DISSERTATION

IN VIVO AND *IN VITRO* ATTRIBUTES OF COPPER, ZINC, AND MANGANESE SOURCES IN BEEF CATTLE NUTRITION

Submitted by

Octavio Guimaraes

Department of Animal Sciences

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Doctoral Committee:

Advisor: Terry E. Engle

John Wagner
Gina Geornaras
Mahesh Nair
Tim Holt

ABSTRACT

IN VIVO AND *IN VITRO* ATTRIBUTES OF COPPER, ZINC, AND MANGANESE SOURCES IN BEEF CATTLE NUTRITION

Trace minerals are not just vital components in the diets of domestic livestock species; they are also essential for ensuring optimal performance, health, reproduction, and a robust immune system. In today's volatile markets, producers and nutritionists face the challenge of meeting predefined goals and targets. They must employ strategies that can maximize production and efficiency. The intricate mechanisms governing the digestion, absorption, and storage of copper (Cu), zinc (Zn), and manganese (Mn) are influenced by various factors, including diet type, breed, life stage, among many others. Research has uncovered intriguing opportunities to enhance the utilization of trace minerals (TM) in ruminant nutrition. To further our comprehension of the digestion, absorption, and retention of modern trace mineral forms, this study conducted 3 experiments aimed at investigating the *in vivo* and *in vitro* characteristics of hydroxy trace minerals (HTM), organic chelated trace minerals (CTM), and sulfate trace minerals (STM) forms of Cu, Zn, and Mn.

This dissertation is the continuation of a Master Thesis submitted by Guimaraes et al., 2021. In the current series of experiments the influence of TM source on digestibility and ruminal solubility was investigated. The objectives of **experiment 1** were to determine the effects of TM

source on 1) nutrient digestibility and ruminal fermentation, 2) concentrations of soluble Cu, Zn, and Mn in the rumen following a pulse dose of TM, and 3) Cu, Zn, and Mn binding strength on ruminal digesta using dialysis against a chelating agent in steers fed a diet formulated to meet the requirements of a high producing dairy cow. Twelve Angus steers fitted with ruminal cannulae were adapted to a diet balanced with nutrient concentrations similar to a diet for a high producing lactating dairy cow for 21 d. Steers were then randomly assigned to dietary treatments consisting of 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either sulfate (STM), hydroxychloride (HTM) or complexed trace minerals (CTM). The experimental design did not include a negative control (no supplemental Cu, Mn, or Zn) because the basal diet did not meet the NRC (Nutrient Requirement for Beef Cattle) requirement for Cu and Zn. Copper, Mn, and Zn are also generally supplemented to lactating dairy cow diets at concentrations approximating those supplied in the present study. Following a 14-d adaptation period, total fecal output was collected for 5-d. Following the fecal collection period, rumen fluid was collected for VFA parameters. On the following day, the same diet was provided for 14 d, without supplemental Cu, Zn, and Mn. This period served as a wash-out period. A pulse dose of 100, 400, and 600 mg of Cu, Zn, Mn, respectively, from either STM, HTM, or CTM, was administered via ruminal cannulae to the steers on day 15. Over a 24-h period ruminal samples were obtained every 2-h. Following centrifugation, the supernatant was analyzed for Cu, Mn, and Zn. Ruminal solid digesta samples from times 0, 12, and 24 hours after bolus dosing were exposed to dialysis against Tris-EDTA. The digestibility of NDF and ADF were lesser in STM vs. HTM and vs. CTM supplemented steers. Steers receiving HTM and CTM had greater total VFA concentrations than STM, and molar proportions of individual VFA were not affected by treatment. Ruminal soluble Cu and Zn concentrations were greater post dosing in STM and CTM supplemented steers at 2, 4, and 6 h for Cu and 4, 6, 8, 10

and 12 h for Zn when compared to HTM supplemented steers. The release of Cu and Zn from ruminal solid digesta following dialysis against Tris-EDTA at 12 and 24 h post-dosing was greater for steers receiving HTM compared to those receiving STM or CTM. Results indicate trace mineral source impacts: 1) how tightly bound Cu and Zn are to ruminal solid digesta; 2) fiber digestion; 3) and ruminal total VFA concentrations.

The objective of **experiment 2** was to evaluate the effects of sources of STM and HTM fed at the same levels on 1) nutrient digestibility and fermentation characteristics, 2) ruminal solubility of Cu, Zn, and Mn following a pulse dose of trace mineral I, and 3) binding strength of Cu, Zn, and Mn on ruminal solid digesta upon dialysis against a chelating agent. Minor dietary changes can have a direct impact on ruminal pH, temperature, microbial population, and redox potential, which will influence fermentation, and ultimately affect nutrient digestibility. These changes can influence or be influenced by the solubility of certain TM in the rumen. Rumen soluble TMs can alter nutrient digestibility, fermentation, and increase chances of antagonistic interaction. Twelve ruminally cannulated Angus steers (BW 587.6 ± 23.1 kg) were adapted to a diet balanced to meet requirements of finishing steers for 21 d. Steers were then randomly assigned to dietary treatments consisting of Cu, Mn, and Zn supplemented at 18, 40, and 90 mg/kg DM, respectively, from STM or HTM sources (n = 6 steers/treatment; experimental unit = steer). Following the 14 days treatment adaptation period total fecal output was collected for 5 d. Digestibility of DM, CP, Starch, NDF, and ADF was not affected ($P > 0.15$) by TM source. On d 6, rumen fluid was collected at 0, 2, and 4 h post feeding for VFA analysis. There were no treatment x time interactions for any VFA measured. However, HTM steers had greater ($P < 0.05$) ruminal molar proportions of propionate and tended ($P < 0.07$) to have greater molar proportions of valerate compared to STM steers. Steers fed STM had greater ($P < 0.05$) molar proportions of butyrate compared to

HTM steers. Steers were then fed the same high concentrate diet without supplemental Cu, Zn, or Mn for 14 d. On d 15 steers received a pulse dose (via rumen cannulae) from either STM or HTM. Ruminal samples were obtained at 2 h intervals starting at -4 and ending at 24 h relative to dosing. There was a treatment x time interaction ($P < 0.05$) for ruminal soluble Cu concentrations. Ruminal soluble mineral concentrations were greater ($P < 0.05$) for Cu at 4, 6, 8, and 16 h post dosing in STM compared to HTM supplemented steers. There was no treatment x time interaction for ruminal soluble Zn or Mn concentrations. The release of Cu, Zn, and Mn from ruminal solid digesta following dialysis against Tris-EDTA at 12 and 24 h post-dosing was greater ($P < 0.01$) for steers receiving HTM compared to those receiving STM. Results of this experiment indicate that trace mineral source affects how tightly bound Cu, Zn, and Mn are to ruminal solid digesta, and that ruminal solubility of Cu differs between STM and HTM sources and may impact rumen fermentation characteristics.

Experiment 3: This study analyzed samples from experiments investigating TM source effects on nutrient digestibility, short-chain fatty acid production, Cu, Zn, and Mn ruminal solubility, and binding strength in rumen insoluble digesta. Twelve Angus steers (5+ years old) participated in three studies: Study 1 (Guimaraes et al., 2020, 2021; medium quality forage diet), Study 2 (Guimaraes et al., 2022; dairy type diet), and Study 3 (unpublished, Chapter 3 of this dissertation; high concentrate diet). Due to impacts on digestibility, fermentation, mineral solubility, and binding in all studies, microbiome analysis was conducted to assess potential bacterial and protozoa population shifts from all studies. In Study 1 (medium quality forage diet), *Papillibacter* was the predominant species in the rumen. Rumen samples showed a significant time point impact on Shannon's PD ($P < 0.05$). Beta diversity analysis using unweighted UniFrac revealed notable differences in community diversity between rumen and fecal samples ($q < 0.001$).

Rumen microbiota clustered at multiple time points. No beta diversity differences between STM and HTM were observed at baseline (Time A, $q = 0.23$), but differences emerged after adaptation (Time C, $q < 0.03$) and during total collection (Time F, $q < 0.01$). ANCOM analysis revealed higher abundance of *Papillibacter*, *Ruminoccoccaceae*, and *Prevotellaceae* in rumen samples, regardless of TM source. No significant differences were found in fecal samples. ANCOM analysis did not detect measurable differential abundance between trace mineral sources in fecal or rumen samples following Alpha and Beta diversity results. In Study 2 (dairy diet), *Moraxellaceae* was the most prevalent species within the rumen. Rumen samples showed a significant time point effect on Shannon's PD ($P < 0.001$), with noteworthy differences between several time points. However, TM source had no impact on Shannon's PD ($P = 0.15$). Fecal samples showed no differences in time points or TM sources. Beta diversity analysis using unweighted UniFrac revealed a significant distinction between microbial community diversity in rumen and fecal samples ($q < 0.001$). Rumen microbiota clustered differently at various time points. Trace mineral source did not affect Beta diversity, but there was a tendency toward greater diversity with HTM supplementation ($q = 0.07$). ANCOM analysis found higher abundance of ASVs *Moraxellaceae*, *Planococcaceae*, *Ruminoccoccaceae*, and *Prevotellaceae* in the rumen environment, regardless of TM source. No significant differential abundance was detected between TM sources in rumen samples, consistent with Shannon's PD diversity analysis. In Study 3 (high concentrate diet), *Moraxellaceae* and *Planococcaceae* were the most abundant in rumen samples. Rumen samples showed a significant impact of collection time point on Shannon's PD ($P < 0.001$), with significant differences between several time points. However, TM source had no effect on Shannon's Diversity ($P = 0.19$). Interestingly, Shannon's Diversity in feces showed significant differences between TM sources ($P < 0.03$), with STM having lesser diversity than HTM. There was no effect of time point in fecal

samples ($P = 0.38$). Beta diversity analysis using unweighted UniFrac revealed clear differences between microbial diversity in rumen and fecal samples ($q < 0.001$). ANCOM analysis found higher abundance of ASVs *Moraxellaceae, Prevotellaceae, Planococcaceae,* and *Ruminoccoccaceae* in the rumen environment, regardless of TM source. In conclusion, this study provides valuable insights into the complex interplay between TM sources, the rumen microbiome, and various aspects of rumen health and function. While TM sources did not significantly alter the rumen microbiome composition, time points demonstrated substantial effects on microbial communities. These findings contribute to our understanding of how dietary factors can influence rumen ecology and function, with potential implications for livestock nutrition and management strategies.

In conclusion, while experiment 1 showed that HTM and CTM sources positively impacted fiber digestibility and ruminal VFA concentrations compared to STM, experiment 2 indicated that the diet type could influence these effects. However, ruminal soluble mineral concentrations remained greater in STM-supplemented steers. Experiment 3 further highlighted the differences in rumen and fecal microbiota diversity between TM sources. These findings underscore the importance of selecting the appropriate TM source based on specific dietary and production requirements. The interactions between TMs, diet type, and the rumen microbiome are complex and require further investigation to optimize ruminant nutrition and overall well-being. This research builds upon previous studies and contributes valuable insights into the utilization of TMs in ruminant nutrition, paving the way for more informed decision-making in livestock production practices.

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DEDICATION

To my beloved Grandpa Jose Roberto de Almeida Guimaraes and my cherished first son, Andre Davi Guimaraes

This journey through life is enriched by the presence of both the past and the future. You, Grandpa, was and have been a steadfast pillar of wisdom and love, a repository of stories that have shaped our family's legacy. Your guidance and enduring spirit have left an indelible mark on my heart.

And to my precious first son, you are the embodiment of hope, promise, and the continuation of our family's story. Your innocence and boundless curiosity show me what is life meant to be, you breathe new life into our family's future.

In this dedication, I honor the wisdom and love of generations past and embrace the bright possibilities of generations to come. You both hold special places in my heart, and I am grateful for the connections you've woven into the tapestry of our family's story.

With love and gratitude,

OG

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Trace minerals have been identified as essential components in the diets of domestic livestock species. Included in the category of essential trace minerals (or micro minerals) are chromium (Cr), cobalt (Co), copper (Cu), iodine (I), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn). Several of these have been heavily studied over the years.

Numerous biochemical reactions have been identified requiring trace minerals for proper function. It has been well documented that deficiencies of various trace minerals can result in metabolic and many other diseases. However, the interactions between trace minerals and metabolic processes are extremely complex, and that complexity makes it difficult to simulate or recreate in the laboratory. As an example, trace minerals have been identified as essential components for carbohydrate, lipid, protein, and vitamin metabolism, and have been shown to be involved in hormone production, immunity, and cellular homeostasis.

In general, trace minerals function primarily as catalysts in enzyme systems within cells. Enzymes requiring metals for proper function can be classified 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 are enzymes that contain a tightly bound metal ion

at or near the active site. The metal ions bound to metalloenzymes are actively involved in catalysis. Removal of the metal ion renders the enzyme 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.

In order to optimize animal performance, ruminant nutritionists have focused primarily on dietary carbohydrate and protein formulation. However, the availability of several feed additives, which includes different trace mineral sources and compounds, offering improvements in feed efficiency, health, and many other aspects of the production system, are forcing a broader knowledge and understanding to better utilization and application.

Depending on the type and source of carbohydrate and protein utilized in rations, supplements are then formulated to supply the balance of nutrients that are not met by the basal ingredients. Trace minerals are typically added to these supplements, and very little consideration was given to the trace minerals contained in the primary dietary ingredients. The requirements of essential trace minerals that are needed to avoid deficiencies are well known, however, the need for research demonstrating optimal levels and sources of trace minerals is still needed (Lindemman 2013; Cohen, 2014). Several experiments have focused on trace mineral supplementation during the feedlot phase of beef production, but results have been highly variable (Rhoads et al., 2003, Malcolm-Callis et al., 2000). Identifying not only the optimal trace minerals, but sources and doses, can be challenging because trace mineral nutrition requires a complete understanding of the animal's mineral requirements as well as an understanding of the mechanisms underlying digestion, absorption, and utilization of the minerals provided in the diets. Regardless of

contradicting data or variability in response variables, the importance of trace minerals for growth, reproduction, and immune response has been repeatedly demonstrated.

The goal of this chapter is to review the literature pertaining to the importance of trace mineral nutrition in cattle production, focusing primarily on Cu, Zn, and Mn and to discuss the general functions underlying digestion, absorption, transport, storage, excretion, and bioavailability of these trace minerals in ruminants. Another aspect of this chapter will be devoted to discussing chemical characteristics of trace mineral sources of Cu, Zn, and Mn supplemented to beef and dairy cattle.

TRACE MINERAL ABSORPTION

Zinc

The trace mineral zinc (Zn) is an essential nutrient for mammals because of its role in enzymes involved in protein synthesis and carbohydrate metabolism. From a structural standpoint, Zn will stabilize the quaternary structure of an enzyme (McDowell, 1992). The small intestine, especially the proximal portion, is the main site of absorption and re-excretion. Absorption and homeostatic control mechanisms can vary depending on age and breed of ruminants (Miller et al., 1970). In addition, throughout the absorption process certain dietary factors can directly impact absorption of Zn in ruminants. Certain trace minerals such as Cu and Fe have an impact on the absorption of Zn. Other factors involved in Zn absorption include the source and dose of Zn.

The majority of Zn absorption research has been conducted in mice and rats with little research conducted in ruminants. Zinc absorption can be divided into four phases according to Cousins (1982). Figure 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 (2-4) pH; 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 blood stream making Zn absorption bidirectional (Hambidge et al., 1986).

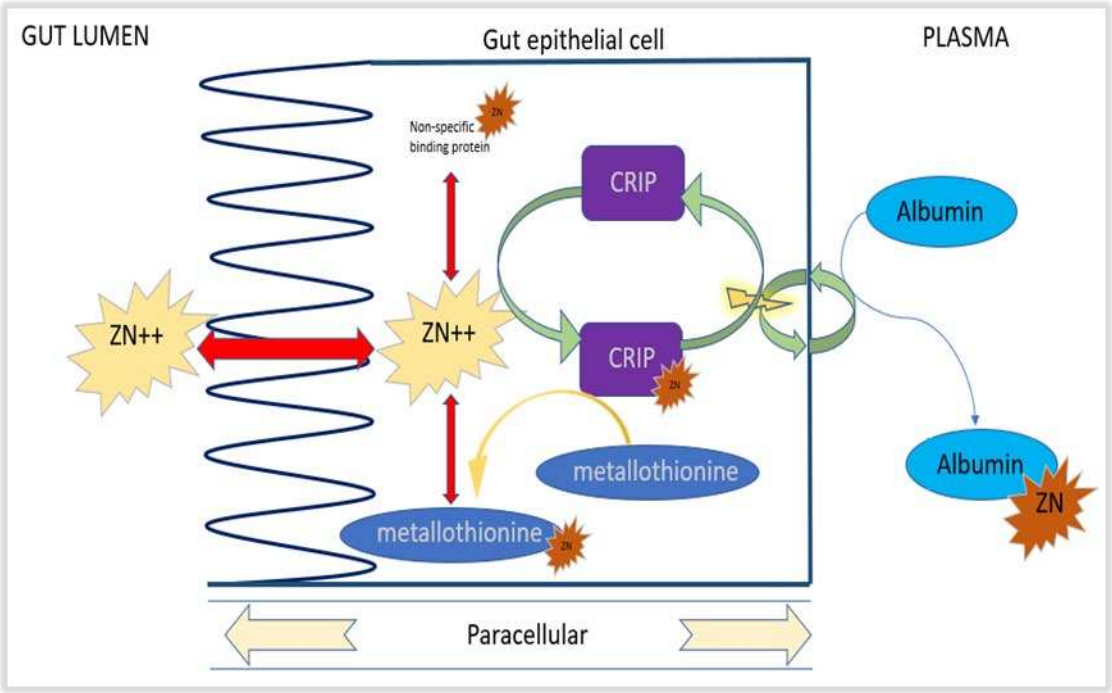

Figure 1. Mechanisms underlying Zn absorption (Adapted from J. NUTR. 122:89-95 (1992))

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 a low molecular weight protein, such as citrate, EDTA, or amino acids that may not require ATP (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). 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 acts 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. Furthermore, McDowell (1992) explains that in another study by Cousins (1996), there is evidence that dietary Zn and plasma Zn regulate the quantity of Zn absorbed by the body, playing a significant role in Zn homeostasis. When dietary Zn concentrations are low, metallothionein synthesis is decreased thus allowing for increased interactions of Zn and intracellular binding proteins such as CRIP that allows for the transport of Zn to the basolateral surface of the enterocyte. Hempe and Cousins (1991) explain that CRIP moves Zn across the enterocyte to the basolateral side of the cell. However, the exact process of Zn chaperoning has not been fully characterized. Zinc is then attached to a carrier molecule such as albumin. After Zn attaches to albumin it is then transported throughout the body (Hempe and Cousins, 1991). Also, Zn that is absorbed through the rumen wall in ruminants can also be

reabsorbed into the lumen of the small intestine. This mechanism of Zn absorption is minimal and secondary to Zn absorption from the small intestine.

Zinc absorption can also be regulated by other dietary factors. For example, inositol hexaphosphates and pentaphosphates are the phytate forms that exert strong negative effects on Zn absorption in non-ruminants (Lonnerdal, 2000). However, in ruminants, with a functional rumen, the amount of phytate found in the diet does not interfere or limit the amount of Zn absorbed (NRC, 2000) due to a phytases that are naturally found in the rumen. Phytases degrade phytate which in turn allows for the release of Zn and other ions that are attached to phytate (NASEM, 2016). In forages, a substantial amount of Zn is bound to plant cell walls (Whitehead et al., 1985), which certain bacteria and protozoa found in the rumen, can process and digest the Zn-associated fiber (Durand and Kawashima, 1980). Nevertheless, it remains unclear whether the interactions between Zn and dietary fiber affects its absorption.

Additionally, the amount of dietary protein has been demonstrated to be positively correlated to Zn absorption (Lonnerdal, 2000). Furthermore, fiber can have a negative effect on Zn absorption which could be due to the fact that most fiber-containing foods also contain phytate (Lonnerdal, 2000). However, 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 fiber has an influence on Zn digestion is contradicted by the NRC (2000) which states that it's unknown whether zinc's association with fiber reduces absorption. Zinc status should however be considered when dealing with a high fiber diet.

Source (organic or inorganic) of Zn has been reported to influence the absorption of Zn. According to Spears et al. (1989) organic and inorganic forms of Zn are metabolized differently following absorption. Organic forms of Zn have been reported to enhance performance and

improve health and reproduction. Data also supports that Zn absorption is similar between Zn methionine and inorganic sources, but evidence exists that Zn provided by Zn methionine is retained in the body more effectively than inorganic Zn (Brown et al., 2004; Spears, 1989). Furthermore, Zn dose has also been of interest when considering the optimum Zn supplementation practices. Interestingly, on average, nutritionists formulate feedlot diets to contain Zn concentrations well above the NRC (2000) requirements (Vasconcelos and Galyean, 2007). Typically, the Zn concentrations in feedlot diets formulated by nutritionists tend to be 3 times the NRC (2000) recommendations (Vasconcelos and Galyean, 2007). Ten years later, a similar survey by Samuelson et al. (2016) provided similar results indicating that consulting nutritionists formulated diets 2 times the NASEM (2016) recommendations for Cu and 3 times for Zn for growing and finishing feedlot cattle. This might seem counterintuitive, as the rate of Zn absorption tends to rise as dietary Zn levels decrease, but decreases with elevated Zn intake, as indicated by Church in 1988. Likewise, research has demonstrated that elevated Zn concentrations negatively affect absorption in comparison to the standard NRC (2000) Zn levels. Additionally, excessive Zn intake could directly influence the absorption of other trace minerals, such as Cu.

The mechanisms governing Zn absorption are intricate, and additional research is required to ascertain the ideal Zn dosage for ruminant diets. Although existing data provide guidance for preventing both toxicities and deficiencies in ruminant nutrition, Zn involvement in multifaceted metabolic functions necessitates the determination of an optimal nutritional concentration. This ensures that Zn can continue to fulfill its role in various metabolic processes effectively, as previously discussed.

Copper

Intestinal absorption of Cu can be through both a passive and active process and is thought to be a similar process in ruminants and non-ruminants (Underwood and Suttle, 1999). Nonetheless, the mechanisms underlying Cu absorption are critical due to Cu being poorly absorbed in most mammals (McDowell, 1992; Underwood, 1977). The absorption of Cu in ruminants is low (<1.0-10%) when compared to non-ruminants (Spears, 2003). As discussed above, Cu absorption, under certain conditions, can be directly influenced by dietary Zn concentrations. The chemical form of Cu can influence the amount of Cu absorbed as explained by McDowell (1992) and Underwood (1977). 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). Copper requirements for ruminants are greatly increased by Mo and S in excess of 2mg Mo/kg dietary DM and 0.25% S in most situations (Smart et al., 1986; Underwood and Suttle, 1999; Spears, 2003; Hansen et al., 2008; Kessler et al., 2012; Drewnoski et al., 2014; Pogge et al., 2014). The antagonist interaction of Mo with Cu metabolism can be even more severe when S is also high in the diet. Considerable evidence suggests that molybdate and sulfide interact to form thiomolybdates in the rumen (Suttle, 1991). Copper is believed to react with thiomolybdates in the rumen to form insoluble complexes that are undigestible. As discussed in NRC (2001), it is difficult to predict the negative impacts that S and Mo can have on Cu digestion, absorption, and metabolism.

The processes underlying Cu absorption is controlled through two mechanisms (Figure 2) which consist of saturable and unsaturable mechanisms relating back to the active transport and simple diffusion process (McDowell, 1992). More recently Cu is thought 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. Like Zn, Cu is solubilized at a low pH (2-4). Once solubilized in the stomach, Cu will enter the small intestine. Briefly, 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. Once 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). The exact mechanism(s) of Cu absorption is still not well understood (Hill and Link, 2009). Furthermore, metallothionein also functions as a regulator of Cu absorption in the epithelial cells of the intestine. Much like Zn, when Cu concentrations in the diet are low then Cu absorption is increased. Copper absorption can also be negatively influenced by the formation of Cu sulfide in the gut (NRC, 2000). To enhance Cu absorption at the intestinal level, its crucial to minimize the interactions between Cu and other dietary components (McDowell, 1992). Dietary Cu recommendations may fluctuate based on the dietary concentrations of other trace minerals and the specific diet composition. This dynamic interplay underscores the importance of tailored Cu recommendations for optimal nutritional outcomes.

It is known that concentrate diets offer more available Cu when compared to high forage diets. Certain environmental, genetic, and dietary factors can also influence animal performance, digestion, and absorption responses to dietary Cu, which might explain drastic variability in experimental results.

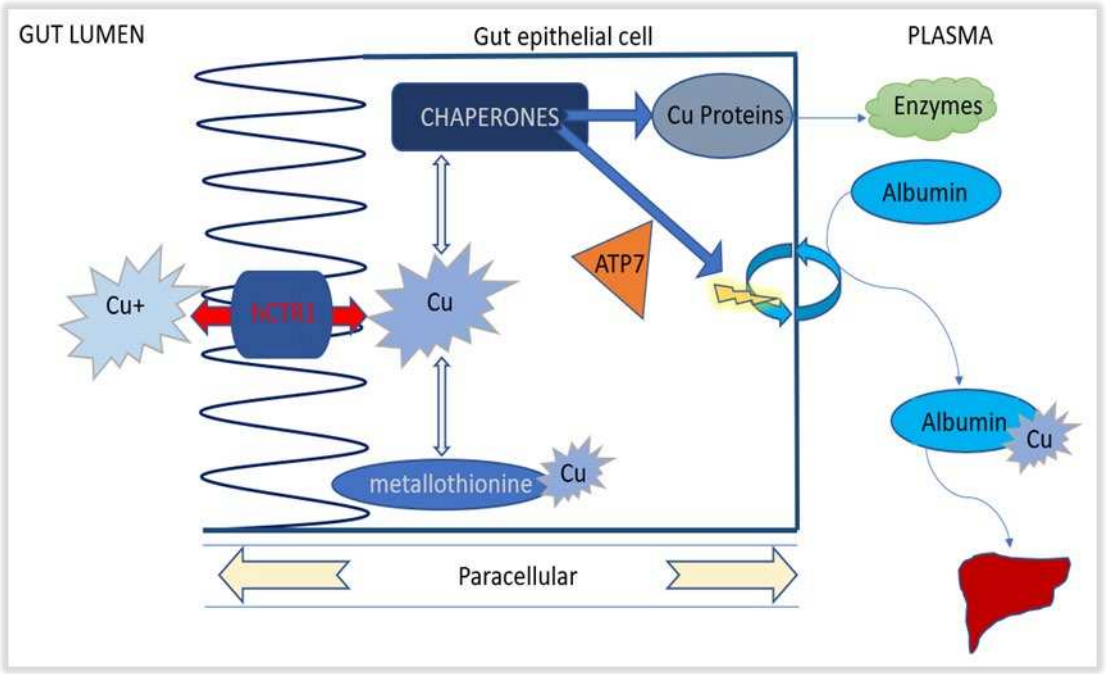

Figure 2. Mechanisms underlying Cu absorption (Adapted from J. NUTR. 122:89-95 (1992))

Manganese

Although little is known concerning dietary factors affecting Mn absorption (NRC, 2000), McDowell (1992) explains that Mn absorption occurs in a two-step process which involves the uptake from the gut lumen, and then transfer across the mucosal cells. Also, much like Zn and Cu, when dietary Mn concentrations are high absorption decreases, and when dietary Mn concentrations are low absorption increases. However, a Mn deficiency can occur in diets composed of normal feed ingredients in swine, poultry, and ruminants (McDowell, 1992). Unlike Zn where there is a regulated absorption and excretion process, Mn homeostasis is primarily regulated through excretion (Thomas, 1970). Manganese is thought to be absorbed 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 is similar to iron. Once absorbed, Mn is transported

throughout the body bound to transferrin (Underwood and Suttle, 1999). However, Mn antagonist in the diet such as Ca and P may cause the Mn requirement in the diet to increase (Hawkins et al., 1955; Dyer et al., 1964; Lassiter et al., 1972; Olson and Hale, 2001; Spears, 2003). Excretion of Mn through bile plays an important homeostasis role, but little excretion of Mn occurs via the urine (Hidiroglou, 1979). 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 under natural conditions in certain areas of the northwestern United States.

Manganese can generally be supplemented as manganese sulfate, manganese oxide, or various organic forms (manganese methionine, manganese proteinate, manganese polysaccharide complex, or manganese amino acid chelate). Manganese sulfate is known to be more available when compared to manganese oxide (Wong-Ville et al., 1989; Henry et al., 1992), however when manganese sulfate is compared to an organic source such as manganese methionine, availability was found to be 20% less.

TRACE MINERAL TRANSPORT

Zinc

Once Zn enters the blood circulatory system a variety of factors will dictate its metabolism. Zinc is transported through the blood stream bound to either albumin, alpha 2 macroglobulin, and also as traces of metallothionein (Underwood and Suttle, 1999) and flows to the liver. Once 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 abundance in liver, pancreas, intestine, and kidney, whereas metallothionein 3 and 4 are found principally 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. Whereas the bond between enterocyte derived metallothionein and Zn is extremely tight.

Therefore, once Zn is in the enterocyte the absorption of Zn into the blood stream is dependent on Zn concentrations of Zn in the circulatory system along with the regulation of metallothionein in the enterocyte. When Zn concentrations in the blood are low, Zn transport into the blood will increase (Hambidge et al., 1986). Yet, the reverse effect occurs when Zn concentrations are high in the blood; less Zn is absorbed from the intestinal cells into the blood stream.

Whether the absorption of Zn is up or down regulated, plasma Zn will dictate only part of Zn status within the animal. This implies that to make accurate assumptions about Zn status within the animal more should be evaluated than blood status alone. A possible approach would be to identify indicators of Zn status in the serum such as metallothionein 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 and is also affected by gut and bone disorders (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 blood stream and from the blood stream into the lumen is a bidirectional process in 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 blood stream (Underwood and Suttle, 1999). The other portion of plasma Zn can be bound to alpha 2 macroglobulin or to metallothionein for example.

Copper

More recent data reports the identification of Cu transporters and chaperones (Hill and Link, 2009). Molecular techniques as well as human metabolic disorders and single-cell organisms have all helped understand the mechanisms underlying Cu metabolism (Hill and Link, 2009). Transporter chaperones are critical for cellular trace mineral homeostasis as well as for the whole animal (Cohen, 2014; Fry et al., 2013). In general, once Cu is absorbed and enters the blood stream it then binds to albumin and/or the amino acid histidine and is then transported throughout the body (McDowell, 1992). If Cu binds to metallothionein in the intestinal mucosa then this interaction could restrict the further translocation of Cu (Cousins, 1985; Underwood and Suttle, 1999). While Cu is transported throughout the body there is a good possibility that antagonists such as molybdenum (Mo), sulfur (S), and iron (Fe) could induce hypokyphosis by forming insoluble Cu complexes in the digestive tract, bloodstream, and tissues of ruminants (Bailey et al., 2001). Therefore, Bailey et al. (2001) emphasizes that it would be advantageous to develop supplementation strategies that would limit these antagonistic effects.

Manganese

Manganese absorption can occur throughout the length of the small intestine in two steps; uptake from the lumen then transfer across the mucosal cells. Absorbed Mn is transported by transferrin to the liver (Davidson, 1989; Underwood, 1999). Manganese is mainly excreted in the

feces via biliary excretion (Thomas, 1970). Manganese being excreted through bile will have a minimal possibility of being reabsorbed (Underwood, 1999). Since bile is a major route for excess Mn; then attempting to understand flow rates of Mn through the digestive tract post oral or ruminal dosing is challenging due to endogenous losses of Mn. Also, Mn excretion via the feces is proportionally greater as the amount of Mn absorbed increases (Underwood, 1999). Nonetheless, Genther and Hansen (2014) report the importance of Mn superoxide dismutase which is an antioxidant found 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 it was demonstrated that trace mineral injection of Cu, Mn, and Zn increased red blood cell lysate Mn superoxide dismutase activity (Genther and Hansen, 2014). However, Genther and Hansen (2014) did report there is still lack of a good biomarker of Mn in cattle, but Mn 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.

TRACE MINERAL STORAGE AND EXCRETION

Zinc

Even though the mechanisms of tissue uptake of Zn have not yet been characterized, Zn complexed with albumin is readily available for uptake by tissues (McDowell, 1992). Yet not all tissues that uptake 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 blood stream 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 accumulation 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 blood stream 80% is present in the erythrocytes, which contain about 1mg Zn per 10^6 cells (Underwood and Suttle, 1999). The pancreas, liver, kidney, 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 in order 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 blood, pancreas, kidney, 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 the synthesis of 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 Cu toxicity (Underwood and Suttle, 1999). Again, much like Zn metallothionein will bind Cu and aid in Cu storage as well as an aid in providing Cu 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 (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 not only excreted through feces, with bile being the main source (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 at 15.8 mg/kg but not when cows were supplemented at 25mg/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 importance of dietary supplementation of cattle feeds with trace minerals is irrefutable, regardless of the source of products commercially available. With rapid advances in research techniques, and data obtained from both in-vitro and in-vivo studies, differences between inorganic and organic minerals have become more apparent. Currently, trace minerals are available in both organic and inorganic forms as well as hydroxyl minerals.

The source of the mineral is of crucial importance. In addition to differing by type, organic, and inorganic, they also differ greatly in terms of how well they are absorbed and utilized by the animal. Research has further highlighted that the low pH environment of the upper gastrointestinal tract reduces the digestibility of inorganic salts by causing dissociation, thereby leaving the minerals susceptible to various nutrient and ingredient antagonisms that impair absorption (Ammerman et al., 1975, Spears et al., 1996). As pH increases in the small intestine, minerals such as Zn and Cu can additionally form insoluble hydroxide precipitates, rendering them unavailable for absorption. Over the last number of years, organic mineral sources have increasingly been used instead of inorganic sources due to their apparent benefits—the organic counterparts are better protected from unwanted interactions in the GI tract and have enhanced bioavailability. Trace minerals defined as inorganic are those that are typically bound to sulfates, carbonates, chlorides, or oxides, while those defined as organic are bound to amino acids or protein complexes, and are referred to as complexes, chelates or proteinates (Spears, 1996). Spears (1996) provides a table (Table 1) from the Association of American Feed control officials providing definitions for various types of mineral products.

Table 1. Definitions of various organic mineral products according to the Association of American Feed Controls Officials.

57.150 Metal amino acid complex - The product resulting from complexing of a soluble metal salt with an amino acid(s).
57.142 Metal amino acid chelate - The product resulting from the reaction of a metal ion from a soluble metal salt with amino acids with a mole ratio of one mole of metal to one to three (preferably two) moles of amino acids to form coordinate covalent bonds. The average weight of the hydrolyzed amino acids must be approximately 150 and the resulting molecular weight of the chelate must not exceed 800.
57.23 Metal proteinate - The product resulting from the chelation of a soluble salt with amino acids and/or partially hydrolyzed protein.
57.29 Metal polysaccharide complex - The product resulting from complexing of a soluble salt with a polysaccharide solution.

J.W. Spears/Animal Feed Science Technology 58 (1996) 151-163

Bioavailability has been defined as the proportion of the element consumed that is utilized for a biochemical or physiological function (O'Dell, 1997; Hill and Link, 2009). The general premise behind increased bioavailability of organic trace minerals is that organic trace minerals are protected from many of the dietary antagonists (as previously mentioned) that can potentially make them unavailable for absorption (Hemken et al., 1996). It has been theorized by some researchers that organic trace minerals remain intact in the gastrointestinal tract, through the sight of absorption, and perhaps beyond absorption. On the other hand, the hydroxyl trace minerals (copper hydroxyl chloride, zinc hydroxyl chloride, and manganese hydroxyl chloride) belong to a separate group of trace minerals because of their unique chemical characteristics. 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 relates to the sulfate group by a weak ionic bond (Mn hydroxy chloride and Cu hydroxyl chloride contain the covalent bonds as well). The covalent bonds are believed to increase the amount of biologically active Zn delivered to the intestine, driving increased Zn absorption (Cohen

and Steward, unknown). The same authors explained how the covalent bonds with the metals are strong enough to limit parasitic reactions, but weak enough to readily hand off the metal to the escort ligands involved to facilitate absorption, thus positively affecting the bioavailability of the hydroxyl trace minerals. In addition, the sulfate sources of Zn, Cu, and Mn contain the ionic bond and are thought to disassociate once contact with moisture occurs allowing the metal ions to bind with many diet antagonists. Thus, tribasic Cu chloride is more bioavailable than CuSO4 when added to diets high in the Cu antagonists Mo and S (Spears et al., 2004).

Several studies have been conducted using in vitro and in vivo techniques to determine the relative bioavailability of trace mineral sources. These experiments typically use an inorganic mineral as a benchmark (100%) and compare other mineral sources to it. Results have been variable, however under certain circumstances organic mineral sources have centered around the theory that they are more bioavailable, which suggests that organic trace minerals may be more similar to forms that occur in the body (Spears, 1996). Since trace minerals that are present in the body function as organic complexes or chelates and not as free inorganic ions then a stable metal chelate that keeps from forming complexes with other dietary components that inhibit absorption would allow for a greater absorption and utilization by the body (Spears, 1996). Furthermore, it is challenging to interpret data from different experiments because different researchers may have used various methods of supplementation, different sources of trace minerals, a variety of different cattle types, and a variety of environmental variables may exist. 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 experiments.

RUMEN MICROBIOME

The rumen plays a pivotal role in the digestive process, and within this complex ecosystem, an involved community of microorganisms collaborates in a symbiotic relationship with their host to break down complex plant materials and other feed stuffs into valuable nutrients. The rumen microbiome consists of bacteria, archaea, fungi, and protozoa, working together to ferment fibrous plant material, converting it into volatile fatty acids and microbial protein that serve as a crucial energy source for the ruminant animal. This intricate network of microorganisms has been a subject of extensive research and continues to intrigue scientists due to its essential role in ruminant nutrition, health, and environmental sustainability. The most remarkable aspect of the rumen microbiome is its staggering diversity (Amos et al., 1978; Akin et al., 1979; Prins and Clarke, 1980). It houses thousands of species of microorganisms, each with its own specific function (Henderson et al., 2015). Bacteria, the most abundant members, are responsible for cellulose and starch degradation, while archaea, specifically methanogens, play a role in methane production, a potent greenhouse gas. Fungi contribute to fiber degradation, breaking down complex lignocellulosic structures, and protozoa predate on bacteria and contribute to nitrogen recycling. This diversity and specialization within the microbiome allow ruminants to efficiently extract nutrients from plant-based diets that are challenging to digest by most other animals.

The composition of the rumen microbiome is highly dynamic and responsive to dietary changes. Altering the diet can rapidly shift the abundance and diversity of various microbial populations (Petri et al., 2013; Gruninger et al., 2019). For instance, transitioning from a high-fiber diet to a grain-based diet can lead to a dramatic increase in starch-degrading bacteria, significantly impacting fermentation patterns and nutrient availability. Such diet-induced shifts

in the microbiome can have profound effects on the overall health and productivity of ruminants. Researchers and nutritionists are continually studying these relationships to optimize animal performance, reduce environmental impacts, and develop more sustainable feeding strategies. A balanced and diverse rumen microbiome is crucial for ruminant health (Auffret et al., 2017). Dysbiosis, or an imbalance in the microbial community, can lead to digestive disorders, reduced nutrient utilization, and decreased animal productivity. These issues can have economic implications for livestock producers and ethical implications for animal welfare. Therefore, understanding the factors that influence the composition and stability of the rumen microbiome is of paramount importance.

Trace minerals commonly supplemented to cattle may impact the rumen microbial community. The extend of the impact will potentially depend on multiple variables, including the type and source of trace minerals, the composition of the diet, and the specific microbial populations present in the rumen. Different forms of trace minerals may influence the diversity of microbial populations in the rumen (Durand and Kawashima, 1979; Faulkner et al., 2017). Organic forms of trace minerals, such as amino acid complexes, have been suggested to promote a more diverse microbial community compared to inorganic forms (Durand and Kawashima, 1979; Moore et al., 1989; Kessler et al., 2003; Broom et al., 2006; Rabiee et al., 2010; Faulkner et al., 2016). Furthermore, microbial diversity may affect efficiency and nutrient utilization. Fiber digestion is dependent on very specific microorganisms, certain sources of trace minerals become more rapidly soluble in the rumen (Caldera et al., 2019; Guimaraes et al., 2020; Guimaraes et al., 2021; Guimaraes et al., 2022), and by becoming soluble, can be toxic to some bacteria and protozoa, thus limiting the rate and extent of fiber digestion (Zhou et al., 2016; Faulkner et al., 2017; Wang et al., 2019). As we continue to delve deeper into the complexities of the rumen microbiome, we gain

valuable insights that can be applied to optimize ruminant nutrition, reduce environmental impacts, and improve animal welfare. This knowledge not only benefits the livestock industry but also contributes to our broader understanding of microbial ecosystems and their critical roles in the natural world. Furthermore, it is challenging to interpret data from different experiments because different researchers may have used various methods of supplementation, different sources of trace minerals, a variety of different cattle types, and a variety of environmental variables may exist. 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 experiments.

CONCLUSIONS

With that in mind, the next chapters will discuss the results obtained when trace mineral sources were fed in combination of different diet types. These experiments focused on investigating the effects of trace mineral sources on nutrient digestibility, fermentation characteristics, mineral soluble concentration, mineral binding strength to digesta, and the rumen microbiome of beef cattle when fed a forage-based diet, dairy type diet, and high concentrated diet.

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CHAPTER II

Trace mineral source influences digestion, ruminal fermentation, and ruminal copper, zinc, and manganese distribution in steer fed a diet suitable for lactating dairy cow.

SUMMARY

High solubility of certain trace minerals (TM) in the rumen can alter nutrient digestibility and fermentation. The objectives of the present studies were to determine the effects of TM source on 1) nutrient digestibility and ruminal fermentation, 2) concentrations of soluble Cu, Zn, and Mn in the rumen following a pulse dose of TM, and 3) Cu, Zn, and Mn binding strength on ruminal digesta using dialysis against a chelating agent in steers fed a diet formulated to meet the requirements of a high producing dairy cow. Twelve Angus steers fitted with ruminal cannulae were adapted to a diet balanced with nutrient concentrations similar to a diet for a high producing lactating dairy cow for 21 d. Steers were then randomly assigned to dietary treatments consisting of 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either sulfate (STM), hydroxychloride (HTM) or complexed trace minerals (CTM). The experimental design did not include a negative control (no supplemental Cu, Mn, or Zn) because the basal diet did not meet the NRC requirement for Cu and Zn. Copper, Mn, and Zn are also generally supplemented to lactating dairy cow diets at concentrations approximating those supplied in the present study. Following a 14-d adaptation period, total fecal output was collected for 5-d. Following the fecal collection period, rumen fluid was collected for VFA parameters. On the following day, the same diet was provided for 14 d, without supplemental Cu, Zn, and Mn. This period served as a wash-out period. A pulse dose of

100, 400, and 600 mg of Cu, Zn, Mn, respectively, from either STM, HTM, or CTM, was administered via ruminal cannulae to the steers on day 15. Over a 24-h period ruminal samples were obtained every 2-h. Following centrifugation, the supernatant was analyzed for Cu, Mn, and Zn. Ruminal solid digesta samples from times 0, 12, and 24 h after bolus dosing were exposed to dialysis against Tris-EDTA. The digestibility of NDF and ADF were lesser in STM vs. HTM and vs. CTM supplemented steers. Steers receiving HTM and CTM had greater total VFA concentrations than STM, and molar proportions of individual VFA were not affected by treatment. Ruminal soluble Cu and Zn concentrations were greater post dosing in STM and CTM supplemented steers at 2, 4, and 6 h for Cu and 4, 6, 8, 10 and 12 h for Zn when compared to HTM supplemented steers. The release of Cu and Zn from ruminal solid digesta following dialysis against Tris-EDTA at 12 and 24 h post-dosing was greater for steers receiving HTM compared to those receiving STM or CTM. Results indicate trace mineral source impacts: 1) how tightly bound Cu and Zn are to ruminal solid digesta; 2) fiber digestion; 3) and ruminal total VFA concentrations.

Keywords: fiber digestibility, mineral solubility, cattle, trace minerals.

INTRODUCTION

Reduction in fiber digestibility can directly impact cattle production. Trace mineral source may influence fiber digestibility (Guimaraes et al., 2021), and trace mineral bioavailability (Spears, 2002). In addition to that, Hydroxychloride (OHCl) sources of copper (Cu) and zinc (Zn) were less soluble in the ruminal environment than sulfate forms in cattle fed a corn silage-based diet (Caldera et al., 2019) and when fed a medium quality grass hay-based diet (Guimaraes et al., 2021). This is consistent with earlier findings that sulfate sources of Cu and Zn are highly soluble in water and in acid solutions, whereas OHCl sources of Cu and Zn are much less soluble in water and have increased solubility as pH becomes more acidic (Cao et al., 2000; Spears et al., 2004). High concentrations of soluble Cu and Zn in the rumen can negatively impact fiber digestion (Durand and Kawashima, 1980), and alter ruminal fermentation (Daniel et al., 2020; Guimaraes et al., 2021). Replacing sulfate trace minerals (STM) with OHCl trace minerals (HTM) improved NDF digestibility in lactating dairy cows, when total fecal output was measured over a 4-d period (Faulkner and Weiss, 2017). Other studies, using indigestible NDF to estimate digestibility, have also reported improved NDF digestibility in lactating dairy cows fed HTM compared with those supplemented with STM (Daniel et al., 2020; Miller et al., 2020) sources of Cu, Mn, and Zn. It is unclear if the greater NDF digestibility in cows fed HTM source of Cu, Mn, and Zn is caused by changes in digestibility of hemicellulose, cellulose, or both.

Organic forms of Cu or Zn may also affect fiber digestibility differently than sulfate forms. Lambs receiving 10 or 20 mg supplemental Cu/kg DM from Cu proteinate had greater digestibility of NDF and ADF than lambs given a similar amount of Cu from Cu sulfate (Dezfoulian et al., 2012). Compared to Zn sulfate, supplementation with organic forms of Zn increased NDF and

ADF digestibility in lambs in some studies (Garg et al., 2008; Alimohamady et al., 2019), whereas results in VanValin et al. (2018) seemed contradictory. Limited research also suggests that Zn provided in the form of amino acid complex may affect the rumen bacterial community differently than Zn sulfate in lambs (Ishaq et al., 2019).

Studies have indicated that Cu and Zn from OHCl forms were more bioavailable than sulfate forms in steers (Spears et al., 2004; Shaeffer et al., 2017). The greater bioavailability of Cu and Zn from OHCl forms may relate to their lower ruminal solubility reducing antagonistic interactions in the ruminal environment. Based on Cu and Zn release from ruminal solid digesta estimated using an in vitro technique (dialysis against Tris-EDTA), Cu and Zn from OHCl forms were less tightly bound to solid digesta than sulfate forms (Caldera et al., 2019). Previous data investigating the influence of TM source on binding characteristics of Cu, Mn, and Zn in the rumen has been conducted with cattle fed either a high forage diet (Guimaraes et al., 2021) or a growing feedlot diet (Caldera et al., 20219). To our knowledge, no research has been conducted investigating the solubility and binding characteristics of Cu, Mn, and Zn from different TM sources in cattle fed a diet formulated to meet the nutrient requirements of a lactating Holstein cow. Therefore, the objectives of the present studies were to determine the influence of TM source on 1) nutrient digestibility and ruminal fermentation characteristics, 2) ruminal soluble concentrations of Cu, Zn, and Mn following a pulse dose of TM, and 3) in vitro release of Cu, Zn, and Mn from ruminal solid digesta using dialysis against a chelating agent in steers fed a diet formulated to meet the requirements of a lactating Holstein dairy cow. We hypothesized that under the ruminal conditions produced by feeding a lactating dairy cow diet, that ruminal solubility of Cu, Mn, and Zn would be greater and fiber digestibility lower in steers fed sulfate compared to those receiving OHCl or organic sources.

MATERIAL AND METHODS

All the experimental procedures described in this study were approved by the Colorado State University Animal Care and Use (IACUC approval #17-7182A) prior to beginning the experiment.

Nutrient digestibility and fermentation characteristics

The present study was conducted at the Colorado State University Agriculture, Research, Development, and Education Center in Fort Collins, CO, United States. Twelve crossbred Angus steers fitted with ruminal cannulae (initial BW 530.9 ± 22.7kg were utilized in this experiment. Steers were previously ranked by BW and allocated to 3 feedlot pens (4 steers per pen) and fed a basal diet without supplemental Cu, Mn or Zn for 21 days. Ingredient and analyzed nutrient composition of the basal experimental diet is shown in Table 2. The basal diet was formulated with nutrient concentrations similar to a diet for a high producing Holstein dairy cow (producing 45 kg milk/d), except for Cu, Mn, and Zn (NRC, 2001). The basal diet contained 6.9, 32.7, and 31.6 mg/kg DM of Cu, Mn, and Zn, respectively.

Following the adaptation phase, steers were assigned to one of three dietary treatments. Supplements containing each treatment were prepared prior to the initiation of the study. Treatments were formulated to provide an additional 10 mg Cu/kg DM, 40 mg Mn/kg DM, and 60 mg Zn/kg DM, from either STM, HTM (IntelliBond® C, M, and Z; Micronutrients USA LLC., Indianapolis, IN), or complexed trace minerals (CTM; Zn methionine, Cu lysine, and manganese methionine; Zinpro Corp., Eden Prairie, MN) sources to the basal diet. Therefore, total dietary Cu, Mn, and Zn concentrations were 16.9, 72.7, and 91.6 mg/kg DM, respectively and exceeded the

Cu, Mn, and Zn requirements for high producing dairy cows (NRC, 2000). Of the total Cu, Mn, and Zn concentrations in the diet, approximately 59%, 55%, and 66% of the Cu, Mn, and Zn came from supplemental sources. Treatments were mixed with ground corn. The appropriate TM supplement was top-dressed to the basal experiment diet at 2.48% of the total diet and mixed by hand, ensuring even supplement distribution within the diet. Supplying the supplement at 2.48% of the diet provided an additional 10, 40, and 60 mg of Cu, Mn, and Zn /kg DM, respectively to the basal diet. After receiving treatments for 7 days, steers were moved indoors and housed in individual pens (2.5 m x 2.5 m pens equipped with automatic waters, individual feeders, and rubber matted floors) and allowed to acclimate to the indoor research facility for 2 days as described by Guimaraes et al. (2021).

Steers were then relocated into individual metabolism stalls (3.0 m x 1.1m pens) equipped with automatic waters, individual plastic feeders, and rubber matted floors for a 5-d acclimation period, where DMI for each steer was determined as described by Guimaraes et al. (2021). At the end of the acclimation period, steers were blocked across treatments based on the stratification procedure described by Caldera et al. (2019). Briefly, steers within each treatment were ranked from lowest to highest DMI. Steers with the lowest DMI in each treatment were block 1. This was repeated until 4 blocks of three steers (1 steers from each treatment) were configured. To allow equal DMI across all steers within a block, the method described by Guimaraes et al. (2021) was used, where 90% of the DMI of the steer with the lowest DMI was used across the block. This allowed for equal DMI across all steers within a block over the 5-d total collection period.

Table 2. Ingredient and nutrient composition of the basal diet fed to steers using a lactation dairy diet.

Item	DM, % as fed
Ingredient	
Corn silage, %	64.5
Alfalfa hay, %	10.2
Supplement, %[1]	25.3
Analyzed chemical composition	
DM, %	51.6
CP, %	14.5
Organic Matter, %	93.8
Ash, %	6.2
Fat, %	4.1
Starch, %	29.4
ADF, %	20.3
NDF, %	32.5
Ca, %	0.95
P, %	0.40
S, %	0.19
Cu, mg/kg	6.9
Mn, mg/kg	32.7
Zn, mg/kg	31.6

[1] On a DM basis consisted of: 64.0% soybean meal, 16.2% dried distillers grains, 9.4% cracked corn, 7.5% limestone, 1.9% salt, 0.63% magnesium oxide, and 0.35% trace mineral premix (provided per kilogram of DM: 0.1 mg Co, 0.50 mg I, and 0.10 mg Se).

Diets were fed twice daily (60% of the ration in the morning and 40% of the ration in the afternoon). Analyzed Cu, Mn, and Zn concentrations in the TM-ground corn supplements are presented in Table 3.

Sample collection and laboratory analysis

Total fecal output was measured daily for individual steers during the 5-d collection period as described by Caldera et al. (2019), Figure 3. Feces collected each day were quantified by wet weight, mixed thoroughly, and sampled (10.0% of wet weight). Duplicate, individual fecal samples were sealed in plastic bags, labeled, and stored at -20º C. Prior to chemical analysis (DM, NDF, ADF, and crude protein) of feces and feed, samples were proportionally composited across all

collection days for each animal. Fecal sampling and nutrient composition were performed and determined as described by Guimaraes et al. (2021).

Following the 5-d fecal collection period (d 20) ruminal samples were collected at 0, 2, and 4 h post-feeding and immediately 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 VFA concentrations via gas chromatography (Agilent 6890N, Santa Clara, CA). Briefly, the gas chromatograph used for analysis was fitted with a fused silica capillary column (30 m x 0.25 µm x 0.25 µm) and a flame ionization detector. The following instrument parameters were used: injection mode = split less; injection volume = 1.0 µl; carrier gas = helium; carrier gas flow = 1.0 ml/min; injector temperature = 250°C; oven ramping program = 100°C for 3 min, 185°C for 11 min; detector temperature 250°C. At the time of ruminal content collection, rumen pH was determined using a portable pH meter (Hanna Instruments, INC; Portable pH/ORP Meter; HI98190; RI 02917) inserted into the geometric center of the rumen.

Figure 3. Metabolism building at the Agricultural Research Development Educational Center (ARDEC), Fort Collins, CO, USA. Twelve fistulated steers were used in a 5-day total fecal and urine collection experiment.

Soluble trace mineral concentrations

At the end of experiment 1, steers were placed in individual pens within the metabolism building and fed the basal diet without supplemental Cu, Mn, and Zn for 14 days. During this time

steers had *ad libitum* access to drinking water and the basal diet was fed as described above. On d 15, steers received a pulse dose of the TM sources being evaluated prior to feeding as described by Guimaraes et al. (2021). Steers were dosed with the same TM source that they received in experiment 1. However, for this experiment, new supplements were formulated in 0.23 kg of ground corn and administered as a single bolus-dose via the rumen fistula to provide 20 mg Cu/kg DM, 60 mg Zn/kg DM, and 40 mg Mn/kg DM for an 8.2 kg DMI. Immediately after bolus dose administration, the rumen contents were thoroughly mixed by hand. Ruminal samples were then obtained as described by Guimaraes et al. (2021): 2 h intervals beginning at -4 and ending at 24 h post-dosing, time zero being the administration of bolus and feeding of the basal diet; rumen pH was determined before each sampling time; after pH determination, ruminal contents were thoroughly mixed by hand and a sample was obtained from the geometric center of the rumen (approximately 250 g); ruminal samples were then centrifuged 28,000 x *g* in graduated centrifuge tubes immediately post collection. Once centrifuged, the volume of supernatant was determined, and the supernatant was frozen at -20°C until TM analysis was performed. The Cu, Mn, and Zn concentrations of the supernatant fraction were considered the soluble fraction of each element per method described by Guimaraes et al. (2021).

Dialysis of ruminal insoluble digesta

Approximately 250 g of ruminal solid digesta samples that were collected at 0, 12, and 24h, from experiment 2, were dried at 60°C for 48 h in a forced air-drying oven, ground using a mortar and pestle to fit through a 2 mm screen, and were analyzed for Cu, Mn, and Zn, and were exposed to dialysis. Following the method described by Caldera et al. (2019), samples were dialyzed against 0.01M ethylenediaminetetraacetate in 0.05 M Tris buffer (Tris-EDTA). Regenerated cellulose dialysis tubing (31.8 mm diameter, 30 µm wall thickness, molecular weight

cut off 6,000 to 8,000; Fisher Scientific, Pittsburgh, PA) was cut into 10 cm segments and treated to remove metal contamination as described previously (Caldera et al., 2019). A 50% ethanol; 50% deionized water; 1mM EDTA solution was used to store dialysis tubing at 4ºC prior to use. The Tris-EDTA buffer was prepared immediately prior to use and the pH adjusted to 6.8. Samples were placed into 10 ml of 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 L of the Tris-EDTA buffer for 16 h at 4ºC with continuous stirring as described by Guimaraes et al. (2021). An industrial walk-in indoor cooler was used to maintain the temperature at 4ºC. The buffer was changed, and dialysis continued for another 6 h, at the same temperature and location. Samples were removed from dialysis bags, placed into pre-weighed acid-washed crucibles, and dried overnight at 60ºC. After drying, samples were weighed and then ashed at 600°C in a Thermo-Fisher Thermolyne muffle furnace overnight. After cooling, ashed samples were weighed and re-suspended in 5 mL of boiling 1.2 M HCl and analyzed for Cu, Mn, and Zn as described above (Guimaraes et al., 2021).

Table 3. Analyzed concentrations of copper, manganese, and zinc in supplements fed to steers using a lactation dairy diet.

	Treatments [1]		
Item	STM	CTM	HTM
Supplement inclusion[2], %	2.48	2.48	2.48
Copper, mg/kg DM	418.9	420.8	414.5
Manganese, mg/kg DM	1,648	1,637	1,659
Zinc, mg/kg DM	2,447	2,488	2,439

[1] Treatments are: sulfate trace minerals (STM), complexed trace minerals (CTM), and hydroxychloride trace minerals (HTM).

[2] Supplement inclusion: % DM

STATISTICAL ANALYSIS

Total tract apparent digestibility of DM, ADF, NDF, and CP 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 ruminal soluble, initial and post dialysis Cu, Mn and Zn concentrations, pH, and VFA concentrations and molar proportions. The fixed effects were treatment, time, and the treatment x time interaction and block were considered as a random effect. 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 ($P < 0.05$) was detected, treatment means were separated using the PDIFF option of the LSMEANS statement of SAS.

RESULTS

Nutrient digestibility and fermentation characteristics

By design, DMI was similar across treatments (Table 4). Steers supplemented with STM had lower ($P < 0.05$) DM digestibility than those given HTM sources of Cu, Mn, and Zn. Dry matter digestibility in steers receiving CTM did not differ from those supplemented STM or HTM sources of Cu, Mn, and Zn. Digestibility of ADF and NDF was greater ($P < 0.05$) in steers

supplemented with HTM or CTM compared with steers that received STM sources of Cu, Mn, and Zn. Crude protein digestibility was not affected by treatment.

Ruminal pH decreased ($P < 0.01$) with time after feeding (6.92, 6.35, and 6.12, at 0, 2, and 4 h, respectively), but no interaction between treatment and time was detected (Table 5). Total VFA concentrations were greater ($P < 0.05$) in steers fed HTM or CTM than those supplemented with STM sources of Cu, Mn, and Zn at 0, 2, and 4 h post-feeding (Table 5). Total VFA concentrations were also affected by a treatment x time ($P = 0.05$) interaction. The interaction was due to VFA concentrations being greater ($P <0.05$) at 2 and 4 h post-feeding compared to 0 h in STM supplemented steers. In steers receiving HTM or CTM sources of Cu, Mn, and Zn, total VFA concentrations at 4 h did not differ from those at 0 or 2 h post-feeding. Molar proportions of individual VFA were not affected by treatment or treatment x time. Compared to 0 h, molar proportion of acetate was lower ($P < 0.05$) at 2 and 4 h, while molar proportions of propionate, butyrate, and valerate increased ($P <0.05$) following feeding.

Table 4. Influence of trace mineral source on nutrient digestibility in steers fed a lactation dairy diet.

	Treatment [1]				
Item	STM	CTM	HTM	SEM	P-value
DMI[2], kg/d	8.2	8.2	8.2	-	-
DM digestibility, %	64.6 [a]	65.7 [a,b]	66.5 [b]	0.53	0.05
CP digestibility, %	63.5	63.7	64.3	0.32	0.20
ADF digestibility, %	29.8 [a]	31.6 [b]	32.4 [b]	0.49	0.05
NDF digestibility, %	43.0 [a]	47.0 [b]	47.6 [b]	1.17	0.05

[a,b] Means within a row with different superscript letters are different (P<0.05).

[1] Treatments are: sulfate trace minerals (STM), complexed trace minerals (CTM), and hydroxychloride traces minerals (HTM)

[2] Dry matter intake.

Table 5. Influence of trace mineral source on ruminal pH, total volatile fatty acid concentrations, and volatile fatty acid molar proportions in steers fed a lactation dairy diet.

Item	Treatment [1]			SEM	*P*-value		
	STM	CTM	HTM		Trt[3]	Time	Trt[3] x Time
Ruminal pH	6.38	6.41	6.59	0.09	0.26	0.01	0.17
Total VFA[2], mmol/L	73.3	78.0	77.4	0.80	0.01	0.01	0.05
0 h	71.5 [a]	77.5 [b]	76.5 [b]				
2 h	73.2 [a]	78.5 [b]	77.9 [b]				
4 h	75.2 [a]	78.0 [b]	77.0 [b]				
Acetate[4]	43.1	42.9	42.3	0.81	0.74	0.01	0.98
Propionate[4]	22.4	22.4	20.5	0.92	0.29	0.01	0.20
Isobutyrate[4]	6.62	6.76	7.36	0.34	0.27	0.12	0.87
Butyrate[4]	18.1	17.6	19.4	0.62	0.16	0.01	0.26
Isovalerate[4]	6.49	6.99	7.13	0.67	0.79	0.13	0.34
Valerate[4]	3.20	3.24	3.31	0.21	0.93	0.01	0.97

[a,b] Means within a row with different superscripts letters are different (P<0.05).

[1] Treatments are: sulfate trace minerals (STM), complexed trace minerals (CTM), and hydroxychloride traces minerals (HTM)

[2] Volatile fatty acid

[3] Treatment

[4] Individual volatile fatty acid are represented in mmol/100 mmol unit.

Soluble trace mineral concentrations

After TM dosing and feeding, ruminal pH was impacted by time ($P < 0.001$) but not by treatment or treatment x time interaction. Prior to feeding and TM dosing, ruminal pH averaged 6.56. Following feeding, ruminal pH decreased reaching a low of 5.55 at 6 h and returned to baseline values by 12 h post-feeding.

Ruminal soluble Cu concentrations were affected by treatment ($P < 0.001$) time ($P < 0.001$), and treatment x time ($P < 0.001$; Figure 4A). Steers receiving STM or CTM had greater ($P < 0.05$) soluble Cu concentrations at 2, 4, 6, and 8 h post-dosing than those dosed with HTM sources of Cu, Mn, and Zn. At 10 h post-dosing, ruminal soluble Cu concentrations were greater

($P < 0.05$) in STM steers compared with those receiving HTM or CTM sources of Cu, Mn, and Zn. Following dosing, ruminal soluble Cu concentrations did not increase in steers receiving HTM sources of Cu, Mn, and Zn at any sampling time. Compared to time 0 h values, soluble Cu concentrations increased ($P < 0.05$) through 10 h in STM steers, and through 8 h in steers dosed with CTM.

Treatment ($P < 0.001$), time post-dosing ($P < 0.001$), and treatment x time ($P < 0.001$; Figure 4B) were significant sources of variation for ruminal soluble Zn concentrations. Ruminal soluble Zn concentrations were lower ($P < 0.05$) in steers dosed with HTM sources of Cu, Mn, and Zn at 4, 6, 8, 10, and 12 h post-dosing compared with those receiving STM or CTM. Steers given CTM had greater ($P < 0.05$) soluble Zn concentrations at 6 and 8 h, but lower ($P < 0.05$) soluble Zn concentrations at 10 h post-dosing than STM steers. Ruminal soluble Zn concentrations were elevated ($P < 0.05$) above 0 h values at 4, 6, 8, 10, and 12 h post-dosing in STM and CTM steers. Steers receiving HTM sources of Cu, Mn, and Zn had greater ($P < 0.05$) soluble Zn concentrations at 4 and 10 h relative to pre-dosing concentrations.

Ruminal soluble Mn concentrations were affected by time ($P < 0.001$) and treatment x time ($P < 0.02$), but not by treatment ($P = 0.81$; Figure 4C). Compared to pre-dosing concentrations, soluble Mn concentrations increased ($P < 0.05$) in all treatment groups through at least 10 h post-dosing. Steers receiving CTM had greater ($P < 0.05$) soluble Mn concentrations at 8 h following dosing than STM steers. At 10 h post-dosing steers given HTM sources of Cu, Mn, and Zn had greater ($P < 0.05$) ruminal soluble Mn concentrations than those receiving STM or CTM. Soluble Mn concentrations were greater ($P < 0.05$) at 14 h post-dosing in steers receiving CTM compared with those dosed with STM.

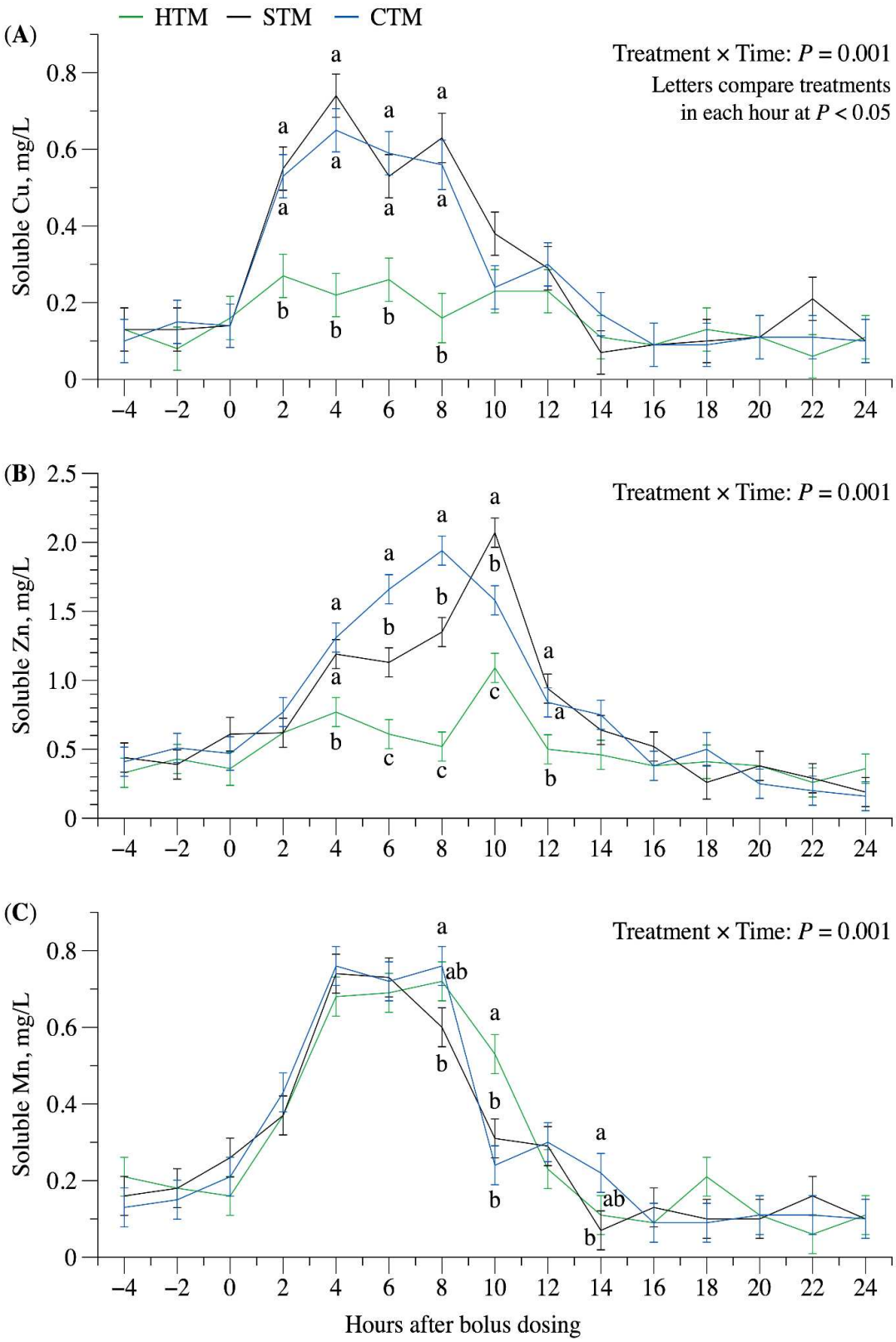
HTM
STM
CTM
(A)
Soluble Cu, mg/L
Treatment × Time: P = 0.001
Letters compare treatments
in each hour at P < 0.05
(B)
Soluble Zn, mg/L
Treatment × Time: P = 0.001
(C)
Soluble Mn, mg/L
Treatment × Time: P = 0.001
Hours after bolus dosing

Figure 4. Influence of trace mineral source on ruminal soluble Cu (A), Zn (B), and Mn (C) concentration in steers. [a, b] Means within a time point with different superscripts letters are different (P<0.05). Treatments are hydroxychloride traces minerals (HTM), sulfate trace minerals (STM), and complexed trace minerals (CTM). Abbreviations: HTM = hydroxychloride trace minerals; STM = sulfate trace minerals; CTM= complexed trace minerals.

Dialysis of ruminal insoluble digesta

Concentrations of Cu, Mn, and Zn in ruminal solid digesta (mg of TM/unit of solid digesta DM) were affected (P < 0.001), time (P < 0.001), and treatment x time (P < 0.001; Figures 5A, B, C). Copper, Mn, and Zn concentrations in ruminal solid digesta prior to TM dosing were similar across treatments. At 12 h post-dosing Cu concentrations in solid digesta were greater (P <0.05) in steers receiving HTM compared with those dosed with STM or CTM sources of Cu, Mn, and Zn. Steers dosed with CTM had similar (P < 0.05) Cu concentrations in solid digesta at 12 h compared with STM steers. Copper concentrations in digesta were similar across treatments at 24 h post dosing. Zinc concentrations in ruminal solid digesta at 12 and 24 h post-dosing was greater (P <0.05) in steers receiving HTM than in those dosed with STM or CTM sources of Cu, Mn, and Zn. Steers receiving STM had lower (P < 0.05) Mn concentrations in solid digesta than those dosed with HTM or CTM sources at 12 h post-dosing.

The percentage of Cu and Zn in ruminal solid digesta released during dialysis against Tris-EDTA was affected (P < 0.001) by treatment, time, and treatment x time (Figures 5A and B). Copper and Zn released from digesta by Tris-EDTA prior to TM dosing (0 h) was similar across treatments. At 12 and 24 h post-dosing, release of Cu and Zn from ruminal solid digesta, after being dialyzed against Tris-EDTA, was much greater (P < 0.05) for steers receiving HTM compared to those receiving STM or CTM sources of Cu, Mn, and Zn. Percentage of Cu released from solid digesta from HTM steers was greater (P < 0.05) at 12 and 24 h than 0 h, while percentage of Cu released following dialysis decreased (P < 0.05) following TM dosing in steers

receiving STM and CTM sources. Zinc released from solid digesta after dialysis against Tris-EDTA was greater ($P < 0.05$) at 12 and 24 h compared with 0 h in all treatments. Manganese released from solid digesta was affected ($P < 0.001$) by time and treatment x time, but not by treatment ($P = 0.84$, Figure 5C). Percentage of Mn released following dialysis was greater ($P < 0.05$) for all treatments at 12 and 24 h post-dosing than at 0 h. In steers receiving STM the percentage of Mn released from solid digesta was greater at 24 h than at 12 h post-dosing, while the percentage of Mn released from digesta of steers receiving HTM or CTM sources of Cu, Mn, and Zn was less ($P < 0.05$) at 24 than at 12 h post-dosing.

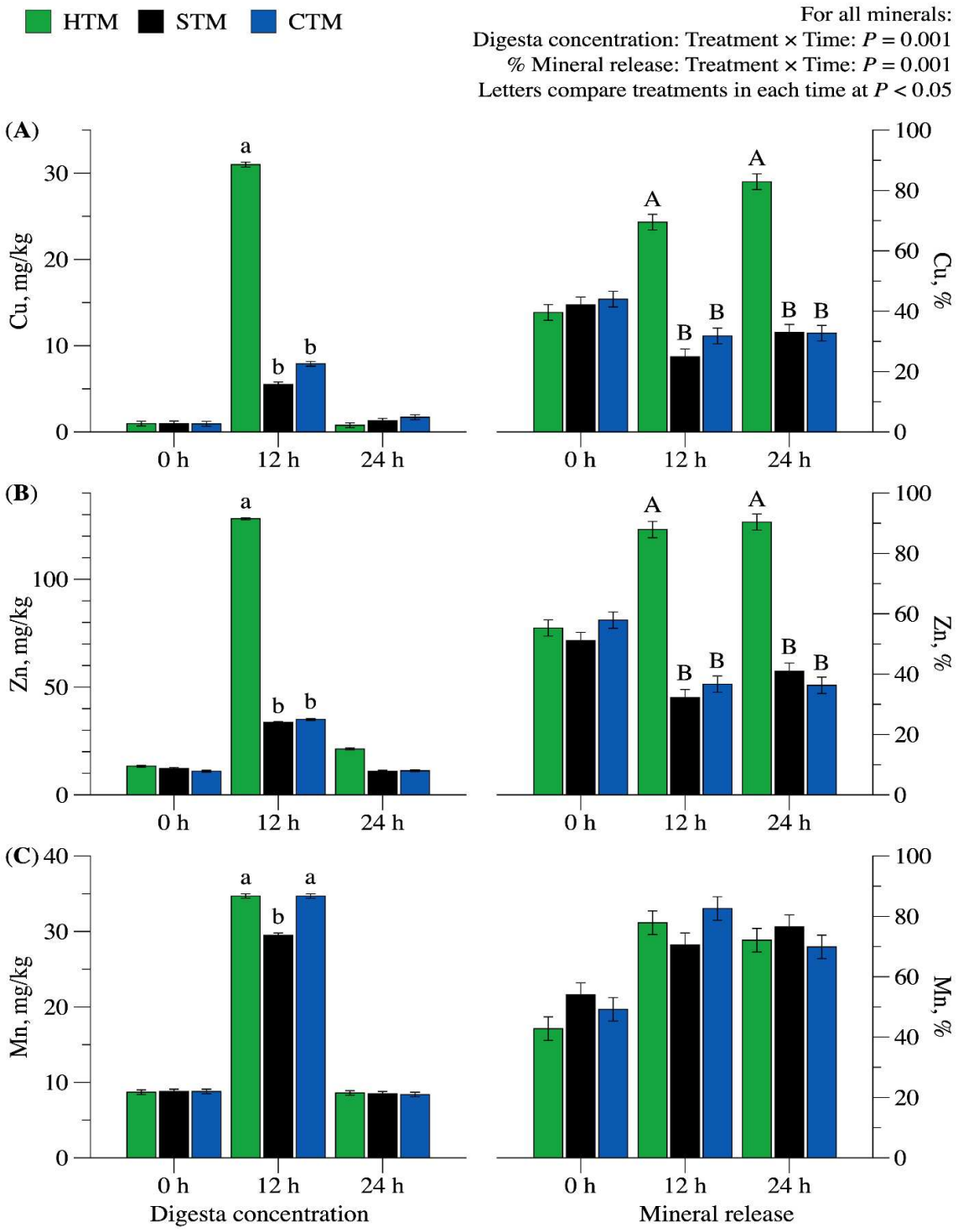

Figure 5. Influence of trace mineral source on the concentration of copper (A), zinc (B), and manganese (C) on ruminal solid digesta and percentage of mineral released on dialysis against Tris-EDTA in steers. a-c Means within time point with different letters differ (P < 0.05). Error bars represent SEM (Exp. 2). Treatments are sulfate trace minerals (STM), complexed trace minerals (CTM), and hydroxychloride traces minerals (HTM). Abbreviations: HTM = hydroxychloride

trace minerals; STM = sulfate trace minerals; CTM= complexed trace minerals; EDTA = ethylenediaminetetraacetate

DISCUSSION

The basal feed ingredients supplied 63, 252, and 61% of the Cu, Mn, and Zn requirement for a dairy cow producing 45 kg of milk per day (NRC, 2001). Because of unknown bioavailability of TM in feed ingredients, Cu, Mn, and Zn are typically supplemented at concentrations similar to those supplied in the present study. The total mixed rations supplied approximately 17.3, 73.6, and 92.3 mg/kg of Cu, Mn, and Zn, respectively. Manganese concentrations greatly exceeded NRC (2001) requirements in the present study. However, recent research (Weiss and Socha, 2005; Hansen et al., 2006) indicates that Mn requirements are underestimated in the Dairy NRC (2001).

Steers supplemented with HTM had greater DM, ADF, and NDF digestibility than those supplemented with STM sources of Cu, Mn, and Zn in the present study. The improvement in NDF digestibility in steers fed HTM vs. STM sources of Cu, Mn, and Zn is consistent with previous studies in lactating dairy cows (Faulkner and Weiss, 2017; Daniel et al., 2020; Miller et al., 2020). Steers fed a medium quality grass hay-based diet and supplemented with HTM also had greater ADF and NDF digestibility than those receiving STM (Guimaraes et al., 2021). In contrast ADF and NDF digestibility did not differ among lambs supplemented with HTM or STM sources of Cu, Mn, and Zn (Van Valin et al., 2018).

Digestibility of ADF and NDF was also greater in steers supplemented with CTM vs. STM, but fiber digestibility was similar in steers receiving CTM and HTM sources of Cu, Mn, and Zn. Lambs consuming a supplement containing 10 or 20 mg Cu/kg DM, from Cu proteinate, had

greater ADF and NDF digestibility than those receiving Cu sulfate (Dezfoulian et al., 2012). Acid detergent fiber digestibility was also greater in lambs supplemented with 20 or 30 mg Zn/kg DM, from Zn proteinate or Zn methionine compared with Zn sulfate (Gang et al., 2008; Alimohamady et al., 2019). Ewes consuming a supplement containing 25 mg Zn/kg DM, from Zn methionine, had greater ADF and NDF digestibility than ewes fed Zn sulfate (Hassan et al., 2011). Lambs fed a supplement with 150 mg Mn/kg DM, from Mn sulfate, tended to have lower NDF digestibility than those receiving a Mn glycine chelate (Gresakova et al., 2018). However, dairy heifers supplemented with STM had similar NDF and ADF digestibility to those supplemented with proteinate TM (Pino and Heinrichs, 2016). It is difficult to determine the impact of improved fiber digestibility on animal productivity. However, improving fiber digestibility by approximately 1-2% could result in less feed being consumed and less manure being produced which would improve overall animal production efficiency.

The experimental design of the present study did not include a negative control (no supplemental Cu, Mn, and Zn) because the basal diet did not meet the NRC (2001) requirement for Cu and Zn. Furthermore, Cu, Mn, and Zn are generally supplemented to lactating dairy cow diets at concentrations approximating those provided in the present study. Since there was not a negative control in the present study, we cannot determine if STM decreased or if HTM and CTM improved NDF and ADF digestibility.

Collectively these data indicate that rumen solubility of Cu, Mn, and Zn from sulfate sources may be influencing fiber digestibility, possibly through modulation of the rumen microbiome. Research by Emmanuel and Staples (1990) suggest that Cu and Zn release from feedstuffs during ruminal fermentation should be sufficient to meet microbial needs for Cu and Zn. Therefore, providing additional soluble minerals above microbial requirements may cause

certain microorganism within the rumen to increase energy expenditure to maintain homeostasis via, regulating metal uptake, efflux, and sequestration. Future research examining the impact of trace mineral solubility in the rumen on microbial function and overall animal production efficiency is warranted.

Molar proportions of individual ruminal VFA were not affected by TM source. However, total VFA concentrations were greater in steers supplemented with HTM or CTM compared to STM sources of Cu, Mn, and Zn. The higher total VFA concentrations in steers receiving HTM or CTM sources of Cu, Mn, and Zn is consistent with the greater fiber digestibility in these groups, however further assumptions cannot be conclusive since rumen volume and passage rate were not measured. Steers fed a medium quality grass hay-based diet supplemented with HTM also had greater ruminal total VFA concentrations than those receiving STM (Guimaraes et al., 2021). Growing steers fed a corn silage-based diet supplemented with 30 or 90 mg Zn/kg DM, from ZnOHCl, had greater total VFA concentrations than those supplemented with the same concentrations of Zn from Zn sulfate (Shaeffer, 2006). In contrast total ruminal VFA concentrations did not differ in lactating dairy cows fed STM or HTM sources of Cu, Mn, and Zn (Daniel et al., 2020). In vitro total VFA concentrations were greater when 10 or 20 µg Zn/mL rumen fluid was added from a Zn amino complex or two Zn proteinates compared to similar additions from Zn sulfate (Wang et al., 2013). Total VFA concentrations relative to the control (0 added Zn) were reduced by the highest Zn concentration (20 µg/mL) from Zn sulfate, but not by the same concentration of Zn from the organic sources. The substrate used in this study, on an as-fed basis, consisted of 50% corn silage, 18% hay, and 32% concentrate mix.

It cannot be determined from the present study, which TM (Cu, Zn, and/or Mn) was responsible for the lower fiber digestibility. Early in vitro studies, using washed suspensions of

rumen microorganisms, indicated that soluble sulfate sources of Cu, Mn, and Zn at certain concentrations can decrease cellulose digestion (Hubbert et al., 1958; Martinez and Church, 1973). The addition of 20 (Mallaki et al., 2015) or 40 mg Zn/kg DM (VanValin et al., 2020) from Zn sulfate to a control diet containing 22 mg Zn/kg DM reduced NDF digestibility in lambs. However, in other lamb studies (Gang et al., 2008; VanValin et al., 2018; Alimohamady et al., 2019) addition of Zn sulfate to a control diet to supply 20 to 40 mg Zn/kg DM did not affect NDF or ADF digestibility. In lambs fed a control diet containing 8.6 mg Cu/kg DM, supplementation of 10 or 20 mg Cu/kg DM (from Cu sulfate) tended to reduce NDF and ADF digestibility (Dezfoulian et al., 2012).

Steers receiving HTM sources of Cu, Mn, and Zn had lower ruminal soluble Cu and Zn concentrations at 10 to 12 h after bolus dosing. Similar results were obtained in steers fed a medium quality grass hay-based diet (Guimaraes et al., 2021) and in steers receiving a 50% corn silage – 50% steam flaked corn diet (Caldera et al., 2019) following dosing with STM or HTM sources of Cu, Mn, and Zn. Interestingly, Mn solubility appeared to be similar across treatments. Furthermore, ruminal soluble Mn increased following dosing with HTM, while ruminal Cu and Zn solubility remained similar to 0 h values. However, dietary excess of Mn coming from the basal diet could have influenced final results of Mn soluble concentrations.

Despite similar effects on fiber digestibility and total ruminal VFA concentrations, the rumen solubility of Cu and Zn in HTM and CTM supplemented cattle differed greatly. Soluble Cu and Zn concentrations following dosing were generally similar in steers receiving STM and CTM source of Cu, Mn, and Zn. Trace minerals can be found in two different forms within the rumen environment: 1) soluble and/or 2) insoluble. Within the soluble fraction, trace minerals can exist in both ionic (free) and complexed forms (Bremner, 1970; Allen et. al., 1987). It is possible that

Cu and Zn from CTM existed as a soluble complex rather than in the ionic form. Studies in sheep have indicated that the majority of Cu, Mn, and Zn is associated with the solid digesta in the rumen, when TM come from forages (Bremner, 1970; Waghorn et al., 1990). In the present study trace minerals were derived from both the basal dietary ingredients as well as the pulse dose supplement. In order to determine the impact of basal diet Cu, Mn, and Zn on fiber digestibility, a control group not receiving supplemental Cu, Mn, and Zn should be implemented in future studies.

Trace minerals derived from feed ingredients and supplements can become soluble in the rumen. Several factors such as feedstuff type, TM composition of a given supplement, and form of TM can impact rumen solubility of TM. Once soluble, TM can interact with a variety of compounds within the rumen environment (e.g., components of feedstuffs, final product of fermentation, microbial cells, etc.) and may form insoluble complexes. Depending on how tightly bound TM are in an insoluble complex will dictate if the TM can become soluble in the small intestine and be absorbed. Therefore, understanding the ability of TM to be released from common products of digestion that can complex TM, is important.

Dialysis against chelating agents has been used to determine the availability of Zn and other elements from feedstuffs (Jones et al., 1985). In the current experiment, Cu and Zn contained within the solid digesta obtained from the rumen in steers dosed with HTM sources of Cu, Mn, and Zn were released to a greater extent when dialyzed against Tris-EDTA at 12 and 24 h post-dosing compared to those receiving STM or CTM. These data suggest that Cu and Zn in ruminal solid digesta from steers receiving STM or CTM have a stronger binding coefficient to ruminal digesta when compared to Cu and Zn from steers receiving the HTM treatment. Therefore, it is possible that a greater binding to solid digesta within the rumen may impact the availability of Cu and Zn in the small intestine.

Concentrations of Cu and Zn in ruminal solid digesta were considerably higher at 12 h post-dosing in steers receiving HTM than those dosed with STM or CTM sources of Cu, Mn, and Zn. This is consistent with CuOHCl and ZnOHCl not being very soluble in the rumen. At 24 h post-dosing Cu concentrations in solid digesta were similar across treatments. Although Cu concentrations in solid digesta were similar across treatments, the release of Cu following dialysis against Tris-EDTA was still greater for steers receiving the HTM treatment at 24 h post-dosing. Zinc concentrations in solid digesta remained higher in HTM supplemented steers at 24 h post-dosing. Although ZnOHCl was of low solubility in the ruminal environment, we did not determine if Zn from ZnOHCl was still in the OHCl form or if the Zn was removed from the OHCl and incorporated into bacterial cells. Studies are needed to determine if bacterial Zn concentrations are affected by Zn source.

CONCLUSION

Supplementation of a lactating dairy cow-based diet with Cu, Mn, and Zn from STM resulted in lower ADF and NDF digestibility and total VFA concentrations in steers than supplementation with HTM or CTM sources of Cu, Mn, and Zn. Following a pulse dose of TM, ruminal soluble concentrations of Cu and Zn were greater for 8 to 12 h post-dosing in steers receiving STM or CTM compared with those dosed with the HTM treatment. Rumen solid digesta Cu concentrations were greater at 12 h and Zn concentrations in solid digesta were greater at 12 and 24 h post-dosing in steers receiving HTM sources of Cu, Mn, and Zn. Copper and Zn from the STM and CTM treatments were more tightly bound to ruminal solid digesta than steers receiving the HTM treatment, based on their release following dialysis against the chelating agent

EDTA. The weaker binding of Cu and Zn to ruminal solid digesta may result in an increased delivery of soluble Cu and Zn to the small intestine.

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CHAPTER III

TRACE MINERAL SOURCE IMPACT RUMEN TRACE MINERAL METABOLISM AND NUTRIENT DIGESTION IN STEERS FED A HIGH CONCENTRATED FEEDLOT DIET

SUMMARY

The objective of this experiment was to evaluate the effects of sources of sulfate trace mineral (STM) and hydroxy trace mineral (HTM) fed at the same levels on 1) nutrient digestibility and fermentation characteristics, 2) ruminal solubility of Cu, Zn, and Mn following a pulse dose of trace mineral I, and 3) binding strength of Cu, Zn, and Mn on ruminal solid digesta upon dialysis against a chelating agent. Minor dietary changes can have a direct impact on ruminal pH, temperature, microbial population, and redox potential, which will influence fermentation, and ultimately affecting nutrient digestibility. These changes can influence or be influenced by the solubility of certain trace minerals (TM) in the rumen. Rumen soluble trace minerals can alter nutrient digestibility, fermentation, and increase chances of antagonistic interaction. Twelve ruminally cannulated Angus steers (BW 587.6 ± 23.1 kg) were adapted to a diet balanced to meet requirements of finishing steers for 21 days. Steers were then randomly assigned to dietary treatments consisting of Cu, Mn, and Zn supplemented at 18, 40, and 90 mg/kg DM, respectively, from either sulfate (STM) or hydroxy trace mineral (HTM) sources (n = 6 steers/treatment; experimental unit = steer). Following the 14 days treatment adaptation period total fecal output was collected for 5 days. Digestibility of DM, CP, Starch, NDF, and ADF was not affected (*P* >

0.15) by trace mineral source. On d 6, rumen fluid was collected at 0, 2, and 4 h post feeding for VFA analysis. There were no treatment x time interactions for any VFA measured. However, HTM steers had greater ($P < 0.05$) ruminal molar proportions of propionate and tended ($P < 0.07$) to have greater molar proportions of valerate compared to STM steers. Steers fed STM had greater ($P < 0.05$) molar proportions of butyrate compared to HTM steers. Steers were then fed the same high concentrate diet without supplemental Cu, Zn, or Mn for 14 days. On d 15 steers received a pulse dose (via rumen cannulae) from either STM or HTM. Ruminal samples were obtained at 2 h intervals starting at -4 and ending at 24 h relative to dosing. There was a treatment x time interaction ($P < 0.05$) for ruminal soluble Cu concentrations. Ruminal soluble mineral concentrations were greater ($P < 0.05$) for Cu at 4, 6, 8, and 16 h post dosing in STM compared to HTM supplemented steers. There was no treatment x time interaction for ruminal soluble Zn or Mn concentrations. The release of Cu, Zn, and Mn from ruminal solid digesta following dialysis against Tris-EDTA at 12 and 24 h post-dosing was greater ($P < 0.01$) for steers receiving HTM compared to those receiving STM. Results of this experiment indicate that trace mineral source affects how tightly bound Cu, Zn, and Mn are to ruminal solid digesta, and that ruminal solubility of Cu differs between STM and HTM sources and may impact rumen fermentation characteristics.

Key words: pH, mineral solubility, feedlot, trace minerals.

INTRODUCTION

Studies have indicated that hydroxychloride (OHCl) sources of copper (Cu) and zinc (Zn) behave differently from sulfate (SO_4) forms in the rumen environment. In studies conducted by Caldera et al. (2019) using a 50/50 corn silage and steam flaked corn-based diet, which ruminal pH averaged 6.23 across all sampling times, Guimaraes et al. (2022) using a dairy type diet, had similar pH of 6.39, and a subsequent study (Guimaraes et al., 2021) using a medium quality grass hay-based diet, pH averaging 6.63. Those studies demonstrated that hydroxychloride trace mineral sources (HTM) are less soluble in the ruminal environment when compared to sulfate trace mineral sources (STM). In the same light, similar differences in solubility of trace mineral sources were also found in earlier studies by Cao et al. (2000) and Spears et al. (2004) showing that hydroxychloride forms of Cu and Zn were less soluble in both water and acidic solutions when compared to sulfate forms. Interestingly, hydroxychloride forms increases in solubility when pH becomes more acidic.

Copper and Zn solubility in the ruminal environment has been shown to have an impact on fiber digestion (Durang and Kawashima, 1980; Faulkner et al., 2017a; Guimaraes et al., 2021; Guimaraes et al., 2022), fermentation parameters (Daniel et al., 2020; Guimaraes et al., 2021; Guimaraes et al., 2022), and indirectly could also have an impact on the fecal microbiome population (Faulkner et al., 2017b). Other studies, where indigestible fiber was utilized to evaluate digestibility, reported similar results showing greater fiber digestibility in lactating dairy cows supplemented with HTM when compared to STM (Daniel et al., 2020; Miller et al., 2020).

Studies have also indicated that Cu and Zn from OHCl forms were more bioavailable than SO_4 forms in steers (Spears et al., 2004; Shaeffer et al., 2017). It can be assumed that providing Cu from sources that increase bioavailability (such as OHCl or organic complexes) and at the same time are less soluble in the rumen could mitigate adverse effects of antagonists like molybdenum and sulfur in ruminants. Based on Cu and Zn release from ruminal solid digesta upon dialysis against Tris-EDTA, Cu and Zn from OHCl forms were less tightly bound to solid digesta than SO_4 forms in studies conducted by Caldera et al. (2019), Guimaraes et al. (2021) and Guimaraes et al. (2022).

In addition, previous studies conducted in monogastric mammals, including mice (Faith et al., 2011), and humans (Walker et al., 2011 and David et al., 2014), it has been demonstrated that microbiota shift occurs within the gut within one day of dietary modification. Dietary change has been reported to affect microbiota, however results showing how this affects other parameters still needs to be investigated. In general, the main reason for microbiome changes is always correlated to diet composition. However, there are several other factors that need to be considered other than diet composition changes or microbiome shift. Alternative mechanisms can be most likely accountable to those changes, such as pH (Walker et al., 2015) and redox potential (Jonas et al., 1999, Feillet-Coudray et al., 2009)

Considering that the diet in this study is very different from that of Guimaraes et al. (2021), and Guimaraes et al. (2022) studies, which provided a different ruminal environment, such as pH, microbiota, and fermentation parameters, the objectives of the present study were to determine the influence of TM source on 1) nutrient digestibility and ruminal fermentation parameters, 2) soluble concentrations of Cu, Zn, and Mn in the rumen following a pulse dose of TM, and 3) binding

strength of Cu, Zn, and Mn from ruminal solid digesta upon dialysis against a chelating agent in steers fed a diet formulated to meet the requirements of a finishing feedlot steer.

MATERIALS AND METHODS

All the experimental procedures described in this study were previously approved by the Colorado State University Animal Care and Use Committee (IACUC approval #17-7182A).

Nutrient digestibility and fermentation characteristics

The present study was conducted at the Colorado State University Agriculture, Research, Development, and Education Center in Fort Collins, CO, United States. Twelve crossbred Angus steers fitted with ruminal cannulae (initial BW 530.9 ± 22.7kg) were utilized in this experiment. Steers were previously ranked by BW and allocated in two feedlot pens (6 steers per pen). Steers were then fed a basal diet without supplemental Cu, Zn, and Mn that was balanced to meet the requirements of finishing feedlot steers (1.53 Mcal/kg, targeting 3.5 lb gain/day), (NRC, 2016), for 21 days. Ingredient and analyzed chemical composition of the basal diet is shown in Table 6.

Following the 21day adaptation period, steers were assigned to one of two treatments. Treatments consisted of 18 mg Cu/kg DM, 60 mg Mn/kg DM, and 90 mg Zn/kg DM, from either STM or HTM (IntelliBond® C, M, and Z; Micronutrients USA LLC., Indianapolis, IN) sources. After receiving the treatments for 7 days, steers were moved indoors and housed in individual pens (2.5 m x 2.5 m pens equipped with automatic waters, individual feeders, and rubber matted floors) for 2 days and allowed to acclimate to their new surroundings. Steers were then relocated into

individual metabolism stalls (3.0 m x 1.1m pens) equipped with automatic waters, individual plastic feeders, and rubber matted floors for a 5-d acclimation period. During the acclimation period, DMI for each steer was determined. At the end of the acclimation period, steers were blocked across treatments based on their mean DMI over the 5-d period. Once animals were appropriately blocked by DMI, each steer within a block was fed the same amount of feed. Feed delivered to each steer within a block was calculated to be 90.0% of the steer within the block with the lowest average DMI during the acclimation period. This ensured equal amounts of feed offered to the individual steers within a block during the 5-d total fecal and urine collection period, as previously described by Guimaraes et al. (2021).

Table 6**.** Ingredient composition of feedlot diet (% DM).

Ingredient	%DM
Steam Flaked Corn	66.9
Corn Silage	10.0
Alfalfa hay	10.0
Corn distillers	10.02
Limestone	1.5
Urea	1.06
Salt - white	0.30
Vit E	0.07
Rumensin	0.016
Vit A	0.015
Tylosin	0.004
Selenium	0.0008
Iodine	0.00005
TOTAL	100.0
Chemical Composition	
Dry Matter, %	78.1
Crude Protein, %	13.7
Organic matter, %	95.54
NEm, Mcal	1.93
Neg, Mcal/kg	1.53
Ash, %	4.46
Fat, %	4.0
Starch, %	35.5

NFC, %	61.84
Acid detergent fiber, %	8.7
Neutral detergent fiber, %	16.0
Roughage NDF, %	6.24
Calcium, %	0.71
Phosphorus, %	0.33
Sulfur, %	0.16
Copper, mg/kg	5.8
Manganese, mg/kg	10.5
Zinc, mg/kg	19.16
Analyzed composition	
Dry matter, %	67.0
Crude Protein, %	14.3
Organic matter, %	94.8
Starch, %	34.8
Fat, %	4.5
ASH, %	5.2
NFC, %	47.15
Acid detergent fiber, %	17.6
Neutral detergent fiber, %	28.85
Roughage NDF, %	65.8
Copper, mg/kg	14.95
Manganese, mg/kg	38.21
Zinc, mg/kg	72.39

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 mixed with ground corn. Immediately after feeding the basal diet, the appropriate TM supplement was top-dressed at 2.96% of the diet and mixed thoroughly by hand for each feeding. Analyzed Cu, Zn, and Mn concentrations in the TM-corn supplements are presented in Table 7.

Sample collection and laboratory analysis

Total fecal output was measured daily for individual steers during the 5-d collection period as described by Caldera et al. (2019). Feces collected each day were quantified by wet-weight,

thoroughly mixed, and sampled (10.0% of wet weight). Duplicate, individual fecal samples were sealed in plastic bags, labeled, and stored at -20º C. Prior to chemical analysis of feces and feed, samples were proportionally composited across all collection days for each animal. Fecal dry matter was determined by placing a known mass of wet material in a forced-air drying oven for 48 h at 100 ºC. 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). Copper, Zn and Mn concentrations in the TM supplements were quantified via inductively coupled plasma-mass spectrometry (NexION 2000 B, PerkinElmer), following dry ashing at 600°C in a muffle furnace. Crude protein was quantified using the TruSpec CN Carbon/Nitrogen LECO system (Leco Corp., St. Joseph, MI). Starch and chemical composition of the basal diet was determined at a commercial laboratory (SDK Laboratories, Inc. Hutchinson, KS).

Following the 5-d fecal collection (d 20), ruminal samples were collected at 0, 2, and 4 h post-feeding for determination of volatile fatty acids (VFA) and pH. Ruminal 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 VFA concentrations via gas chromatography (Agilent 6890N, Santa Clara, CA). Ruminal pH was determined using a portable pH meter (Hanna Instruments, INC; Portable pH/ORP Meter; HI98190; RI 02917), as described by Guimaraes et al. (2021a).

Table 7**.** Analyzed concentrations of copper, manganese, and zinc in supplements fed to steers using a finishing feedlot diet.

Item	STM	HTM
Supplement inclusion[2], %	2.96	2.96
Copper, mg/kg DM	620.5	619.5

Manganese, mg/kg DM	2,000.0	2,001.1
Zinc, mg/kg DM	3,020.2	3,021.0

[1] Treatments are: sulfate trace minerals (STM), and hydroxychloride trace minerals (HTM).

[2] Supplement inclusion, % DM.

Soluble trace mineral concentrations:

At the end of experiment 1, steers were placed in individual pens within the metabolism building and fed the basal diet without supplemental Cu, Mn, and Zn for 14 days. During this time steers had *ad libitum* access to drinking water and the basal diet was fed as described above. On d 15, steers received a pulse dose of the TM sources being evaluated. Steers were dosed with the same TM source that they received in experiment 1. 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 18 mg Cu/kg DM, 90 mg Zn/kg DM, and 60 mg Mn/kg DM. Immediately after bolus dose administration, the rumen contents were thoroughly mixed by hand. Ruminal samples were then obtained at 2 h intervals beginning at -4 and ending at 24 h post-dosing; time zero being the administration of bolus and feeding of the basal diet. Before each sampling time, ruminal 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 at 28,000 x *g* in graduated centrifuge tubes. Once centrifuged, the volume of supernatant was determined, and the supernatant was frozen at -20° C until TM analysis was performed. The Cu, Mn, and Zn concentrations of the supernatant and pellet fractions were considered the soluble and 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 ruminal digesta collected was dried at 60°

C for 48 h in a forced air-drying oven, ground using a mortar and pestle to fit through a 2 mm screen, analyzed for Cu, Mn, and Zn, and dialyzed against 0.01M ethylenediaminetetraacetate in 0.05 M Tris (Tris-EDTA) buffer. Regenerated cellulose dialysis tubing (31.8 mm diameter, 30 μm wall thickness, molecular weight cut off 6,000 to 8,000; Fisher Scientific, Pittsburgh, PA) was cut into 10 cm segments and treated to remove metal contamination as described previously (Caldera et al., 2019). Dialysis tubing was stored in 50% ethanol; 50% deionized water; 1mM EDTA at 4 ºC prior to use. The Tris-EDTA buffer was prepared immediately prior to use and the pH adjusted to 6.8. Samples were placed into 10 ml of 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 L of the Tris-EDTA buffer for 16 h at 4 ºC 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 ºC. After drying, samples were weighed, and then ashed at 600 °C in a Thermo-Fisher Thermolyne muffle furnace overnight. After cooling, ashed samples were 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, NDF, Starch and CP 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 ruminal soluble, initial and post dialysis Cu, Mn and Zn concentrations, pH, and VFA concentrations and molar proportions. 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

Nutrient digestibility and fermentation characteristics

Following a similar design from Guimaraes et al. (2021) and Guimaraes et al. (2022), DMI was similar across treatments (Table 8). There were no treatment effects on DM, NDF, ADF, CP, or starch digestibility between steers receiving the STM versus HTM supplement. Ruminal pH decreased ($P < 0.001$) with time after feeding (6.23, 5.60, and 5.27, at 0, 2, and 4 h, respectively), but no interaction between treatment and time was detected (Table 9).

Table 8. Influence of trace mineral source on dry matter, acid detergent fiber, neutral detergent fiber, and crude protein digestibility on steers receiving a high concentrated diet.

	Treatment			
Item	STM	HTM	SEM	P<
No. of animals	6	6	---	---
DMI, kg DM/steer/d	8.1	8.1	---	---
DM dig., %	75.8	79.5	1.75	0.16
ADF dig. %	27.2	30.5	1.47	0.15
NDF dig., %	41.2	41.0	0.40	0.71
CP dig, %	69.8	68.4	1.13	0.40
Starch dig, %	96.7	96.1	0.63	0.52

There were no treatment x time interactions for any VFA measured. However, HTM steers had greater ($P < 0.05$) ruminal molar proportions of propionate and tended ($P < 0.07$) to have greater molar proportions of valerate compared to STM steers. Steers fed STM had greater ($P < 0.05$) molar proportions of butyrate compared to HTM steers.

Table 9. Influence of trace mineral source on short chain fatty acid production and pH at 0, 2, and 4 hours post feeding.

	Treatment			P<		
Item	STM	HTM	SEM	Trt	Time	Trt*time
pH, s.u.						
0h	6.21	6.25	0.06	0.98	0.0001	0.93
2h	5.60	5.61	---	---	---	---
4h	5.35	5.32	---	---	---	---
Short chain fatty acid, mM/100mM						
Acetic acid						
0h	31.95	29.62	2.59	0.64	0.07	0.47
2h	31.88	31.70	---	---	---	---
4h	30.27	27.35	---	---	---	---
Propionic acid						
0h	30.33	38.50	3.12	0.05	0.0001	0.42
2h	38.00	47.53	---	---	---	---
4h	38.65	50.98	---	---	---	---
Butyric acid						
0h	16.47	12.12	1.77	0.05	0.53	0.17
2h	18.18	11.63	---	---	---	---
4h	17.50	11.20	---	---	---	---
Valeric acid						
0h	9.33	11.11	2.11	0.67	0.02	0.07
2h	9.85	7.25	---	---	---	---
4h	12.33	9.48	---	---	---	---
Total short chain fatty acids, mM						
0h	114.60	115.90	2.74	0.80	0.69	0.61
2h	116.43	122.23	---	---	---	---
4h	120.17	116.20	---	---	---	---

Soluble trace mineral concentrations

There was a treatment x time interaction ($P < 0.05$) for ruminal soluble Cu concentrations. Ruminal soluble mineral concentrations were greater ($P < 0.05$) for Cu at 4, 6, 8, and 16 h post dosing in STM compared to HTM supplemented steers (Figure 6a). There was no treatment x time interaction for ruminal soluble Mn and Zn concentrations (Figure 6b and 6c, respectively).

Dialysis of ruminal insoluble digesta

Concentrations of Cu, Mn, and Zn in ruminal solid digesta (mg of TM/unit of solid digesta DM) were affected by treatment ($P < 0.001$), time ($P < 0.001$), and treatment x time ($P < 0.001$; Table 10). Copper, Mn, and Zn concentrations in ruminal solid digesta prior to TM dosing were similar across treatments. At 12 h post-dosing Cu, Mn, and Zn concentrations in solid digesta were greater ($P < 0.001$) in steers receiving HTM compared with those dosed with STM. Copper and Mn concentrations in digesta were similar across treatments at 24 h post dosing. Zinc concentrations in ruminal solid digesta at 24 h post-dosing was greater ($P < 0.001$) in steers receiving HTM than in those dosed with STM.

The percentage of Cu, Mn, and Zn in ruminal solid digesta released during dialysis against Tris-EDTA was affected ($P < 0.001$) by treatment and treatment x time (Table 10). Copper, Mn, and Zn released from digesta by Tris-EDTA prior to TM dosing (0 h) was similar across

treatments. At 12 and 24 h post-dosing, release of Cu, Mn, and Zn from ruminal solid digesta, after being dialyzed against Tris-EDTA, was much greater ($P < 0.001$) for steers receiving HTM compared to those receiving STM sources of Cu, Mn, and Zn.

Table 10. Influence of dialysis on copper, manganese, and zinc release from rumen solid digesta 0, 12, and 24 hours 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.

	Trace mineral source			
	HTM	STM	SEM	P <
Initial concentration of digesta, mg/kg DM				
0 hour				
Copper	0.85	0.83	0.28	0.97
Manganese	7.5	7.6	0.95	0.84
Zinc	10.9	11.8	0.99	0.63
12 hours				
Copper	26.6	4.7	4.01	0.001
Manganese	29.7	25.3	4.67	0.001
Zinc	109.8	28.9	10.10	0.001
24 hours				
Copper	0.66	1.20	0.45	0.21
Manganese	7.4	7.3	2.35	0.81
Zinc	18.2	9.7	10.24	0.001
Tris-EDTA, % mineral released				
0 hour				
Copper	23.6	27.4	1.27	0.14
Manganese	31.9	32.2	1.93	0.92
Zinc	56.1	50.1	2.67	0.10
12 hours				
Copper	61.4	27.3	1.61	0.001
Manganese	64.5	79.5	16.21	0.001
Zinc	91.7	35.3	12.41	0.001
24 hours				
Copper	80.3	28.9	13.65	0.001
Manganese	90.5	98.1	10.18	0.01
Zinc	89.8	33.3	14.93	0.001

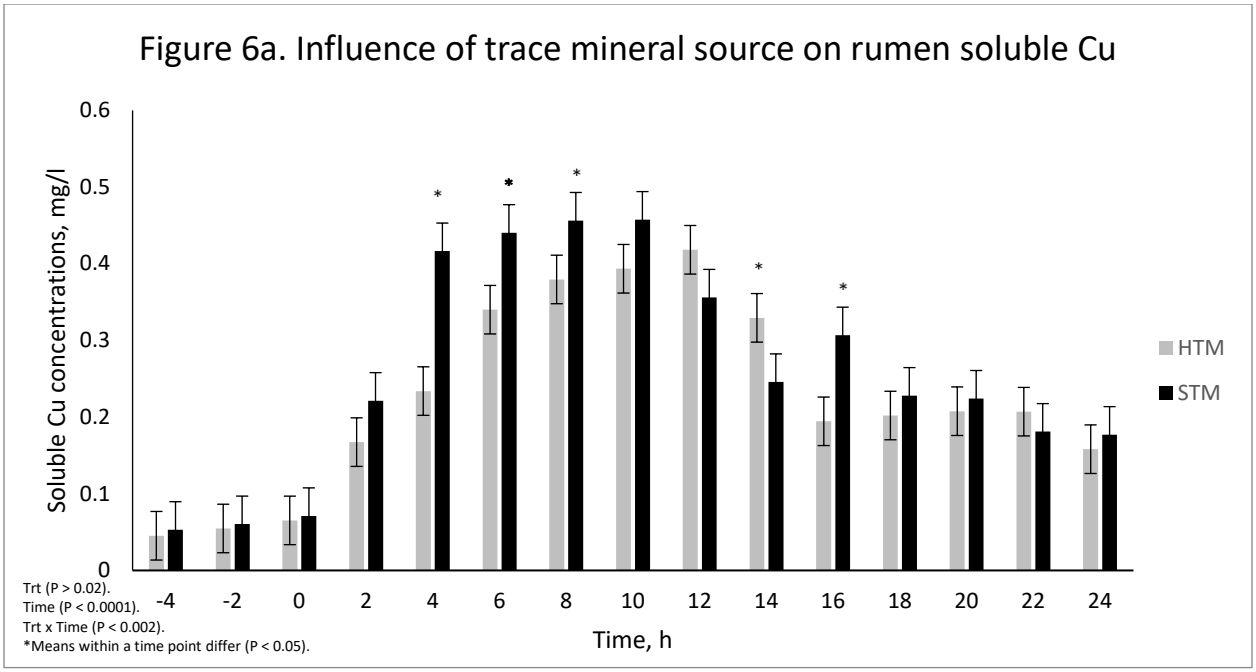

Figure 6a. The influence of trace mineral source on soluble Cu within the ruminal contents of steers receiving a pulse dose of either sulfate trace minerals (STM; 18 mg Cu/kg DM from CuSO4; 40 mg Mn/kg DM from MnSO4; 90 mg Zn/kg DM from ZnSO4 or hydroxy trace minerals (HTM; 18 mg Cu/kg DM from CuOHCl; 40 mg Mn/kg DM from MnOHCl; 90 mg Zn/kg DM from ZnOHCl). The x-axis denotes sampling time in hours, the y-axis denotes rumen soluble Cu. Error bars represent standard errors.

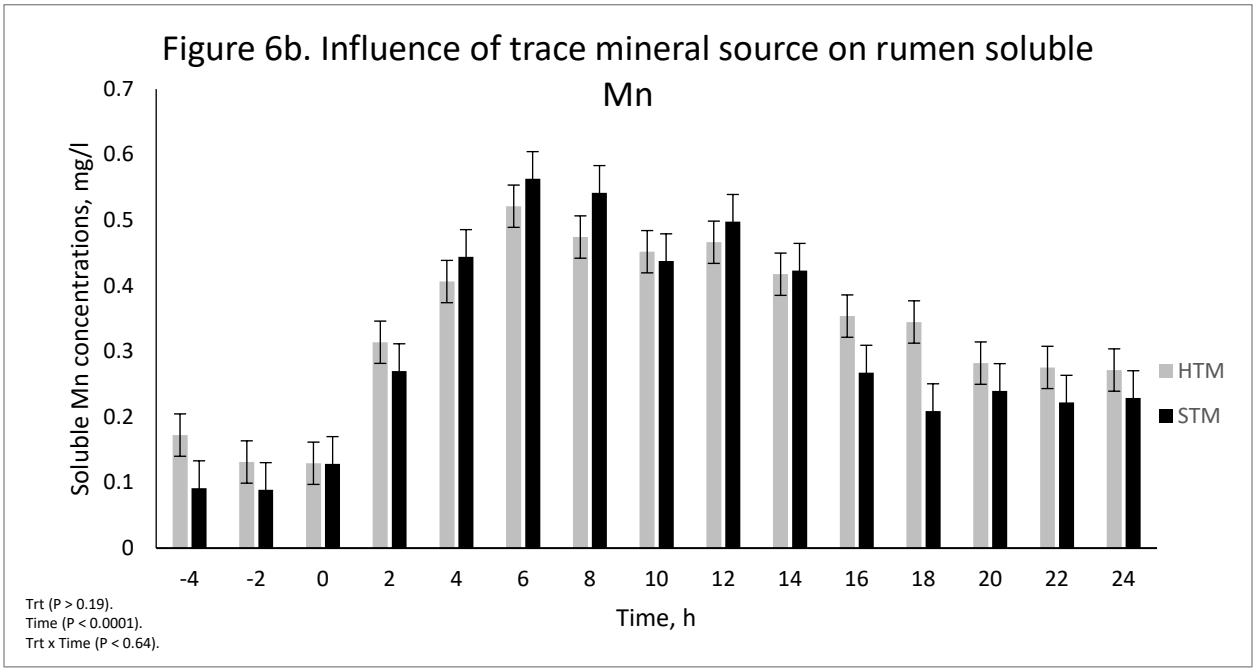

Figure 6b. The influence of trace mineral source on soluble Mn within the ruminal contents of steers receiving a pulse dose of either sulfate trace minerals (STM; 18 mg Cu/kg DM from CuSO4; 40 mg Mn/kg DM from MnSO4; 90 mg Zn/kg DM from ZnSO4 or hydroxy trace minerals (HTM; 18 mg Cu/kg DM from CuOHCl; 40 mg Mn/kg DM from MnOHCl; 90 mg Zn/kg DM from ZnOHCl). The x-axis denotes sampling time in hours, the y-axis denotes rumen soluble Mn. Error bars represent standard errors.

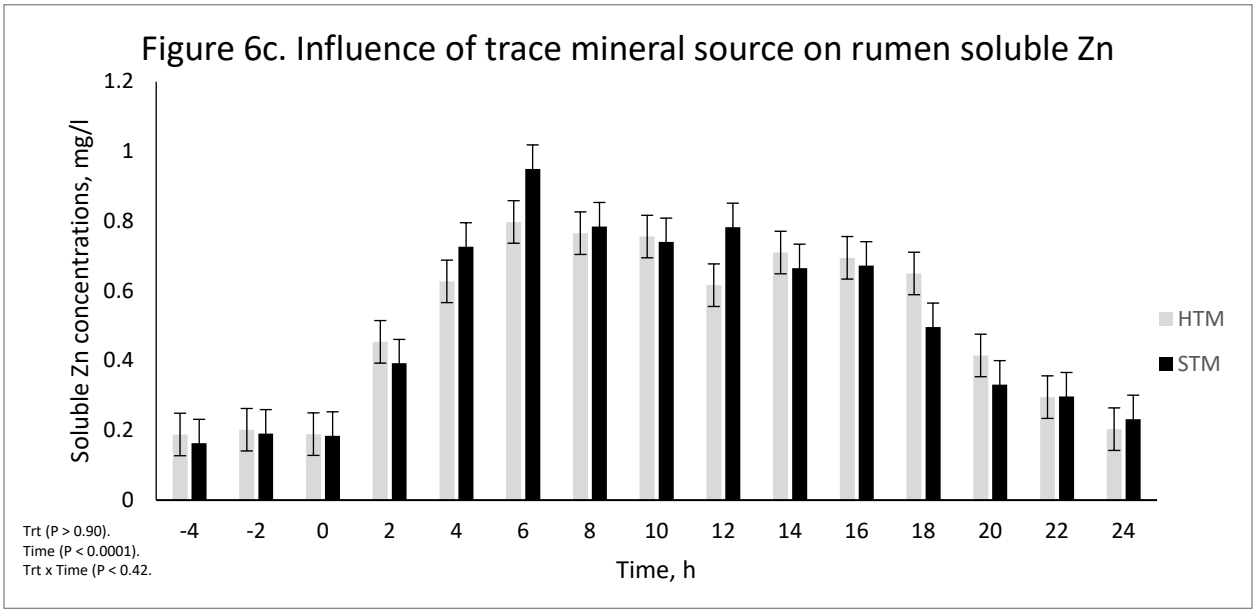

Figure 6c. The influence of trace mineral source on soluble Zn within the ruminal contents of steers receiving a pulse dose of either sulfate trace minerals (STM; 18 mg Cu/kg DM from CuSO4; 40 mg Mn/kg DM from MnSO4; 90 mg Zn/kg DM from ZnSO4 or hydroxy trace minerals (HTM; 18 mg Cu/kg DM from CuOHCl; 40 mg Mn/kg DM from MnOHCl; 90 mg Zn/kg DM from ZnOHCl). The x-axis denotes sampling time in hours, the y-axis denotes rumen soluble Zn. Error bars represent standard errors.

DISCUSSION

The basal feed ingredients supplied 40, 91, and 140% of the Cu, Mn, and Zn requirement for a finishing feedlot steer target to gain 3.5 lb/day (NASEM, 2016). Due to the uncertain bioavailability of trace minerals (TM) in feed ingredients, Cu, Mn, and Zn are typically added to diets at levels similar to those used in this study. The total mixed rations provided approximately 33.2, 98, and 159 mg/kg of Cu, Mn, and Zn, respectively. Notably, concentrations greatly exceeded the NASEM 2016 recommendations in this study. However, several researchers have proven the

extent of unavailable trace in basal ingredients, feedlot nutritionists tend to not account for dietary, and formulate based on supplemental mineral (Galyean et al., 2007; Samuelson et al., 2017).

Steers supplemented with HTM had no impact on nutrient digestibility in the present study. Other studies (Faulkner and Weiss, 2017; Daniel et al., 2020; Miller et al., 2020) have proven the contrary, where improvement in NDF digestibility was observed in dairy animals fed HTM vs. STM sources of Cu, Mn, and Zn. Beef steers fed a medium quality grass hay-based diet and supplemented with HTM also had greater ADF and NDF digestibility than those receiving STM (Guimaraes et al., 2021). However, there were no variations in ADF and NFD digestibility between lambs that received supplementation from HTM versus STM sources of Cu, Mn, and Zn, as reported by Van Valin et al. (2018). In addition to that, studies comparing organic sources versus sulfate also observed greater ADF and NDF digestibility by sheep receiving CTM sources (Hassan et al., 2011; Dezfoulian et al., 2012; Garg et al., 2008; Gresakova et al., 2018; Alimohamady et al., 2019). Great variation related to the diet type need to be mentioned between those sheep studies. Van Valin et al. (2018) fed a higher proportion of concentrates leading to higher energy and crude protein in the diet, compared to the other studies which fed a higher forage-based diet.

Dairy heifers that received STM supplementation exhibited comparable NDF and ADF digestibility to those supplemented with proteinate TM, as observed in the study conducted by Pino and Heinrichs in 2016. Assessing the precise influence of enhanced fiber digestibility on animal performance can be challenging. Enhancing fiber digestibility by approximately 1-2% could potentially lead to reduced feed intake, lessened manure production, and consequently, an increase in overall feed efficiency, leading to better growth performance.

The current study did not include a negative control (no supplemental Cu, Mn, and Zn) because the primary intention was to simulate industry practice. Moreover, Cu, Mn, and Zn are

typically added in feedlot diets, from receiving to finishing phase. However, due to the absence of a negative control in our study, it remains unclear whether supplemented trace minerals that become soluble in the rumen affect fiber digestion. Taking into consideration that all the studies mentioned above were conducted with much higher levels of forage, there is great indication that the pH tended to be maintained above the present study (average of 5.6, as low as 5.3 was recorded after 6 h post feeding). Repeatedly data shows that inorganic trace minerals, such sulfate and oxide, by being highly soluble in the rumen impact certain microorganisms, leading to fermentation discrepancy, and possibly reduced digestion. Since there was not a negative control in the present study, we cannot determine to a precise extent if both treatments by becoming soluble in the rumen, had an negative impact on fiber digestibility.

Taken together, these findings suggest that the solubility of Cu, Mn, and Zn regardless of source, might have an impact on fiber digestibility when high diets are fed, potentially by altering the composition of the rumen microbiome. Research conducted by Emmanuel and Staples in 1990 implies that the release of Cu and Zn from feed ingredients during ruminal fermentation should be adequate to meet the microbial requirements for these minerals. Consequently, providing additional soluble minerals beyond the microbial needs could prompt certain microorganisms in the rumen to increase their energy expenditure in order to maintain homeostasis. This regulation may involve processes such as metal uptake, efflux, and sequestration. There is a need for future research to explore the effects of trace mineral solubility in the rumen on microbial function and, consequently, its impact on overall animal production efficiency.

Total volatile fatty acid concentration was not influenced in this study, however HTM steers had greater ($P < 0.05$) ruminal molar proportions of propionate and tended ($P < 0.07$) to

have greater molar proportions of valerate compared to STM steers. Steers fed STM had greater ($P < 0.05$) molar proportions of butyrate compared to HTM steers.

The greater ruminal molar proportions of propionate and valerate in steers receiving HTM is somewhat consistent with other studies that observed improvements on fermentation characteristics by HTM. The increase on propionate and valerate in this experiment did not translate to differences in total VFA concentration. Other studies (Shaeffer et al., 2006; Daniel et al 2020; Guimaraes et al 2021; Guimaraes et al., 2022) observed an increase in total VFA concentration, following improvements in fiber digestibility. However, further assumptions cannot be conclusive since rumen volume and passage rate were not measured in any of these studies. In short, it cannot be determined from the present study, which TM (Cu, Zn, and/or Mn) was responsible for the greater propionate and valerate production.

Ruminal mineral solubility results in this study were less in magnitude compared to the other diet types reported in the literature. In steers receiving STM sources, soluble Cu concentrations were greater at 4, 6, 8, and 16h after bolus dosing. Steers that received HTM sources of Cu, Mn, and Zn had reduced concentrations of soluble Cu and Zn in the rumen during the 10 to 12-hour period following bolus dosing (Guimaraes et al., 2022). Similar outcomes were observed in steers fed a medium-quality grass hay-based diet (Guimaraes et al., 2021) and in steers consuming a diet composed of 50% corn silage and 50% steam-flaked corn (Caldera et al., 2019) after receiving dosages of STM or HTM Cu, Mn, and Zn sources. Interestingly, Mn solubility levels seemed to remain consistent among the treatments. Moreover, ruminal soluble Mn concentrations increased following dosing with HTM, while the solubility of Cu and Zn in the rumen remained comparable to their initial levels at 0 h. However, it's worth noting that dietary

excess of Mn and Zn originating from the basal diet may have influenced the final results of Mn and Zn soluble concentrations.

Trace minerals in the rumen environment can assume two distinct forms: 1) soluble and/or 2) insoluble. Within the soluble fraction, these minerals can exist either as ionic (free) or complexed forms (Bremner, 1970; Allen et al., 1987). It is plausible that in the case of CTM, copper (Cu) and zinc (Zn) existed in a soluble complex state rather than in the ionic form. Studies conducted in sheep have suggested that the majority of Cu, Mn, and Zn becomes associated with the solid digesta in the rumen when these trace minerals originate from forages (Bremner, 1970; Waghorn et al., 1990). In our study, trace minerals were sourced from both the basal dietary ingredients and the pulse dose supplement. To understand the impact of trace mineral on nutrient digestibility, it would be helpful to include a negative control group in future studies that does not receive supplemental Cu, Mn, and Zn supplementation. This would help to elucidate the specific effects of the basal diet on trace mineral availability and nutrient digestion.

Trace minerals obtained from both feed ingredients and supplements can undergo solubilization in the rumen. Several factors, including the type of feedstuff, the composition of trace minerals within a specific supplement, and the form of trace minerals, can influence their solubility within the rumen. Once they become soluble, these trace minerals can engage with various compounds present in the rumen environment, such as components of feedstuffs, the end products of fermentation, microbial cells, and more. Consequently, they may form insoluble complexes. The extent to which trace minerals are tightly bound within these insoluble complexes determines whether they can become soluble in the small intestine and be absorbed. Therefore, it is crucial to comprehend the ability of trace minerals to be released from common digestion byproducts that can form complexes with them.

Dialysis involving chelating agents has been used to assess the accessibility of Zn and other elements in feedstuffs, as noted by Jones et al. (1985). In the present study, Cu, Mn, and Zn found within the solid digesta extracted from the rumen of steers administered HTM sources of these minerals exhibited a more pronounced release when subjected to dialysis against Tris-EDTA at 12 and 24 h after dosing, compared to steers receiving STM. These findings imply that Cu, Mn, and Zn within the ruminal solid digesta of steers supplemented with STM exhibit a stronger binding affinity to ruminal digesta compared to Cu, Mn, and Zn in steers subjected to the HTM treatment. Consequently, it is plausible that a more robust binding to solid digesta within the rumen could influence the accessibility of Cu, Mn, and Zn in the small intestine, leading to decrease in absorption and lesser bioavailability.

The concentrations of Cu, Mn, and Zn in ruminal solid digesta were similar at the 0-hour mark after dosing. This observation aligns with the fact that both sources were highly soluble in the rumen environment. However, by the 12 h post-dosing point, Cu, Mn, and Zn concentrations in solid digesta were greater for HTM. Despite this similarity in Cu concentrations, the release of Cu following dialysis against Tris-EDTA was still greater in steers that received the HTM treatment at the 24 h post-dosing time frame.

Regarding Zn, its concentrations in solid digesta remained elevated in the HTM-supplemented steers at the 24 h post-dosing point. However, this did not examine whether Zn from ZnOHCl remained in the OHCl form or if the Zn was separated from OHCl and incorporated into bacterial cells. Further studies are necessary to investigate whether bacterial Zn concentrations are influenced by the source of Zn.

CONCLUSIONS

This study sheds light on the complexities of trace mineral supplementation in cattle diets. The basal feed ingredients provided a substantial portion of the Cu, Mn, and Zn requirements for finishing feedlot steers. However, due to the uncertain bioavailability of trace minerals in these ingredients, Cu, Mn, and Zn are typically supplemented at levels similar to those employed in this study, resulting in concentrations that exceed recommended levels. The impact of trace mineral supplementation on nutrient digestibility appears to vary depending on the source and context. While some studies have shown improvements in fiber digestibility with specific trace mineral sources, others have not demonstrated significant differences. Enhancing fiber digestibility, even by a modest percentage, can potentially lead to benefits such as reduced feed intake, lower manure production, and improved overall feed efficiency, which can ultimately enhance growth performance. It is important to note that this study did not include a negative control group without supplemental Cu, Mn, and Zn, as it aimed to replicate industry practices. However, future research with a negative control group would help elucidate the specific effects of trace mineral supplementation on fiber digestion.

The solubility of Cu, Mn, and Zn, regardless of their source, may have an impact on fiber digestibility, potentially by altering the composition of the rumen microbiome. Research suggests that providing excess soluble minerals beyond microbial requirements may prompt certain rumen microorganisms to increase energy expenditure for homeostasis. Further investigations are needed to explore the effects of trace mineral solubility in the rumen on microbial function and its implications for overall animal production efficiency. The study also observed differences in ruminal fermentation characteristics between HTM and STM supplementation, with HTM steers showing greater proportions of propionate and valerate. However, these differences did not

translate into changes in total volatile fatty acid concentration. Further research is required to understand the specific effects of different trace minerals on ruminal fermentation.

In summary, this study highlights the need for a comprehensive understanding of trace mineral supplementation in cattle diets. Factors such as source, solubility, and interactions with rumen microbiota all play a role in shaping the nutritional outcomes of trace mineral supplementation in beef cattle diets. Continued research is essential to optimize trace mineral supplementation strategies and enhance the efficiency and sustainability of beef production.

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CHAPTER IV

CHARACTERIZING THE INFLUENCE OF TRACE MINERAL SOURCE AND DIET TYPE ON THE FECAL AND RUMEN MICROBIOME IN BEEF CATTLE

SUMMARY

This study analyzed samples from experiments investigating trace mineral source effects on nutrient digestibility, short-chain fatty acid production, Cu, Zn, and Mn ruminal solubility, and binding strength in rumen insoluble digesta. Twelve Angus steers (5+ years old) participated in three studies: Study 1 (Guimaraes et al., 2020, 2021; low quality forage diet), Study 2 (Guimaraes et al., 2022; dairy type diet), and Study 3 (unpublished, Chapter 3 of this dissertation; high concentrate diet). Due to impacts on digestibility, fermentation, mineral solubility, and binding in all studies, microbiome analysis was conducted on rumen and fecal samples collected to assess potential bacterial and protozoa population shifts. In Study 1 (low quality forage diet), *Papillibacter* was the predominant species in the rumen. Rumen samples showed a significant time point impact on Shannon's PD ($P < 0.05$). Beta diversity analysis using unweighted UniFrac revealed notable differences in community diversity between rumen and fecal samples ($q < 0.001$). Rumen microbiota clustered at multiple time points. No beta diversity differences between STM and HTM were observed at baseline (Time A, $q = 0.23$), but differences emerged after adaptation (Time C, $q < 0.03$) and during total collection (Time F, $q < 0.01$). ANCOM analysis revealed higher abundance of *Papillibacter*, *Ruminoccoccaceae*, *Prevotellaceae*, and *Saccharofermentans*

in rumen samples, regardless of trace mineral source. No significant differences were found in fecal samples. ANCOM analysis did not detect measurable differential abundance between trace mineral sources in fecal or rumen samples following Alpha and Beta diversity results. In Study 2 (dairy diet), *Moraxellaceae* was the most prevalent species within the rumen. Rumen samples showed a significant time point effect on Shannon's PD ($P < 0.001$), with noteworthy differences between several time points. However, trace mineral source had no impact on Shannon's PD ($P = 0.15$). Fecal samples showed no differences in time points or trace mineral sources. Beta diversity analysis using unweighted UniFrac revealed a significant distinction between microbial community diversity in rumen and fecal samples ($q < 0.001$). Rumen microbiota clustered differently at various time points. Trace mineral source did not affect beta diversity, but there was a tendency toward greater diversity with hydroxy trace mineral supplementation ($q = 0.07$). ANCOM analysis found higher abundance of ASVs *Moraxellaceae*, *Planococcaceae*, *Ruminoccoccaceae*, and *Prevotellaceae* in the rumen environment, regardless of trace mineral source. No significant differential abundance was detected between trace mineral sources in rumen samples, consistent with Shannon's PD diversity analysis. In Study 3 (high concentrate diet), *Moraxellaceae* and *Planococcaceae* were the most abundant in rumen samples. Rumen samples showed a significant impact of collection time point on Shannon's PD ($P < 0.001$), with significant differences between several time points. However, TM source had no effect on Shannon's Diversity ($P = 0.19$). Interestingly, Shannon's Diversity in feces showed significant differences between TM sources ($P < 0.03$), with STM having lesser diversity than HTM. There was no effect of time point in fecal samples ($P = 0.38$). Beta diversity analysis using unweighted UniFrac revealed clear differences between microbial diversity in rumen and fecal samples ($q < 0.001$). ANCOM analysis found higher abundance of ASVs *Moraxellaceae*, *Prevotellaceae*,

Planococcaceae, and *Ruminoccoccaceae* in the rumen environment, regardless of trace mineral source. In conclusion, this study provides valuable insights into the complex interplay between trace mineral sources, the rumen microbiome, and various aspects of rumen health and function. While trace mineral sources did not significantly alter the rumen microbiome composition, time points demonstrated substantial effects on microbial communities. These findings contribute to our understanding of how dietary factors can influence rumen ecology and function, with potential implications for livestock nutrition and management strategies.

INTRODUCTION

The microbiome, per its definition, refers to the collection of genomes, or collection of DNAs, of all the microorganisms in a specific environment. These microorganisms living in an environment interact directly as a community, microorganisms of which include bacteria, archaea, microbial eukaryotes such as fungi and amoeba, and viruses. The microbiota, on the other hand, refers to microbes that live in a specific environment. For instance, the microbiota on human skin is different than the microbiota that lives in the gastrointestinal tract. Diet and environment have a large impact on our microbiota; the same is true for ruminants and other mammals.

The rumen microbiome consists of a specialized anaerobic microbial community that provides its host with enzymes required to digest plant material, and ultimately volatile fatty acids for energy. A study of 742 rumen samples representing 32 animal species and 35 countries showed that rumen microbiomes include several "core" taxa including the bacteria *Prevotellaceae*, *Butyvivibrio*, *Ruminococcus*, unclassified *Ruminoccoccaceae*, unclassified *Bacteroidales*, and unclassified *Clostridiales*; the archaea *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium*; and the protozoa *Entodinium* and *Epidinium* (Henderson et al., 2015). Overall trends

suggest that archaeal diversity is lower compared to bacterial diversity, and protozoan communities exhibit the highest inter-individual variability.

Understanding the complexity and dynamic ruminal microbial ecosystem has been heavily studied the past couple of years. Especially how this ecosystem responds to dietary changes; going from a high forage-based diet to high concentrated finishing, or by simply switching additive; these changes can have a direct impact on the rumen microbiome, which could impact digestion and rumen fermentation. It is well known that diet appears to drive differences primarily in bacterial microbial communities, possibly because of bacteria's diverse metabolic capabilities compared to archaea and protozoa. The significance of minerals in the microorganism realm has been an area of study for decades. However, current technology has allowed the investigation process to further deepen the knowledge highlighting later findings on catalytic, structural, and stabilizing roles played by mineral elements within microbial cells (Zembayashi, 1974; Weinberg, 1977; Wetzel and Menke et al., 1978), along with their ability to either trigger or suppress certain secondary metabolic processes, as well as their uptake and storage by these microorganisms (Durand and Kawashima, 1980; Spears, 2003; Zhao et al., 2022; Spears et al., 2023). Surprisingly, the role of mineral elements in rumen microbial activities has not garnered substantial focus in studies addressing mineral metabolism in ruminants to this date. The truth is that major minerals such as Cu, Zn, and Mn, contribute to the regulation of not only metabolic functions, but have a direct impact on the regulation of important physicochemical characteristics of the rumen environment such as osmotic pressure, buffering capacity, redox potential, dilution rate, all of which affects rumen fermentation, and digestion of nutrients. Lactating dairy cows supplemented with hydroxy TM (HTM) had greater NDF digestibility than those supplemented with sulfate TM (STM) sources (Faulkner and Weiss,

2017). The authors suggested that the impact of trace mineral source on fiber digestion may be due to differences in ruminal solubility of Cu and Zn. In agreement with these findings, NDF digestibility tended to be lower in steers fed STM compared to those receiving HTM (Caldera et al., 2019). Furthermore, ruminal soluble Cu and Zn concentrations were greater (at multiple time points over a 24 h period) in steers following a single bolus dose of Cu, Mn, and Zn from STM compared to steers receiving HTM (Caldera et al., 2019). Rumen pH averaged 6.23 in the aforementioned experiment. These findings agree with earlier studies indicating that HTM forms of Cu and Zn are relatively insoluble under slightly acidic pH conditions and increase in solubility as pH decreases, while STM forms of Cu and Zn are almost completely soluble in water and acidic conditions (Cao et al., 2000; Spears et al., 2004).

Collectively, these data suggest that ruminal solubility of Cu and Zn may influence ruminal fermentation. Therefore, the objective of the current experiment was to examine the influence of TM source on the microbial ecology of the rumen of cattle receiving different diet types.

MATERIALS AND METHODS

Prior to the initiation of this experiment, the Animal Care and Use Committee at Colorado State University previously approved all animal care and procedures described here (IACUC approval #17-7182A). Twelve crossbred Angus steers fitted with ruminal cannulae (initial BW 452.8 ± 6.1 kg) were utilized in three consecutive studies.

This was a retrospective study, analyzing the microbiome of samples obtained from three digestibility experiments investigating the influence of trace mineral source on fiber digestion in

fistulated steers receiving a: 1) medium quality high roughage-based diet (Guimaraes et al., 2020 [MS Thesis]; Guimaraes et al., 2021); 2) a high lactating dairy cow diet (Guimaraes et al., 2022); and 3) a high concentrate finishing diet (Chapter 3 in this dissertation). Steers were housed at the Colorado State University Agriculture, Research, Development, and Education Center (ARDEC) in Fort Collins, CO. Initially, steers were housed in two pens (6 steers per pen) for experiments 1 and 3 and three pens (4 steers per pen) for experiment 2. For all experiments, steers were fed their respective basal diets for 22 d prior to beginning any experimental trace mineral treatments.

Sampling and collection methods

After the basal diet adaptation phase for each experiment, rumen and fecal samples were collected from each of the 12 fistulated steers per experiment at different timepoints. Time point A represented the first day of the experiment (day 0- or 22-day post adjustment to the appropriate basal diet), in which steers were only receiving the experimental basal diets without supplemental trace minerals. After a 7-day period, trace mineral treatments were introduced to the animals. Time point B represents the beginning of treatments (day 8). Therefore, time point A and B provided a baseline of the ruminal and fecal microbiome communities and allowed for baseline inter-individual rumen variability quantification. Time point C (day 10) and time point D (day 14) were intermediate collection points during the treatment adaptation phase (from day 8 through day 14). These samples allowed for assessment of possible early changes of the ruminal microbiome community due to treatment. On day 15 (time point E), steers were moved into the metabolism barn. Following a 3-d adaptation period in the metabolism barn (dry matter intake measurements), total fecal and urine collection phase (time point F through J) was initiated. Rumen and fecal samples were collected for microbiome analysis over the 5-day total collection period.

For each sample collection event, ruminal liquid/digesta material were collected in a 15 ml falcon tube. For fecal samples from each individual steer, animals were observed until defecation and fresh feces were swabbed using a sterile BD BBL CultureSwab (Becton Dickson, USA). This procedure avoided rectal lumen specific microbial communities.

Sample analysis

DNA extraction, PCR amplification, and sequencing protocols followed Earth Microbiome Project standard protocols as outlined on the website http://www.earthmicrobiome.org/protocols-and-standards/ . For DNA extraction, we included 12 mock/negative extractions for a total of 276 DNA extractions.

Research on the microbiome has exploded in the past decade due to a revolution in DNA sequencing technologies (next-generation sequencing), which resulted in a rapid decline in the cost of sequencing. These DNA sequencing advances coupled with similar advances in computational power and bioinformatics have made microbiome data accessible broadly across the sciences. Additionally, large-scale efforts such as the Earth Microbiome Project (earthmicrobiome.org) have standardized data generation protocols to allow for comparable data across laboratories.

Several data types exist for describing a microbial community. These include gene amplicon sequencing, shotgun metagenomics sequencing, and RNA sequencing (also known as transcriptomic). Gene amplicon sequencing is the most common data type (currently) in the microbiome field because it is very inexpensive and provides useful taxonomic data. In this approach a taxonomically informative gene (16S rRNA for bacteria and archaea, 18S rRNA for microbial eukaryotes such as protozoa and fungi, and the internal transcribed spacer (ITS) for fungi specifically) is amplified via PCR and the resulting amplicons are sequenced generating a

taxonomic profile of the microbial community. Shotgun metagenomics is the method of sequencing all DNA molecules in a sample. This approach provides information both about the community taxonomy and its functional potential (i.e. functional genes), but it is more expensive and can be problematic for host-associated samples with high host DNA content (e.g. a lung or liver sample). Finally, a transcriptomic analysis is highly useful as it reveals the portion of the microbial community that is active and the genes that are being transcribed into proteins. However, working with RNA presents challenges, and it is more expensive. For the present study, only gene amplicon sequencing of the 16S rRNA gene was performed. Therefore, protozoa and fungi taxa were not identified.

The steps included: 1) sample collection 2) DNA extraction 3) PCR of the 16S rRNA gene using primers that attach a unique barcode to each sample for multiplexing 4) pooling PCRs and sequencing the pool 5) bioinformatically assigning each sequence to a sample (since they were multiplexed) 6) generating a table of sequences 7) assigning taxonomy and 8) statistical assessment.

Experiment 1

Steers (n = 6/treatment) were fed a high fiber, medium quality hay (chopped) diet balanced to meet the nutrient requirements of growing steers. The diet was supplemented with protein, salt, Ca, P, Se, I, Co, and vitamins A, D, and E to meet requirements for grazing beef cows. Treatments consisted of 60 ppm Zn, 20 ppm Cu, and 40 ppm Mn per kg of DM, from either sulfate or hydroxy sources.

Experiment 2

Steers (n = 4/treatment) were fed a dairy-type diet balanced to meet requirements for high producing lactating dairy cows. Zinc, Cu, and Mn were supplemented at 60, 10, and 40 ppm, respectively. Treatments consisted of 1) sulfate, 2) organic, and 3) hydroxy trace mineral sources.

Experiment 3

Steers (n = 6/treatment) were fed a high concentrate finishing diet balanced to meet the requirements for finishing steers. Treatments consisted of 90 ppm Zn, 18 ppm Cu, and 40 ppm Mn from either sulfate or hydroxy sources.

Data Generation

The DNA extraction was performed using the PowerMag Soil DNA Isolation Kit (Qiagen, Cat no. 27000-4 KF) according to manufacturer's instructions in a 96-well plate. Two positive controls, six negative controls, and two tip controls were included. The DNA extraction was performed in a PCR hood using KingFisher Equipment.

Polymerase chain reaction targeting the V4 region of the 16S rRNA bacterial gene, and V4 region of the 18SRNA protozoa gene was performed with the 515F forward primer, barcoded [AATGATACGGCGACCACCGAGATCTACACGCT *(5' Illumina adapter)* XXXXXXXXXXXX *(Golay barcode)* TATGGTAATT *(Forward primer pad)* GT *(Forward primer linker)* GTGYCAGCMGCCGCGGTAA *(Forward primer (515F)*] and the 806R reverse primer [CAAGCAGAAGACGGCATACGAGAT *(Reverse complement of 3' Illumina adapter)* AGTCAGCCAG *(Reverse primer pad)* CC *(Reverse primer linker)* GGACTACNVGGGTWTCTAAT *(Reverse primer (806)*]. Sequencing of amplicons was performed at CSU's NGS Metcalf Lab with the Illumina Platform using a 500 cycle Illumina kit for 2x250 forward and reverse reads.

Data analysis

Multiplexed EMP-paired-end sequences were imported in Qiime2 (Boylen et al., 2019) and demultiplexed using the qiime2 demux emp-paired tool (Hamday et al., 2009; Hamday et al., 2008). After demultiplexing, a visualization file was generated to assess the quality of the forward and reverse reads. Following, demultiplexed sequences were denoised using DADA2 plugin of qiime2 for paired-end sequences (Callahan et al., 2016) to generate a feature table, a representative sequence file, and denoised statistics. The dada2 method was considered truncating at 256 for experiment 1, 251 bp for experiment 2, and 245 bp for experiment 3.

After denoising, taxonomy classification was performed using the SILVA database and the pre-fitted sklearn-based classifier (Pedregosa et al., 2011). Following, taxa bar plots were generated using the feature table, taxonomy classification, and metadata. ASV's assigned to mitochondria and chloroplasts were filtered out. We assessed the control quality from the feature count table and the taxa bar plot. A phylogenetic tree was created using the fragment insertion command of Qiime2 by inserting the representative sequence file into the SEPP reference phylogeny sequence file (Janssen et al., 2019). The analysis approach considered comparisons of trace mineral sources within rumen and fecal samples. Additionally, the overall effect of sample type was reported.

The diversity metrics used were Shannon's PD for alpha diversity and Unweighted UniFrac for beta diversity. Differential relative abundance was determined using ANCOM (Janssen et al., 2018). First, differential relative abundance was calculated for the rumen and fecal sample types across all trace mineral sources.

Shannon's PD within sample were performed using Kruskal-Wallis pairwise comparisons. The q-value for multiple comparison was use for hypothesis testing ($q < 0.05$). Differences in dispersion between trace mineral sources within sampling site and collection time points were tested using PERMANOVA.

Results

Experiment 1 – Low Quality Forage Diet

Alpha Diversity: After filtering out controls, the total number of sequences was 1,855,789 for 180 samples. There were 1,624 unique ASVs. *Papillibacter* was the most abundant ASV in the rumen. From the rumen samples, there was an impact of collection point on Shannon's PD observed ($P < 0.05$, Figure 7).

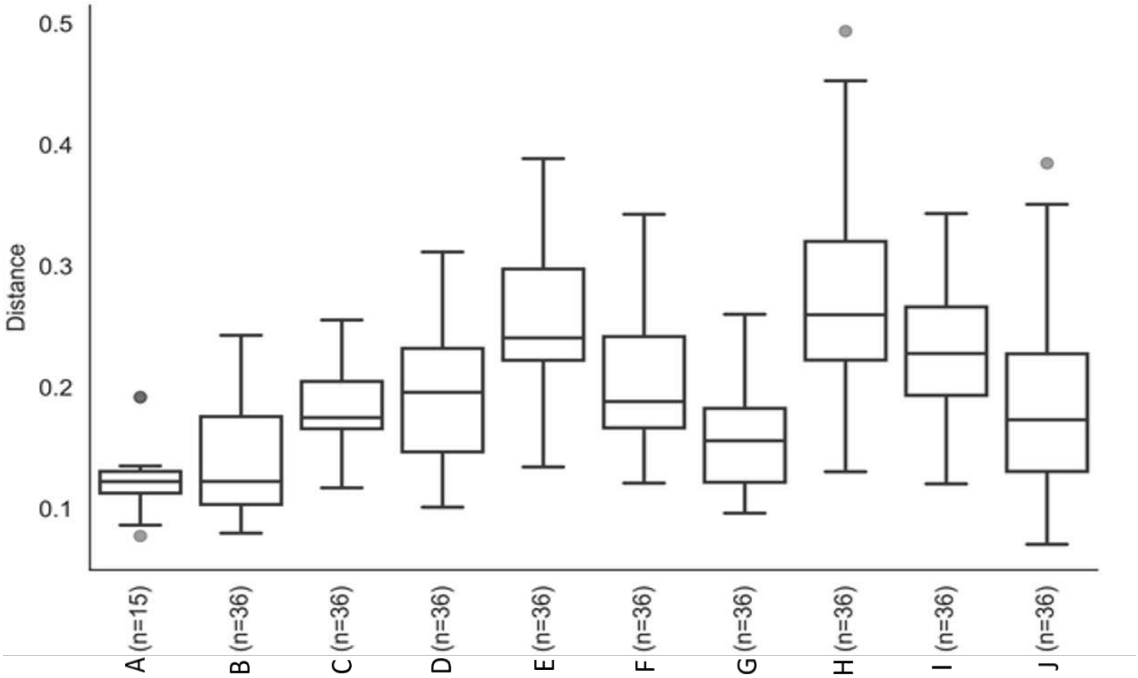

Figure 7. Time point A represents the first day on trial (d 0), which steers were only receiving the basal diet without test treatments. After the 7d adaptation period, trace mineral treatments were introduced to the animals. Time point B represents the start of treatments (d 8). Time point C (d 10) and time D (d 14) were intermediate collection points during the treatment adaptation phase (from d 8 through 14). On d 15 (time point E) steers were moved into the metabolism barn. Following 3 d of adaptation in the metabolism barn (dry matter intake measurements) fecal and urine collection was initiated. Time points F through J were collected over the 5d fecal and urine collection period

Beta Diversity: Beta diversity was assessed using unweighted UniFrac between trace mineral sources within both fecal and rumen samples. Not surprising, significant differences were observed between the rumen and fecal community diversity ($q < 0.001$). The PCoA plot containing both fecal and rumen samples (Figure 8) show clustering of both sampling sites, however, more evident clusters in fecal samples were observed.

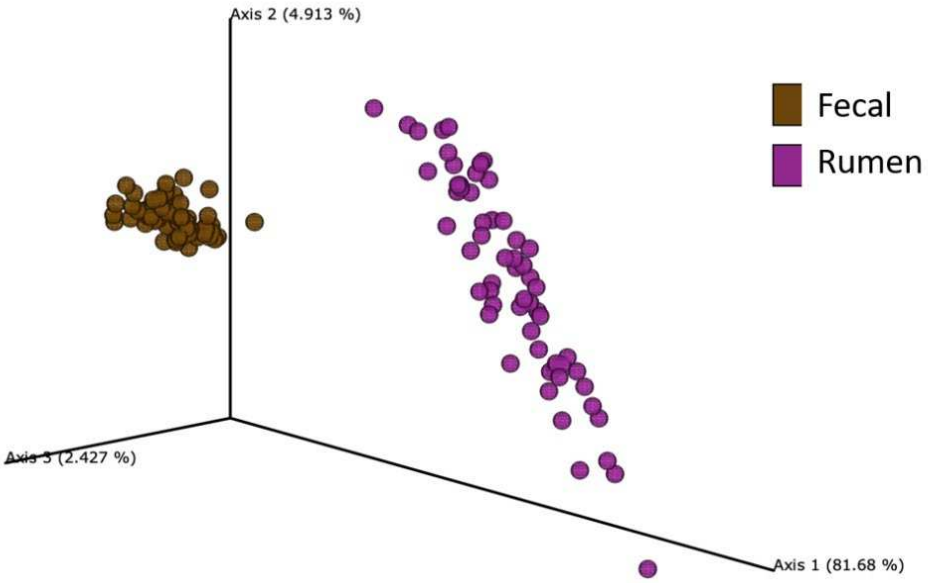

Figure 8. PCoA Unweighted UniFrac plot from both fecal (brown) and rumen (purple) samples. Sample site exhibited an effect on microbiota diversity when comparing samples collected from the rumen and fecal swabbing locations

Additionally, collection time points showed a clustering on rumen microbiota at time point A, C, F, H, and J (Figure 9). This agrees with the Alpha Diversity analysis as discussed previously.

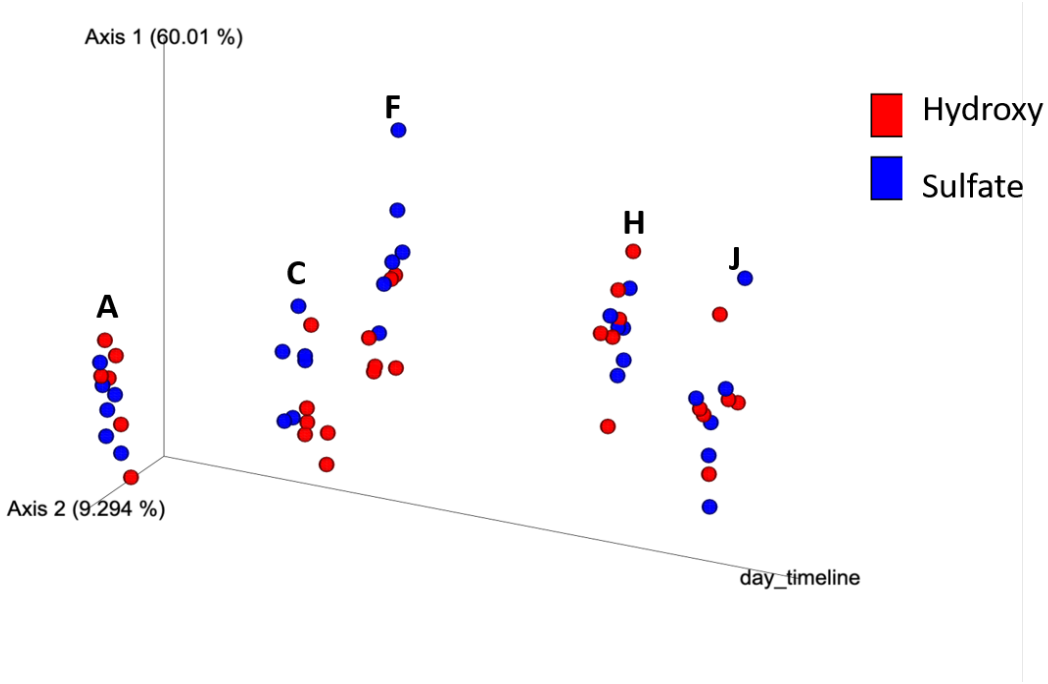

Figure 9. PCoA Unweighted UniFrac plot from rumen samples. Collection point exhibited an effect on microbiota diversity. Differences in microbial diversity are more evident between treatments at time point C and F.

Beta diversity was assessed using Unweighted UniFrac significance between trace mineral source and collection points within rumen samples. Table 11 shows the PERMANOVA results from the rumen.

Table 11. Permanova Unweighted Unifrac significance on treatment by collection time in the rumen of steers fed a forage-based diet.

TRT x Collect. Point	TRT x Collect. Point	Sample size	Permutations	pseudo-F	p-value	q-value
HTM - A	STM - A	24	999	1.456	0.209	0.23
HTM - A	STM – C	24	999	3.654	0.021	0.03
HTM - C	STM – C	24	999	4.191	0.019	0.03
HTM - F	STM – F	24	999	11.356	0.006	0.01
HTM - C	STM – A	24	999	6.389	0.003	0.01
HTM - A	HTM – C	24	999	4.543	0.017	0.02
HTM - F	STM – C	24	999	5.454	0.005	0.01
HTM - A	HTM – F	24	999	6.493	0.004	0.01
HTM - C	HTM - F	24	999	12.384	0.007	0.01

Time point A represents the first day on trial (d 0), which steers were only receiving the experimental basal diet without test treatments. After a 7d adaptation period, trace mineral treatments were introduced to the animals. Time point B represents the start of treatments (d 8). Time C (d 10) and time D (d 14) were intermediate collection points during treatment adaptation phase (from d 8 through 14). On d15, (time point E) steers were moved into the metabolism barn. Following 3 d of adaptation in the metabolism barn (dry matter intake measurements) fecal and urine collection was initiated. Time points F through J were collected over the 5d fecal and urine collection period.

Relative Differential Abundance: Comparison of differential relative abundance of fecal and rumen samples was established using ANCOM. The ASVs *Pappilibacter* (w = 644), *Ruminoccoccaceae* (w = 555), *Prevotellaceae* (w = 448), and *Saccharofermentans* (w = 441), were determined to be more abundant in the rumen samples regardless of the trace mineral source (Figure 10). There were no taxa determined to be more abundant across treatments in the fecal swabs.

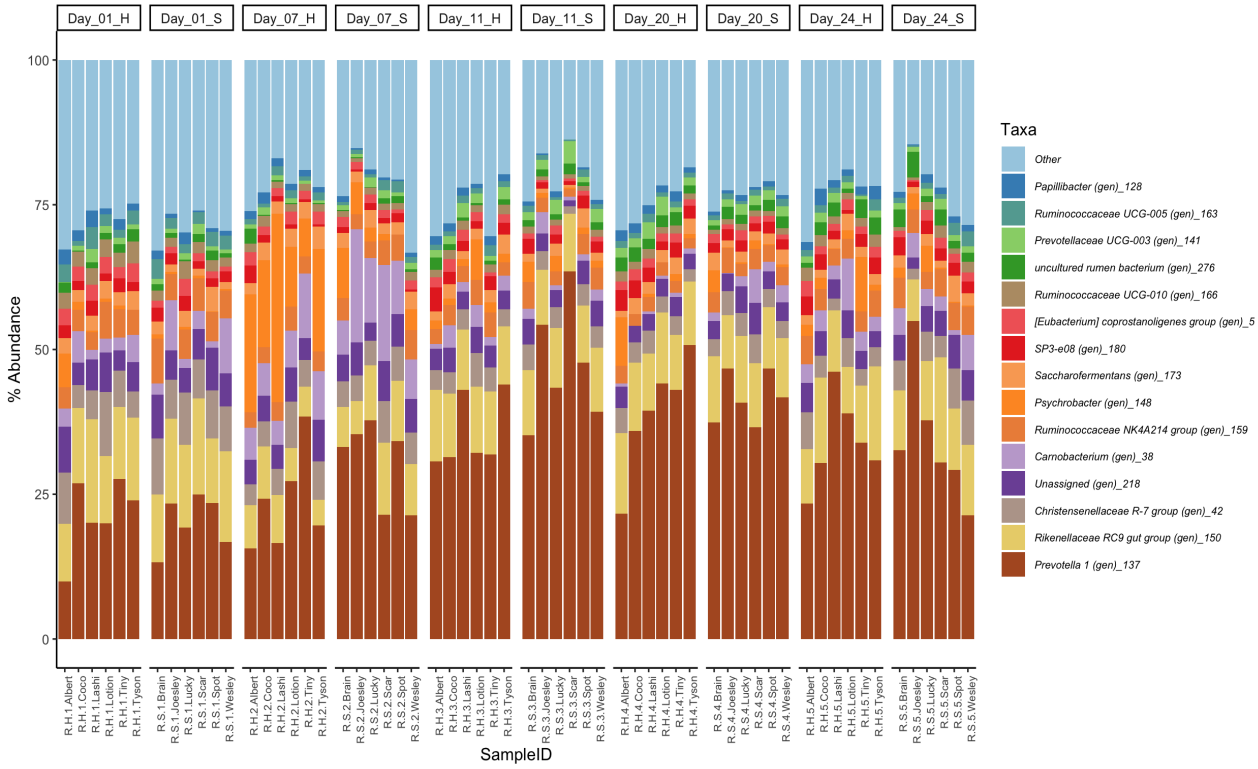

Figure 10. Relative abundance taxonomic plot sorted by collection time point in rumen samples of steers fed a forage-based diet.

Following Alpha and Beta diversity results, ANCOM (Analysis of composition of microbiome) was performed to determine different taxa between trace mineral source and sampling site. The ANCOM analysis did not detect any measurable differential abundance between trace mineral source in the fecal or rumen samples.

Experiment 2 – Dairy Diet

Alpha Diversity: After filtering out controls, the total number of sequences was 1,513,770 for 180 samples. There were 1,243 unique ASVs. *Moraxellaceae* was the most abundant ASV in the data set.

From the rumen samples, there was an effect of collection time point ($P < 0.001$) on Shannon's PD. Consequently, significant differences were observed between collection time point B and D ($q < 0.013$), B and F ($q < 0.002$), B and G ($q < 0.002$), and B and J ($q < 0.02$) as shown in Figure 11.

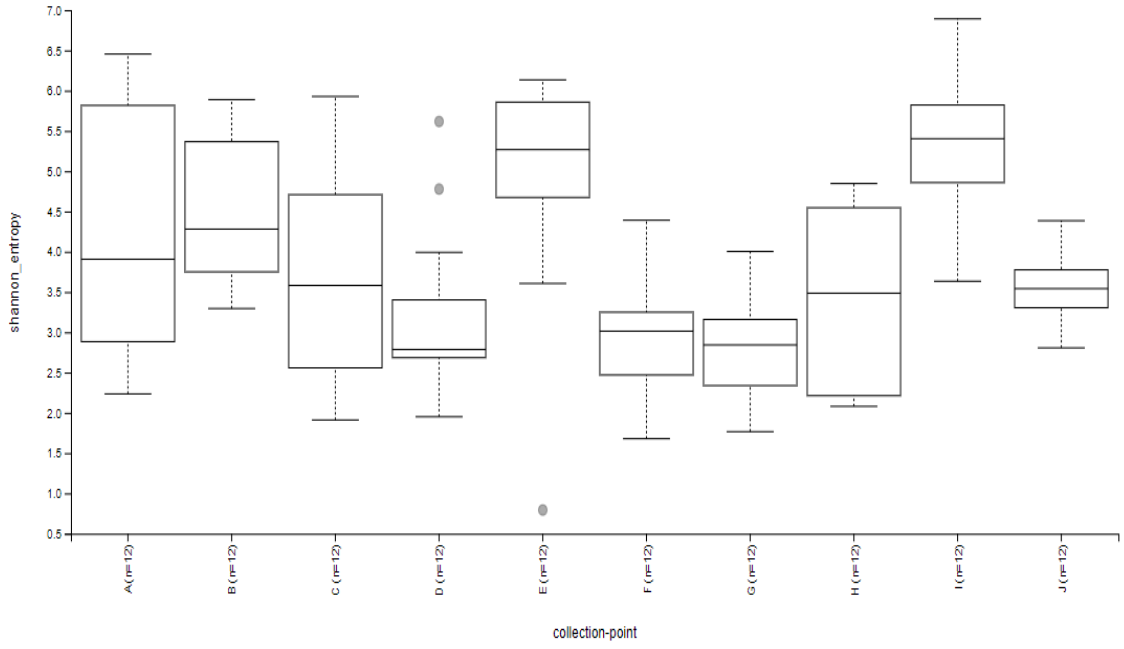

Figure 11. Shannon's PD measuring richness of rumen samples obtained from steers fed a dairy type over 25 d period. Time point A represents the first day on trial (d 0), which steers were only receiving basal diet without test treatments. After the 7-day adaptation period, trace mineral treatments were introduced to the animals. Time point B represents the start of treatments (d 8). Time point C (d 10) and time point D (d 14) were intermediate collection points during treatment adaptation phase (from d 8 through 14). On d 15 (time point E) steers were moved into the metabolism barn. Following 3 d of adaptation in the metabolism barn (dry matter intake

measurements), fecal and urine collection was initiated. Time points F through J were collected over the 5-day fecal and urine collection period.

There was no effect of trace mineral source on Shannon's PD observed ($P = 0.15$; Figure 12).

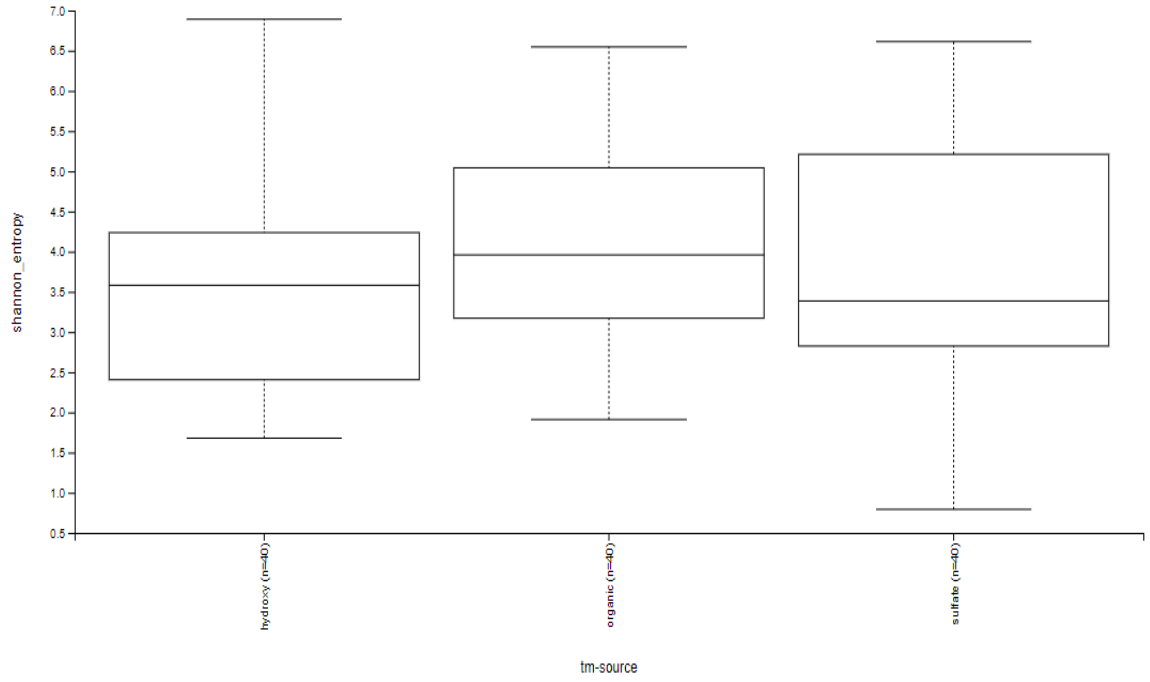

Figure 12. Shannon's Phylogenetic Diversity measuring richness across trace mineral source in the rumen samples of steers fed a dairy-type diet over a 25d period.

Fecal collection time point ($P = 0.51$, Figure 13) and trace mineral source ($P = 0.41$, data no shown) had no impact on Shannon's PD.

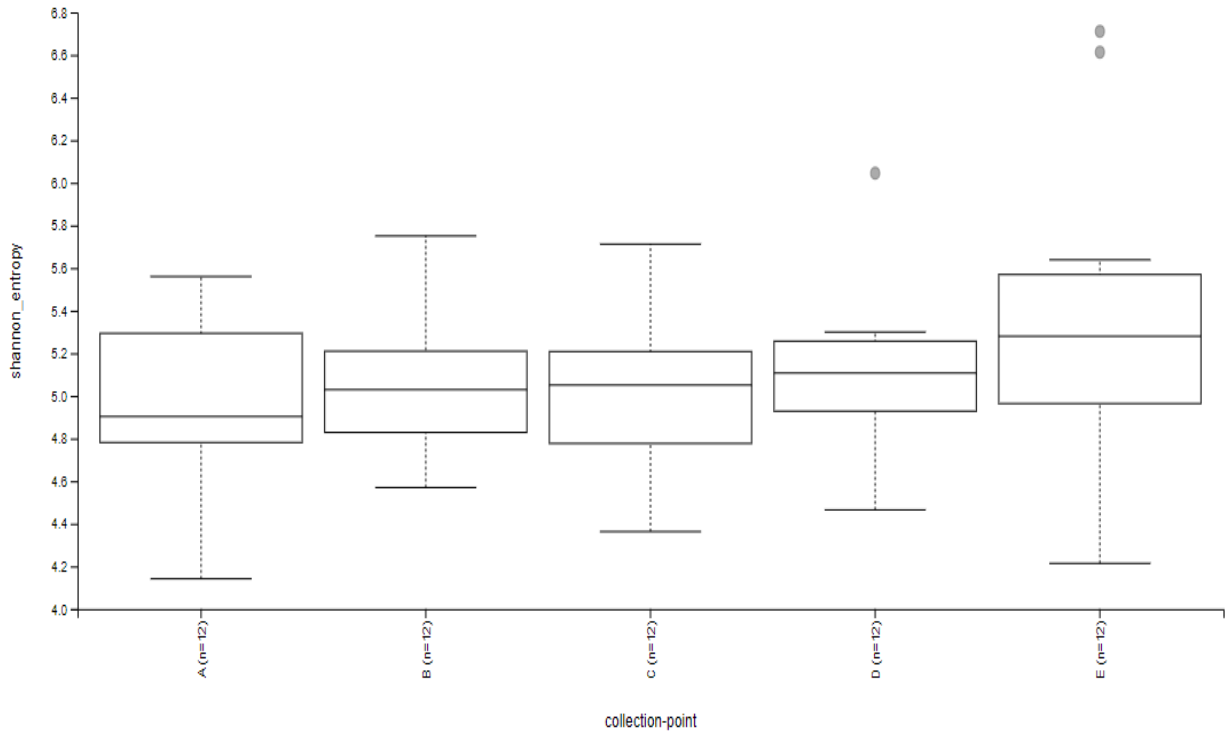

Figure 13. Shannon's PD measured richness in feces obtained from steers fed a dairy type of diet over 25 day period.

Beta Diversity: Beta diversity was assessed using unweighted UniFrac between trace mineral sources within both fecal and rumen samples. Not surprising, a significant difference was observed between the rumen and fecal microbial community diversity ($q <$ 0.001). The PCoA plot containing both fecal and rumen samples (Figure 14) show clustering of both sampling sites, however, there was a more evident cluster in fecal samples.

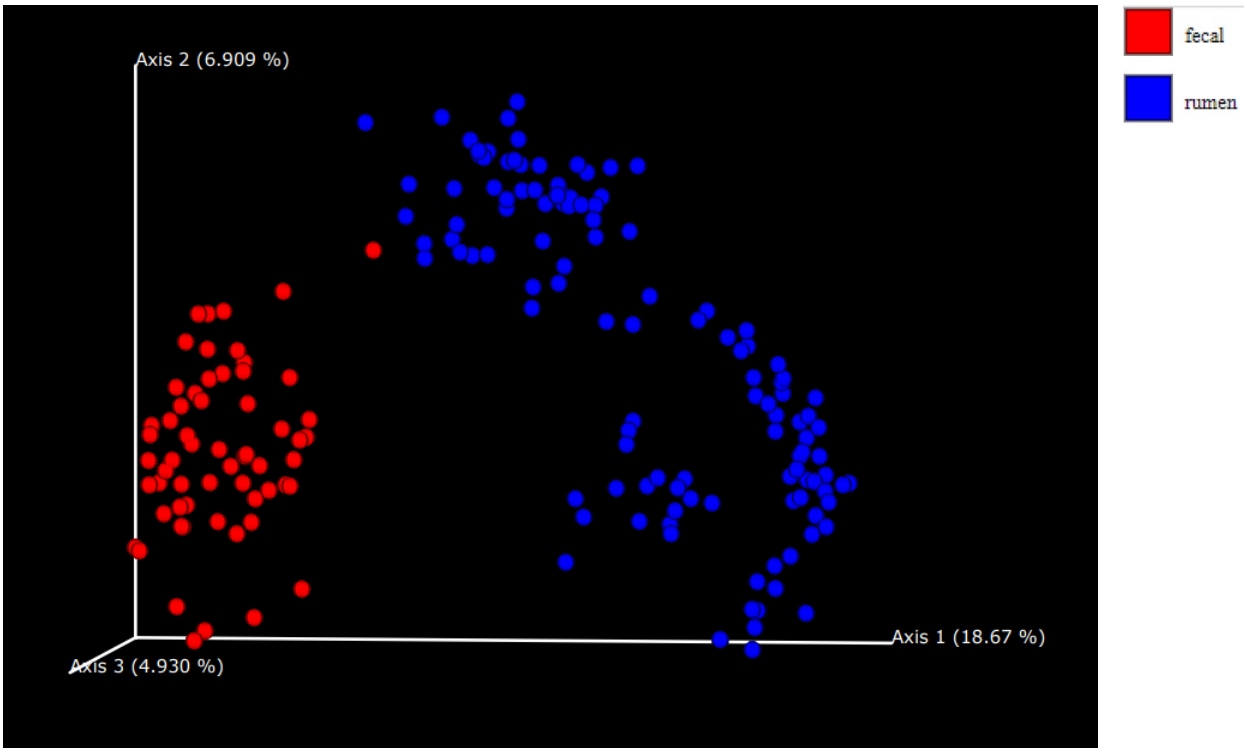

Figure 14. PCoA unweighted UniFrac plot from both fecal (red) and rumen (blue) samples. Sample location exhibited an effect on microbiota diversity when comparing samples collected from the rumen and fecal swabbing locations.

Additionally, collection time points showed a clustering of rumen microbiota at time points A, B, C, and D which differ from time points F, G, I, and J (Figure 15). These results agree with what was reported in the Alpha Diversity analysis.

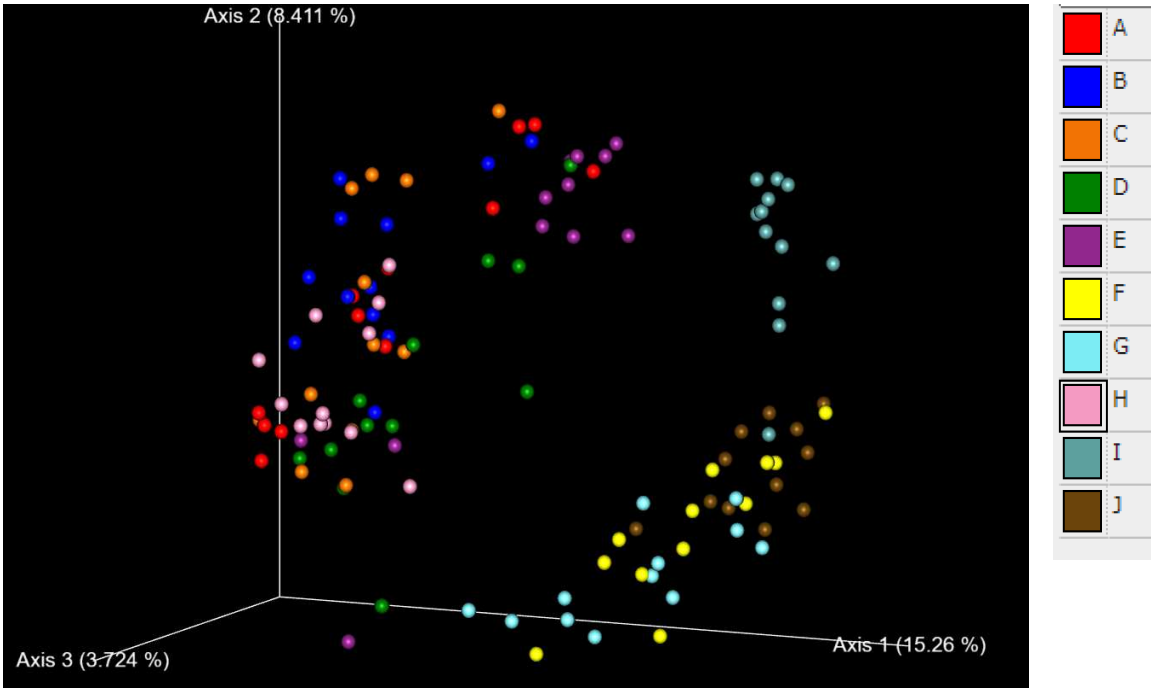

Figure 15. PCoA unweighted UniFrac plot from rumen samples of steers fed a dairy-type diet. Collection point exhibited an effect on microbiome diversity.

Beta diversity was assessed using unweighted UniFrac significance between collection time points within the rumen. Table 12 shows the PERMANOVA results from the rumen.

Table 12. Unweighted UniFrac significance of the rumen on collection time point of steers fed a dairy-type diet.

Collect. Point	Collect. Point	Sample size	Permutations	pseudo-F	p-value	q-value
A	E	24	999	4.403	0.02	0.026
A	F	24	999	4.514	0.008	0.01
A	I	24	999	15.299	0.001	0.002
A	J	24	999	3.475	0.02	0.026
B	D	24	999	3.294	0.029	0.03
B	E	24	999	8.395	0.006	0.01
B	F	24	999	9.574	0.001	0.002
B	G	24	999	7.931	0.002	0.003
B	H	24	999	3.265	0.019	0.02
B	I	24	999	21.516	0.001	0.002
B	J	24	999	6.637	0.001	0.002
C	E	24	999	11.716	0.001	0.002
C	F	24	999	9.510	0.001	0.002
C	G	24	999	8.793	0.001	0.002
C	H	24	999	4.779	0.007	0.01
C	I	24	999	36.897	0.001	0.002
C	J	24	999	8.353	0.002	0.003
E	F	24	999	15.587	0.001	0.002
E	G	24	999	10.425	0.001	0.002
E	H	24	999	10.274	0.002	0.003
E	I	24	999	9.447	0.001	0.002
E	J	24	999	7.864	0.002	0.003
F	H	24	999	4.906	0.006	0.01

F	I	24	999	55.022	0.001	0.002
F	J	24	999	2.371	0.03	0.03
G	H	24	999	3.713	0.019	0.02
G	I	24	999	40.251	0.001	0.002
H	I	24	999	29.676	0.001	0.002
H	J	24	999	3.927	0.005	0.009
I	J	24	999	26.386	0.001	0.002

Time point A represents the first day on trial (d 0), which steers were only receiving the experimental basal diet without test treatments. After the 7d adaptation period, trace mineral treatments were introduced to the animals. Time point B represents the start of treatments (d 8). Time point C (d 10) and time point D (d 14) were intermediate collection time points during treatment adaptation phase (from d 8 through 14). On d 15, (time point E) steers were moved into the metabolism barn. Following 3 d of adaptation in the metabolism barn (dry matter intake measurements) fecal and urine collection was initiated. Time points F through J were collected over the 5d fecal and urine collection period.

Trace mineral source had no impact on beta-diversity in rumen and fecal samples. However, a tendency was observed between beta-diversity in the hydroxy and sulfate source (Table 13). Steers supplemented with hydroxy trace minerals tended ($q = 0.07$) to express a greater beta-diversity when compared to sulfate trace mineral supplemented steers.

Table 13. Unweighted Unifrac significance in the rumen within trace mineral source supplemented to steers fed a dairy-type diet.

TRT	TRT	Sample size	Permutations	pseudo-F	p-value	q-value
Hydroxy	Sulfate	80	999	1.638	0.02	0.07
Hydroxy	Organic	80	999	1.187	0.20	0.20
Sulfate	Organic	80	999	1.302	0.10	0.16

Steers supplemented with hydroxy trace mineral sources expressed a tendency for greater beta-diversity when compared to sulfate supplemented steers.

Relative Differential Abundance: Finally, a comparison of differential relative abundance on fecal and rumen samples within trace mineral sources was established using ANCOM. The ASVs *Moraxellaceae* (w = 495), *Planococcaceae* (w = 488), *Ruminoccoccaceae* (w = 481), and *Prevotellaceae* (w = 480), were determined to be more abundant in the rumen environment regardless of the trace mineral source. ANCOM analysis did not indicate any measurable differential abundance between trace mineral sources in the rumen samples. This was also previously indicated by Shannon's PD diversity of the rumen samples by treatment as shown in Figure 16.

Experiment 3 – High Concentrate Diet

Alpha Diversity: After filtering out controls, the total number of sequences was 1,255,889 for 180 samples. There were 1,224 unique ASVs. *Moraxellaceae* and *Papillibacter* were the most abundant ASVs in the rumen samples. There was an impact of collection time point on Shannon's PD observed ($P < 0.001$) in the rumen environment. Therefore, significant differences were observed between collection time point B and D ($q < 0.01$), B and F ($q < 0.001$), B and G ($q < 0.001$), and B and J ($q < 0.02$) as shown in Figure 16.

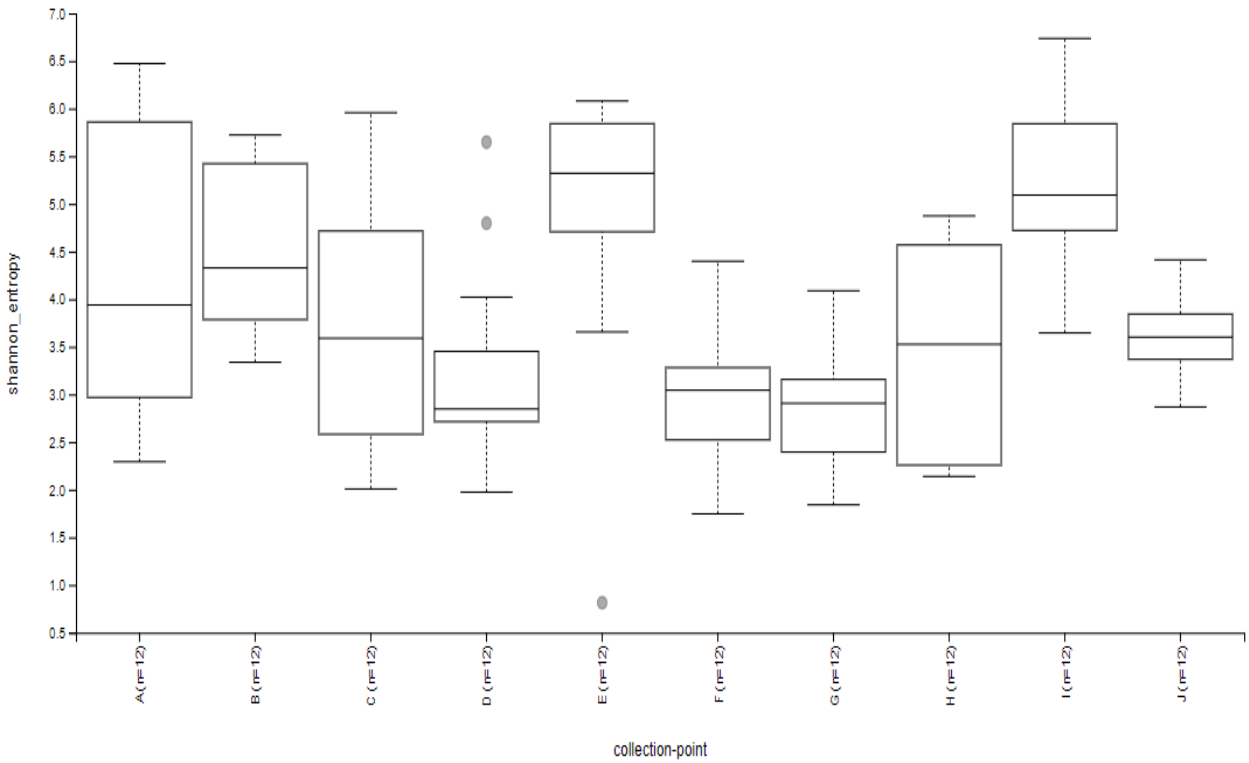

Figure 16. Time point A represents the first day on trial (d 0), in which steers were receiving the basal experimental diet without test treatments. After a 7d adaptation period, trace mineral treatments were introduced to the animals. Time point B represents the start of treatments (d 8). Time point C (d 10) and time point D (d 14) were intermediate collection time points during treatment adaptation phase (from d 8 through 14). On d 15 (time point E) steers were moved into the metabolism barn. Following 3 d of adaptation in the metabolism barn (dry matter intake measurements) fecal and urine collection was initiated. Time points F through J were collected over the 5d fecal and urine collection period.

Besides the impacts that were observed for Alpha Diversity by collection time point within the rumen, there was no impact of trace mineral source on Shannon's Diversity observed (P = 0.19; Figure 17).

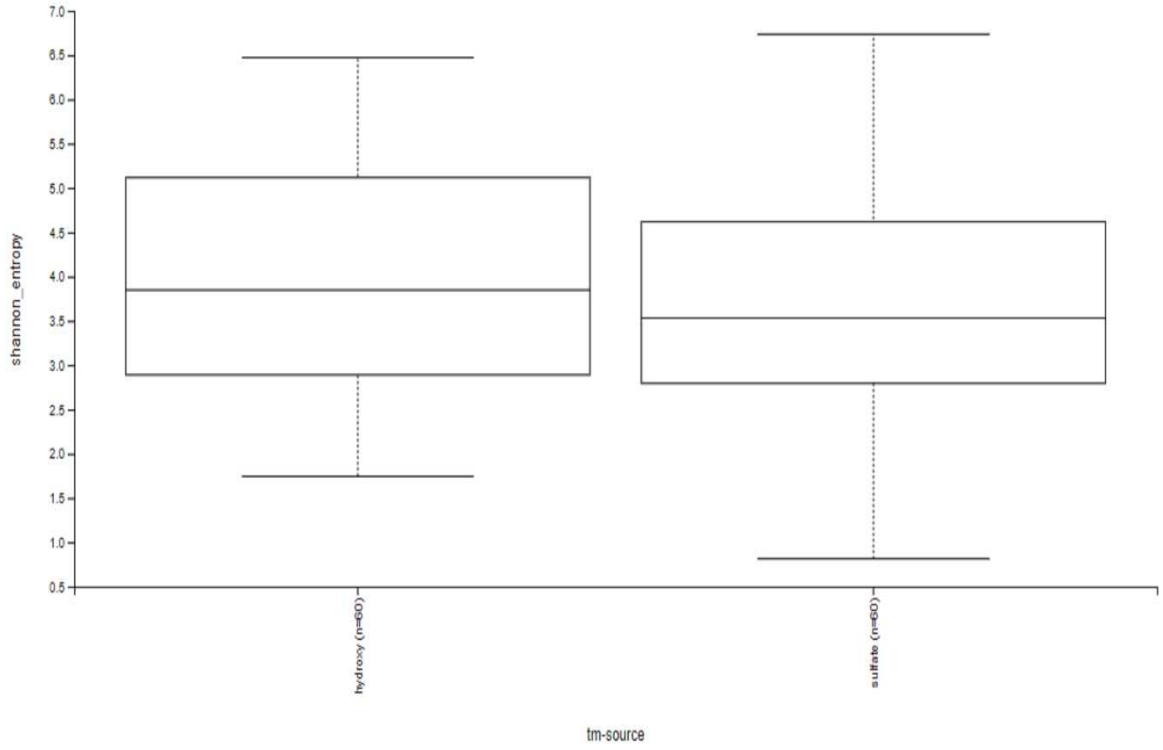

Figure 17. Shannon's PD measured richness across trace mineral source of the rumen samples in steers fed a feedlot-type diet over a 25d period. A similarity across trace mineral source and alpha diversity was observed in the rumen samples.

Interestingly, results showed significant differences in Shannon's Diversity between trace mineral sources in feces (P < 0. 03), where sulfate trace mineral source had lesser diversity compared to hydroxy trace mineral sources (Figure 18). However, there was no effect on collection time point in fecal samples (P = 0.38, data not shown).

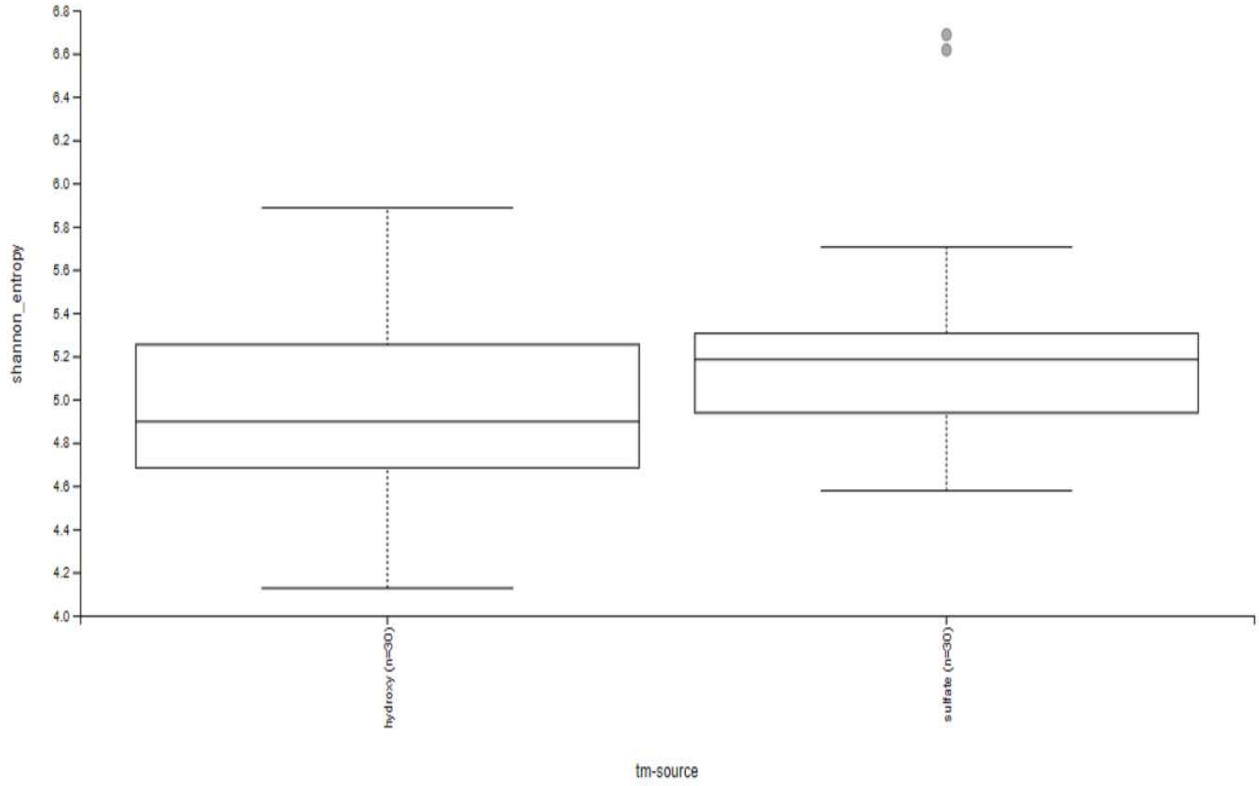

Figure 18. Shannon's PD measured richness across trace mineral source of the fecal samples in steers fed a feedlot-type diet over a 25d period. A similarity across trace mineral source and alpha diversity was observed in the fecal samples.

Beta Diversity: Beta diversity was assessed using unweighted UniFrac between trace mineral sources within fecal and rumen samples. Similar to the studies above, clear differences were observed between the microbial diversity in rumen and fecal samples ($q < 0.001$). The PCoA plot containing both fecal and rumen samples (Figure 19) show clustering of both sampling sites with a more evident cluster in fecal samples.

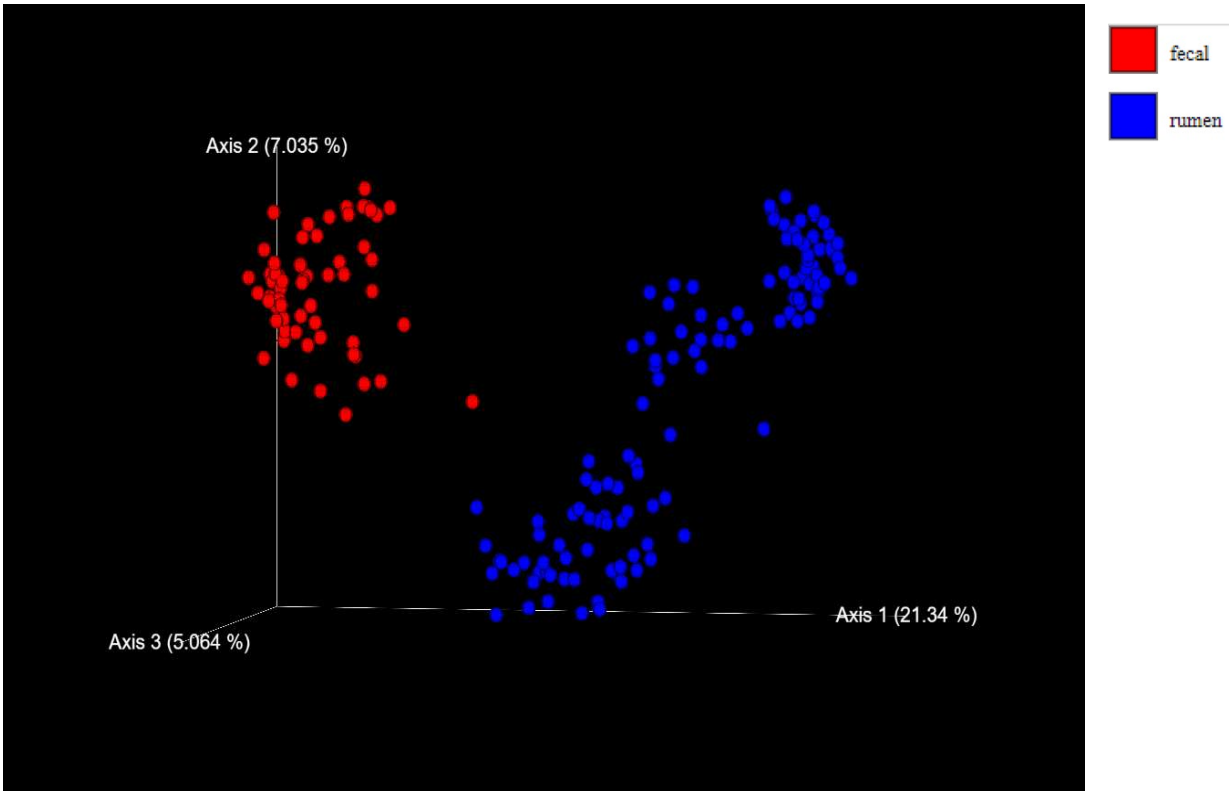

Figure 19. PCoA unweighted UniFrac plot from both fecal (red) and rumen (blue) samples. Sample site exhibited an effect on microbiota diversity when comparing samples collected from the rumen and fecal swabbing locations.

Additionally, also in agreement with the previous diets, collection time points had an impact on rumen microbiota samples collected at the initiation of the trial, such as time points A, B, C, and D which differed from timepoints F, G, I, and J (Figure 20).

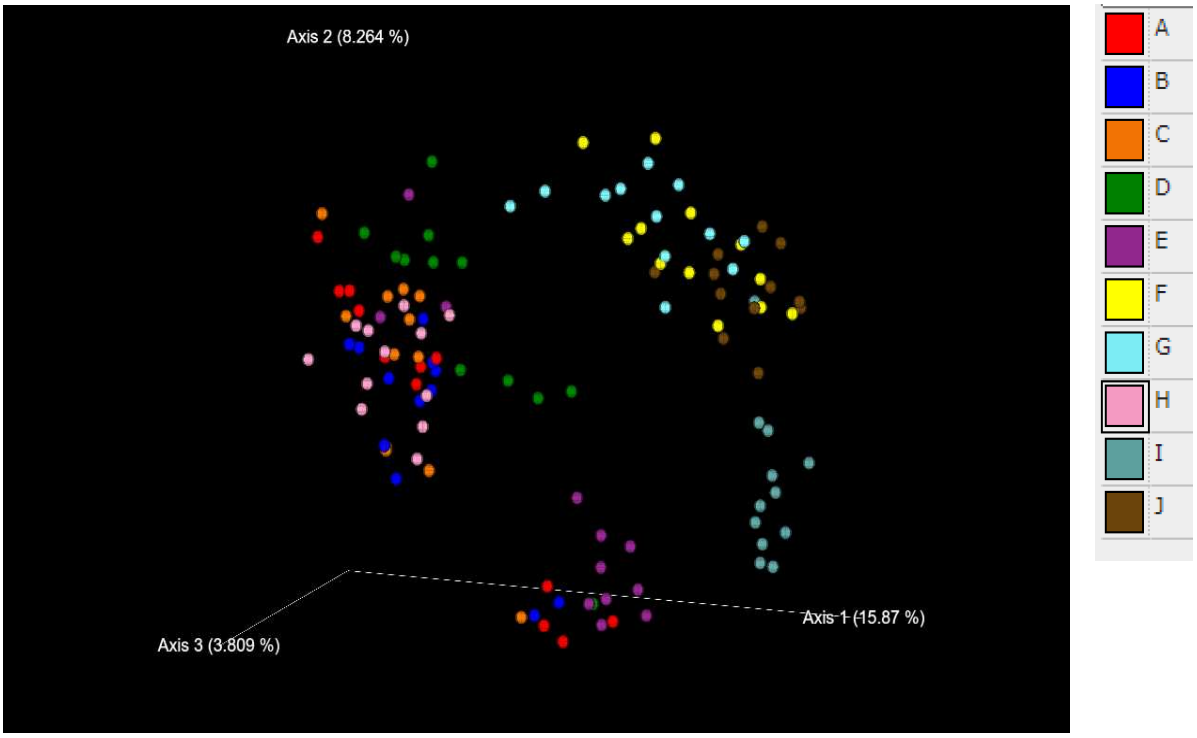

Figure 20. PCoA unweighted UniFrac plot from rumen samples. Collection time point exhibited an effect on microbiota diversity.

Therefore, beta diversity was assessed using unweighted UniFrac significance between collection points within rumen samples. Table 14 shows the PERMANOVA results from the rumen samples that were collected at 10 time points across the 25 days of experiment.

Table 14. Unweighted UniFrac significance of the rumen samples by collection time point on steers fed a finishing feedlot-type diet.

Collect. Point	Collect. Point	Sample size	Permutations	pseudo-F	p-value	q-value
A	D	24	999	1.592	0.04	0.05
A	E	24	999	2.102	0.01	0.01
A	F	24	999	5.354	0.001	0.001
A	G	24	999	5.167	0.001	0.001
A	H	24	999	3.188	0.001	0.001
A	I	24	999	6.353	0.001	0.001
A	J	24	999	6.005	0.001	0.001
B	D	24	999	1.902	0.002	0.002
B	E	24	999	3.028	0.002	0.002
B	F	24	999	6.650	0.001	0.001
B	G	24	999	6.611	0.001	0.001
B	I	24	999	3.360	0.001	0.001
B	J	24	999	7.950	0.001	0.001

Time point A represents the first day on trial (d 0), in which steers were receiving experimental basal diet without test treatments. After the 7 d adaptation period, trace mineral treatments were introduced to the animals. Time point B represents the start of treatments (d 8). Time C (d 10) and time D (d 14) were intermediate collection time points during treatment adaptation phase (from d 8 through 14). On d 15, (time point E) steers were moved into the metabolism barn. Following 3 d of adaptation in the metabolism barn (dry matter intake measurements) fecal and urine collection was initiated. Time points F through J were collected over the 5 d fecal and urine collection period.

Trace mineral source had no impact on beta-diversity within the rumen ($P = 0.21$) and feces ($P = 0.27$).

Relative Differential Abundance: Finally, a comparison of differential relative abundance of fecal and rumen samples from steer fed a high concentrate finishing feedlot diet were established using ANCOM. The ASVs *Moraxellaceae Psychrobacter* (w = 525), *Prevotellaceae*

(w = 500), *Planococcaceae* (w = 488), and *Ruminococcaceae* (w = 480) were determined to be more abundant in the rumen environment regardless of the trace mineral source.

ANCOM analysis was performed to determine if taxa differed between trace mineral source and collection time point. The ANCOM analysis did not indicate any measurable differential abundance of taxa between trace mineral source in rumen samples.

Protozoa

Tables 15, 16, and 17 describe the relative abundance of identified rumen protozoa in cattle receiving high forage, high concentrate, or dairy type diets, respectively. Approximately 60 % of the ruminal protozoa were composed of the genus *Entodinium* in steers fed a high forage diet. Steers receiving HTM had lesser *Diplodinium* ($P < 0.05$) and *Eudiplodinium* ($P < 0.02$) in HTM vs. STM supplemented steers (Table 15).

Table 15. Relative abundance of identified rumen protozoa in cattle receiving a high forage diet supplemented with hydroxy or sulfate copper, manganese, and zinc.

		Treatment			*P*<
Taxa level	Classification	STM[a]	HTM[b]	SEM	Trt
Genus	Entodinium	59.34	63.4	4.39	0.72
Genus	Isotricha	1.29	1.7	0.64	0.57
Genus	Dasytricha	6.44	6.6	2.94	0.91
Genus	Ostracodinium	2.81	3.77	0.62	0.27
Genus	Diplodinium	0.92	0.97	0.1	0.67
Genus	Diploplastron	5.37	6.02	1.27	
Genus	Eudiplodinium	11.38	9.07	1.01	0.05
Genus	Epidinium	12.47	8.47	0.95	0.02
Genus	Metadinium	0	0	---	---
Genus	Ophryoscolex	0	0	---	---
Genus	Polyplastron	0	0	---	---

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

Steers receiving a high concentrate diet supplemented with HTM had lesser ruminal *Diplodinium* ($P < 0.05$) and *Eudiplodinium* ($P < 0.02$) when compared to STM supplemented steers (Table 16). The genus *Entodinium* tended ($P < 0.09$) to be greater and the genera *Dasytricha* ($P < 0.05$) and *Polyplastron* ($P < 0.04$) were lesser in HTM vs STM supplemented steers.

Table 16. Relative abundance of identified rumen protozoa in cattle receiving a high concentrate diet supplemented with hydroxy or sulfate copper, manganese, and zinc.

		Treatment			*P*<
Taxa level	Classification	STM[a]	HTM[b]	SEM	Trt
Genus	Entodinium	92.0	95.2	2.194	0.09
Genus	Isotricha	1.23	0.98	0.399	0.95
Genus	Dasytricha	2.69	0.7	0.408	0.05
Genus	Ostracodinium	0.12	0.21	0.076	0.98
Genus	Diplodinium	0.98	1.85	0.083	0.03
Genus	Diploplastron	0.13	0	---	---
Genus	Eudiplodinium	0	0	---	---
Genus	Epidinium	0	0	---	---
Genus	Metadinium	0	0	---	---
Genus	Ophryoscolex	0.65	0	---	---
Genus	Polyplastron	1.21	0.57	0.24	0.04

[a]Sulfate trace minerals: 18 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/kg DM from $MnSO_4$; 90 mg Zn/kg DM from $ZnSO_4$.
[b]Hydroxy trace minerals: 18 mg Cu/kg DM from CuOHCl; 40 mg Mn/kg DM from MnOHCl; 90 mg Zn/kg DM from ZnOHCl.

Steers receiving a dairy diet supplemented with CTM and HTM had lesser ($P < 0.04$) ruminal *Dasytricha* when compared to STM supplemented steers (Table 17). Steers supplemented with CTM and HTM had similar *Dasytricha.* All other identified protozoa were similar across treatments.

Table 17. Relative abundance of identified rumen protozoa in cattle receiving a dairy diet supplemented with sulfate, complexed, or hydroxy copper, manganese, and zinc.

		Treatment				P <
Taxa level	Classification	STM[a]	CTM[b]	HTM[c]	SEM	Trt
Genus	Entodinium	80.60	80.19	80.74	2.74	0.81
Genus	Isotricha	1.15	1.42	1.30	0.43	0.74
Genus	Dasytricha	2.27	1.72	1.10	0.74	0.04
Genus	Ostracodinium	1.37	1.21	1.10	0.29	0.64
Genus	Diplodinium	0.92	0.81	0.85	0.08	0.38
Genus	Diploplastron	2.42	2.46	2.64	0.53	0.86
Genus	Eudiplodinium	5.89	5.71	5.59	0.42	0.78
Genus	Epidinium	4.66	5.87	5.94	0.40	0.62
Genus	Metadinium	---	---	---	---	---
Genus	Ophryoscolex	0.25	0.14	0.23	0.01	0.54
Genus	Polyplastron	0.46	0.46	0.50	0.10	0.76

[a]Sulfate trace minerals: 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$

[b]10 mg Cu/kg DM from Cu lysine; 40 mg Mn/kg DM from Mn methionine; 60 mg Zn/kg DM from Zn methionine.

[c]Hydroxy trace minerals: 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl.

DISCUSSION

Trace mineral were supplemented to exceed (approximately 2×) for Cu, Zn, and Mn requirements (NASEM 2016 for Study 1 and 3, and NRC, 2001 for Study 2). The degree of TM solubility plays a crucial role in determining the concentration of metallic minerals that come into contact with rumen microbes, thereby influencing their interactions (Genther and Hansen, 2015). For instance, Cu, Zn, and Mn sourced from HTM exhibits lower solubility in the rumen (Caldera et al., 2019; Guimaraes et al., 2021; Guimaraes et al., 2022) but comparable solubility to sulfates in the acidic abomasum (Genther and Hansen, 2015), or high concentrate diet, pH 5.3 such as in Chapter 3. It's worth noting that even a minute concentration of 1.5 mg/L Cu in solution is known to be toxic to cellulolytic bacteria (Hubbert et al., 1958). The selective solubility and different

binding strength in various gut compartments is one way in which HTM diverge from STM. Additionally, the sulfate component itself is a factor to consider. When a diet contains Cu, Zn, and Mn sulfates to meet the requirements of feedlot steers, of 18 mg/kg Cu, 90 mg/kg Zn, and 60 mg/kg Mn, these TM supplements introduce approximately 72 mg/kg of sulfur by themselves, that wouldn't be present in an equivalent HTM. However, in comparison to the recommended dietary sulfur content of 0.2% of DM (2,000 mg/kg; NASEM, 2021), this extra sulfur amounts to just a 2.7% increase in supply. As such, it is unlikely that this slight increment in sulfur plays a significant role in driving differential responses between TM sources.

Zinc plays a vital role in bacterial metabolism and contributes to enhancing the stability of the bacterial cell wall when it forms bonds with the outer bacterial membrane (Durand and Kawashima, 1980). This TM holds particular significance for bacteria like *Campylobacter jejuni*, a commensal microorganism commonly found in chickens. Notably, the presence of the Zn transporter ZnuA is indispensable for this bacterium as it is incapable of replication or successful colonization in the gastrointestinal tract when Zn is deficient (Gielda and DiRita, 2012; Frirdich et al., 2012). Furthermore, it is important to mention that extremely high Zn supplementation, reaching levels as high as 3,100 mg/kg in the form of Zn oxide administered to weanling pigs increased intestinal IgA levels while simultaneously reducing the size of mesenteric lymph nodes. This phenomenon might be attributed to alterations in cytokine profiles within the gut-associated lymphatic tissue and the neutralization of bacteria (Broom et al., 2006). This finding is in agreement with recent human research, which explains the direct impacts the microbiome in the gastrointestinal tract can have on diseases such as Parkinson’s and Alzheimer’s, a phenomenon called the brain-gut-microbiome axis (Martin et al., 2018; Barrio et al., 2022).

Copper has the potential to exert toxicity on rumen bacteria, as indicated in prior research (Durand and Kawashima, 1980). On dairy farms, the use of Cu sulfate footbaths is a common practice for the treatment of hoof diseases. This practice obviously uses higher concentrations of Cu STM compared to how much a feedlot diet would have, but still a good indication that certain TM within specific sources can have a negative impact on microorganisms.

In the present study, feeding Cu, Zn, and Mn as STM or HTM did not have an impact on specific genera of bacteria, however some impact may have occurred on protozoa. *Dasytricha* is a genus of ciliated protozoa that belongs to the order *Vestibuliferida*, which is within the class *Litostomatea*. *Dasytricha* protozoa are characterized by their elongated and slipper-shape, commonly found in the rumen. They play a role in the digestion of cellulose and other fibrous plant materials, helping break down these complex substances into simpler compounds through fermentation, making them more digestible for the host, simultaneously benefiting from the nutrients available in the rumen and provide the host with enhanced digestion of plant material (Soares et al., 2019). These protozoa also play a very important role related to nitrogen recycling, primarily through engulfing and digesting other microbes, including bacteria and fungi. Through the digestion process, release of amino acids and peptides occurs, which will be further utilized by another microorganism or bypass the rumen and end up being absorbed in the small intestine (Soares et al., 2019). In summary, *Dasytricha* protozoa in the rumen play a vital role in breaking down complex plant materials, fermenting them into VFAs, recycling nitrogen, and providing a source of microbial protein and energy for the host ruminant animal. Their activities contribute to the overall efficiency of digestion in ruminants. These protozoa were found to be in lesser proportion in HTM fed steers when compared to STM for Study 2 (dairy diet) and 3 (feedlot diet). Further shotgun analysis along with metabolomics analysis would be necessary in order to make

further conclusions, even though taxonomic differences were observed, the results cannot be extrapolated to a biological level. However, another class of protozoa, *Diplodinium* was found to be in a greater proportion for HTM steers compared to STM. This class of protozoa plays a similar role in fiber digestion and nitrogen recycling in the rumen. Interestingly, similar proportions of these two protozoa were found in the study testing the high concentrated diet (Study 2). Further investigation is warranted to learn how specific trace mineral sources have on the determined class of protozoa, and if any competitive correlation exists between those 2 classes.

CONCLUSIONS

Overall, these findings shed light on the intricate microbial dynamics within the rumen of cattle fed various diets supplemented with diverse trace mineral sources. The observed shifts in microbial diversity and relative abundance underscore the complexity of rumen microbiota and highlight the need for further research to elucidate the functional implications of these microbial variations in ruminant nutrition and health.

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