn is soluble, it then binds to a Zrt- and Irt-like protein -4 (Zip4) transporter located on the apical membrane of the enterocyte (Cousins et al., 2006). Zrt- and Irt-like proteins are a family of solute-linked carrier 39 (SLC39) proteins responsible for increasing cytosolic Zn concentrations (Cousins et al., 2006). Once Zn is transported to the cytosol of the enterocyte, it binds to a cysteine-rich intestinal binding protein (CRIP) and is transported to the basolateral portion of the enterocyte where a solute-linked carrier 30 protein (ZnT1; SLC30A) transports Zn out of the enterocyte where it is bound to albumin and transported throughout the body. Although much of the Zn is absorbed from the lumen, Zn within the mucosal cells can also be derived from Zn reabsorbed from the bloodstream making Zn absorption bidirectional (Hambidge et al., 1986).

Absorption of Zn through the small intestine is regulated by a variety of low molecular weight binding ligands (McDowell, 1992). The low molecular weight binding ligands include low molecular weight proteins, such as citrate, EDTA, or amino acids that may not require ATP for absorption (Hambidge et al., 1986). Metallothionein (a binding ligand) is a metal binding protein synthesized by hepatic and intestinal tissues and can be influenced by dietary Zn and

plasma Zn concentrations (McDowell, 1992). The function of intestinal metallothionein is to limit the absorption of Zn within the intestinal mucosal cells when dietary Zn concentrations are high (Cousins, 1996; Underwood and Suttle, 1999). Therefore, when dietary Zn is high, the production of metallothionein increases. Metallothionein binds excess Zn and prevents further absorption into the blood. Also, by binding to excess Zn, the metallothionein is acting as a Zn regulator in achieving homeostasis. However, metallothionein also functions as a regulator of Cu absorption in the epithelial cells of the intestine. Metallothionein production is induced by excess Zn but has a higher binding affinity for Cu. Therefore, high concentrations of dietary Zn would then influence Cu absorption when Cu is at normal concentrations. In addition, Zn that becomes soluble in the rumen can be absorbed through the rumen wall, and then could be reabsorbed into the lumen of the small intestine. However, this mechanism of Zn absorption is minimal and secondary to Zn absorption from the intestine.

When dealing with ruminants, the issues with fiber-containing foods would only be an issue in non-functional ruminants such as young calves. The idea that high fiber diets decrease Zn absorption is contradicted by the NRC (2000) which states that it is unknown whether Zn's association with fiber reduces absorption. However, Zn source, levels, and status should be considered when dealing with a high fiber diet.

Galyean (1996) and Wedekind et al. (1992) indicate that Zn source and concentration should be addressed to optimize feedlot cattle performance. It has been suggested that inorganic and organic forms of Zn are metabolized differently following absorption (Galyean, 1996; NRC, 1996). Galyean (1996) showed that amino acid-based trace minerals have lower solubility/availability in the rumen. However, polysaccharide complex organic trace minerals may be more available to rumen bacteria (Kennedy, 1993). It has been shown that from the

inorganic forms, sulfate forms of Zn seem to be the most soluble and available in the rumen. Additionally, organic sources tend to have equal or greater availability than sulfate forms (Wedekind et al., 1992).

According to Spears et al. (1989), organic forms of Zn have been reported to enhance performance, improve health, and reproduction. This study also supports that Zn absorption is similar between Zn methionine and inorganic sources. However, evidence exists that Zn provided by Zn methionine is retained in the body more efficiently than inorganic Zn (Brown et al., 2004; Spears, 1989). Zinc absorption and the mechanisms involving absorption and retention are highly complex. Thus, further research is warranted to fully understand the optimum levels and sources of Zn to be applied in ruminant diets.

Copper

Intestinal absorption of Cu can be through both a passive and active process and has been shown to be a similar process between ruminants and non-ruminants (Underwood and Suttle, 1999). In ruminants, the relatively low rate of Cu absorption is unique compared to non-ruminants, primarily due to the presence of well-known antagonists such as sulfur (S) and molybdenum (Mo), and the conversion of sulfate to sulfite in the rumen (Underwood, 1977; Underwood and Suttle, 1999). When Cu is bound to either sulfide or molybdate, it is almost completely unabsorbed by ruminants (Huisingh and Matrone, 1976). Taking into consideration that the absorption of Cu in ruminants is low (<1.0-10%) when compared to non-ruminants (Spears, 2003), the importance of understanding how antagonists impact Cu absorption and metabolism is important. Furthermore, the chemical form of Cu can influence the amount of Cu absorbed as explained by McDowell (1992) and Underwood (1977).

Other Cu antagonists that reduce Cu absorption and could potentially cause Cu deficiency are Fe, calcium (Ca), cadmium (Cd), and silver (Ag; Underwood and Suttle, 1999; Underwood, 1977). As discussed above, Cu absorption, under certain conditions, can also be influenced by dietary Zn concentrations. For instance, the concentration of Zn in the lumen of the intestine negatively affects Cu uptake into mucosal cells of the small intestine (Oestreicher and Cousins, 1985).

The process involving Cu absorption is controlled through two main mechanisms which consist of saturable and unsaturable mechanisms relating back to the active transport and simple diffusion process (McDowell, 1992). More recently, Cu was found to be absorbed primarily in the duodenum where it is transferred across the brush border into the enterocyte (Hill and Link, 2009; Cater and Mercer, 2006). However, most of the research investigating Cu absorption has been conducted in rodents, similar to that for Zn.

Copper is solubilized at a low pH (2-4). Once solubilized in the stomach, Cu will enter the small intestine. Figure 1.2 briefly shows the process of Cu absorption through the enterocyte into the bloodstream. Once soluble and in the lumen of the small intestine, Cu will bind to a high affinity copper transport protein (hCTR1) that is expressed on the apical membrane of the enterocyte. After Cu is transported into the intestinal cell, a P-type ATPase MNK protein chaperones Cu to the basolateral surface of the enterocyte where Cu is bound to albumin and transported throughout the circulatory system (Pena et al., 1999). Furthermore, the same metallothionein participating during Zn absorption, also functions as a regulator of Cu absorption in the epithelial cells of the intestine. Following the same concept as Zn, when Cu concentrations in the diet are low, then Cu absorption efficiency is increased. Copper absorption can be negatively influenced by the formation of Cu sulfide in the gut (NRC, 2000). Potentially

minimizing the interactions between Cu and other components in the diet, especially antagonists, would increase the absorption of Cu at the intestinal level (McDowell, 1992). However, the full mechanism(s) of Cu absorption is still not well understood (Hill and Link, 2009).

Supplemental Cu in the form of CuO would be the least available form when compared to Cu - sulfate, carbonate, chloride, chelates, and proteinates. One of the organic forms of Cu such as a Cu proteinate had a greater bioavailability than $CuSO_4$ when fed to calves receiving diets high in the Cu antagonist Mo (Kincaid et al., 1986; McDowell, 1992).

Manganese

The absorption of Mn is very low (possibly only 3 or 4%; Henry, 1995) in nearly all species, making the presence or absence of Mn deficiency dependent on variability in availability among feeds or supplemental sources (Underwood and Suttle, 1999; McDowell, 1992).

There are several Mn antagonists that have been identified including phytate and fiber (which can substantially decrease availability; Wedekind et al., 1991), P, Ca, Fe, and Co (McDowell, 1992). Since the most common Mn antagonist, phytate, is mainly degraded in the rumen, it is assumed that Mn absorption is probably higher (around 15%) in ruminants, especially when Mn intake is low. However, in one study (Abrams et al., 1977), the availability of Mn in cattle was reported to be lower (approximately 1%) compared to the expected absorption rates mentioned above.

Although little is known concerning dietary factors affecting Mn absorption (NRC, 2000), McDowell (1992) and NRC (2016) explain that Mn absorption occurs in a two-step process which involves the uptake of Mn from the gut lumen and then transfer across the mucosal cells. Also, much like Zn and Cu, when dietary Mn concentrations are high, absorption

efficiency decreases, and when dietary Mn concentrations are low, absorption efficiency increases.

Manganese absorption is thought to go from the intestinal lumen via a divalent metal transporter 1 (DMT1) which is located on the apical surface of the enterocyte. The DMT1 transports divalent metal ions such as Mn, Fe, Zn, etc. into the cytosol of the enterocyte. Manganese absorption was shown by Underwood and Suttle (1999), to be like that of Fe. Once absorbed, Mn is transported throughout the body bound to transferrin.

Manganese deficiency can occur in animals consuming diets composed of normal feed ingredients low in Mn (McDowell, 1992). It is quite controversial whether Mn homeostasis is primarily regulated through excretion (Thomas, 1970) or managed via absorption and fecal excretion via bile (NCR, 1996; Watson et al., 1973). On the other hand, Zn has been shown to be regulated through the absorption and excretion process.

Manganese antagonists in the diet, such as Ca and P, may cause the Mn requirement in the diet to increase (Olson and Hale, 2001). Typically, Mn is thought to be poorly absorbed because of the substantial surplus of Mn provided by most practical rations (Underwood and Suttle, 1999); however, deficiencies have been noted in beef cattle, even under natural conditions in certain areas of the northwestern United States.

TRACE MINERAL TRANSPORT AND STORAGE

Once absorbed at the site of the small intestine, trace minerals are loosely bound to albumin and amino acids in the circulatory system (Underwood, 1977). Serum albumin and amino acids can also bind trace minerals when released by tissues based on physiological needs elsewhere in the body (Underwood, 1977). Once a trace mineral reaches the liver (via absorption

from the diet or tissue release) it is either stored in the liver, typically via the incorporation into mitochondria, microsomes, nuclei, and/or parenchymal cells, released for immediate incorporation into metalloenzymes, or excreted from the body, usually via bile (Underwood, 1977). If absorbed and not bound to albumin, it is believed that trace minerals bind tightly to alpha-2 macroglobulin, with some additional metals becoming oxidized in the circulation and binding transferrin (Hambidge et al., 1986; Hidiroglou, 1979).

Storage of trace minerals in body tissue can vary between species, age, diet composition (antagonists, sources, availability), disease conditions, and environment, while distribution of trace minerals in the body has been shown to only be affected by species, age, and trace mineral status (Underwood, 1977).

Zinc

Most mammalian tissues contain 30 to 250 mg Zn/kg, whereas body Zn concentration in cattle ranges from 20 to 30 mg Zn/kg (Hambidge et al., 1986). Once Zn is absorbed and reaches the liver, a substantial portion is sent back into the blood and incorporated into various tissues such as bone or central nervous tissue, and once incorporated into theses tissues, is unavailable for use by other tissues (McDowell, 1992). While in the liver, Zn is primarily bound to metallothionein. Metallothionein in the liver is the major storage form of Zn, and can be mobilized during metabolic need (McDowell, 1992). There are four isoforms of metallothionein present in mammals: metallothionein 1 and 2 which have ubiquitous tissue distribution with particular abundance in the liver, pancreas, intestine, and kidneys, whereas metallothionein 3 and 4 are found primarily in the brain and skin (Davis and Cousins, 2000). The binding of Zn to liver metallothionein is relatively weak, thus giving liver metallothionein the ability to acquire and

release Zn. By contrast, the bond between enterocyte derived metallothionein and Zn is extremely tight, thus preventing absorption.

When Zn enters the enterocyte, the absorption of Zn into the bloodstream is essentially dependent on concentrations of Zn itself in the circulatory system, in addition to the regulation of metallothionein in the enterocyte. The same would occur when Zn concentrations are high in the blood; less Zn is absorbed from the intestinal cells into the bloodstream.

Whether the absorption of Zn is up or down regulated, Zn found in the plasma will most likely dictate only part of the Zn status within the animal. With that said, in order to assess Zn status within the animal, more should be evaluated than only blood status. A reasonable approach would be to find and identify indicators of Zn status in the serum, such as metallothionein and/or serum alkaline phosphatase activity. Alkaline phosphatase activity has been shown to fall during Zn deficiency but follows a similar time course to serum Zn (Underwood and Suttle, 1999).

Since Zn can be transported across the small intestine and transported in the body by albumin, the attachment of Zn to methionine may alter its mode of absorption and transport in the animal's body compared to Zn from Zn oxide (Greene et al., 1988). Transport of Zn from the intestinal lumen into the bloodstream and from the bloodstream into the lumen is a bidirectional process for which the mechanism remains unknown (Hambridge et al., 1986). The metabolism of Zn in the blood after it is absorbed is affected by the ligands involved which can vary depending on Zn status (McDowell, 1992; Underwood and Suttle 1999). Only about two thirds of plasma Zn is bound to albumin in the portal bloodstream (Underwood and Suttle, 1999). The other portion of plasma Zn can be bound to macroglobulin or to metallothionein for example.

Additionally, besides the liver, Zn can also be stored in the pancreas, kidneys, and spleen (McKenney et al., 1962; Feaster et al., 1954). However, the ability of stored Zn to be readily

available for use by other tissues is limited in most species (Underwood, 1977). During cases of low intake of Zn, the hepatic metallothionein Zn storage is primarily of immediate use (Richard and Cousins, 1976).

Copper

In the liver, adults of most species contain approximately 10 to 50 mg Cu/kg DM, with most animals containing 15 to 30 mg Cu/kg DM (Underwood, 1997). Adult ruminants store about 100 to 400 mg Cu/kg DM in the liver, possibly due to an improved capacity for sheep and cattle to bind Cu in the liver (Underwood, 1977). The liver is the primary storage site for excess Cu. Therefore, diagnosing Cu deficiency is commonly done via analysis of liver Cu concentrations (Underwood and Suttle, 1999). Over half of the total body Cu is contained in muscle and bone, even though the concentrations found in the liver are very high. Additionally, Cu can also be stored in the blood, heart, kidneys, brain, lungs, and skin (Underwood, 1977).

Newer research, using molecular techniques as well as human metabolic disorders and single-cell organisms, have improved our understanding of the mechanisms underlying Cu metabolism and storage (Hill and Link, 2009). Transporter chaperones are essential for cellular and whole animal Cu homeostasis (Cohen, 2014; Fry et al., 2013). Once Cu is absorbed and enters the bloodstream, it binds to albumin and/or the amino acid histidine and is transported to the liver (McDowell, 1992). If Cu binds to metallothionein in the intestinal mucosa, then this interaction could restrict the further translocation of Cu into the bloodstream (Cousins, 1985; Underwood and Suttle, 1999). While Cu is transported throughout the body, antagonists such molybdenum (Mo), sulfur (S), and iron (Fe), could induce hypocuporsis by forming insoluble Cu complexes in the digestive tract, bloodstream, and tissues of ruminants (Bailey et al., 2001), thus affecting further storage and availability. Therefore, Bailey et al. (2001) emphasizes that it would

be advantageous to develop supplementation strategies that would limit these antagonistic effects.

Manganese

When compared to Cu and Zn, concentrations of Mn in tissues of livestock are considerably low, typically ranging from 0.5 to 3.9 mg Mn/kg in sheep and cattle carcasses (Underwood and Suttle, 1999). Manganese is commonly found in the liver, bones (which has a low concentration of Mn but contains approximately 25% of the total body Mn), pancreas, and kidney tissue, while very little Mn is found in muscle. It is believed that most Mn in the body is stored in the mitochondria, supported by evidence that tissues rich in mitochondria (liver and kidneys) had greatest retention rates of Mn in growing lambs (Watson et al., 1973). Although bone reserves are the largest in the body, Mn from bone is not as available as it is from the different storage tissues, especially during low Mn intake situations (McDowell, 1992). Interestingly, storage capacity for Mn in the liver has been shown to be limited compared to other trace minerals such as Cu and Zn (McDowell, 1992).

After being absorbed, most of the Mn is transported by transferrin to the liver (Davidson, 1989; Underwood, 1999). Since bile is a major route of excretion for excess Mn, attempting to understand flow rates of Mn through the digestive tract is challenging. Also, Mn excretion via the feces is proportionally greater as the amount of Mn absorbed increases (Underwood, 1999). Additionally, Genther and Hansen (2014) reported the importance of Mn superoxide dismutase which is an antioxidant in the mitochondria that catalyzes the conversion of the superoxide radical to less reactive hydrogen peroxide. Beef steers were utilized in a trace mineral repletion study and the authors reported that trace mineral injection of Cu, Mn, and Zn increased red blood cell lysate Mn superoxide dismutase activity (Genther and Hansen, 2014). However, the same study showed there was still a lack of a good biomarker of Mn in cattle. Superoxide dismutase

activity did reflect Mn supplementation via injection in their study and by dietary intake in other animal studies, suggesting it has a potential to be used as a biomarker of Mn status. There is also evidence that liver Mn concentration in various tissues responds very little or not at all to Mn supplementation (Carter et al., 1974), except in calves lacking a functional rumen (Howes and Dyer, 1971).

TRACE MINERAL EXCRETION

Zinc

Even though the mechanisms of tissue uptake of Zn have not yet been well characterized, Zn complexed with albumin is readily available for uptake by tissues (McDowell, 1992). Yet not all tissues that take up Zn make the Zn available to other tissues. For example, the uptake of Zn in bone and the central nervous system is relatively slow and firmly bound once acquired by these tissues, making the Zn unavailable to other tissues (McDowell, 1992).

The distribution of Zn throughout the body is well understood, but the mechanisms involved in Zn uptake by other tissues beyond the liver are not well known (Cousins, 1996; Underwood and Suttle, 1999). Subsequently, Zn is released back into the bloodstream after approximately 30-40% of the Zn entering through the hepatic venous supply is extracted by the liver (McDowell, 1992). The circulating Zn enters various extrahepatic tissues at differing rates, which consist of different rates of Zn turnover (Underwood and Suttle, 1999). Body tissues will exhibit different concentrations and turnover rates following oral administration and subsequent absorption (Miller et al., 1970). After an oral dosing, plasma Zn concentrations reach their peak within 1 to 3 d followed by a rapid decline for 3 to 4 weeks and a subsequent very slow decrease (Miller et al., 1970). Even though Zn tends to accumulate very slowly in some tissues, the amount in red blood cells, muscle, and bone continues to increase for several weeks after a single

oral dose (Miller et al., 1970). In the bloodstream, 80% is present in the erythrocytes, which contain about 1 mg Zn per 106 cells (Underwood and Suttle, 1999). The pancreas, liver, kidneys, and spleen have the most rapid accumulation and turnover of retained Zn (McKinney et al., 1962).

Most of the intracellular Zn is found in the cytosol (60-80%), with some Zn found in the crude nuclear fraction (10-20%) and small amounts in the microsomal and mitochondrial fractions (Saylor and Leach, 1980; McDowell, 1992). Zinc found in the cytosol is primarily bound to proteins whereas other fractions of Zn may be found on the cell membrane (McDowell, 1992). However, Hempe et al. (1991) identified a low molecular mass, intracellular constituent from rat intestinal mucosa that binds Zn during transmucosal Zn transport. The low molecular mass was not metallothionein, based on the Cd-hemoglobin affinity assay (Hempe et al. 1991), indicating the possibility of other cellular homeostatic mechanisms for Zn.

Zinc storage within an animal is minimal, leading to complications during a dietary Zn deficiency. Although Zn is widely distributed throughout the body, animals have limited capacity for storing Zn in a form where it can be mobilized rapidly to prevent a deficiency (McDowell, 1992). Along with its importance in Zn absorption, metallothionein is also involved with being the major storage form of Zn within the liver (Richards and Cousins, 1976). Spears and Samsell (1986) reported that Zn retention was greater for lambs fed Zn methionine compared with those fed a control or Zn oxide-supplemented diet. Even though the absence of recognized stores exist, Zn may be redistributed from large pools found in bone and muscle during a deficiency (Underwood and Suttle, 1999). Also, when Zn is fed in large amounts, the Zn content greatly increases in some tissues including the blood, pancreas, kidneys, bone, hair, and liver, but may have little impact on other tissues such as muscle (Miller et al., 1970).

Copper

The liver is the major storage organ for Cu where it is then released for incorporation into various enzymes (McDowell, 1992). While other organs contribute to the storage of Cu, approximately 20% of the animal's Cu supply is stored in the liver and remains as the main site for storage (McDowell, 1992). Copper is typically stored in the liver in the form of mitochondrial cuprein (McDowell, 1992). Within the body, Cu will be stored in the liver in ruminant animals to a greater extent when compared to non-ruminants. This indicates that ruminants are at a greater risk of a Cu toxicity (Underwood and Suttle, 1999). Again, much like Zn, metallothionein will bind Cu and aid in Cu storage as well as aid in providing Cu to the rest of the body during a Cu deficiency (McDowell, 1992).

Copper can be excreted through urine, bile, and sloughed intestinal cells, but the main excretory route is through feces (McDowell, 1992). Non-ruminants will excrete Cu at a greater extent when compared to ruminant animals because they tend to not be as susceptible to a Cu deficiency and will excrete more Cu via the bile and maintain lower concentrations of liver Cu (Underwood and Suttle, 1999).

Manganese

Manganese is mainly stored in the lowest concentrations of all trace minerals within tissues (Underwood and Suttle, 1999). Unlike Cu, the storage capacity for Mn in the liver is minimal (McDowell, 1992). Studies have shown fluctuations in liver Mn when dietary Mn is elevated; however, liver Mn seems to resist change when liver Mn reaches a certain concentration (Watson et al., 1973; McDowell, 1992). Bone growth is affected when a Mn deficiency is present resulting in shorter and thicker bones (McDowell, 1992). Reproductive effects were among the first signs of a Mn deficiency to be observed in livestock (McDowell,

1992); therefore, a good understanding of storage and excretion rates is necessary to maintain optimal reproduction.

Manganese deficiency is most likely a result of limited concentrations of circulating Mn (McDowell, 1992). Manganese is primarily excreted through feces with bile being the main source of fecal Mn (Thomas, 1970) but also through pancreatic juice as well as secretion from the intestinal wall (McDowell, 1992). Also, the majority of Mn found in the body is found in the skeleton and is not readily available during a Mn deficiency. In calves, there were bone abnormalities noted when cows were supplemented with Mn at 15.8 mg/kg but not when cows were supplemented at 25 mg/kg (Rojas et al., 1965; NRC, 2000). The NRC (2000) also reports that Mn requirements for growth and skeletal development are less than for reproduction. Therefore, it seems that the excretion of Mn is critical regarding maintaining concentrations within narrow limits (Ahola et al., 2005).

BIOAVAILABILITY OF TRACE MINERAL SUPPLEMENTS

The number of studies that have compared availability of different trace mineral sources is excessive, but the results are highly variable. It has been confirmed that differences in availability based on chemical changes must occur since Cu from fresh forage is less effective at increasing Cu status than Cu from cured/silage hay, even when the concentration of Cu is similar in both forage sources (Underwood, 1977).

Historically, trace minerals were only supplemented to cattle as inorganic salts (Spears, 1996), with the trace mineral usually bound to a sulfate, carbonate, oxide, or chloride group. However, new technologies have been developed which enable the replacement of an inorganic group with one or even several amino acids, to various trace minerals (Cu, Mn, Zn, Co, and Se).

So far, there are five types of organic minerals that have been defined by the Association of American Feed Control Officials: 1) metal specific amino acid complex, 2) metal amino acid complex, 3) metal amino acid chelate, 4) metal proteinate, and 5) metal polysaccharide complex. Interest in the use of organic trace minerals has increased in recent years, mostly due to reports of improved growth, reproduction, and health in ruminants receiving organic trace minerals (Spears, 1996). When comparing the impact of various forms of trace minerals on beef cattle performance, results have been highly inconsistent. Reasons for observed differences in performance have not been fully identified.

The theory that organic trace minerals are more available than inorganic trace minerals is based on the thought that organic forms are more like the physiological forms found in the body (Spears, 1996). It is hypothesized that the differences in availability may be due to variability of absorption of organic trace minerals and that they are able to remain intact until the absorption site is reached. However, no data have been reported to validate this theory (Spears, 1996). Additionally, based on *in vitro* evaluation of the solubility and structural integrity of organic trace minerals with gel filtration, at a low pH, a metal that was once bound to a proteinaceous ligand became dissociated (Brown and Zeringue, 1994). The same authors therefore hypothesized that it is unlikely that organic trace minerals, once at the site of absorption, are absorbed differently from inorganic trace minerals.

Currently, trace minerals are available in both organic and inorganic forms as well as hydroxyl minerals. The hydroxyl trace minerals (copper hydroxychloride, zinc hydroxychloride, and manganese hydroxychloride) having their unique chemical characteristics, belong to a separate group of trace minerals. Zinc hydroxychloride differs from Zn sulfate because of the covalent bonds located between the Zn ion, multiple hydroxyl groups and the chloride ions, but

Zn sulfate contains an ionic bond where the Zn ion is connected with the sulfate group by a weak ionic bond (Mn hydroxychloride and Cu hydroxychloride contain the covalent bonds as well). Therefore, the covalent bonds are thought to increase the amount of biologically active Zn delivered to the intestine, resulting in higher Zn absorption (Cohen and Steward). The sulfate sources of Zn, Cu, and Mn contain the ionic bond and perhaps disassociate once in contact with moisture allowing the metal ions to bind with many diet antagonists (Cohen and Steward, unknown). Limited research has indicated that tribasic Cu chloride is more bioavailable than $CuSO_4$ when added to diets high in the Cu antagonists Mo and S (Spears et al., 2004).

Source and amount of trace mineral can heavily influence rumen fermentation (Faulkner and Weiss, 2017). Organic trace minerals (Cu, Zn, Mn, Se, and Co) had no effect on nutrient digestibility by dairy heifers, however, increased total volatile fatty acid (VFA) production was noted compared with sulfate minerals (Pino and Heinrichs, 2016). Excessive *in vitro* Cu (Durand and Kawashima, 1980) and Zn (Aerlovich et al., 2000; Eryavuz and Dehority, 2009) supplementation negatively affects rumen microbial populations, rumen fermentation, and nutrient digestibility. Ruminal solubility of trace mineral is likely a factor that influences how trace minerals affects rumen fermentation and microbial populations, which may in turn affect nutrient digestibility (Faulkner and Weiss, 2017). Therefore, reducing the concentration of soluble trace minerals, particularly Cu, by feeding hydroxy minerals may increase ruminal digestibility (Faulkner et al., 2017).

Hydroxy Cu and Mn are less soluble in the rumen when compared to sulfate trace mineral sources, whereas differences in solubility of hydroxy Zn and Zn sulfate are inconsistent (Cao et al., 2000; Genther and Hansen, 2015; Caldera et al., 2018). Furthermore, it is very challenging to interpret data from different studies because researchers have used various

methods of supplementation, different sources of trace minerals, a variety of different cattle types, and highly variable experimental designs. Moreover, breed of cattle, antagonists present in the diet, as well as physiological status of the animal must be taken into consideration when comparing the results from trace mineral source studies. Therefore, the objectives of the current study were to examine the influence of trace mineral source on: 1) fiber digestion and rumen fermentation characteristics in cattle fed a low-quality, high fiber diet, 2) rumen solubility of Cu, Mn, and Zn, and 3) the relative binding strength of trace minerals located in the rumen insoluble digesta fraction.

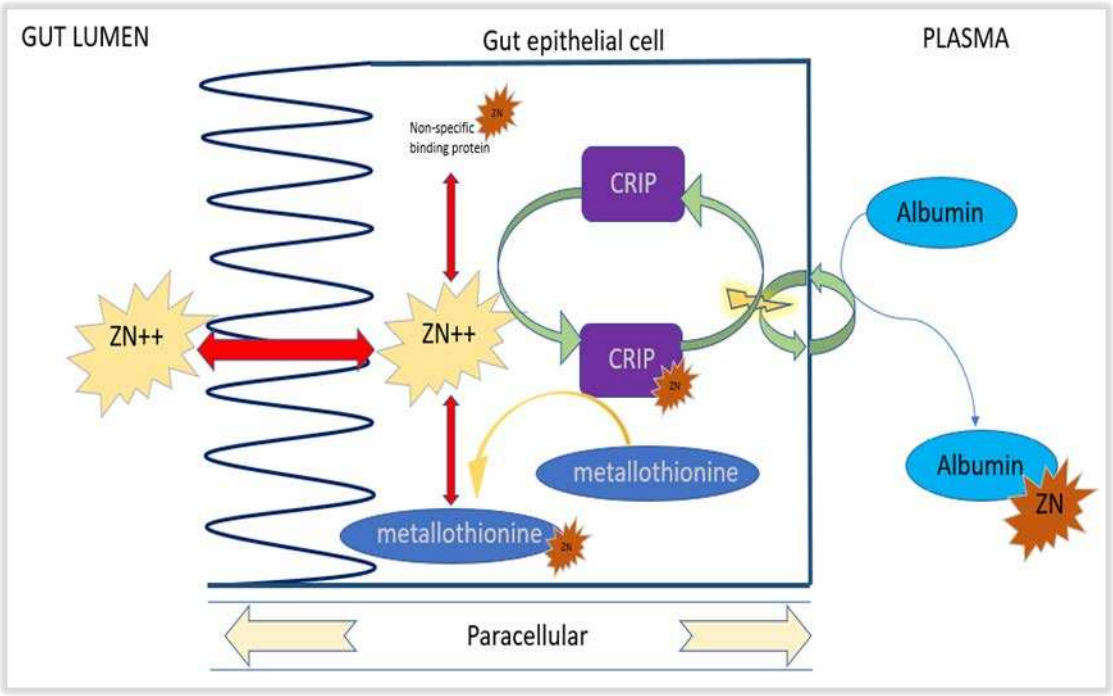

Figure 1.1. Mechanisms underlying Zn absorption [Adapted from J. NUTR. 122:89-95 (1992)].

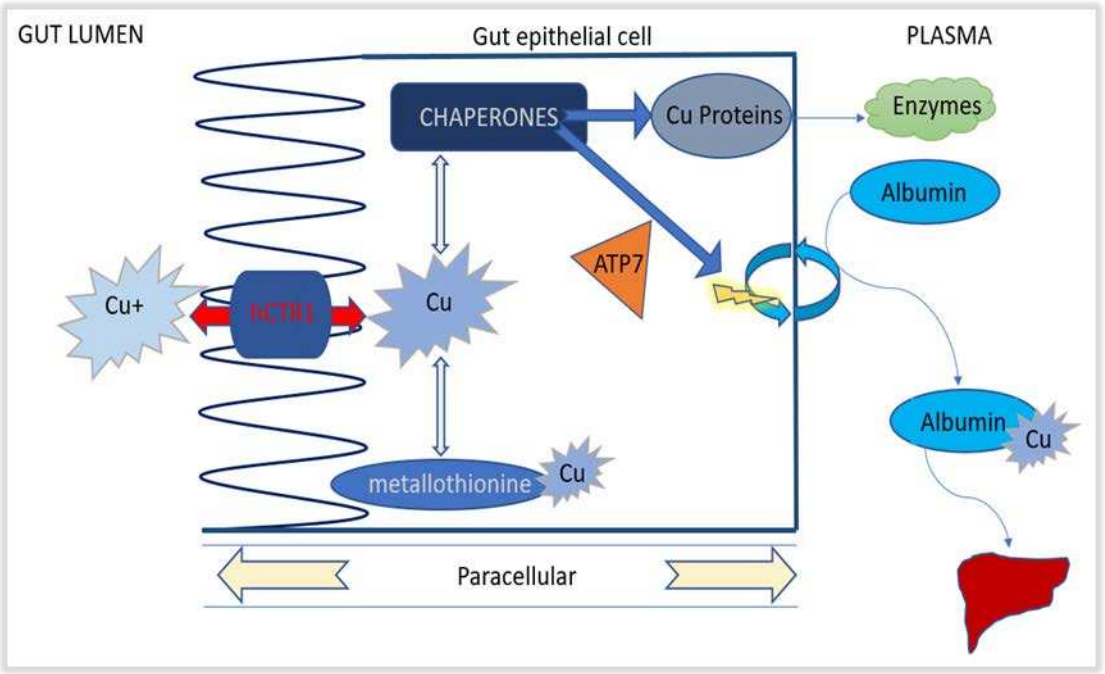

Figure 1.2. Mechanisms underlying Cu absorption [Adapted from J. NUTR. 122:89-95 (1992)].

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CHAPTER 2 – TRACE MINERAL SOURCE IMPACTS RUMEN TRACE MINERAL DISTRIBUTION AND FIBER DIGESTION IN STEERS FED A LOW-QUALITY FORAGE-BASED DIET

SUMMARY

Twelve Angus steers (BW 452.8 ± 21.8 kg) fitted with ruminal cannulae were used to determine the impact of trace mineral (TM) source on neutral detergent fiber (NDF) digestibility, short chain fatty acid (SCFA) production, ruminal solubility of Cu, Zn, and Mn, and relative binding strength of trace minerals located in the rumen insoluble digesta fraction. Steers were fed a low-quality grass hay diet (DM basis: 10.8% CP, 63.1% NDF, 6.9 mg Cu/kg, 65.5 mg Mn/kg, and 39.4 mg Zn/kg) supplemented with protein for 21 d. Treatments consisted of 20, 40, and 60 mg supplemental Cu, Mn, and Zn/kg DM, respectively, from either sulfate (STM) or hydroxy (HTM) sources (n=6 steers/treatment). Following a 21-d adaptation period, total fecal output was collected for 5 d. Dry matter digestibility tended ($P < 0.07$) to be reduced (51.9 vs. 53.4 ± 0.52%) and NDF digestibility was reduced ($P < 0.04$; 40.4 vs.42.7 ± 0.67%) in STM vs. HTM supplemented steers. On d-6, rumen fluid was collected at 0, 2, and 4 h post feeding and analyzed for SCFA. There were no treatment x time interactions for any response variables measured. However, treatment was a significant ($P < 0.05$) source of variation for butyric acid and total SCFA production. Steers receiving HTM had less ($P < 0.02$) butyric acid and greater ($P < 0.05$) total SCFA than STM supplemented steers. Steers were then fed the same low-quality grass hay diet without supplemental Cu, Zn, or Mn for 14 d. On d-15, steers received a pulse dose of 20 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either STM or HTM sources (n=6 steers/treatment). Ruminal samples were obtained at 2-h intervals starting at 4 h and ending at 24 h relative to dosing. There was a treatment x time interaction for ruminal soluble Cu, Mn, and Zn concentrations. Ruminal soluble mineral concentrations were greater ($P < 0.05$) for Cu at 4, 6, 8,

10, 12, and 14 h; for Mn at 4 and 6 h; and for Zn at 4, 6, and 8 h post dosing in STM compared to HTM supplemented steers. Concentrations of Cu and Zn in ruminal solid digesta were also affected by treatment, time, and treatment x time. At 12 h post dosing, Cu and Zn concentrations were greater ($P < 0.05$) in HTM supplemented steers when compared to STM supplemented steers. Upon dialysis against Tris-EDTA the % Zn released was greater at 12 h ($P < 0.03$) and 24 h ($P < 0.05$) and the % Cu released was greater ($P < 0.02$) at 24 h post dosing when compared to STM supplemented steers. Results indicate that Cu and Zn from HTM have low solubility in the rumen, may improve fiber digestibility and appear to be less tightly bound to ruminal solid digesta than Cu and Zn from STM.

INTRODUCTION

In 2017, Faulkner and Weiss reported that lactating dairy cows supplemented with hydroxy TM (HTM) had greater NDF digestibility than those supplemented with sulfate TM (STM) sources. The authors suggested that the impact of trace mineral source on fiber digestion may be due to differences in rumen solubility of Cu and Zn (Faulkner and Weiss, 2017). In agreement with these findings, Caldera et al. (2019) reported that NDF digestibility tended to be lower in STM compared to HTM supplemented steers. Furthermore, Caldera et al. (2019) reported that rumen soluble Cu and Zn concentrations were greater (at multiple time points over a 24 h period) in STM compared to HTM supplemented steers following a single bolus dose of Cu, Mn, and Zn from either STM or HTM sources. Diets used by Faulkner and Weiss (2017) and Caldera et al. (2019) were primarily composed of corn silage and corn and ranged from 28.3 – 36.4% NDF. Although not measured by Falkner and Weiss (2017), rumen pH averaged 6.23 in the Caldera et al. (2019) study. The finding of Caldera et al. (2019) agrees with earlier *in vitro* and *in vivo* research indicating that HTM forms of Cu and Zn are relatively insoluble under basic pH conditions and increase in solubility as pH decreases. By contrast, STM forms of Cu and Zn are almost completely soluble under basic and acidic conditions (Spears et al., 2004; Shaeffer, 2006).

Collectively, these data suggest that rumen solubility of Cu and Zn may influence rumen fermentation. Based on these data we hypothesized that rumen solubility of HTM forms of Cu and Zn would be low and that NDF digestibility would be greater with lower rumen solubility of Cu and Zn in cattle fed low-quality, high fiber diets. Therefore, the objectives of the current study were to examine the influence of TM source on: 1) fiber digestion and rumen fermentation characteristics in cattle fed a low-quality, high fiber diet, 2) rumen solubility of Cu, Mn, and Zn,

and 3) the relative binding strength of trace minerals located in the rumen insoluble digesta fraction.

MATERIALS AND METHODS

Prior to the initiation of this study, all animal care, handling, and procedures described herein were approved by the Colorado State University Animal Care and Use Committee (IACUC approval #17-7182A).

Cattle and feeding procedures: Twelve crossbred Angus steers fitted with ruminal cannulae (initial BW 452.8 ± 21.8 kg) were utilized in this study. Steers were housed at Colorado State University's Agriculture, Research, Development, and Education Center (ARDEC) in Fort Collins, CO. Steers were initially stratified by BW and housed in two feedlot pens (6 steers per pen) and fed a high fiber, low-quality hay (chopped) diet balanced to meet the CP, Na, Cl, Ca, P, Se, I, Co, and vitamin A, D and E requirements for growing steers (Table 2.1) with no supplemental Cu, Mn, or Zn for 21 d. After the 21-d adaptation period, steers received one of two treatments. Treatments consisted of 20 mg Cu/kg DM, 40 mg Mn/kg DM, and 60 mg Zn/kg DM, from either sulfate (SO_4) or hydroxy (IntelliBond C, M, and Z; Micronutrients USA LLC., Indianapolis, IN) sources. After receiving treatments for 7 d, steers were moved to individual pens in the metabolism building (2.5 m x 2.5 m pens equipped with automatic waters, individual feeders, and rubber matted floors) for 2 d and allowed to acclimate to their new surroundings. Steers were then relocated into individual metabolism stalls (3.0 m x 1.1 m pens equipped with automatic waters, individual plastic feeders, and rubber matted floors) for a 5-d acclimation period. During the acclimation period, Dry matter intake (DMI)for each steer was determined. At the end of the acclimation period, steers were paired across treatments based on their mean DMI

over the 5-d period. Once animals were appropriately paired by DMI, each steer within a pair was fed the same amount of feed. Feed delivered to each steer within a pair was calculated to be 90.0% of the steer within the pair with the lowest average DMI during the acclimation period. This ensured equal amounts of feed intake for individual steers within a pair (block) during the 5-d total fecal and urine collection period.

Diets were fed twice daily (60% of the ration in the morning and 40% of the ration in the afternoon). Appropriate TM treatment supplements were manufactured prior to the initiation of the study. Soybean meal corn was used as the carrier for the TM treatments. Immediately after feeding the basal diet, the appropriate TM supplement amounts (60% of the ration in the morning and 40% of the ration in the afternoon) were top-dressed and mixed thoroughly by hand for each feeding within a day.

Sample collection and analysis: Total fecal and urinary output was measured daily for individual steers during the 5-d collection period as described by Caldera et al. (2019). Briefly, 100 ml of 6N HCl was added daily to carboys used for urine collection to prevent N loss. Feces and urine collected each day (over a 24-h period) were quantified by wet-weight (feces) or volume (urine), thoroughly mixed, and sampled (10.0% of wet weight or volume). Duplicate, individual fecal samples were sealed in plastic bags, labeled, and stored at -20ºC. Urine samples were stored in acid washed polypropylene storage containers. Prior to DM, NDF, ADF, and nitrogen analysis of feces, urine, and feed, samples were proportionally composited across all collection days for each animal. Dry matter analysis was determined by placing a known mass of wet material in a forced-air drying oven for 48 h at 100ºC. After drying, samples were allowed to cool in a desiccator and then weighed. Neutral detergent fiber and ADF were analyzed using an Ankom 200 Fiber analyzer (Ankom Technology Corp.; Van Soest et al., 1991). All TM were

quantified via inductively coupled plasma-mass spectrometry (PerkinElmer; NexION 2000 B) and N was quantified using the TruSpec CN Carbon/Nitrogen LECO system (Leco Corp., St. Joseph, MI).

Following the 5-d fecal and urine collection (i.e., day 6) rumen samples were collected at 0, 2, and 4 h post-feeding for determination of short chain fatty acids (SCFA) and rumen pH. Rumen contents were centrifuged at 28,000 x *g* at 5°C for 30 min. A 2.0 ml aliquot of the supernatant was acidified with 25% (vol/vol) meta-phosphoric acid, and frozen at -80°C until analyzed for SCFA concentrations via gas chromatography. Rumen pH was determined by inserting a portable pH meter (EcoTestr pH 2+; Oaktron 153 Instruments, Vernon Hills, IL) into the geometric center of the rumen at the time of rumen content collection.

Rumen soluble concentration: At the end of the experiment, steers were placed in individual pens within the metabolism building and fed the basal diet without supplemental Cu, Mn, and Zn for 7 d. During this time, steers had *ad libitum* access to drinking water and the basal diet was fed as described above. On d-8, steers received a pulse dose of the TM sources being evaluated. Individual trace mineral treatments were thoroughly mixed with 0.23 kg of ground corn and administered as a single bolus-dose via the rumen fistula to provide two times the NASEM (2016) requirement for Cu (20 mg Cu/kg DM), Mn (40 mg Mn/kg DM), and Zn (60 mg Zn/kg DM). Immediately after bolus dose administration, the rumen contents were thoroughly mixed by hand. Rumen samples were then obtained at 2 h intervals beginning at 4 h and ending at 24 h post dosing; time zero being the administration of bolus + feeding of the basal diet. Before each sampling time, the rumen contents were thoroughly mixed by hand and a sample was obtained from the geometric center of the rumen (approximately 250 g). After each collection time, ruminal samples were centrifuged 28,000 x *g* in graduated centrifuge tubes.

Once centrifuged, the volume of the supernatant was determined and frozen at -20°C until TM analysis was performed. The Cu, Mn, and Zn concentrations of the supernatant and pellet fractions were considered to be the soluble and solid (insoluble) fractions of these elements, respectively.

Dialysis of ruminal insoluble digesta: Ruminal solid digesta samples from three different collection times (0, 12, and 24 h) were exposed to dialysis. Briefly, the insoluble fraction of the rumen digesta collected was dried at 60°C for 48 h in a forced air drying oven, ground in a Wiley mill to fit through a 2 mm screen, analyzed for Cu, Mn, and Zn, and dialyzed against 0.01M ethylenediaminetetraacetate (EDTA) in 0.05 M Tris (Tris-EDTA). Regenerated cellulose dialysis tubing (31.8 mm diameter, 30 µm wall thickness, MWCO 6,000 to 8,000; Fisher Scientific) was cut into 10 cm segments and treated to remove metal contamination. Dialysis tubing was stored in solution comprised of 50% ethanol; 50% deionized water; 1mM EDTA at 4°C prior to use. The chelating buffer was as follows: 0.01M EDTA in 0.05 M Tris (Tris-EDTA). The diluted buffer was prepared immediately prior to use and the pH adjusted to 6.8. Samples were placed into 10 ml of the appropriate buffer, then placed into dialysis tubing pre-wet with deionized water, and the tubing was then sealed with clips. The samples were then dialyzed against 1.0 liter of the same buffer for 16 h at 4°C with continuous stirring. The buffer was changed, and dialysis continued for another 6 h. Samples were removed from dialysis bags, placed into pre-weighed acid-washed crucibles, and dried overnight at 60°C. After drying, samples were weighed, and then ashed at 600°C in a Thermo-Fisher Thermolyne muffle furnace overnight. After cooling, ashed samples were then weighed and re-suspended in 5 ml of boiling 1.2 M HCl and analyzed for Cu, Mn, and Zn as described above.

STATISTICAL ANALYSIS

Total tract apparent digestibility of DM, ADF, and NDF and initial and post dialysis Cu, Mn, and Zn concentrations of insoluble digesta at times 0, 12, and 24 h were analyzed using a mixed effects model (PROC MIXED, SAS Inst. Inc., Cary, NC) for a completely randomized block design. A mixed effects model repeated measures analysis (PROC MIXED) for a completely randomized block design was used to analyze rumen soluble Cu, Mn and Zn concentrations, pH, and SCFA proportions and total concentrations. The fixed effects were treatment, time, and the treatment x time interaction. For all response variables measured, individual animal was considered the experimental unit. Several covariance structures were compared to determine the most appropriate covariance structure for data analysis. For all response variables, significance was determined at $P \leq 0.05$ and tendencies were determined at $P > 0.05$ and ≤ 0.10. When a significant treatment × time interaction was detected, treatment means were separated using the PDIFF option of the LSMEANS statement of SAS.

RESULTS

The influence of TM source on dry matter, ADF, NDF, and CP digestibility is shown in Table 2.2. By design, dry matter intake was similar across the treatments. Dry matter ($P < 0.07$; 51.9 vs. 53.4±0.52%) and CP ($P < 0.06$; 51.2 vs. 54.3 ±0.58%) digestibility tended to be reduced and NDF ($P < 0.04$; 40.4 vs. 42.7 ±0.67%) and ADF ($P < 0.05$; 32.4 vs. 34.1 ±0.49%) digestibility were reduced in STM vs. HTM supplemented steers.

The influence of trace mineral source on rumen soluble Cu, Mn, and Zn concentrations of steers receiving a pulse dose of TM is presented in Figure 2.1a-c. There was a treatment x time interaction for Cu ($P < 0.03$), Mn ($P < 0.03$), and Zn ($P < 0.02$). Ruminal soluble concentrations

were greater ($P < 0.05$) for Cu at 4, 6, 8, 10, 12, and 16 h post dosing in STM compared to HTM supplemented steers. Manganese rumen soluble concentrations were greater for STM supplemented steers at 4 and 6 h post dosing and lower at 18 h post dosing when compared to HTM supplemented steers. Rumen soluble Zn concentrations were greater in STM supplemented steers at 4, 6, and 8 h post dosing compared to HTM steers.

Table 2.3 shows the influence of trace mineral source on rumen pH and SCFA production at 0, 2, and 4 h post feeding. There were no treatment x time interactions detected so overall treatment main effects are presented in Table 2.3. Steers receiving HTM had less ($P < 0.02$) butyric acid and greater ($P < 0.05$) total SCFA than STM supplemented steers. Isovaleric acid tended ($P < 0.09$) to be greater in HTM compared to STM supplemented steers. Rumen pH and all other SCFA measured were similar across treatments.

The influence of dialysis against Tris-EDTA on the percent of Cu, Mn, and Zn released from the rumen solid digesta at 0, 12, and 24 h post bolus dose administration is shown in Table 2.4. Initial (0 h) rumen solid digesta Cu, Mn, and Zn concentrations were similar across treatments. At 12 h post bolus dose administration, Cu concentrations of rumen solid digesta were greater ($P < 0.05$) in HTM compared to STM supplemented steers. Manganese and Zn concentrations were similar across treatments. At 24 h post bolus dose administration, Cu ($P < 0.03$) and Zn ($P < 0.001$) concentrations were greater in HTM compared to STM supplemented steers, while Mn concentrations were similar across treatments.

Following dialysis against Tris-EDTA, the percentages of Cu, Mn, and Zn released at 0 h were similar across treatments. At 12 h post bolus dose administration, the percentage Zn released was greater ($P < 0.03$) and Cu tended ($P < 0.06$) to be greater in HTM vs STM steers. At 24 h post bolus dose administration, the percentage Cu ($P < 0.02$) and Zn ($P < 0.05$) were

greater in HTM compared to STM supplemented steers. The percentage of Mn released at 12 h and 24 h post bolus dose administration was similar between treatments.

DISCUSSION

The role that trace minerals have in rumen microbial fermentation is not well understood. However, *in vitro*, and *in vivo* studies have reported that Cu and Zn concentrations supplemented above NASEM (2016) requirements can reduce fiber digestion (Durand and Kawashima, 1980; Arelovich et al., 2000). Furthermore, data indicate that practical diets fed to ruminants without Cu and Zn supplementation can meet the microbial Cu and Zn requirements (Emmanuel and Staples, 1990).

Most research suggests that excessive supplemental trace minerals (specifically Cu and Zn) can negatively affect rumen microorganism populations, which could potentially reduce nutrient digestion. However, omission of trace minerals, specifically Zn, can decrease protozoal growth in pure cultures. As reported by Faulkner and Weiss (2017), the NDF fiber digestibility increase in cows supplemented with hydroxy trace minerals compared to that of cows supplemented with sulfate trace minerals was suggested to be caused by reduced concentrations of soluble Cu, thereby reducing inhibitory effects of rumen bacteria. This could explain the improvement in fiber digestibility in steers receiving HTM when compared to STM in the present study. These data also agree with data reported by Caldera et al. (2019) in a study conducted with beef cattle, where NDF fiber digestibility tended to also be greater in steers fed HTM compared to steers fed STM. The lower ruminal soluble TM concentration observed in steers dosed with HTM is most likely the main affect for differences in fiber digestibility.

Trace minerals coming from feedstuff or supplemental sources can become soluble in the rumen and interact with rumen metabolites, other feed ingredients, and microorganisms. These

interactions can produce insoluble complexes. Trace minerals that end up insoluble in the ruminal environment could be less accessible for absorption in the small intestine. The solubility data presented in the current study agrees with those presented by Shaeffer et al. (2017). It has been well established that Zn and Cu HTM sources are insoluble in water and highly soluble at lower pH. Cao et al. (2000) reported that Zn hydroxychloride had lower solubility in water than $ZnSO_4$, and Spears et al. (2004) reported that Cu from Cu hydroxychloride was relatively insoluble (0.6%) in water (pH 7.0) and highly soluble (81.4%) at a low pH (2.2), whereas Cu from $CuSO_4$ was almost completely soluble in both water (94.5%) and at a low pH (97.6%). Several *in vitro* studies have indicated that Zn and Cu can negatively affect fiber digestion (Durand and Kawashima, 1980).

Ruminal soluble Cu concentrations reported in the present study were increased at 4 h post dosing through 12 h post dosing in STM compared to HTM supplemented steers. Additionally, the percent soluble Mn was greater at 4 h and 6 h post dosing in STM dosed steers. Furthermore, soluble Zn concentrations were greater at 4, 6, and 8 h post dosing in STM than in HTM steers. A study by Genther and Hansen (2015) also reported ruminal concentrations of soluble Cu that were greater in steers fed diets supplemented with $CuSO_4$ compared with CuOHCl. Steers supplemented with $ZnSO_4$ had greater ruminal soluble Zn concentrations at 2 h post feeding than those receiving ZnOHCl (Shaeffer, 2006). In addition, Caldera et al. (2019) demonstrated that soluble concentrations of Zn and Cu were greater from 2 – 10 h post-dosing, whereas percent soluble Cu and Zn was greater at 6 and 12 h post dosing, respectively in STM dosed steers. However, Genther and Hansen (2015) reported that steers supplemented with ZnOHCl had greater ruminal soluble Zn concentrations than those supplemented with $ZnSO_4$. The difference in findings from the two studies may be related to how ruminal content

collections were processed after sampling. In the present study, and in the Caldera et al. (2019) and Shaeffer (2006) studies, ruminal samples were centrifuged shortly after being collected and the supernatant was collected before storing at -20°C for later determination of ruminal soluble TM. In the Genther and Hansen (2015) study, ruminal samples were frozen prior to centrifugation and separation of the soluble and insoluble fractions. Freezing and thawing may have altered the distribution of Cu and Zn between the soluble and insoluble fraction in the Genther and Hansen (2015) study.

Steers dosed with MnOHCl had greater soluble Mn concentrations relative to 0 h values at all times post dosing. It is uncertain if Mn released from MnOHCl in the ruminal soluble fraction was present in the ionic form or as a soluble complex. Compared to baseline 0 h values, ruminal soluble Mn concentrations in steers dosed with STM did not increase until 4 h and remained greater only until 6 h. In contrast, ruminal soluble Zn and Cu concentrations were greater by 2 h post dosing but returned to pre-dosing values by 10 to 16 h post dosing in steers given STM. Differences among TM sources in ruminal distribution were also smaller for Mn than those observed for Zn and Cu. Similar results were found by Genther and Hansen (2015) reporting that steers supplemented with $MnSO_4$ had greater ruminal soluble Mn concentrations than those fed diets supplemented with MnOHCl.

Trace minerals derived from dietary feedstuffs or supplemental sources can become soluble in the rumen. As solubility increases these elements can interact with ruminal contents to form insoluble complexes. Depending on how strong the binding is, TM becoming insoluble in the ruminal environment most likely would be less available for absorption in the small intestine than forms that remain soluble. In some cases, the insoluble complexes are so strongly bound that the compounds formed never become absorbed. In the present study, ruminal solid digesta

(pellet) was dialyzed against two chelating agents to assess the strength in which TM were bound in digesta. Histidine is a relatively weak chelator while EDTA is a stronger chelating agent. Previous research has shown that the ability of chelators to remove Zn from protein sources during dialysis may be useful in estimating *in vivo* bioavailability (Jones et al., 1985). Dialysis data from the current study suggest that Cu and Zn in the ruminal solid fraction from steers dosed with STM was more tightly bound and less available for absorption in the small intestine. These data are similar to those reported by Caldera et al. (2019). Strength of binding to the solid rumen digesta appeared to be in the order of Cu > Zn > Mn based on the extent of dissolution of the metals with acid or EDTA (Bremner, 1970). By contract, Caldera et al. (2019) reported the binding strength to the solid digesta in steers receiving STM was Cu > Zn > Mn ($P < 0.05$) when dialyzed against EDTA at 6 and 12 h post dosing. In the present study, binding strength to the solid digesta was reported to be Cu > Zn > Mn based on the mineral release percentage.

In summary, results from this study indicate that Cu and Zn from HTM have low solubility in the rumen. Increased NDF digestibility when hydroxy minerals were fed could indicate that HTM supplementation is favorable for cellulolytic bacteria, and perhaps protozoa growth. However, a more promising explanation is that sulfate trace minerals are inhibitory to fiber digesting microorganisms. Although further research is needed to understand the effects of trace mineral supplementation using different sources on microbiome populations, it appears that increased rumen solubility of Cu and Zn can decrease fiber digestion.

Table 2.1. Ingredient composition of high forage diet (% DM)

Ingredient	%DM
Grass hay	90.0
Soybean meal (46%)	4.7
Beet Pulp	4.6
Salt - white	0.35
Urea	0.18
Vit A	0.03
Vit E	0.013
Selenium (06)	0.003
Cobalt Sulfate	0.00005
Iodine (EDDI)[a]	0.00005
TOTAL	100.00
Chemical Composition[b]	
Dry Matter, %	91.8
Crude Protein, %	13.0
NEm, Mcal/kg	1.25
Neg, Mcal/kg	1.4
Fat, %	2.8
Acid detergent fiber, %	29
Neutral detergent fiber, %	49.0
Calcium, %	0.65
Phosphorus, %	0.30
Sulfur, %	0.30
Copper, mg/kg	6.6
Manganese, mg/kg	58.4
Zinc, mg/kg	37.4

[a]Iodine ethylenediamine dihydroiodide.
[b]Chemical composition of the basal diet.

Table 2.2. Influence of trace mineral source on dry matter (DM), acid detergent fiber (ADF), neutral detergent fiber (NDF), and crude protein (CP) digestibility in steers fed a low-quality high forage diet.

	Treatment			
Item	HTM[a]	STM[b]	SEM	*P* <
n =	6	6	---	---
DM intake, kg DM/hd/d	7.4	7.4	---	---
DM digestibility, %	53.4	51.9	0.52	0.07
ADF digestibility, %	34.1	32.4	0.49	0.05
NDF digestibility, %	42.7	40.4	0.67	0.04
CP digestibility, %	54.3	51.2	0.58	0.06

[a]Hydroxy trace minerals: 20 mg Cu/kg DM from CuOHCl; 40 mg Mn/kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl.
[b]Sulfate trace minerals: 20 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

Table 2.3. Influence of trace mineral source on short chain fatty acid production at 0, 2, and 4 h post feeding steers a high forage diet.

	Treatment			*P* <		
Item	HTM	STM	SEM	Trt	Time	Trt*time
n =	6	6	---	---	---	---
pH, s.u.	6.68	6.59	0.087	0.47	0.01	0.57
<u>Short chain fatty acid, mM/100mM</u>						
Acetic acid	49.15	48.89	0.539	0.74	0.05	0.92
Propionic acid	21.21	22.38	0.824	0.34	0.44	0.45
Isobutyric acid	5.80	5.57	0.232	0.51	0.001	0.61
Butyric acid	14.93	16.28	0.346	0.02	0.001	0.93
Isovaleric acid	5.09	4.08	0.374	0.09	0.001	0.43
Valeric acid	3.83	3.71	0.220	0.71	0.001	0.91
Total short chain fatty acids, mM	72.26	59.81	3.93	0.05	0.85	0.86

[a]Hydroxy trace minerals: 20 mg Cu/kg DM from CuOHCl; 40 mg Mn/kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl.
[b]Sulfate trace minerals: 20 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

Table 2.4. Influence of dialysis on copper, manganese, and zinc release from rumen solid digesta collected at 0, 12, and 24 h after receiving a pulse dose of 20 mg copper, 40 mg manganese, and 60 mg zinc/kg DM from either hydroxy or sulfate trace mineral sources.

	Treatment			
Item	HTM[a]	STM[b]	SEM	*P* <
Digesta, initial mineral concentration, mg/kg DM				
0h				
Copper	1.4	1.5	0.34	0.95
Manganese	8.4	8.6	1.1	0.91
Zinc	9.3	10.2	0.91	0.78
12h				
Copper	21.2	6.5	2.8	0.05
Manganese	15.3	14.9	2.9	0.65
Zinc	47.3	21.3	7.2	0.11
24h				
Copper	28.2	5.1	2.3	0.03
Manganese	28.3	24.5	4.2	0.89
Zinc	112.3	30.2	10.3	0.001
Mineral released, %[c]				
0h				
Copper	24.5	29.3	1.9	0.82
Manganese	37.2	41.2	4.3	0.72
Zinc	54.3	46.9	3.8	0.73
12h				
Copper	61.2	28.3	7.1	0.06
Manganese	71.2	75.9	21.3	0.82
Zinc	92.3	37.2	12.3	0.03
24h				
Copper	84.5	24.3	18.4	0.02
Manganese	91.2	94.3	15.3	0.73
Zinc	92.3	28.6	12.7	0.05

[a]Hydroxy trace minerals: 20 mg Cu/kg DM from CuOHCl; 40 mg Mn/kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl.
[b]Sulfate trace minerals: 20 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.
[c]Dialyzed against Tris-EDTA (0.01M ethylenediaminetetraacetate in 0.05M tris-hydroxymethyl-aminomethane) at 4°C for 22 h.

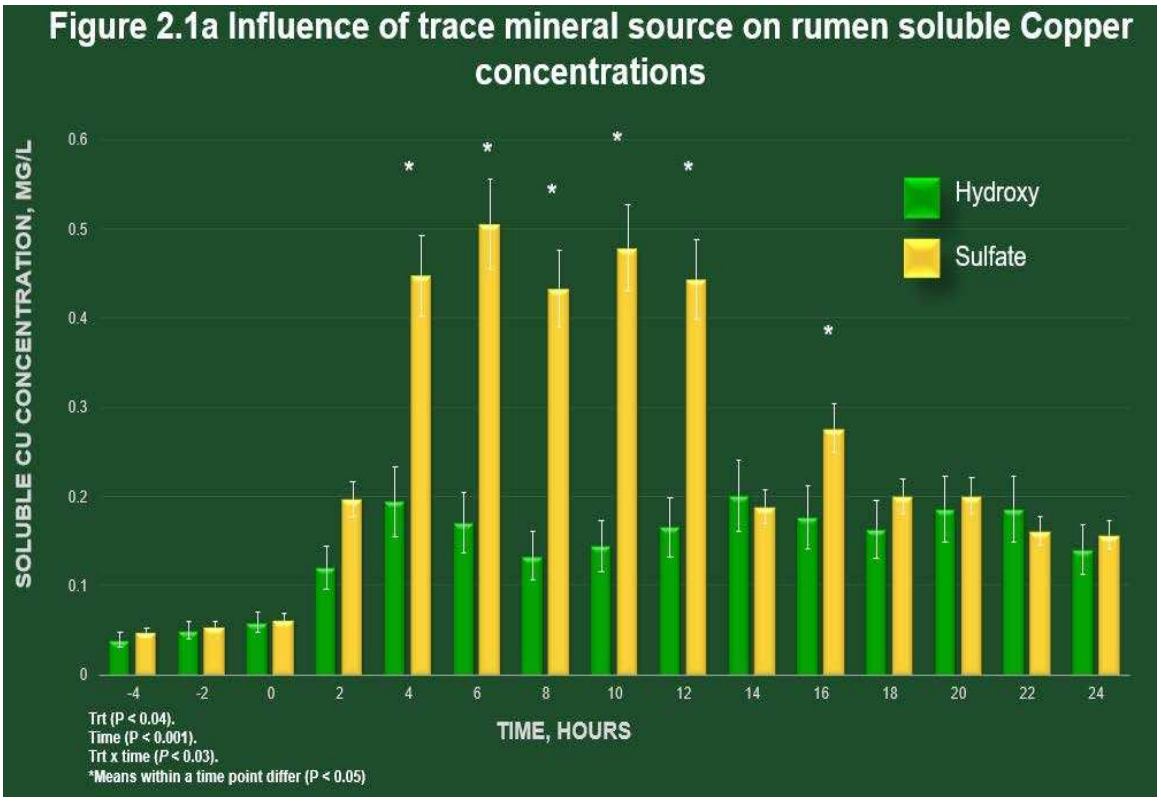

Figure 2.1a: The influence of trace mineral source on soluble copper, within the ruminal contents of steers receiving a pulse dose of either sulfate trace minerals (STM; 20 mg Cu/kg DM from $CuSO_4$; or hydroxy trace minerals (HTM; 20 mg Cu/kg DM from CuOHCl; The x-axis denotes sampling time in hours, the y-axis denotes rumen soluble copper. Error bars represent standard errors.

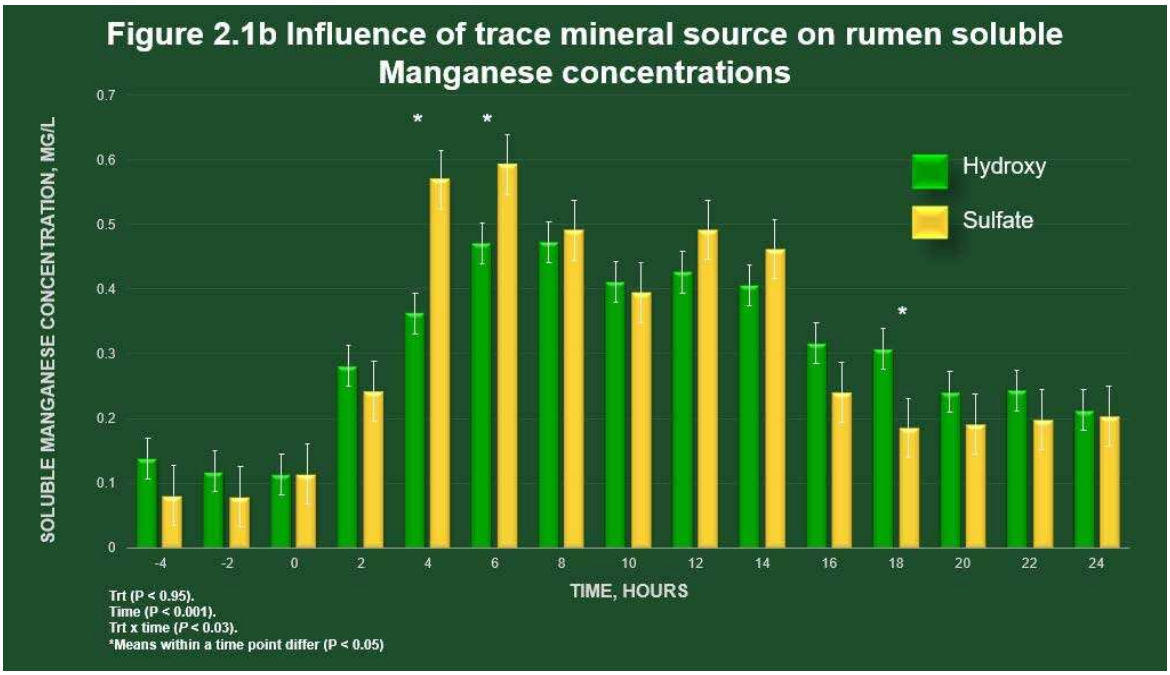

Figure 2.1b: The influence of trace mineral source on soluble manganese, within the ruminal contents of steers receiving a pulse dose of either sulfate trace minerals (40 mg Mn/kg DM from $MnSO_4$) or hydroxy trace minerals (HTM; 40 mg Mn/kg DM from MnOHCl). The x-axis denotes sampling time in hours, the y-axis denotes rumen soluble manganese. Error bars represent standard errors.

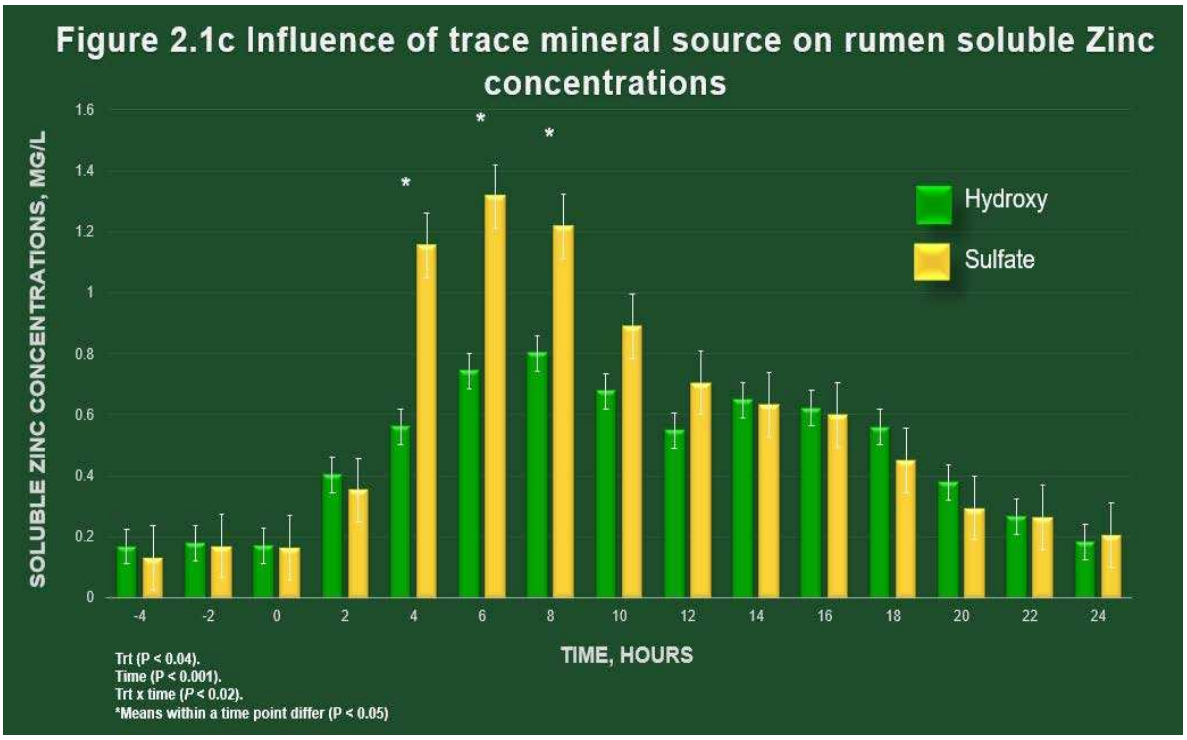

Figure 2.1c: The influence of trace mineral source on soluble zinc, within the ruminal contents of steers receiving a pulse dose of either sulfate trace minerals (STM; 60 mg Zn/kg DM from $ZnSO_4$) or hydroxy trace minerals (HTM; 60 mg Zn/kg DM from ZnOHCl). The x-axis denotes sampling time in hours, the y-axis denotes rumen soluble zinc. Error bars represent standard errors.

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