DISSERTATION

# THE EFFECT OF TRACE MINERAL SOURCE ON SOLUBILITY, RUMEN FERMENTATION CHARACTERISTICS, AND TRACE MINERAL CONCENTRATION IN PROTOZOA AND BACTERIA OF STEERS CONSUMING A LACTATION DAIRY TYPE DIET

Submitted by

Huey Yi Loh

Department of Animal Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2024

Doctoral Committee:

Advisor: Terry Engle

Mahesh Narayanan Nair
Stephen Coleman
Camille Torres-Henderson

ABSTRACT

THE EFFECT OF TRACE MINERAL SOURCE ON SOLUBILITY, RUMEN FERMENTATION CHARACTERISTICS, AND TRACE MINERAL CONCENTRATION IN PROTOZOA AND BACTERIA OF STEERS CONSUMING A LACTATION DAIRY TYPE DIET

A series of experiments were conducted to investigate the influence of trace mineral (TM) source on TM solubility, rumen fermentation characteristics, and trace mineral concentration in protozoa and bacteria of steer consuming a lactation dairy-type diet. Experiments 3 and 4 were classified as experiments 1 and 2 in chapter 3 for publication purposes in academic journal.

In the first experiment, hydroxychloride TM (HTM) and sulfate TM (STM) sources of Cu, Mn, and Zn (n = 4/element/source; N = 24) were incubated separately in water for 24 h. Initial pH was measured after adding the TM to the solution, then the tubes were incubated at 39°C with agitation. After a 24-h incubation, samples were filtered to obtain the filtrate for TM analysis, and final pH readings were taken. Initial pH of each solution was greater ($P < 0.03$) for HTM compared to STM for all elements. Final pH tended to be greater for Cu ($P = 0.09$) and Zn ($P = 0.07$) from HTM compared to STM. Water solubility of Cu, Mn, and Zn from STM was greater ($P < 0.01$) than HTM sources. These data indicate that TM source influences pH and solubility of Cu, Mn, and Zn in water.

In Experiment 2, eight steers fitted with rumen cannula were blocked by body weight and randomly assigned to treatments consisting of 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from

either STM or HTM sources (n = 4/treatment). Steers were individually fed a cracked corn-corn silage-based diet. Treatments were top-dressed daily. Rumen contents were collected at 0, 2, and 4 h post-feeding on d 1 and 14. On d 15, strained ruminal fluid (SRF) and particle-associated microorganisms (PAO) were obtained. Digesta from HTM-supplemented steers has a lesser ($P < 0.01$) Mn concentration than STM-supplemented steers on d 14 of the trial. Steers supplemented with STM had a greater ($P = 0.0016$) soluble Cu concentration in the rumen on d 14 than those fed HTM. Zinc was more tightly bound ($P = 0.01$) to the digesta in HTM-supplemented steers compared to STM on d 14. The data suggest that dietary TM source can affect rumen soluble Cu concentrations and binding strength of Zn to solid digesta.

In Experiment 3, three cannulated steers were adapted to a diet formulated to meet the nutrient requirements for lactating dairy cows. Strained RF was obtained by straining rumen content through 2 layers of cheesecloth. Half of the remaining digesta was washed with McDougall's buffer and filtered through 2 layers of cheesecloth to obtain PAO. Both SRF and PAO were filtered again through 8 layers of cheesecloth. Strained RF was mixed with either McDougall's buffer (SRF) or PAO (SRF+PAO) at a ratio of 1:2 or 1:4 and incubated at 39°C for 12 h using the ground basal diet as the substrate. Digestibility of DM was greater ($P < 0.05$) in digestion tubes containing SRF and SRF+PAO at a 1:2 ratio.

In Experiment 4, eight steers fitted with ruminal cannula were blocked by body weight and assigned to one of two treatment groups. Treatments consisted of 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either 1) sulfate (STM) or 2) hydroxychloride (HTM) sources. Steers were housed in individual pens and fed the same diet as described in experiment 1. Dietary TM treatments were mixed with dried distillers grains and mixed in the diet by hand, immediately after basal diet delivery. Dietary treatments were fed for 14 d. On day 15, SRF+PAO was

collected from each steer (STM-RF and HTM-RF) and used in a series of in vitro crossover experiments. In vitro substrates (S) used were the ground diets consumed by the animals on each treatment (STM-S and HTM-S). Incubations containing HTM-S had greater ($P < 0.01$) total VFA concentration and propionic acid molar proportions, but lesser ($P < 0.01$) acetic acid molar proportions than STM-S. Rumen fluid from steers supplemented with HTM had a greater ($P < 0.03$) total VFA than STM-RF at 24h post incubation. After 12 h post incubation, the molar proportion of propionic acid in HTM-RF was lesser ($P = 0.04$) than STM-RF. After simulated abomasal digestion, soluble Mn concentration in HTM-S was greater ($P < 0.01$) than STM-S. These data indicate that the source of trace minerals can influence in vitro rumen fermentation characteristics and Mn solubility under simulated abomasal conditions.

# ACKNOWLEDGEMENTS

Firstly, I would like to express my deepest gratitude to my advisor, Dr. Terry Engle, for his unwavering support, guidance, and encouragement throughout my Ph.D. journey. Your advice, insightful feedback, and guidance have been invaluable for the completion of this dissertation. In addition, your personality, kindness, and mentorship have shaped me into a better scientist, researcher, and person. I am incredibly fortunate to have had the opportunity to work under your mentorship and I am forever indebted to you.

I would also like to thank my committee members, Dr. Mahesh Narayanan Nair, Dr. Stephen Coleman, and Dr. Camille Torres-Henderson for their time, effort, and valuable suggestions. Your expertise and advice have been critical for the completion of this journey, and I genuinely appreciate you being my committee member.

Furthermore, I would like to thank other faculty and friends at Colorado State University who have been essential to my graduate journey: Dr. Caitlin Cadaret, Dr. Lily Edwards-Callaway, Dr. Gina Georgnaras, Dr. Shawn Archibeque, Dr. Catie Cramer, Dr. Pedro Carvalho, Dr. Octavio Guimaraes, Dr. Meghan Thorndyke, Paxton Sullivan, Briana Tangredi, Tyler Thomas, Lauren Dean, Harrison Hallmark, Allison Vautier, Colton Smith, Claire Okoren, Alison Kuderka, Julia Baroni Alves, and countless others. I appreciate all the help, support, and goofiness you have provided me. I am grateful for the stimulating discussions, the shared moments of both frustration and triumph, and the sense of community we have built together. This journey wouldn't be as fun and interesting without all of you.

Lastly, to my family, words cannot express how grateful I am for your unwavering love, encouragement, and belief in me. To my parents, Boon Kuang Loh and Pat Moy Sum, and my

siblings, Zheng Ken, Wan Yi, Zheng Yao, Zheng Hao, Chin Yi, I would not be where I am without you, thank you for always being my biggest supporters and for instilling in me the values of hard work and perseverance. I am deeply appreciative of the sacrifices you have made and the strength you have given me. A heartfelt thank you to my friends, Emmanuel Meneses, Ellie Teo, Hailey Teo, Joyce Jiang, Iverson Chong, Zi Fan Lim, Chee Jun Wong, Chong Han Lee, Stephanie Chu, and Nicole Lee, who are always by my side, your support, words of encouragement, and countless moments of laughter have been a source of strength and comfort throughout this journey.

Lastly, I would like to thank everyone who, in one way or another, has contributed to the completion of this dissertation. Your support, whether big or small, has not gone unnoticed, and I am sincerely grateful.

TABLE OF CONTENTS

# CHAPTER 1-REVIEW OF LITERATURE

## Introduction

Copper (Cu), manganese (Mn), and zinc (Zn) are essential trace minerals (TM) that are required in small amounts by cattle as they are involved in various biochemical reactions in the biological system. This chapter consists of a review of literature on Cu, Mn, and Zn focusing on their physical and chemical properties, how they are digested, absorbed, and excreted in the biological systems, their physiological functions, deficiency and toxicity signs, and the techniques that are commonly utilized to investigate the mechanism(s) of nutrient absorption.

## Physical and Chemical Properties

### *Copper*

Copper, the 29$^{th}$ element on the periodic table, has an atomic number of 29 and a standard atomic weight of 63.546u. Copper has a melting point of 1085°C, so it generally exists as a metallic form with the appearance of orange-red or brown color in nature. Due to its high thermal and electrical conductivity and ductility, Cu is commonly used in house appliances and wires. Copper is a transition metal with an electron arrangement of $[Ar]3d^{10}4s^{1}$. As the d-shells of this element are filled, most of the interactions with other elements will occur through the s-electron. Copper has a standard reduction potential of +0.34V and a standard oxidation potential of -0.34V, which led to high redox potential characteristics. While Cu is commonly used in wire and electronic components, it's also one of the essential TM required by most of the living things on earth to survive. Its bio-essentiality is due to its interchangeable oxidation states. Copper can exist in various oxidation forms in animals' bodies, which are cuprous ($Cu^{+}$), cupric ($Cu^{2+}$), and

possibly $Cu^{3+}$ (Bremner and Beattie, 1995). The reversible changes between oxidized ($Cu^{2+}$) and reduced ($Cu^{+}$) states of Cu allow it to play an essential role in biology (Messerschmidt, 2010; Festa and Thiele, 2011). As Cu ionizes in the body, different oxidative states display different levels of affinity toward the chemical compounds in the biological system (Festa and Thiele, 2011). $Cu^{+}$ has a higher affinity towards compounds that contain thiol (-SH) and thioether (R-S-R') functional groups, such as cysteine or methionine amino acids (Festa and Thiele, 2011). On the other hand, $Cu^{2+}$ will most likely interact with oxygen or imidazole nitrogen groups, which can be found in glutamate and histidine (Festa and Thiele, 2011). However, Cu needs to be regulated in the body as excessive Cu has a high potency to generate oxygen radicals, which can lead to oxidative stress in cells (Bremner and Beattie, 1995).

***Manganese***

Manganese is the $25^{th}$ element on the periodic table with an atomic weight of 54.94u, and an electron configuration of [Ar] $3d^5 4s^2$. Manganese is a silvery-gray metal that was widely used to produce iron, steel, and aluminum alloys in the metal industry. Due to its high melting point, Mn also exists in solid form at room temperature. Common oxidation states of Mn are +2, +3, +4, and +7, with +2 being the most stable form, but oxidation states of -3 to +7 have been observed (Kolb, 1988; Cotton et al., 1999). Mn (II) is soluble in water and will form a pink aqueous solution with a +2 charge. Complexes such as Mn sulfate ($MnSO_4$), Mn chloride ($MnCl_2$), and Mn carbonate ($MnCO_3$) are the common Mn (II) compounds. Mn (III) is formed as an intermediate during the oxidation of Mn (II) to Mn (IV) by bacteria through multicopper oxidase in the ocean (Webb et al., 2005). Mn (III) is unstable in a normal environment, so it will rapidly oxidize and reduce Mn (II) and Mn (IV) as it is both a strong oxidizing agent and reducing agent (Johnson, 2006). The oxidation of Mn (II) to Mn (IV) is thermodynamically favorable and may also be able to prevent the generation of reactive oxygen species (ROS) or

free radicals in the cells (Daly, 2009; Geszvain et al., 2012). Mn (IV) can rapidly oxidize organic material to carbon sources for microorganisms and can serve as the terminal electron acceptors in the cells (Daly, 2009; Geszvain et al., 2012).

### *Zinc*

Zinc (Zn) is the 30$^{th}$ element on the periodic table with an atomic weight of 65.38u. As a transition element, Zn has a relatively low melting point which is 419.5°C, but it exists as a silvery-gray solid at room temperature. Zinc has an electron configuration of $[Ar]3d^{10}4s^2$ and a high reduction potential, which enables it to be used as a reducing agent in various scenarios (Goodwin, 2012). The redox potential of Zn is -0.724V, so it will not participate in biological redox reactions as it will generally exist in $Zn^{2+}$ oxidation state (Reyes, 1996). The most stable oxidation state of Zn ion is +2 under standard temperature and pressure, but oxidation states of -2, +1, and +2 have been observed. By having a completed d subshell and 2 additional s electrons, the chemically bound form of Zn is always the +2 oxidative state (Riordan, 1976). Zn (II) forms many binary compounds except the element from the noble gases group due to its reactivity with negatively charged groups (Vallee, 1959). Common inorganic compounds of Zn (II) include but are not limited to Zn nitrate, Zn sulfate, Zn phosphate, and Zn molybdate (Perry, 2016). Zinc usually binds to sulfur or oxygen in nature, and cadmium was found in Zn ores (Vallee, 1959).

## Digestion, Absorption, and Excretion

### *Copper*

Copper, due to its redox nature, needs to be absorbed, transported, and regulated systematically through Cu transporter and Cu chaperones (López-Alonso and Miranda, 2020). Most of the research on Cu metabolism in mammals has been conducted in humans and mice. Therefore, our understanding of ruminant Cu metabolism is very limited. Nevitt et al. (2012)

reviewed Cu transport and metabolism in eukaryotes from yeast to mammals and concluded that across species, yeast, and mammals shared similarities in basic Cu transport and cellular metabolism. In ruminants, however, Cu absorption can be complicated or altered due to the diet they consume and their difference in the digestive system. Ruminants developed a 4-chambers digestive system, which has a rumen containing a large population of microorganisms to help break down fiber materials for energy. Due to the presence of rumen microbes, a reducing environment was created as microbes donate electrons when they digest fiber materials. The reducing environment in the rumen can promote the formation of insoluble compounds (e.g. thiomolybdates), which leads to a decrease in copper (or other trace elements) availability for absorption. The reduction in bioavailability of copper (or other nutrients) can lead to modifications in the Cu transporters in the small intestine to uptake copper more efficiently. In 1965, van Campen and Mitchell discovered that in rats, Cu absorption started in the stomach, but the greatest amount of Cu was absorbed in the small intestine. The possible reason for Cu digestion and absorption occurring in the stomach is due to the low pH environment in the stomach. Copper is liberated from the food/feed particles, hence free Cu ions can diffuse through the stomach wall and be absorbed into the blood (Tompsett, 1940; Wapnir, 1998; Underwood and Suttle, 1999). Copper deficiency was reported after gastric bypass surgery in humans, which suggests that the gastric stomach may play a role in Cu digestion and/or absorption (Myint et al., 2018). However, another Cu absorption study carried out by Fields et al. (1986) failed to observe Cu absorption in rat stomachs that were being fed Cu-containing diets and water. While Cu absorption in the stomach remains debatable, various studies in rats and humans confirm that most of the Cu absorption occurs in the small intestine, specifically in the duodenum and ileum (van Campen and Mitchell, 1965; Stern et al., 2007).

Copper ions in the gastrointestinal (GI) tract will generally be digested and released from food particles as $Cu^{2+}$. Cupric ions will then need to be reduced into their most active form, Cu (I), by Cu (II) reductase (cytochrome B reductase 1; Cybrd1), to be transported through copper transporter 1 (CTR1) into enterocyte cytosol (Hassett and Kosman, 1995; Clarkson et al., 2020). Approximately 70% of Cu will be absorbed through a specific membrane protein, CTR1, and the remaining Cu will be absorbed through nonspecific divalent metal transporter 1 (DMT-1) as $Cu^{2+}$ (Rolfs and Hediger, 1999; López-Alonso and Miranda, 2020). Copper transport 1 protein is the first Cu-transport gene that was discovered in yeast to facilitate Cu transport (Dancis et al., 1994). Copper transport 1 gene was further discovered in HeLa cells in humans and hepatocytes and germ cells in bovids (Zhou and Gitschier, 1997; Anchordoquy et al., 2017). Gunshin et al. (1997) discovered that DMT-1 (also called DCT-1 and nRAMP2) is a metal transporter that can bind to various divalent cations, which are iron ($Fe^{2+}$), $Zn^{2+}$, $Mn^{2+}$, cobalt ($Co^{2+}$), cadmium ($Cd^{2+}$), nickel ($Ni^{2+}$), and lead ($Pb^{2+}$). Transportation of Cu from the lumen of the intestine into enterocytes through DMT-1 could be limited as Cu needs to compete with other divalent cations (López-Alonso and Miranda, 2020).

After entering the enterocyte, Cu chaperone proteins will bind to free Cu and transport it to other proteins or enzymes. Examples of cytosolic Cu chaperones that are present in the enterocyte are superoxide dismutase (SOD), Cu chaperones for SOD (CCS), antioxidant 1 (ATOX1), cyclo-oxygenase 17 (COX17), glutathione (GSH), amino acids, adenosine triphosphate (ATP), and Cu metallochaperones (Suttle, 2012; Maryon et al., 2013; Clarkson et al., 2020). Glutathione is important in handling free Cu at the entry step of Cu absorption, ATOX1 is a cytosolic protein that moves Cu ions throughout the cytosol, CCS incorporates

cytosolic Cu and deliver it to SOD, while COX17 will deliver Cu to cytochrome c oxidase (CCO) located in mitochondria. (Suttle, 2012; Maryon et al., 2013; Clarkson et al., 2020).

Copper in the enterocytes will first be utilized by the enterocyte to generate enzymes and for ion balance. Once Cu concentration in enterocytes reaches above the cell's requirement, it will need to be secreted out from the cells via the Golgi body, also known as the secretory pathway (Nevitt et al., 2012; Suttle, 2012; Clarkson et al., 2020). Once Cu enters the secretory pathway, Cu will first bind to metallothionein (MT), a cysteine-rich protein located in lysosome that is responsible for detoxifying and storing free cellular Cu (Cousins, 1985; Nevitt et al., 2012; Clarkson et al., 2020). Metallothionein is the common detoxifying agent in the cells that is also known to bind a variety of metals such as cadmium, Cu, mercury, and Zn, to convert harmful radical ions into non-harmful materials (Fogel and Welch, 1982; Cousins, 1985). Metallothionein synthesis is induced by high metal ion concentration in the cell, including but not limited to Zn and Cu ions, to protect the cell from free metal ion-induced-cellular damage (Bremner and Davies, 1975; Cousins, 1985). Metallothionein also plays an important role in regulating Cu and Zn metabolism during fetal and neonatal development (Klein et al., 1991). Once the Cu concentration in metallothionein reaches saturation, excess Cu in the Golgi apparatus will be secreted out from the enterocytes through ATP7A (ATPase Cu Transporting Alpha; Chelly et al., 1993; Das et al., 1995; Nevitt et al., 2012; Suttle, 2012; Clarkson et al., 2020). ATP7A is a P-type cation-transporting ATPase and in humans, this gene is transcribed in all cell types except in the liver (Vulpe et al., 1993). However, a recent study by Tillquist et al. (2022) discovered that ATP7A was transcribed in the bovine liver and hepatocyte cell culture, but protein analysis was not conducted to confirm the presence of this protein in the liver. When the Cu ion passes through interstitial fluid, the oxidation of $Cu^{+}$ into $Cu^{2+}$ will occur spontaneously without the

requirement of oxidase due to the oxygen tension in the interstitial fluid and will bind to transporters in the interstitial fluids to enter the circulation (Gulec and Collins, 2014). Upon exiting enterocytes, Cu will bind to albumin, transcuprein, amino acids, or ceruloplasmin present in circulation, then transported to the liver for metabolism, distribution, and storage (Bligh et al., 1992; Linder and Hazegh-Azam, 1996; Stern et al., 2007).

Albumin is synthesized by the liver during the phase of adequate nutrition and possibly stimulated by thyroid hormone and cortisone (Rothschild et al., 1977). Serum albumin has various functions, such as maintaining osmotic pressure and transportation of a variety of substances or nutrients including Cu (Rothschild et al., 1988). Most of the Cu will be transported from the intestine to the liver through blood circulation by binding to the N-terminal histidine residue in albumin protein (Bremner and Beattie, 1995). Although Zn is known to have an antagonist effect on Cu, it will not affect the transportation of Cu in animals' bodies, as Zn (II) and Cu (II) will bind to different sites of the albumin (Masuoka and Saltman, 1994). Since the binding of one mineral will not affect the binding of another mineral, a higher concentration of Zn uptake will not affect the rate of Cu transport in the blood. The concentration of metallothionein increases in species that lack Cu binding sites in the albumin, which suggests that metallothionein might also play a role in Cu transportation (Bremner and Beattie, 1995). However, Cu is not a significant inducer in signaling metallothionein synthesis when compared to Zn and cadmium (Peterson and Mercer, 1988). This indicates that although metallothionein is responsible for Cu transport, only a small portion of Cu will be transported by metallothionein to the liver (Peterson and Mercer, 1988; Bremner and Beattie, 1995). The concentration of albumin is higher than transcuprein in blood plasma, but transcuprein has a higher affinity for Cu and is responsible for one-third of Cu transport from the enterocytes in the circulation (Weiss and

Linder, 1985; Linder and Hazegh-Azam, 1996; Clarkson et al., 2020). As certain amino acids are charged under physiological pH, they can bind to free Cu ions and assist in transport to the liver to prevent the production of ROS in the bloodstream.

After Cu is absorbed into the circulatory system, Cu will be transported to the liver. The liver (hepatocytes) is the major site of Cu storage in mammals. Copper is transported to the liver by serum Cu transporters mentioned above and reduced to $Cu^{+}$ form by NADH oxidase upon arrival (Crisponi et al., 2010). CTR1 is responsible for transporting $Cu^{+}$ into hepatocytes, and the mechanism of Cu regulation and transportation within hepatocytes is similar to enterocytes (Zhou and Gitschier, 1997; Kim et al., 2009; Clarkson et al., 2020). After Cu enters the liver, it will be regulated to either be distributed throughout the body, excreted out through bile, or retained in the liver by parenchymal cells (Luza and Speisky, 1996). Hepatocytes have potentially three storage pools, with varying levels of availability. Most of the Cu will be in the first pool, which is the readily available extractable Cu pool, the remaining Cu will be in a less readily available pool in soluble form, and some Cu will potentially be stored in the third pool, a potential subset of the second pool, which is a non-extratable, insoluble Cu pool (McArdle et al., 1989; Bingham et al., 1997; Clarkson et al., 2020). When Cu enters the hepatocytes, it will first move to the first pool, then move to the second or third pools after approximately 2h (Bingham et al., 1997). GSH, COX17, CCS, ATOX1, SOD, and Golgi apparatus are the Cu chaperones in the hepatocytes to transport Cu within hepatocyte, the minor difference between hepatocyte and enterocyte is instead of ATP7A protein in Golgi apparatus, ATP7B (~76% homologous to ATP7A) is responsible for Cu efflux in the hepatocytes (Bull et al., 1993; Tanzi et al., 1993; Huster et al., 2003; Clarkson et al., 2020). The majority of ATP7B is expressed in the liver, but the expression in the kidney, placenta, lower levels in the brain, heart, and lungs were observed

(Bull et al., 1993; Tanzi et al., 1993; La Fontaine and Mercer, 2007). Copper chaperones for SOD traffic Cu to cytosolic Zn-Cu-SOD in the cytosol, while COX17 chaperones Cu into mitochondria for metabolic function (Prohaska, 2008; Hepburn et al., 2009; Suttle, 2012; Clarkson et al., 2020). ATOX1 will bind to Cu and deliver it to the Golgi apparatus that contains ATP7B, which will then incorporate Cu into ceruloplasmin for distribution to other tissues (Klomp et al., 1997; de Bie et al., 2005; Suttle, 2012; Clarkson et al., 2020).

From the liver, Cu will be transported to other body tissues by ceruloplasmin, a member of multicopper oxidase enzyme family, that is synthesized by liver that plays a role in the distribution of Cu (Bremner and Beattie, 1995; Hellman and Gitlin, 2002). More than 75% of total plasma Cu present in blood was found to be bound to ceruloplasmin in ruminants, which also suggests that ceruloplasmin plays a big role in Cu transport and distribution (Luza and Speisky, 1996). Ceruloplasmin can be a good indicator to measure Cu deficiency in cattle and sheep, but it does not represent Cu concentration in the liver (Blakley and Hamilton, 1985). Although ceruloplasmin serves as an effective transport protein for Cu, it is not a good Cu donor for hepatocytes (Luza and Speisky, 1996). With that, when ceruloplasmin-Cu from peripheral tissues return to the liver, the whole molecule of ceruloplasmin will be absorbed into the liver for destruction and excretion through bile (Irie and Tavassoli, 1986; Tavassoli et al., 1986; Harris, 2000; Clarkson et al., 2020).

In order to incorporate Cu into tissues for physiological function, Cu-containing ceruloplasmin will interact with the cell surface to allow Cu separation from ceruloplasmin (Ramos et al., 2016). In humans, CTR1 was found across various tissues in human body, suggesting that CTR1 plays an essential role in incorporating Cu into the cell (Eisses and Kaplan, 2005). However, Cu will generally be in a $Cu^{2+}$ state when it is being transported by

ceruloplasmin throughout the circulation (Ramos et al., 2016). As CTR1 protein only allows the passing of $Cu^{1+}$, reductase must be present on the cell surface to assist with Cu entry into the cells through CTR1 (Ramos et al., 2016). In addition, simple diffusion following concentration gradients might also be the additional method for Cu to enter the cell. This hypothesis is proposed by Tong and McArdle (1995), as they observed that micromolar concentration of Cu ions can diffuse through high-affinity phospholipid bilayer membrane into the cell to be utilized for metabolic function. They concluded that the passive carrier system is utilized by cytotrophoblast cells for the uptake of Cu (Tong and McArdle, 1995). When Cu is being transported to cells for utilization, cellular organelles will play a role in Cu storage and regulation. Lysosomes were found to be responsible for the storage of Cu in liver tissue and the excretion of Cu when the Cu concentration is high (Humbert et al., 1982; Gross et al., 1989; Polishchuk and Polishchuk, 2016). As lysosome organelle is found in most of the cells in the body, this might suggest that Cu might be regulated by lysosome organelle in generic cells.

One of the mechanisms of biliary excretion of Cu is the sequestering of Cu by lysosome in the liver, and the lysosome exocytosis their contents into biliary canaliculi to be excreted out of the body (Gross et al., 1989). ATP7B plays an essential role in Cu excretion as it incorporates Cu in the cytosol into the trans-Golgi networks (TGN), which can then either bind to ceruloplasmin for distribution or bile for elimination (Payne and Gitlin, 1998; Wijmenga and Klomp, 2004). ATP7B is localized in TGN under normal physiological conditions but also in different cellular compartments depending on the Cu status of the cells (Hung et al., 1997; Wijmenga and Klomp, 2004). When the Cu concentration in the hepatocytes increases, ATP7B is rapidly translocated from TGN to vesicular compartments throughout the cells but will rapidly return to TGN once the Cu level drops (Hung et al., 1997; Roelofsen et al., 2000). In

hepatocytes, excretion of excessive Cu through bile required Cu metabolism MURR1 domain (COMMD1), which level was regulated by X-linked inhibitor of apoptosis protein (XIAP), binding to the N-terminal region of ATP7B (Prohaska, 2008; Collins et al., 2010; Clarkson et al., 2020). In humans, studies found about 80% of Cu is excreted through bile, whereas approximately only 3% of the daily intake of Cu is excreted through the kidney (Winge and Mehra, 1990; Harris, 1991). However, in sheep, Cu excretion through urine and bile is very low, which can explain the high proneness of Cu toxicity in sheep (Søli and Rambæk, 1978). The main route of Cu elimination in sheep is hypothesized to be through the excretion of Cu into the GI tract from liver (Søli and Rambæk, 1978).

***Zinc***

Zinc absorption and transport is a time, temperature, and pH-sensitive process, and the uptake of Zn is performed by both saturable and nonsaturable processes (Reyes, 1996). Zinc transportation may require energy, or it can diffuse across the membrane as a free ion (Reyes, 1996). In humans, Zn absorption rate is the highest in the jejunum, followed by the duodenum, and the lowest in the ileum (Lee et al., 1989; Maares and Haase, 2020). In calves, most of the Zn was absorbed in the upper small intestine and reabsorbed in the lower section of the small intestine (Hiers et al., 1968). Some Zn absorption occurs in the rumen, reticulum, and via saliva (Weston and Kastelic, 1967; Hiers et al., 1968). Zinc absorption increases significantly by glucose addition, and higher Zn concentration will increase glucose absorption, but reduce sodium and water absorption in the small intestine (Lee et al., 1989). Zinc absorption in the small intestine is mainly mediated by Zrt-, Irt-like protein 4 (ZIP4; also known as SLC39A4), which carries ionic Zn from the lumen into enterocytes (Wang et al., 2002). Zinc transporter 5 (ZnT-5) variant B (SLC30A5B) is located at the apical membrane of enterocytes, and it can transport Zn from the lumen into enterocyte or vice versa (Cragg et al., 2002; Valentine et al., 2007). As ZnT-

5B is a bidirectional transporter, it might play a role in the regulatory mechanism of Zn homeostasis (Jackson et al., 2007; Maares and Haase, 2020). DMT-1 expression increases with iron deficiency, but it has higher specificities for Zn, Cd, Mn, and Cu than Fe (McMahon and Cousins, 1998a). DMT-1 can bind to Zn, but it is not the main transporter for Zn uptake in the small intestine, as Zn absorption was not affected by decreased DMT-1 expression (Tandy et al., 2000; Yamaji et al., 2001). Messenger RNA of DMT-1 is the highest in the enterocytes lining the crypts and lower villi of the duodenum, instead of the villi tips (McMahon and Cousins, 1998a).

Zinc within the cell will mainly be bound to proteins, stored in vesicles, or exist in free ionic Zn form (Maares and Haase, 2020). Free Zn (II) ions are important as they serve as the signaling ion intra- and intercellular communication, but excess amount of free Zn ions can be toxic to cells, so they need to be tightly regulated (Bozym et al., 2010; Maret, 2015). Free Zn in the cytosol will bind to small molecule ligands and be considered a biologically active form of the ion (Bozym et al., 2010; Maret, 2015). The second Zn transporter (ZnT-2) was identified in rat kidneys, and its function as the facilitator of Zn into endosomal/lysosomal compartments and prevent Zn toxicity in the cell (Palmiter et al., 1996a). ZnT5A and ZnT-7 are mainly located in the Golgi apparatus, and they transport Zn into this organelle, which can be essential for dietary Zn absorption and the functionality of Zn metalloenzymes (Kirschke and Huang, 2003; Suzuki et al., 2005; Huang et al., 2007; Wang and Zhou, 2010). Metallothionein, mainly MT-1 and MT-2, synthesis in hepatocytes and enterocytes were upregulated by Zn supplementation through injection, and diminished with dietary Zn restriction (Krebs, 2000). Metallothionein functions in limiting free Zn concentration by binding to it, and acting as a Zn pool (Davis et al., 1998). Metallothionein expression in enterocytes is elevated with higher cellular free Zn concentration via metal regulatory transcription factor 1 (MTF-1; Andrews, 2000). Higher MT concentration

will decrease luminal Zn absorption, which then lowers serum and body Zn levels (Coppen and Davies, 1987; Hoadley et al., 1988; Hinskens et al., 2000; Maares and Haase, 2020).

In order to reach the bloodstream, Zn ions need to be released from the basolateral side of enterocytes into the bloodstream. The uptake of dietary Zn into enterocytes is very rapid but the transportation of Zn into the bloodstream is reserved (Sahagian et al., 1967). ZnT-1 (SLC30A1) is the transporter that excretes Zn out of the cells (Palmiter and Findley, 1995). ZnT-1 is located at the basolateral surface of enterocytes, and it is highly expressed in the duodenum and jejunum, but not present in the ileum or colon of the intestine (McMahon and Cousins, 1998a). Zinc efflux capacity by ZnT-1 is sodium and ATP independent, and the action of this protein is at the level of plasma membrane (Palmiter and Findley, 1995; McMahon and Cousins, 1998a; McMahon and Cousins, 1998b). ZnT-1 can be found in enterocytes, hepatocytes, and renal tubular cells, and it is involved in Zn efflux, reabsorption, and regulation (Victery et al., 1981; McMahon and Cousins, 1998a). ZIP5 (SLC39A5) and ZIP14 (SLC39A14) are also localized at the basolateral side of enterocytes but their function is to import Zn from blood circulation into enterocytes (Wang et al., 2004; Guthrie et al., 2015; Maares and Haase, 2020).

Albumin acts as the major transporter for Zn in serum (60%), followed by α2-macroglobulin (30%), but 12 other transport proteins also bind to Zn (Scott and Bradwell, 1983). Zinc was localized mainly in skeletal muscle (~57%), followed by bone (~29%), skin (6%), and liver (5%), but the validity of these calculations is limited as these are the approximation from biopsies and sections of tissues (Jackson, 1989; Maares and Haase, 2020). Even though the Zn concentration in skeletal muscle and bone is the highest in the body, the turnover rate is slower, so it is not readily available for utilization (Brown et al., 2001). Serum Zn is rapidly exchangeable, so it is important for Zn distribution throughout the body (Brown et al., 2001).

Zinc excretion is an important part of Zn homeostasis to prevent Zn accumulation or toxicity. Zn excretion through urinal output is minimal under normal physiological conditions due to extensive tubular reabsorption (Victery et al., 1981; McMahon and Cousins, 1998a). In dogs, pancreatic secretion contains a large amount of Zn, but most of it will be reabsorbed along the small intestine (Montgomery et al., 1943; Miller, 1970). Most species excrete Zn through fecal output as their major Zn excretion pathway, with minimal excretion through urine (Miller, 1970). Other Zn transporters are involved in Zn homeostasis in the body. ZnT-3, first discovered in 1996, is fairly similar to ZnT-2, and its function is to facilitate the accumulation of Zn in the synaptic vesicles (Palmiter et al., 1996b). The expression of ZnT-3 is restricted to the brain and testis, which suggests the importance of Zn at these sites (McMahon and Cousins, 1998a). The function of ZnT-3 in the vesicular packaging of Zn can be important in spermatogenesis and insulin secretion (Silva and Williams, 2001). ZnT-4, which shares ~60% similarities with ZnT-2 and ZnT-3, was discovered in mice, with a high abundance in mammary epithelia and brain (Huang and Gitschier, 1997). ZnT-4 aids in Zn efflux, compartmentalization, and transport, and it also distributes Zn into the maternal milk supply (Lee et al., 1992; McMahon and Cousins, 1998a).

***Manganese***

Manganese from the diet will be solubilized in the stomach and released into the duodenum to be absorbed (Bai et al., 2008). Manganese is mainly absorbed in the small intestine through enterocytes in duodenum and jejunum in Holstein calves (Miller et al., 1972). In broilers, the main site of Mn absorption is the ileum (Ji et al., 2006). Divalent metal transporter 1 (DMT-1) has a relatively high affinity to Mn, which suggests that it is the major transmembrane protein for Mn transportation from the lumen into the enterocytes (Canonne-Hergaux et al., 1999; Roth et al., 2002). The transportation of Mn by DMT-1 can be categorized into transferrin-

dependent and transferrin-independent pathways, and transferrin-independent pathway is preferred on the apical side of the enterocytes (Mackenzie and Garrick, 2005; Roth et al., 2013). ZIP8 (SLC39A8) and ZIP14 (SLC39A14), members of the solute carrier 39 (SLC39), also have the ability to transport Mn into other cell types (Jenkitkasemwong et al., 2012). ZIP8 is most abundant in lung, testis, and kidney tissues while ZIP14 is mainly present in the liver and pancreas, and both of them are present in the duodenum to aid in intestinal Mn absorption (Chua and Morgan, 1997; Jenkitkasemwong et al., 2012; Wang et al., 2012). ZIP14 was identified to be the primary transporter for Mn uptake at basolateral membrane of CaCo-2 cells, which is a human intestinal epithelial cell line (Scheiber et al., 2019). Mn was shown to compete with $Ca^{2+}$ to travel through calcium channels into cells, but DMT-1 is still the major pathway for Mn absorption (Anderson, 1983; Roth et al., 2002). In broilers, Mn absorption is likely to be a carrier-mediated process in duodenum and jejunum but was an unregulated diffusion process in the ileum (Bai et al., 2008). Another study that supports the previous finding is the high concentration of DMT-1 in the duodenum and the concentration decreases in the subsequent segments of the small intestine (Collins, 2006; Roth et al., 2013; Chen et al., 2015).

Manganese will be transferred to the basolateral surface once they enter the enterocytes, but the mechanism is not fully defined (Roth et al., 2013; Scheiber et al., 2019). Four transporters were identified to play a role in the exportation of Mn, they are ATPase 13A2 (ATP13A2 or PARK9), solute carrier family 30 member 10 (SLC30A10), ferroportin (FPN1 or SLC40A1), and secretory pathway $Ca^{2+}$- ATPase1 (SPCA1; Yin et al., 2010; Tan et al., 2011; Madejczyk and Ballatori, 2012; Leyva-Illades et al., 2014; Chen et al., 2015). ATP13A2 is a vacuolar/lysosomal transmembrane protein that transports Mn from cytosol to the lumen of lysosomes and exports Mn out of the cells (Tan et al., 2011; Chen et al., 2015). SLC30A10 is one

of the members of the SLC30 family, which is the cation diffusion facilitator ion transporters (Huang and Tepaamorndech, 2013). SLC30A10 is located on the cell surface and acts as the Mn efflux transporter that reduces cellular Mn levels to prevent Mn toxicity (Leyva-Illades et al., 2014). Ferroportin is a transmembrane protein that was widely expressed in duodenum, jejunum, ileum, and endothelial cells in the central nervous system (CNS; Wu et al., 2004). Ferroportin is suggested to play a role in the excretion of Mn, and it's regulated by hepcidin produced by the liver (Yin et al., 2010; Madejczyk and Ballatori, 2012). Transportation via Ferroporin is regulated by pH and transmembrane ion or electrical gradients (Madejczyk and Ballatori, 2012). SPCA1 is a transport protein localized in the Golgi apparatus that mediates Mn efflux (Madejczyk and Ballatori, 2012).

After absorption of Mn into the body, Mn transportation within the plasma is mainly bound to gamma-globulin and albumin as $Mn^{2+}$ form, and a small portion (20%) of $Mn^{3+}$ will bind to transferrin (Tf), which is the iron-carrying protein (Aisen et al., 1969; Aschner and Aschner, 2005; Studer et al., 2022). Manganese will be delivered to organs that have high mitochondria concentration such as liver, pancreas, and pituitary, and the main storage site for Mn is the liver (Doyle and Spaulding, 1978; Studer et al., 2022). Mn will first be transported to the liver and absorbed into the liver through the SLC39A14 on the basolateral membrane of hepatocytes (Liu et al., 2021). Within the liver, DMT-1 is distributed evenly across hepatocytes, which might also aid in Mn transportation into the liver (Trinder et al., 2000).

Tissues such as brain, retina, and dark skin, which have high energy demand and high pigment content, have the highest Mn concentration (Aschner and Aschner, 2005). Bone, liver, pancreas, and kidney also have high Mn concentrations (Aschner and Aschner, 2005). Mn in the circulation is rapidly cleared by the liver, pancreas, kidney, and intestine (Liu et al., 2021).

Excess Mn in the liver will be excreted into bile through SLC30A10 at the apical membrane (Tuschl et al., 2012; Liu et al., 2021). In calves, most of the Mn ingested will be excreted through biliary excretion (Abrams et al., 1977). Manganese will be removed from the blood in the liver and conjugate with bile, then released into the intestine to be excreted via feces, but some Mn will be reabsorbed (Schroeder et al., 1966; Aschner and Aschner, 2005; Liu et al., 2021). Mn will be reabsorbed from bile back into the liver by SLC39A8 at the apical membrane of hepatocytes to prevent Mn deficiency (Lin et al., 2017; Liu et al., 2021). In neonatal animals, biliary excretion is poorly developed, so over supplement of Mn during neonatal period can lead to various neurological problems (Cotzias et al., 1976). In general, excess Mn excretion is mainly through bile, but Mn excretion through pancreatic secretion and sloughed intestinal cells was observed in various species with different routes of Mn intake (Burnett et al., 1952; Bertinchamps et al., 1966; Papavasiliou et al., 1966; Schroeder et al., 1966; Abrams et al., 1977; Davis et al., 1993; Aschner and Aschner, 2005). Only 1% of the Mn will be excreted out from the body by bile in mice (Malecki et al., 1996).

High dietary Mn intake can reduce Mn absorption in the GI tract, enhance liver metabolism, and increase biliary excretion of Mn (Britton and Cotzias, 1966; Abrams et al., 1977; Davis et al., 1993; Malecki et al., 1996). Manganese absorption rate can be different by sex and age. In humans, women absorb more Mn than men and have higher plasma ferritin concentrations, which suggests that the difference in Mn absorption and metabolism may be related to iron status (Finley et al., 1994). The absorption and retention of Mn are higher during neonatal period as compared to adult (Keen et al., 1986; Zlotkin et al., 1995). Inorganic forms of minerals (sulfate, chlorides, carbonates, oxides) can form insoluble complexes with digesta and then be excreted out from the body, which reduces the availability of minerals that can be

absorbed and utilized (Acda and Chae, 2001; Studer et al., 2022). Organic minerals on the other hand can utilize peptides and amino acids to promote the absorption of minerals in the intestine (Ashmead, 1993; Acda and Chae, 2001; Studer et al., 2022). Mn and Fe have antagonistic effects on each other as they were both mediated into the enterocytes through DMT-1 (Gunshin et al., 1997; Malecki et al., 1999). Iron deficiency can promote Mn absorption as iron can inhibit 75% of Mn absorption in the small intestine (Roth et al., 2002). The addition of calcium into human milk decreases Mn absorption as well (Davidsson et al., 1991).

**Physiological Functions of Copper, Manganese, and Zinc**

Copper, Mn, and Zn are classified as essential trace elements (TM) for most living things, which include humans, animals, plants, and microorganisms (NASEM, 2016). In the early 20$^{th}$ century, Cu, Mn, Zn, nickel, and cobalt were suggested to be important to soil, plants, and animals for their vital functions, such as optimal growth and overall health (McHargue, 1925). Other than growth, TM are essential for reproductive features such as embryonic and fetal development in livestock as TM serves as the cofactor of many enzymatic and metabolic pathways in the body (Hostetler et al., 2003). Levels of Zn, Cu, and Mn were several-fold greater in fetus, which indicates that these minerals may be important for fetus development, growth, and survival (Hostetler et al., 2003).

Copper in living systems was first discovered in the 19$^{th}$ century through the mineral analysis of the ash of plants, animal blood, and foods (Meissner, 1816; Harless, 1847; Linder and Goode, 1991). In general, Cu is required for growth, neurological function, muscle function, iron metabolism, enzyme function, and more. Copper is important for the myelination of the central nervous system, but the mechanism is still unknown (Wintrobe et al., 1953; Klevay, 2013). Plasma Cu is elevated in early pregnancy and continues to increase until the third trimester, liver

Cu concentration in newborns is also higher than in adult humans, suggesting that Cu may be essential for fetal development (Fay et al., 1949). In humans, fetus hepatocytes store and regulate 6-8 times higher concentrations of Cu compared to adult hepatocytes (Luza and Speisky, 1996). The pattern of declining Cu storage capacity along with aging is also observed in other species such as cattle and pigs (Cunningham, 1931). In cattle specifically, liver Cu concentration of the fetus, newly born calf, and adult cattle are approximately 260mg/kg, 470mg/kg, and 77mg/kg respectively (Cunningham, 1931). The high Cu concentration in fetuses and newborns correlates to the low Cu centration in the milk of those species, so a high concentration of Cu storage during birth is important for their growth and development (Luza and Speisky, 1996). During adult period, Cu concentration will be reduced to an optimum level where excess Cu will be readily excreted out from the body by fully developed homeostasis systems (Luza and Speisky, 1996).

Copper ion was also required for the functionality of enzymes including but not limited to cytochrome oxidase (COX), lysyl oxidase, Cu-Zn SOD-1, extracellular Cu-Zn SOD (SOD3), ferroxidase enzymes, hephestin, and ceruloplasmin (Linder and Hazegh-Azam, 1996; Myint et al., 2018; Espinosa and Stein, 2021). It is also important for iron transportation, as Cu-metalloprotein, hephaestin, is responsible for oxidizing ferrous ions into ferric ions at the duodenal mucosa to be absorbed (Myint et al., 2018). After absorption, iron will be released by ferroportin into the blood, which then can bind to transferrin for hemoglobin synthesis (Myint et al., 2018). Ceruloplasmin is another enzyme that aids in iron transfer in blood plasma and protects cells against oxidative damage by serving as an oxygen-radicals scavenging agent (Linder and Goode, 1991; Linder and Hazegh-Azam, 1996). Cytochrome oxidase is important for the survival of prokaryotes and eukaryotes, where it catalyzes $4H^+ + 4\ e^- + O_2 \rightleftharpoons 2H_2O$ and

conserves the energy released from this exergonic reaction for pH gradient conservation (Capaldi, 1990). Cytochrome oxidase is also the terminal enzyme in the electron transport chain (ETC), where it utilizes oxygen for energy production in mitochondria (Little et al., 2018).

Copper supplementation to steers can improve dry matter intake, feed efficiency, reduce back fat, and improve longissimus muscle areas (Ward and Spears, 1997). Finishing steers that received higher Cu concentration also show a lower total serum cholesterol concentration and longissimus muscle cholesterol concentration, while having higher unsaturated fatty acid composition (Engle and Spears, 2000). Pharmacological levels of Cu were supplemented to the young nursery pigs to increase average daily gain and improve waste odor quality (Barber et al., 1955; Bunch et al., 1965; Armstrong et al., 2000). A reduction in lipid percentage in the longissimus muscle was observed in pigs that were fed a pharmacological level of Cu (Armstrong et al., 2001). Optimum Cu supplementation can improve neutral detergent fiber (NDF) digestibility and growth in cashmere goats (Zhang et al., 2007). Copper is also important for the keratinization of wool and oxygen-carrying capacity in sheep (Marston and Lee, 1948).

Zinc is an essential TM that is required for development, growth, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein synthesis, and enzyme functions (Riordan, 1976; Prasad, 1995). The essentiality of Zn in the biological system was first shown in the mid-19$^{th}$ century when it was found to be important for the growth of eukaryotes, *Aspergillus niger* (Raulin, 1869). Zinc was then recognized to be important for normal growth, development, and survival in animals by using mice and rats models (Bertrand and Bhattacherjee, 1935; Stirn et al., 1935). There are approximately 2800 proteins that require Zn for their regulatory, structural, and catalytic functions (Andreini et al., 2006). Malformation in the cell within the CNS indicates that DNA synthesis, especially in the head region, is suppressed during Zn deficiency (Eckhert and

Hurley, 1977). Zinc is also important for the stability of both RNA and DNA molecules by binding to the nucleoside bases (Shin and Eichhorn, 1968; Shin, 1973). Zinc is important for proper growth because of its essential role in the cell cycle, where all the phases in this cycle, G1→S, S→G2, and G2→mitosis, require the presence of Zn (Riordan, 1976). Zinc is also essential for leukocyte function as leukemic cells contain 10% less Zn than normal leukocytes (Vallee, 1959). Zinc concentration in human fetal liver is 5-fold higher than in neonatal liver, which serves as an indication of the essentiality of Zn in fetal development (Klein et al., 1991). High Zn concentration in the ocular tissues was observed in sheep, cattle, dogs, foxes, martens, and humans (Underwood, 1977; Karcioglu, 1982). It was hypothesized that Zn is essential to maintain the structure and activity of ocular metalloenzymes in the eyes or stabilize the light-activated proteins in the eyes (Karcioglu, 1982; Shuster et al., 1996). Another hypothesis suggests that it may be due to the role of Zn in maintaining the structure and double membrane integrity of ocular tissues that were comprised of stacks of double membranes (Grahn et al., 2001).

Zinc (II), due to its intrinsic acid properties, is required for many enzymes to function. It's known to be a co-enzyme for the functionality of more than 300 metalloenzymes (Dardenne, 2002). The first Zn enzyme that was identified was carbonic anhydrase, where the activity of this enzyme increases as the Zn concentration increases (Keilin and Mann, 1939). Carbonic Anhydrase aids in $H_2CO_3 \rightleftharpoons CO_2 + H_2O$, which is important for the carbon dioxide transport in the blood (Keilin and Mann, 1939). After the discovery of the first Zn protein, carboxypeptidase in bovine pancreatic cells was identified to be another Zn metalloprotein (Vallee and Neurath, 1954). More research was conducted, and Zn enzymes were classified into several types, which are DNA and RNA polymerases, alkaline phosphatases, peptidases, carbonic anhydrases, and

alcohol dehydrogenases (Kimura, 1993). The essentiality of Zn in DNA and RNA synthesis and cell division was discovered in the 1970s (Vallee, 1977). Zinc is required for the function of DNA polymerase, thymidine kinase, and DNA-dependent RNA polymerase, which are the enzymes involved in nucleic acid synthesis (Dardenne, 2002). Protein synthesis requires Zn finger DNA binding protein, which is also a Zn metalloprotein (Dardenne, 2002).

Pharmacological levels of Zn supplementation (3000 mg/kg DM) to weanling piglets were reported to reduce diarrhea and increase daily gain and feed intake (Hahn and Baker, 1993; Poulsen, 1995). The improvement in performances may be correlated to the stability of intestinal microflora and coliform diversity or the reduced incidence of *E.coli* diarrhea observed when Zn was supplemented for piglets (Mores et al., 1998; Katouli et al., 1999). Supplementation of Zn to ram lambs and rats improves their feed intake, weight gain, testicular growth, and sperm production (Underwood and Somers, 1969; Gilabert et al., 1996). Zinc supplementation improves average daily gain (ADG) in growing steer and improves quality grade, yield grade, marbling, and back fat in the carcass (Spears and Kegley, 2002; Genther-Schroeder et al., 2016). Supplementation of Zn in steroid implanted steer improves the effectiveness of the steroid, where a higher growth response stimulated by implant was observed in Zn-supplemented steers (Messersmith et al., 2021). Zinc supplementation is effective in treating itching tail root eczema in cattle (Haaranen, 1962).

Manganese's irreplaceable function for reproduction in rats was first discovered in 1931 (Orent and Mccollum, 1931). Manganese is important for growth, skeletal development, reproduction, testicular development, ovulation, and litter health (Orent and Mccollum, 1931; Wilson, 1952; Plumlee et al., 1956; Eckhert and Hurley, 1977; Hostetler et al., 2003). Manganese is a redox-active metal, which allows it to be utilized as a cofactor of various metalloenzymes

within the body and it's required for enzyme activation (Culotta et al., 2005). Some examples of Mn metalloenzymes are oxidases, dehydrogenases, kinases, decarboxylases, sugar transferases, DNA polymerases, and RNA polymerases (Sigel, 2000). Manganoproteins, which are the group of enzymes that require Mn for activation, include glutamine synthetase, arginase, pyruvate decarboxylase, serine/threonine phosphatase, and Mn superoxide dismutase (MnSOD; Fridovich, 1995; Christianson, 1997). As an important co-factor for the functionality of many enzymes, Mn directly or indirectly regulates various processes in the body such as skeletal system development, cholesterol synthesis, energy metabolism, function of the nervous and immune systems, reproductive hormone function, and many more (Santamaria, 2008; Wang et al., 2018). Mn is essential for immune system against pathogens, it's important for cytokine, type I interferon (IFN), and improve sensitivity in the recognition of foreign DNA (Wang et al., 2018).

Mn supplementations in swine are higher than the requirement value during gestation, lactation, and nursery through finishing diets, however, the benefit of this practice is still unknown (Flohr, 2016). One hypothesis is that Mn can influence insulin release and gluconeogenesis, which affect carbohydrate metabolism, blood glucose level, and insulin level (Hurley et al., 1984). Supplementation of Mn to cattle and sheep diets improved pregnancy rates (Wilson, 1966; Egan, 1972). Manganese may also play a role in the metabolic and functional properties of the corpus luteum, uterine horn, and caruncles, which can influence progesterone secretion in ewes (Hidiroglou and Shearer, 1976). Mn supplementation can also increase insulin secretion (increase glucose tolerance) and reduce oxidative stress and NADPH oxidase in the liver of patients or experimental rats with type II diabetes (Lee et al., 2013; Burlet and Jain, 2017). Physiological supplementation of Mn was shown to provide antiviral defenses against pathogens (Zhang et al., 2023).

Copper, Zn, and Mn are important cofactors of superoxide dismutase, which helps to protect the body from harmful radical metabolites (Marklund et al., 1982). Superoxide such as ROS interacts with metal-bound SOD and will be decomposed into non-toxic substances, which helps to prevent or reduce oxidative stress (Pajarillo et al., 2021). Mitigation of oxidative stress by SOD has been observed in bacteria, eukaryotes, mammals, and plants (Farr et al., 1986; Alscher et al., 2002; Pajarillo et al., 2021). The evolution of SOD and their metallic center might be related to the oxygen level and the order of the transition metals appearing in the biosphere (Bannister et al., 1991). The first SOD is theorized to contain iron ($Fe^{2+}$) at its active site, and with the increase in oxygen level in the atmosphere, Mn (III) replaced iron at the active site (Bannister et al., 1991). Copper (II)-containing SOD was introduced after the full plenish of oxygen in the atmosphere, where the electronic structure of SOD is completely different from Mn- and Fe-SOD, then Zn (II) was incorporated into the Cu-SOD later to increase stability to the protein structure (Bannister et al., 1991). Manganese-SOD (Mn-SOD or SOD2) is normally found in the mitochondria, peroxisomes, and respiratory cells while Cu-Zn containing SOD (CuZn-SOD) can be found in the cytosol of the cells (SOD1) or the extracellular space (SOD3; Alscher et al., 2002; Sah et al., 2020). The function of SOD isoforms in cells are different, and one cannot replace the other (Kim et al., 2005). The chemical mechanism of Mn-SOD in the disposal of ROS is theorized to be the redox cycling between trivalent and divalent states (Christianson, 1997). The cycling processes occur between $Mn^{3+} + O_2^- \rightarrow Mn^{2+} + O_2$ and $Mn^{2+} + O_2^- \rightarrow Mn^{3+} + H_2O_2$, so the net reaction is $2\ O_2^- \rightarrow O_2 + H_2O_2$ (Christianson, 1997). The mechanism of MnSOD in dealing with superoxide is not fully understood due to its short half-life and high reactivity, but MnSOD exhibits product inhibition properties (Azadmanesh and Borgstahl, 2018). With that property, MnSOD can bind to superoxide, $O_2^{\bullet -}$–$Mn^{2+}$– $H_2O$, and

oxidize it to form oxidized peroxide-bound Mn, $O_2^{2-}$–$Mn^{3+}$– ($OH^-$), and does not let the peroxide disassociate, which can keep superoxide inactive (Azadmanesh and Borgstahl, 2018). The active enzyme will then further oxidize the complex into $HO^{2-}$–$Mn^{3+}$–($OH^-$), where the disassociation of the hydroperoxyl group is favorable, and the disassociated hydroperoxyl can bind to the free carboxylate group from amino acid (Azadmanesh and Borgstahl, 2018). CuZnSOD has the ability to donate an electron to reduce the oxygen radicals ($O_2^{\bullet-}$), which then can react with a proton to form hydrogen peroxide ($H_2O_2$; Alscher et al., 2002; Rahman and Biswas, 2006). Elevated levels of $H_2O_2$ will inactivate CuZnSOD, then it will be reduced and converted to water and oxygen by glutathione peroxidase (GPx) and catalase (Rahman and Biswas, 2006). GPx and catalase will be conserved by SOD through the reduction of $O_2^{\bullet-}$ to $H_2O_2$, and through his feedback loop, cells will be protected against radical molecules (Rahman and Biswas, 2006).

## Trace Mineral Deficiency and Toxicity

### *Copper*

Copper is used as a growth promoter in pig diets with a concentration of 10 times higher than the requirement, which suggests that Cu is well-regulated in pigs (Barber et al., 1955; Bunch et al., 1965; Armstrong et al., 2000). Copper imbalance is more likely to occur in ruminants, approximately 40% of dairy cattle in Britain have been reported to have excessive liver Cu levels (Kendall et al., 2015). A report in 1993 showed that Cu poisoning resulted in 14% of deaths in Holstein cows, where they were showing signs of acute anorexia, weakness, mental dullness, chocolate-colored blood, and more (Bradley, 1993). Supplementation of Cu to cattle has always been a challenge as the range between Cu deficiency and toxicity is very small. The incidents of cattle being over-supplemented with Cu to prevent Cu deficiency have increased

worldwide in the past few years, especially in dairy cattle (López-Alonso and Miranda, 2020). In addition to the narrow margin between deficiency and toxicity, antagonists of Cu in the diet also increase the difficulty of Cu supplementation.

Ruminants, unlike monogastric mammals such as humans and mice, are prone to Cu deficiency due to the presence of ruminal microbial populations that reduce the metal ions. Reduced metal ions can interact with other minerals or compounds in the rumen, which makes Cu supplementation in cattle challenging. Potential antagonistic interaction between Cu, molybdenum, and sulfur in ruminants was first proposed by Dick in 1954 where both dietary molybdenum and sulfur limit Cu accumulation in the liver, which was further confirmed by other research (Hogan et al., 1968; Kline et al., 1971). Copper bioavailability will primarily be affected by the three-way interaction between Cu, molybdenum, and sulfur (Dick, 1954; Hogan et al., 1968; Kline et al., 1971; Suttle, 1977; Suttle, 1991). The presence of microorganisms in the rumen rapidly reduces sulfate (from consumption of water, soil, or diet) into sulfide, which can then react with molybdate to form thiomolybdates (TMD; Dick et al., 1975; Gooneratne et al., 1989; Gould and Kendall, 2011). Thiomolybdates will then combine with Cu to form insoluble Cu-TMD in the rumen, which will limit the absorption of Cu (Dick et al., 1975). Among all the TMD formation, tri- ($MoS_3$/$TMD_3$) and tetrathiomolybdate ($MoS_4$/$TMD_4$) will be more likely to bind to Cu and reduce Cu bioavailability in the rumen, hence reducing Cu absorption (Suttle and Field, 1983; Suttle, 1991). The formation of monothiomolybdate (MoS/TMD) and dithiomolybdate ($MoOS_2$/$TMD_2$) in the rumen, due to a low sulfur concentration environment, will not impair Cu absorption (Suttle, 1975; Suttle, 1991). Specifically, consumption of molybdenum, with high concentrations of dietary sulfur and low rumen pH will lead to the formation of $MoS_4$/ $TMD_4$ in the rumen (Suttle, 1991). These findings suggest that Cu absorption

will only decrease with the presence of sulfur and molybdenum in a higher concentration in the rumen environment (Suttle and Field, 1983; Suttle, 1991). With low level/absence of Cu in the rumen, di-, tri-, and tetrathiomolybdate can be absorbed through the rumen and small intestine into circulation, and transported throughout the body (Kelleher et al., 1983; Price et al., 1987; Gooneratne et al., 1989; Gould and Kendall, 2011). Thiomolybdates in the bloodstream can then bind to free Cu and form insoluble Cu-TMD, hence leading to the reduction in Cu uptake by cells or binding to Cu-containing enzymes for their functionality (Dick et al., 1975; Gould and Kendall, 2011). In addition, thiomolybdate will also increase the secretion of Cu in sheep through bile and kidney, and remove Cu from Cu metalloenzymes (Gooneratne et al., 1985; Kumaratilake and Howell, 1989; Suttle, 1991).

Besides molybdenum and sulfur, Zn also has an antagonist effect on Cu absorption. Bremner et al. (1976) utilized high Zn supplementation concentration to suppress the development of Cu toxicity and decrease the amount of liver damage in sheep, which suggests that excessive Zn concentration will inhibit or decrease Cu absorption and metabolism in the small intestine and placental. The reduction in Cu toxicity may be due to the depressed absorption of Cu or redistribution of Cu in the liver with increased metallothionein protein production due to a high level of Zn (van Campen and Scaife, 1967; Bremner and Marshall, 1974). Both decreases in absorption and retention of Cu might also be one of the causes that can lead to Cu deficiency in animals. Other minerals such as tin, calcium, phosphorus, cadmium, and molybdenum, also have a minor antagonist effect on Cu absorption (Lönnerdal, 1996; Park et al., 2002). High dietary iron greatly reduces Cu concentration in the liver and plasma (Bremner et al., 1987; Phillippo et al., 1987). One potential mechanism of iron reduces Cu bioavailability is by reacting with sulfide and Cu in the rumen and forming Cu complexes that are not absorbable

by the animals, but no evidence is available to support this hypothesis (Standish and Ammerman, 1971; Gould and Kendall, 2011; López-Alonso and Miranda, 2020).

Some amino acids such as histidine and cysteine will interact with Cu and form less soluble/absorbable Cu complexes, hence, decreasing Cu absorption in the small intestine (Lönnerdal, 1996). The bioavailability and solubility of Cu might also be influenced by its chemical and physical form when ingested (Johnson et al., 1998). For example, Cu acetate and Cu sulfate are more soluble and have higher bioavailability in the gut compared to cupric oxide (Johnson et al., 1998). As the liver is the major site for both Cu homeostasis and storage, diet can greatly influence hepatic Cu concentration. Hepatic Cu concentration was elevated when fed a high Cu diet and declined when Cu intake was low (Underwood, 1971; Cousins, 1985). Ruminant diets that are low in Cu and sometimes high in molybdenum and sulfur might also be one of the major reasons that ruminants are prone to Cu deficiency (Suttle, 2012).

Ruminants, including cattle, are susceptible to Cu toxicity because they have a strong capacity to store Cu, especially when they are given a low roughage diet (Suttle, 2012). Some early clinical symptoms of Cu toxicity are sunk eyes, methemoglobin in the vein, animal becoming dull, refusing to eat, but consuming excessive amounts of water (Todd, 1969). Chronic Cu toxicity can lead to enteritis, scouring, severe abdominal pain, and eventually lead to death if not treated properly (Todd, 1969). Excess Cu concentration in the cell can be dangerous due to its high redox potential, as Cu oxidizes/reduces between $Cu^{+}$ and $Cu^{2+}$ states, hydroxyl radicals that can damage proteins, nucleic acids, and lipids might be generated (Festa and Thiele, 2011). In addition, Cu has the ability to displace other metals from their binding site in metalloproteins, which can disrupt protein structure and/or inhibit its functionality and activity (Festa and Thiele, 2011). As liver is the main storage site for Cu, excessive Cu will most likely accumulate there

and inhibit essential enzymes, hence leading to liver malfunction and necrosis (Gummow et al., 1991). The injured liver will then release excess Cu and oxidative substances into the bloodstream, which can lead to vascular damage, lysis of erythrocytes, kidney failure, and necrosis in tubular cells (Gummow et al., 1991).

Cattle that suffer from mild Cu deficiency normally show symptoms of losing healthy coat conditions and poor growth (Ferguson et al., 1943; Suttle, 1991). Those who suffer from severe Cu deficiency display stiff gait, infertility, diarrhea, and emaciation (Ferguson et al., 1943; Phillippo et al., 1982; Suttle, 1991). Merino sheep that were deficient in Cu experience anemia, where the oxygen-carrying capacities decrease significantly (Marston and Lee, 1948). Demyelination in the corpus callosum, internal capsules, white matter of occipital and frontal lobes, and motor pathways in the spinal cord were observed with Cu deficient in lambs (Wintrobe et al., 1953). This is because the brain is rich in Cu-transport protein and needs to utilize a great amount of oxygen for its function, so the enzyme is not able to function properly to prevent oxidative stress with the depletion of Cu (Davies et al., 2013; Klevay, 2013). Depletion of Cu also leads to a decrease in the rate of wool production and quality(Marston and Lee, 1948). Copper deficiency and also lead to enzootic neonatal ataxia (swayback), hypochromic microcytic anemia, hypoferremia, hypocupremia, leukopenia, and bone marrow normoblastic hyperplasia (Bennetts and Chapman, 1937; Wintrobe et al., 1953). To determine Cu toxicity or deficiency in the animal, hepatic Cu analysis through liver biopsy can be a relatively good way to measure the Cu status in the liver (Pfeiffer, 2007).

One of the significant genetic diseases that influence Cu absorption in the small intestine is Menkes disease (MD). Menkes disease is an X-linked recessive disease that leads to multiple mutations in the ATP7A protein-encoding gene (Chelly et al., 1993). As Menkes disease leads to

mutation(s) in the X-linked ATP7A gene, males are more susceptible to Menkes disease as they only have a single X chromosome. Menkes disease can exhibit various severity, where Cu absorption in the small intestine is suppressed due to the mutation in ATP7A (Horn and Tümer, 2002). Mutation in ATP7A will then lead to lower serum Cu and ceruloplasmin concentration, which indicates Cu deficiency (Das et al., 1995). As Menkes disease is a genetic disease, Menkes patients normally suffer from severe Cu deficiency since they are born, which leads to neurological degeneration, hypothermia, and hypopigmentation in early life (Horn and Tümer, 2002; Kaler et al., 2020). Without proper development and growth due to severe Cu deficiency, MD patients are observed with spontaneous bone fractures, and cephalohematomas at birth and early death within 3 years of life due to infection, vascular diseases, or neurological degeneration (Tümer and Møller, 2010). The definite proof of MD is the detection of genetic defects in ATP7A protein (Tümer and Møller, 2010). The general concept of MD treatment is to provide Cu to meet the Cu requirement for proper functions of enzymes and tissues in the body as the patient is not able to absorb Cu from their diet (Tümer and Møller, 2010). Copper histidine has been proven to be the most effective administration form to provide extra Cu to the patient (Kreuder et al., 1993). There is not a lot of discovery of Menkes disease in cattle, but this does not mean that Menkes disease will not happen in cattle. Calves that are born with Menkes disease will normally die at birth or within a few months of age, so no adult cattle that are diagnosed with Cu-deficient are caused by Menkes disease.

Another genetic disease that will influence Cu storage and regulation is Wilson's disease (WD). Wilson disease is an autosomal recessive genetic disease that led to mutation(s) in the ATP7B gene, which is a protein that is essential for Cu regulation in the liver (Ala et al., 2007). Excessive Cu accumulation in the liver due to the mutated ATP7B gene will lead to an increased

amount of free Cu in the body, which is exceptionally dangerous as free Cu can lead to irreversible cellular damage (Pfeiffer, 2007). In addition, once the accumulation of Cu exceeds liver storage capacity, free Cu will leak out from the liver and be deposited in other organs such as kidney, hence, an increase in urinary Cu excretion can be a good indication of Wilson's disease (Pfeiffer, 2007). Although some of the Cu will be excreted out through urine, it is insufficient to accommodate the excess Cu level in the body (Pfeiffer, 2007). Over time, mutations in ATP7B will eventually lead to excessive Cu accumulation in organs such as the liver, brain, cornea, and kidneys (Zudenigo and Relja, 1990). The first clinical sign of WD disease is typically hepatic dysfunction, where the excess Cu induces permanent damage to the liver, which decreases liver's overall function (Pfeiffer, 2007). Dysfunctional liver may lead to multiple metabolic diseases such as osteoporosis, hemolytic anemia, renal tubular dysfunction, hyperpigmentation of skin, cardiovascular dysfunction, and many more (Pfeiffer, 2007). Although WD is recessive, 10-20% of heterozygous WD patients were diagnosed with mild Cu toxicity, they do not show clinical symptoms, but they have a lower ceruloplasmin level (Brewer, 2001). For WD, genetic testing of ATP7B mutations will be the most accurate way to predict and diagnose WD (Ala et al., 2007; Pfeiffer, 2007). Liver transplant is the only available cure for WD, but there are other treatments available such as restriction on Cu intake or Zn or tetra-thiomolybdate administration as both compounds can reduce dietary Cu absorption in the small intestine (Pfeiffer, 2007). Wilson disease-like symptoms were discovered in cattle in 1995, where it is also characterized by similar metabolic manifestations such as liver fibrosis and spongy degeneration of the central nervous system (Wada et al., 1995).

***Zinc***

Zinc availability in the diet and absorption in the small intestine can be influenced by various factors. Zinc concentration in the diet, long-term Zn intake level, Zn status, and age can

influence Zn absorption rate (Sandström and Cederblad, 1980; Sandström et al., 1988; August et al., 1989). When the Zn content in the diet decreases, the percentage of Zn that will be absorbed increases (Miller et al., 1968; Miller, 1969). In humans, Zn absorption increases with a higher intake of protein (Sandström et al., 1980). Phytate, a stored form of phosphorus in plant material, will bind to Zn and decrease its availability in the gut, hence decreasing Zn absorption (O'dell, 1969; Lönnerdal et al., 1984). Fiber has little effect on Zn absorption, but because most of the fiber contains phytate, fiber was mistaken to reduce Zn absorption for a long time (Knudsen et al., 1996; Lönnerdal, 2000). Calcium and Cu are unlikely to cause an effect on Zn absorption, but toxic levels of cadmium can inhibit Zn absorption (Lönnerdal, 2000). The calcium content in phytate-containing diets may influence Zn absorption as calcium tends to form complexes with phytate, which then increase Zn availability in the small intestine (Lönnerdal et al., 1984; Lönnerdal, 2000). Iron has a competitive interaction with Zn, so higher iron can reduce or inhibit the absorption of Zn (Solomons and Jacob, 1981). Ligands/chelators, amino acids, and organic acids can be used to improve Zn bioavailability (Lönnerdal, 2000).

Zinc toxicity can lead to nausea, vomiting, epigastric pain, lethargy, and fatigue (Fosmire, 1990). High Zn intake can also lead to Cu deficiency, which then leads to anemia, neutropenia, impaired immune function, and adverse effects on the ratio of low-density-lipoprotein to high-density-lipoprotein (LDL/HDL) cholesterol (Fosmire, 1990). Cattle have a relatively high tolerance (~500 ppm) for Zn but will be susceptible to Zn deficiency if fed a lower than recommended amount (Miller, 1970).

Zinc deficiency might lead to a compromised immune system resulting in atrophy of thymus and disruption of lymphoid tissues (Skrajnowska and Bobrowska-Korczak, 2019). Zinc deficiency also leads to a decrease in interleukin 2 production by T lymphocytes, or

abnormalities in T-lymphocytes subpopulation (Prasad, 1995). Depletion in Zn can lead to hyperkeratosis and parakeratosis, where impaired vision, thickening and roughening of the skin, dry hair, alopecia, stiff gait, general dermatitis, swelling and reddening around the mouth, nostrils, hocks, and knees, undersized testicles, and inflamed mouth and nose are observed as the clinical signs of Zn deficiency in calves (Miller and Miller, 1960; Miller and Miller, 1962; Miller and Miller, 1963; Ott et al., 1965a). The homeostatic mechanism for Zn is effective in calves, as there is only moderate lower Zn in the liver, pancreas, and bone, and no change of Zn concentration in the muscle and brain during a severe Zn deficiency (Miller, 1970). Wound healing ability was suppressed, and parakeratosis was observed in calves with Zn deficiency (Miller et al., 1965a). Zinc deficiency also led to lower feed efficiency, reduced serum alkaline phosphatase, and lower blood hemoglobin in calves (Miller et al., 1965b). In sheep, the clinical signs of Zn deficiency include but are not limited to anorexia, reduced feed intake, weight gain, and feed efficiency, loose wool, scabby lesions of skin above the hoof and around the eyes, general dermatitis, and swollen hocks (Ott et al., 1964; Ott et al., 1965b).

A genetic disease termed acrodermatitis enteropathica (AE) is an inherited autosomal defect in dietary Zn absorption (VanWouwe, 1989). Patient with this disease has a defective Zn transporter gene, SLC39A4, which encodes Zn transporter ZIP4, that transports Zn across the duodenal mucosa (VanWouwe, 1989; Maverakis et al., 2007). The mutation in this gene leads to a reduction in Zn absorption in the gut (VanWouwe, 1989). Some clinical symptoms of this disease include but are not limited to dermatitis, alopecia, reduced growth rate, intermittent diarrhea, and can be lethal in severe cases (VanWouwe, 1989). Human milk, which has better Zn bioavailability, is commonly used as a nutritional treatment, but Zn supplementation was able to cure all the symptoms (Moynahan, 1974; VanWouwe, 1989).

***Manganese***

The exposure to high levels of Mn through oral, parenteral, or ambient air in humans, can lead to an elevation in tissues Mn concentration, or even Mn toxicity (Santamaria, 2008). Neurological damage at globus pallidus was observed in humans who were exposed to Mn, which led to a Parkinsonian-like disorder known as "Manganism" (Pal et al., 1999; Witholt et al., 2000; Culotta et al., 2005; Tuschl et al., 2008). Hereditary familial Mn-induced parkinsonism is due to the mutation in Mn export protein, SLC30A10, which leads to Mn accumulation in the liver, basal ganglia, and brain (Tuschl et al., 2008; Quadri et al., 2012; Chen et al., 2015). Other symptoms that arise due to the mutation of this protein are hypermanganesemia, dystonia, polycythemia, and chronic liver disease (Tuschl et al., 2008). Dopamine oxidation by Mn is the potential mechanism of neurotoxicity due to the accumulation of $Mn^{2+}$ in the basal ganglia, which is rich in dopamine (Sloot et al., 1996). Mn will inhibit complex I and ATPase complex in the electron transport chain, with $Mn^{3+}$ being the more effective inhibitor (Archibald and Tyree, 1987; Gavin et al., 1999). The predominant oxidative state is $Mn^{2+}$, but an excessive amount of Mn has a higher risk of producing $Mn^{3+}$ spontaneously, which can negatively impact the cell (Archibald and Tyree, 1987; HaMai et al., 2001).

Feeding high iron to animals can potentially lead to Mn deficiency, as high dietary iron downregulates DMT-1, which can reduce Mn absorption in the small intestine (Miret et al., 2003; Hansen et al., 2010). Mice that were deficient in Mn have a lower cytokine production and are more susceptible to diseases caused by DNA viruses (Wang et al., 2018). Manganese deficiency during early development can lead to impaired growth, skeletal abnormalities, irreversible ataxia, compromised reproductive function, abnormal glucose tolerance, and altered lipid and carbohydrate metabolism (Keen et al., 1999; Aschner and Aschner, 2005; Rodríguez-Rodríguez et al., 2011; Li and Yang, 2018). Impaired growth and lower feed intake were observed in chicks

that are Mn deficient (Bolze et al., 1985). Manganese-deficient cows exhibited neonatal deformities, reduced breaking strength and length of the humerus bone, and reduced serum alkaline phosphatase levels (Rojas et al., 1965). Manganese deficiency can also lead to lameness in pigs (Miller et al., 1940). Manganese deficiency in guinea pigs leads to reduced liter size, a higher percentage of dead young or premature delivery, and ataxic symptoms at birth (Everson et al., 1959).

**Trace mineral source for supplementation of cattle**

Trace minerals (TM) were supplemented to cattle to prevent TM deficiency, but over-supplementation of TM can lead to TM toxicity. Trace mineral supplementation in ruminants has been challenging due to the antagonistic relationship between elements, which can decrease the bioavailability of TM. Trace mineral antagonists, such as Fe, Mo, and S are commonly found in grazing environments at varying levels, which makes TM supplementation more complicated to satisfy animal's needs. Trace mineral was often supplemented to grazing cattle as TM-fortified, salt-based, or free-choice supplements. In a feedlot setting, TM was normally mixed into the diet and fed to cattle. In all scenarios, supplements can be formulated using a variety source of TM ingredients that have different chemical characteristics, which contribute to different solubility at different sections of the GI tract and eventually contribute to differences in bioavailability.

There are 2 main classifications of TM supplementation, which are inorganic TM (ITM) and organic TM (OTM; Arthington and Ranches, 2021). Inorganic TM is the most commonly used source due to its market availability and lower cost compared to organic TM. Although ITM is commonly used for ruminant supplementation formulation, its bioavailability and stability are lesser compared to other mineral sources. Inorganic TM are the complexes ionically bound to oxygen, chlorine, sulfur, or other non-carbon-based compounds. In general, sulfate and chloride

forms of TM are the most bioavailable among ITM, followed by carbonates, with oxides being the least bioavailable. Organic TM was introduced around the mid-1990s and was suggested to have a higher bioavailability compared to ITM. Seven different complexes were defined as OTM by the American Association of Feed Control Officials (1998), they are metal (specific amino acid) complexes, metal amino acid complexes, metal amino acid chelates, metal proteinates, metal polysaccharide complexes, metal propionates, and yeast derivative complexes. Hydroxychloride source of TM, specifically Cu, Mn, and Zn, is a newer source of ITM that became available to the feed industry recently. It has lower solubility in water and rumen (Spears et al, 2004; Loh et al. 2024), and solubilizes to a similar extent in the abomasum when compared to ITM, which is believed to have a higher bioavailability compared to ITM and traditional OTM. Other than the increase in bioavailability, other advantages of utilizing OTM are still unknown due to conflicting research data.

**Technologies for mRNA analysis**

The ability to measure or analyze nucleic acid in polynucleotide chain, which ultimately contains all the information for hereditary and biochemical functions of life, is essential for biological research. Deoxyribonucleic acid was first discovered as a 3D double helix structure in 1953 by Watson and Crick, from the data produced by Rosalind Franklin and Maurice Wilkins (Zallen, 2003). DNA replication and protein encoding ability were discussed but the technology to map the sequence of DNA was not developed at that time (Heather and Chain, 2016). Initial efforts for sequencing focus on pure RNA species, as they can be bulk produced and are shorter than DNA molecules, but they were only able to obtain the composition of the RNA, not order (Holley et al., 1961; Heather and Chain, 2016). In 1965, the first whole alanine transfer RNA (tRNA) sequence in yeast was determined, and the technique to read the sequence of nucleotide

was developed within the same year, which allowed researchers to start mapping ribosomal RNA (rRNA) and tRNA (Holley et al., 1965; Sanger et al., 1965; Brownlee and Sanger, 1967; Cory et al., 1968; Dube and Marcker, 1969). With this method, the first complete protein-coding gene sequence was produced in 1972, followed by full RNA genome mapping 4 years later (Jou et al., 1972; Fiers et al., 1976; Heather and Chain, 2016). This method was slowly adapted to DNA sequencing, but the determination of bases was still limited to shorter fragments of DNA (Wu and Kaiser, 1968; Padmanabhan et al., 1972; Sanger et al., 1973; Heather and Chain, 2016). In 1977, Sanger and colleagues made a breakthrough in DNA sequencing technology by developing a method called Sanger's "chain-termination" or dideoxy technique and with this new technology, they were able to sequence the DNA of bacteriophage ϕX174 ('PhiX'), which still serve as the positive control in many sequencing experiments today (Sanger et al., 1977a; Sanger et al., 1977b). This technology incorporates radio- or fluorescent-labeled dideoxynucleotides (ddNTPs) into the DNA pool (containing a fraction of deoxyribonucleotides (dNTPs), the monomers of DNA strands), and uses autoradiography to detect the nucleotides in the DNA (Heather and Chain, 2016). The accuracy, robustness, and ease of use of this method, Sanger sequencing (dideoxy chain termination/first-generation sequencing), made it the most common technology used to sequence DNA for many years. Development of polymerase chain reaction (PCR) and recombinant DNA technologies further advanced genomic sequencing by providing the ability to create purer DNA (Jackson et al., 1972; Cohen et al., 1973; Saiki et al., 1988; Heather and Chain, 2016).

Polymerase chain reaction, a method that amplifies specific pieces of DNA for more than a billionfold, was developed by Kary Mullis in the 1980s, which enables researchers to manipulate DNA molecules more easily (Mullis and Faloona, 1987; Mullis, 1990; Valasek and

Repa, 2005). The development of real-time quantitative PCR (qPCR) utilizes the sensitivity of PCR and combines it with the monitoring of 'real-time' PCR products as they were generated (Higuchi et al., 1992; Higuchi et al., 1993; Valasek and Repa, 2005). Quantitative PCR often conducted along with reverse transcription (RT), termed RT-qPCR, is one of the popular methods for quantifying differences in gene expression levels from diverse fields of science, including agriculture (Taylor et al., 2010). PCR experiment guidelines were published in 2009 to ensure the consistency of qPCR data (Bustin et al., 2009; Lefever et al., 2009; Taylor et al., 2010).

Second-generation sequencing (pyrosequencing) was developed by replacing the radio- or fluorescent labeling with luminescent method to measure pyrophosphate synthesis (Heather and Chain, 2016). This method consists of two enzyme processes in which pyrophosphate will be converted into ATP, which is then used as the substrate for luciferase to produce light proportion of the amount of pyrophosphate (Nyrén and Lundin, 1985). Both first- and second-generation sequencing are 'sequence-by-synthesis (SBS)', which requires direct action of DNA polymerase to produce measurable output (Nyrén, 1987; Ronaghi et al., 1996; Heather and Chain, 2016). The challenge faced by this method is the non-linear noise produced with 4-5 identical nucleotides, which limits the ability to measure how many same nucleotides are in a row (Ronaghi et al., 1996; Heather and Chain, 2016). Pyrosequencing served as the building block of commercial 'next-generation sequencing (NGS)' that is widely used in research today (Heather and Chain, 2016). Next-generation sequencing increases the amount of DNA that can be sequenced per run and is able to produce reads around 400-500 base pairs (bp) long (Margulies et al., 2005). High-throughput sequencing was then introduced to perform a huge number of parallel sequencing reactions on a micrometer scale (Shendure and Ji, 2008). Solexa method (Ilumina) utilizes flow-cell to bind to adapter-bracketed DNA molecules and form neighboring clusters of clonal

population through PCR reaction (Fedurco et al., 2006; Bentley et al., 2008; Voelkerding et al., 2009). By having a reversed orientation of DNA strands in a flow cell (produced during the cloning process), paired-end (PE) reading is made possible to provide a greater amount of information and improve the accuracy of sequencing data (Heather and Chain, 2016). This method was further improved by HiSeq, a machine that can provide greater read length and depth but is expensive to use (Balasubramanian, 2011; Quail et al., 2012). Alternatively, MiSeq machine is available as a low-cost option, with lower throughput but longer read lengths (Balasubramanian, 2011; Quail et al., 2012). There is no clear cut between second- and third-generation sequencing as they utilize the same principle, but in general, those that are capable of sequencing single molecules, without the need for DNA amplification (required by all previous technologies) are classified as third-generation sequencing (Heather and Chain, 2016; Kulski, 2016). The most used third-generation sequencing is the single molecule real time (SMRT), DNA nucleotides will be imaged one at a time, which allows for the detection of modified bases (Flusberg et al., 2010; van Dijk et al., 2014; Heather and Chain, 2016).

"Omics" was derived from the word "genomics", which represents the structure and function of genes, and comparative hereditary relationships and evolution within and between different species when this concept was first introduced (Kuska, 1998). After the advancement of NGS, genomics has been narrowed towards mapping genomic sequences, and "-omics" were added to integrated fields with large or genome-wide scale studies such as transcriptomics, epigenomics, proteomics, metabolomics, and more. (Hocquette et al., 2009). RNA sequencing (RNA-seq) is the NGS method to sequence transcriptome (all the RNA transcripts expressed in cells, tissues, and organs' genome) with a high level of sensitivity and accuracy (Wang et al., 2009; Marguerat and Bähler, 2010; Ozsolak and Milos, 2011). At least 90% of the mammalian

genome will be transcribed into RNA, including but not limited to those that support protein translation such as ribosomal, transfer, and small nucleolar RNAs (Clamp et al., 2007; Marioni et al., 2008). A near complete snapshot of transcriptome can be obtained with a greater sequencing depth (100-1000 reads per base pair) in NGS, but the sequence reads obtained from it is relatively short (35-500bp), so the reconstruction of the full-length transcripts is essential (Metzker, 2010; Martin and Wang, 2011).

Martin and Wang (2011) summarized the essential steps of RNA-seq, starting with the data generation, which is the extraction of RNA and removal of DNA contamination with DNase, then the pure RNA will be broken up into short fragments to make RNA selection (mRNA, tRNA, rRNA, etc.). The RNA will be reverse transcribed into copy DNA (or complementary DNA; cDNA) and sequencing adaptors will be ligated to both ends of the molecule, and another fragment size selection can be made (Martin and Wang, 2011). Then, the end of cDNA can be sequenced, and paired-end reads can be generated if the sequencing read was conducted from both ends. After sequencing, low-quality reads, adaptor sequence, and PCR contaminant are filtered to ensure the quality of the data, and the filtered reads will be assembled into transcripts (Martin and Wang, 2011). Polymerase chain reaction has the tendency to introduce GC (nucleotide pair of Guanine (G) and Cytosine (C)) bias during library preparation, which is a major source of errors and unwanted variation during sequencing, but alternative PCR-free methods were introduced to elevate the issues (Kozarewa et al., 2009; Aird et al., 2011). The expression level of each transcript can be estimated by counting the number of reads that align to each transcript after assembly (Martin and Wang, 2011).

Transcriptomic analysis of mineral transporters is a widely used technology in various species (Bai et al., 2008). DMT-1 mRNA levels at the mucosal level and/or different intestinal

segments in mice and broilers were evaluated by using qPCR technique (Tchernitchko et al., 2002; Bai et al., 2008). In cattle, much research was conducted to understand the mechanism of nutrient absorption (Fry et al., 2013; Tillquist et al., 2022). Messenger RNA expression of Cu-responsive genes, such as CTR1, ATP7A, ATOX1, COX17, and Commd1 was measured to understand gene responsiveness to Cu treatment and breed differences (Fry et al., 2013). Tillquist et al. (2022) utilized qPCR techniques to investigate the mRNA level of 15 Cu-responsive genes in the bovid liver and hepatocytes cultured cells. A follow-up study is usually required to confirm the findings from transcriptomic and/or PCR data. Proteomic is one of the follow-up studies that can be used to confirm the presence of the protein. It is a study on the structure, identification, and characterization of peptides and proteins, which provides information on the protein abundance, modification, variations, and interactions to help researchers identify the protein involved in certain biological processes (Berggård et al., 2007; Hocquette et al., 2009; Malm et al., 2014; Kulski, 2016).

**Cell culture**

Cell culture models were first developed by Harrison in 1906 and have been widely utilized for research since then. All the cell culture experiments since then were conducted on a two-dimensional (2D) model, which is a flat 2D surface, made from polystyrene or glass (Haycock, 2011; Jensen and Teng, 2020). In 1997, more than 50% of research on drug absorption utilized intestinal epithelial cell culture, predominantly Caco-2 cells (Artursson and Borchardt, 1997). Epithelial cell culture is gaining popularity due to its ease of performing under controlled conditions and its ability to provide new insight into drug absorption mechanisms that weren't observed before (Artursson and Borchardt, 1997; Tavelin et al., 2002). Information from epithelial cell line experiments is normally easy to translate into fundamental principles, as it's

less complex than whole tissue models (Artursson and Borchardt, 1997; Tavelin et al., 2002). Recent knowledge on active drug transport and passive transcellular and paracellular drug transport mechanisms was obtained from studies utilizing epithelial cell culture (Artursson and Borchardt, 1997). The variation between cell cultures is something worth paying attention to, as there are always some differences in permeability between Caco-2 cells from different laboratories (Artursson and Borchardt, 1997). The isolation of cells from tissues and transfer to a 2D condition may alter their morphology, mode of cell division, and loss of diverse phenotype, which can potentially lead to disrupted function, organization of the structure inside the cell, cell secretion, and cell signaling (Von Der Mark et al., 1977; Petersen et al., 1992; Nelson and Bissell, 2006; Kilian et al., 2010; Kapałczyńska et al., 2018).

In late 1990 and early 2000, much research suggested that complex biological responses such as receptor expression, transcriptional expression, cellular migration, and apoptosis studied using the 2D model differ significantly from their original organ or tissues, hence suggesting that the 2D model is not an accurate representation of mechanism in native tissues (Weaver et al., 1997; Abbott, 2003; Lee et al., 2008; Haycock, 2011). To bridge the gap between a 2D model and the whole animal, three-dimensional (3D) methods were developed to incorporate the spatial organization of the cell into the culture environment, which are more representative of what happens in living organisms (Cukierman et al., 2001; Abbott, 2003; Pampaloni et al., 2007). Three dimension models were predominantly used in cancer research when it was first introduced, as many researchers were able to discover cellular responses to drugs in 3D models that were not observed in 2D models (Weaver et al., 1997; Wang et al., 1998). One of the reasons is because of the absence of extracellular matrix (ECM) in 2D models, hence researchers might miss certain mechanisms due to the absence of this complex mechanical and biochemical

interplay (Weaver et al., 1997; Wang et al., 1998; Abbott, 2003). The extracellular matrix contains various proteins, such as collagen, elastin, and laminin, that provide tissues with mechanical properties, aid in communication between cells, and interact with membrane receptors to determine how the cells interpret biochemical cues from their surroundings (Roskelley et al., 1994; Roskelley and Bissell, 1995; Weaver et al., 1997; Abbott, 2003; Kleinman et al., 2003). Cell differentiation, proliferation, and cellular function that required ECM, cell-to-cell, and cell-to-matrix interaction are missing in the 2D cell culture model (Bissell et al., 2003; Pampaloni et al., 2007; Ravi et al., 2015; Jensen and Teng, 2020). However, the 3D cell culture model has poorer efficiency, life span, repeatability, and comfort of work compared to 2D systems (Kapałczyńska et al., 2018).

There are many techniques available for 3D modeling cell culture and users should determine which to use based on cell types and research questions. The 3D models that are commonly available include intact animals (both embryos and adults), organ slices, and cell cultures in ECM gels (Pampaloni et al., 2007; Kapałczyńska et al., 2018). Rani et al. (2023) summarized the 3D cell culture techniques available nowadays and categorized them into two main types, which are scaffold-based techniques and scaffold-free techniques.

The scaffold-based technique is essential for larger or more complex cell cultures as the cells will be cultured on substrates that represent ECM, which is closer to its native environment. This technique promotes oxygen, nutrients, and waste transportation, which assist cells in proliferation, migration, and adhesion within the scaffold. There are two types of scaffolds available: 1) In-vitro scaffolds for cell culturing and experimental applications (drug testing); and 2) Biomedical engineering scaffolds that were utilized in tissue regeneration applications. Hydrogel scaffolds are cross-linked polymers of hydrophilic molecules that provide a hydrated

environment for cell colonization and infiltration, which is important to provide a template for cell adhesion, proliferation, and differentiation (Tibbitt and Anseth, 2009; Li and Deming, 2010; Rodrigues et al., 2015; Langhans, 2018). Collagen scaffolds are the commonly used matrix in hydrogel for osteoblast and chondrocytes culture or biomedical applications (Lee et al., 2001; Zhou et al., 2006; Negri et al., 2007; Jensen and Teng, 2020).

In scaffold-free techniques, cells were grown without solid support, such as the forced floating method, hanging drop method, and agitation-based methods. Spheroids (spherical cellular aggregation) are one of the most common 3D cell culture techniques because it's easy to visualize by light or fluorescence microscopy. Hence, this model is critical for experimental research on cancer and therapeutics studies and has aided tremendously in understanding cancer mechanisms and the development of cancer drugs (Rani et al., 2023). In forced floating methods, normally a 96-well plate will be coated with 0.5% poly-2-hydroxyethyl methacrylate (poly-HEMA) and dried for 3 days. This modifies the surface of the plate so the cell cannot adhere to it, which promotes cell-to-cell contact that forces the cells to form 3D spheroids. This method is highly reproducible, accessible, and consistent, so it has been utilized by many cancerous studies and drug testing (Kelm et al., 2003; Breslin and O'Driscoll, 2013). In the hanging drop method, a drop (small aliquot) of cell suspension is added to a microtiter plate, and the plate will be inverted to keep the cells at the tip of the drop and remain in place due to gravity and surface tension (Kelm et al., 2003; Timmins and Nielsen, 2007). This method is easy, reproducible, and produces tightly packed spheroids, but is limited by the volume of liquid used to generate spheroids (Kelm et al., 2003; Rani et al., 2023). In agitation-based method, cell suspension is cultured in a container that remains in continuous motion by either continuous stirring of the cells or the rotation of the container. These motions enhance cell-to-cell communication and

adhesion to each other to form spheroids, and not adhere to the surface of the container (Kelm et al., 2003). This method aids in the transportation of nutrients and waste and for long-term culturing, but the motion can affect cellular physiology (Rani et al., 2023).

Stem cell culturing is ineffective in 2D models but provides valuable response and insight in 3D models with spheroids formation (Jensen and Teng, 2020; Rani et al., 2023). Organotypic explants (cultured piece(s) of tissue/organ removed from the host) were used if the complete information is required in whole animals (i.e. fruit fly and zebrafish), which provide critical data as the cells are physically located in their native niche (Ravi et al., 2015). Organoids can be obtained from either embryonic stem cells (ECSs), induced pluripotent stem cells (iPSCs), or stem cells from neonatal/adult (ASCs; Lancaster and Knoblich, 2014; Huch and Koo, 2015; Corrò et al., 2020). Organoid cell self-organization requires the activation of various signaling pathways by intrinsic or extrinsic cellular components such as ECM and media (Corrò et al., 2020). This 3D cell culture model is mainly used for brain and neural tissues and is grown on gels or semi-permeable membranes with growth medium or isotonic solution (Rani et al., 2023).

Bovine enterocyte cell cultures will be essential to understand cell physiology and absorption mechanism in bovine small intestine (Zhan et al., 2020). Primary bovine intestinal epithelial cell culture (BIEC) was conducted by Zhan et al. in 2017, where they successfully culture primary BIEC and established a novel clone cell method. Primary cell culture is the culture of cells, either in suspension (3D) or adhesion (2D), freshly obtained from the host, instead of the culture of immortalized cell lines, similar to organoids. (Peehl, 2005; Funel, 2014). Organoids will self-organize and result in “mini-organs”, while primary cells generate spheroids, multicellular structures that formed through cell-to-cell communication (Kumari, 2020). Primary

cell culture in ruminant fields was first introduced in 2001 to investigate the interaction of E. coli 157:H7 and bovine cells in the rumen, ileum, colon, and rectum (Dibb-Fuller et al., 2001).

The nutrient absorption mechanism in bovine is worth investigating separately from mice and human cell culture models due to the differences in the digestion system. Matthews and Webb (1995) investigated the absorption of amino acids through gastrointestinal epithelial tissues by mounting the tissues between two chambers (mucosal and serosal orientation) that contain treatment buffer on the mucosal side and Krebs Ringer Phosphate buffer (KRP) on the serosal side under physiological conditions and measure the uptake of the nutrient by the tissues. Absorption of Zn from different sources through rumen and omasum was also investigated by Wright et al. (2008), with a similar method, but the exact mechanism of Zn uptake was not provided. With the establishment method for BIEC, nutrient absorption, regulation, and immune regulation after pathogen infection in bovine can be investigated more intensively (Dibb-Fuller et al., 2001; Zhan et al., 2017; Zhan et al., 2020). Bovine intestinal epithelial cell culture was successfully used to investigate the effect of propionate on key genes that are involved in the gluconeogenic pathway in bovine jejunum (Zhan et al., 2020).

## References


AAFCO. 1998. Official Publication of the Association of American Feed Control Officials Incorporated. Bachman, P.M., Ed. Association of American Feed Control Officials. Oxford, IN, USA. 237–238.

Abbott, A. 2003. Biology's new dimension. Nature. 424:870–872. doi:10.1038/424870a.

Abrams, E., J. W. Lassiter, W. J. Miller, M. W. Neathery, R. P. Gentry, and D. M. Blackmon. 1977. Effect of Normal and High Manganese Diets on the Role of Bile in Manganese Metabolism of Calves. Journal of Animal Science. 45:1108–1113. doi:10.2527/jas1977.4551108x.

Acda, S. P., and B. J. Chae. 2001. A Review on the Applications of Organic Trace Minerals in Pig Nutrition. Pakistan J. of Nutrition. 1:25–30. doi:10.3923/pjn.2002.25.30.

Aird, D., M. G. Ross, W.-S. Chen, M. Danielsson, T. Fennell, C. Russ, D. B. Jaffe, C. Nusbaum, and A. Gnirke. 2011. Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries. Genome Biology. 12:R18. doi:10.1186/gb-2011-12-2-r18.

Aisen, P., R. Aasa, and A. G. Redfield. 1969. The Chromium, Manganese, and Cobalt Complexes of Transferrin. Journal of Biological Chemistry. 244:4628–4633. doi:10.1016/S0021-9258(18)93670-7.

Ala, A., A. P. Walker, K. Ashkan, J. S. Dooley, and M. L. Schilsky. 2007. Wilson's disease. The Lancet. 369:397–408. doi:10.1016/S0140-6736(07)60196-2.

Alscher, R. G., N. Erturk, and L. S. Heath. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53:1331–1341. doi:10.1093/jexbot/53.372.1331.

Anchordoquy, J. P., J. M. Anchordoquy, A. M. Pascua, N. Nikoloff, P. Peral-García, and C. C. Furnus. 2017. The copper transporter (SLC31A1/CTR1) is expressed in bovine spermatozoa and oocytes: Copper in IVF medium improves sperm quality. Theriogenology. 97:124–133. doi:10.1016/j.theriogenology.2017.04.037.

Anderson, M. 1983. Mn ions pass through calcium channels. A possible explanation. Journal of General Physiology. 81:805–827. doi:10.1085/jgp.81.6.805.

Andreini, C., L. Banci, I. Bertini, and A. Rosato. 2006. Counting the Zinc-Proteins Encoded in the Human Genome. J. Proteome Res. 5:196–201. doi:10.1021/pr050361j.

Andrews, G. K. 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochemical Pharmacology. 59:95–104. doi:10.1016/S0006-2952(99)00301-9.

Archibald, F. S., and C. Tyree. 1987. Manganese poisoning and the attack of trivalent manganese upon catecholamines. Archives of Biochemistry and Biophysics. 256:638–650. doi:10.1016/0003-9861(87)90621-7.

Armstrong, T. A., J. W. Spears, T. E. Engle, and M. T. See. 2001. Effect of pharmacological concentrations of dietary copper on lipid and cholesterol metabolism in pigs☆ ☆Use of trade names in this publication does not imply endorsement by the North Carolina ARS or criticism of similar products not mentioned. Nutrition Research. 21:1299–1308. doi:10.1016/S0271-5317(01)00332-3.

Armstrong, T. A., C. M. Williams, J. W. Spears, and S. S. Schiffman. 2000. High dietary copper improves odor characteristics of swine waste. Journal of Animal Science. 78:859–864. doi:10.2527/2000.784859x.

Arthington, J. D., and J. Ranches. 2021. Trace Mineral Nutrition of Grazing Beef Cattle. Animals. 11:2767. doi:10.3390/ani11102767.

Artursson, P., and R. T. Borchardt. 1997. Intestinal Drug Absorption and Metabolism in Cell Cultures: Caco-2 and Beyond. Pharm Res. 14:1655–1658. doi:10.1023/A:1012155124489.

Aschner, J. L., and M. Aschner. 2005. Nutritional aspects of manganese homeostasis. Molecular Aspects of Medicine. 26:353–362. doi:10.1016/j.mam.2005.07.003.

Ashmead, H. D., ed. 1993. The Roles of amino acid chelates in animal nutrition. Noyes Publications, Park Ridge, N.J., U.S.A.

August, D., M. Janghorbani, and V. R. Young. 1989. Determination of zinc and copper absorption at three dietary Zn-Cu ratios by using stable isotope methods in young adult and elderly subjects. Am J Clin Nutr. 50:1457–1463. doi:10.1093/ajcn/50.6.1457.

Azadmanesh, J., and G. E. O. Borgstahl. 2018. A Review of the Catalytic Mechanism of Human Manganese Superoxide Dismutase. Antioxidants (Basel). 7:25. doi:10.3390/antiox7020025.

Bai, S. P., L. Lu, X. G. Luo, and B. Liu. 2008. Kinetics of Manganese Absorption in Ligated Small Intestinal Segments of Broilers1. Poultry Science. 87:2596–2604. doi:10.3382/ps.2008-00117.

Balasubramanian, S. 2011. Sequencing nucleic acids: from chemistry to medicine. Chemical Communications. 47:7281–7286. doi:10.1039/C1CC11078K.

Bannister, W. H., J. V. Bannister, D. Barra, J. Bond, and F. Bossa. 1991. Evolutionary Aspects of Superoxide Dismutase: The Copper/Zinc Enzyme. Free Radical Research Communications. 12:349–361. doi:10.3109/10715769109145804.

Barber, R. S., R. Braude, K. G. Mitchell, and J. Cassidy. 1955. High copper mineral mixture for fattening pigs. Chemistry and Industry. Available from: https://www.cabdirect.org/cabdirect/abstract/19551405864

Bennetts, H. W., and F. E. Chapman. 1937. Copper deficiency in sheep in Western Australia; a preliminary account of the aetiology of enzootic ataxia of lambs and an anaemia of ewes. Australian Veterinary Journal. 13:138–149.

Bentley, D. R., S. Balasubramanian, H. P. Swerdlow, G. P. Smith, J. Milton, C. G. Brown, K. P. Hall, D. J. Evers, C. L. Barnes, H. R. Bignell, J. M. Boutell, J. Bryant, R. J. Carter, R. Keira Cheetham, A. J. Cox, D. J. Ellis, M. R. Flatbush, N. A. Gormley, S. J. Humphray, L. J. Irving, M. S. Karbelashvili, S. M. Kirk, H. Li, X. Liu, K. S. Maisinger, L. J. Murray, B. Obradovic, T. Ost, M. L. Parkinson, M. R. Pratt, I. M. J. Rasolonjatovo, M. T. Reed, R. Rigatti, C. Rodighiero, M. T. Ross, A. Sabot, S. V. Sankar, A. Scally, G. P. Schroth, M. E. Smith, V. P. Smith, A. Spiridou, P. E. Torrance, S. S. Tzonev, E. H. Vermaas, K. Walter, X. Wu, L. Zhang, M. D. Alam, C. Anastasi, I. C. Aniebo, D. M. D. Bailey, I. R. Bancarz, S. Banerjee, S. G. Barbour, P. A. Baybayan, V. A. Benoit, K. F. Benson, C. Bevis, P. J. Black, A. Boodhun, J. S. Brennan, J. A. Bridgham, R. C. Brown, A. A. Brown, D. H. Buermann, A. A. Bundu, J. C. Burrows, N. P. Carter, N. Castillo, M. Chiara E. Catenazzi, S. Chang, R. Neil Cooley, N. R. Crake, O. O. Dada, K. D. Diakoumakos, B. Dominguez-Fernandez, D. J. Earnshaw, U. C. Egbujor, D. W. Elmore, S. S. Etchin, M. R. Ewan, M. Fedurco, L. J. Fraser, K. V. Fuentes Fajardo, W. Scott Furey, D. George, K. J. Gietzen, C. P. Goddard, G. S. Golda, P. A. Granieri, D. E. Green, D. L. Gustafson, N. F. Hansen, K. Harnish, C. D. Haudenschild, N. I. Heyer, M. M. Hims, et al. 2008. Accurate whole human genome sequencing using reversible terminator chemistry. Nature. 456:53–59. doi:10.1038/nature07517.

Berggård, T., S. Linse, and P. James. 2007. Methods for the detection and analysis of protein–protein interactions. PROTEOMICS. 7:2833–2842. doi:10.1002/pmic.200700131.

Bertinchamps, A., S. Miller, and G. Cotzias. 1966. Interdependence of routes excreting manganese. American Journal of Physiology-Legacy Content. 211:217–224. doi:10.1152/ajplegacy.1966.211.1.217.

Bertrand, G., and R. C. Bhattacherjee. 1935. Combined action of zinc and vitamins in animal diets. Ann. Inst. Pasteur. 55:265–272.

de Bie, P., B. van de Sluis, L. Klomp, and C. Wijmenga. 2005. The Many Faces of the Copper Metabolism Protein MURR1/COMMD1. Journal of Heredity. 96:803–811. doi:10.1093/jhered/esi110.

Bingham, M. J., A. M. Sargeson, and H. J. McArdle. 1997. Characterization of intracellular copper pools in rat hepatocytes using the chelator diamsar. American Journal of Physiology-Gastrointestinal and Liver Physiology. 272:G1400–G1407. doi:10.1152/ajpgi.1997.272.6.G1400.

Bissell, M. J., A. Rizki, and I. S. Mian. 2003. Tissue architecture: the ultimate regulator of breast epithelial function. Curr Opin Cell Biol. 15:753–762.

Blakley, B. R., and D. L. Hamilton. 1985. Ceruloplasmin as an indicator of copper status in cattle and sheep. Can J Comp Med. 49:405–408.

Bligh, S. W., H. A. Boyle, A. B. McEwen, P. J. Sadler, and R. H. Woodham. 1992. 1H NMR studies of reactions of copper complexes with human blood plasma and urine. Biochem Pharmacol. 43:137–145. doi:10.1016/0006-2952(92)90270-s.

Bolze, M. S., R. D. Reeves, F. E. Lindbeck, S. F. Kemp, and M. J. Elders. 1985. Influence of Manganese on Growth, Somatomedin and Glycosaminoglycan Metabolism. The Journal of Nutrition. 115:352–358. doi:10.1093/jn/115.3.352.

Bozym, R. A., F. Chimienti, L. J. Giblin, G. W. Gross, I. Korichneva, Y. Li, S. Libert, W. Maret, M. Parviz, C. J. Frederickson, and R. B. Thompson. 2010. Free zinc ions outside a narrow concentration range are toxic to a variety of cells in vitro. Exp Biol Med (Maywood). 235:741–750. doi:10.1258/ebm.2010.009258.

Bradley, C. H. 1993. Copper poisoning in a dairy herd fed a mineral supplement. Can Vet J. 34:287–292.

Bremner, I., and J. H. Beattie. 1995. Copper and zinc metabolism in health and disease: speciation and interactions. Proceedings of the Nutrition Society. 54:489–499. doi:10.1079/PNS19950017.

Bremner, I., and N. T. Davies. 1975. The induction of metallothionein in rat liver by zinc injection and restriction of food intake. Biochem J. 149:733–738. doi:10.1042/bj1490733.

Bremner, I., W. R. Humphries, M. Phillippo, M. J. Walker, and P. C. Morrice. 1987. Iron-induced copper deficiency in calves: dose-response relationships and interactions with molybdenum and sulphur. Animal Science. 45:403–414. doi:10.1017/S0003356100002890.

Bremner, I., and R. B. Marshall. 1974. Hepatic copper–and zinc-binding proteins in ruminants: 2. Relationship between Cu and Zn concentrations and the occurrence of a metallothionein-like fraction. Br J Nutr. 32:293–300. doi:10.1079/BJN19740082.

Bremner, I., B. W. Young, and C. F. Mills. 1976. Protective effect of zinc supplementation against copper toxicosis in sheep. British Journal of Nutrition. 36:551–561. doi:10.1079/BJN19760108.

Breslin, S., and L. O'Driscoll. 2013. Three-dimensional cell culture: the missing link in drug discovery. Drug Discovery Today. 18:240–249. doi:10.1016/j.drudis.2012.10.003.

Brewer, G. J. 2001. Copper Control as an Antiangiogenic Anticancer Therapy: Lessons from Treating Wilson's Disease. Exp Biol Med (Maywood). 226:665–673. doi:10.1177/153537020222600712.

Britton, A., and G. Cotzias. 1966. Dependence of manganese turnover on intake. American Journal of Physiology-Legacy Content. 211:203–206. doi:10.1152/ajplegacy.1966.211.1.203.

Brown, K. H., S. E. Wuehler, and J. M. Peerson. 2001. The Importance of Zinc in Human Nutrition and Estimation of the Global Prevalence of Zinc Deficiency. Food Nutr Bull. 22:113–125. doi:10.1177/156482650102200201.

Brownlee, G. G., and F. Sanger. 1967. Nucleotide sequences from the low molecular weight ribosomal RNA of Escherichia coli. Journal of Molecular Biology. 23:337-IN9. doi:10.1016/S0022-2836(67)80109-8.

Bull, P. C., G. R. Thomas, J. M. Rommens, J. R. Forbes, and D. W. Cox. 1993. The Wilson disease gene is a putative copper transporting P–type ATPase similar to the Menkes gene. Nat Genet. 5:327–337. doi:10.1038/ng1293-327.

Bunch, R. J., J. T. Mccall, V. C. Speer, and V. W. Hays. 1965. Copper Supplementation for Weanling Pigs. Journal of Animal Science. 24:995–1000. doi:10.2527/jas1965.244995x.

Burlet, E., and S. K. Jain. 2017. Manganese supplementation increases adiponectin and lowers ICAM-1 and creatinine blood levels in Zucker type 2 diabetic rats, and downregulates ICAM-1 by upregulating adiponectin multimerization protein (DsbA-L) in endothelial cells. Mol Cell Biochem. 429:1–10. doi:10.1007/s11010-016-2931-7.

Burnett, W. T., R. R. Bigelow, A. W. Kimball, and C. W. Sheppard. 1952. Radiomanganese Studies on the Mouse, Rat and Pancreatic Fistula Dog. American Journal of Physiology-Legacy Content. 168:620–625. doi:10.1152/ajplegacy.1952.168.3.620.

Bustin, S. A., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, and C. T. Wittwer. 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry. 55:611–622. doi:10.1373/clinchem.2008.112797.

van Campen, D. R., and E. A. Mitchell. 1965. Absorption of Cu64, Zn65, Mo99, and Fe59 from Ligated Segments of the Rat Gastrointestinal Tract. The Journal of Nutrition. 86:120–124. doi:10.1093/jn/86.2.120.

van Campen, D. R., and P. U. Scaife. 1967. Zinc Interference with Copper Absorption in Rats. The Journal of Nutrition. 91:473–476. doi:10.1093/jn/91.4.473.

Canonne-Hergaux, F., S. Gruenheid, P. Ponka, and P. Gros. 1999. Cellular and Subcellular Localization of the Nramp2 Iron Transporter in the Intestinal Brush Border and Regulation by Dietary Iron. Blood. 93:4406–4417. doi:10.1182/blood.V93.12.4406.

Capaldi, R. A. 1990. STRUCTURE AND FUNCTION OF CYTOCHROME c OXIDASE. Annual Review of Biochemistry. 59:569–596. doi:10.1146/annurev.bi.59.070190.003033.

Chelly, J., Z. Tümer, T. Tønnesen, A. Petterson, Y. Ishikawa-Brush, N. Tommerup, N. Horn, and A. P. Monaco. 1993. Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. Nat Genet. 3:14–19. doi:10.1038/ng0193-14.

Chen, P., S. Chakraborty, S. Mukhopadhyay, E. Lee, M. M. B. Paoliello, A. B. Bowman, and M. Aschner. 2015. Manganese homeostasis in the nervous system. Journal of Neurochemistry. 134:601–610. doi:10.1111/jnc.13170.

Christianson, D. W. 1997. Structural chemistry and biology of manganese metalloenzymes. Progress in Biophysics and Molecular Biology. 67:217–252. doi:10.1016/S0079-6107(97)88477-5.

Chua, A. C. G., and E. H. Morgan. 1997. Manganese metabolism is impaired in the Belgrade laboratory rat. J Comp Physiol B. 167:361–369. doi:10.1007/s003600050085.

Clamp, M., B. Fry, M. Kamal, X. Xie, J. Cuff, M. F. Lin, M. Kellis, K. Lindblad-Toh, and E. S. Lander. 2007. Distinguishing protein-coding and noncoding genes in the human genome. Proceedings of the National Academy of Sciences. 104:19428–19433. doi:10.1073/pnas.0709013104.

Clarkson, A. H., S. Paine, J. Martín-Tereso, and N. R. Kendall. 2020. Copper physiology in ruminants: trafficking of systemic copper, adaptations to variation in nutritional supply and thiomolybdate challenge. Nutrition Research Reviews. 33:43–49. doi:10.1017/S0954422419000180.

Cohen, S. N., A. C. Y. Chang, H. W. Boyer, and R. B. Helling. 1973. Construction of Biologically Functional Bacterial Plasmids In Vitro. Proceedings of the National Academy of Sciences. 70:3240–3244. doi:10.1073/pnas.70.11.3240.

Collins, J. F. 2006. Gene chip analyses reveal differential genetic responses to iron deficiency in rat duodenum and jejunum. Biol Res. 39:25–37. doi:10.4067/S0716-97602006000100004.

Collins, J. F., J. R. Prohaska, and M. D. Knutson. 2010. Metabolic crossroads of iron and copper. Nutrition Reviews. 68:133–147. doi:10.1111/j.1753-4887.2010.00271.x.

Coppen, D. E., and N. T. Davies. 1987. Studies on the effects of dietary zinc dose on 65Zn absorption in vivo and on the effects of Zn status on 65Zn absorption and body loss in young rats. Br J Nutr. 57:35–44. doi:10.1079/bjn19870007.

Corrò, C., L. Novellasdemunt, and V. S. W. Li. 2020. A brief history of organoids. American Journal of Physiology-Cell Physiology. 319:C151–C165. doi:10.1152/ajpcell.00120.2020.

Cory, S., K. A. Marcker, S. K. Dube, and B. F. C. Clark. 1968. Primary Structure of a Methionine Transfer RNA from Escherichia coli. Nature. 220:1039–1040. doi:10.1038/2201039a0.

Cotton, F. A., G. Wilkinson, C. A. Murillo, and M. Bochmann. 1999. Advanced Inorganic Chemistry. John Wiley and Sons, Inc. Available from: http://repository.vnu.edu.vn/handle/VNU_123/85212

Cotzias, G. C., S. T. Miller, P. S. Papavasiliou, and L. C. Tang. 1976. Interactions Between Manganese and Brain Dopamine. Medical Clinics of North America. 60:729–738. doi:10.1016/S0025-7125(16)31856-9.

Cousins, R. J. 1985. Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. Physiological Reviews. 65:238–309. doi:10.1152/physrev.1985.65.2.238.

Cragg, R. A., G. R. Christie, S. R. Phillips, R. M. Russi, S. Küry, J. C. Mathers, P. M. Taylor, and D. Ford. 2002. A Novel Zinc-regulated Human Zinc Transporter, hZTL1, Is Localized to the

Enterocyte Apical Membrane *. Journal of Biological Chemistry. 277:22789–22797. doi:10.1074/jbc.M200577200.

Crisponi, G., V. M. Nurchi, D. Fanni, C. Gerosa, S. Nemolato, and G. Faa. 2010. Copper-related diseases: From chemistry to molecular pathology. Coordination Chemistry Reviews. 254:876–889. doi:10.1016/j.ccr.2009.12.018.

Cukierman, E., R. Pankov, D. R. Stevens, and K. M. Yamada. 2001. Taking Cell-Matrix Adhesions to the Third Dimension. Science. 294:1708–1712. doi:10.1126/science.1064829.

Culotta, V. C., M. Yang, and M. D. Hall. 2005. Manganese Transport and Trafficking: Lessons Learned from Saccharomyces cerevisiae. Eukaryotic Cell. 4:1159–1165. doi:10.1128/EC.4.7.1159-1165.2005.

Cunningham, I. J. 1931. Some biochemical and physiological aspects of copper in animal nutrition. Biochem J. 25:1267–1294. doi:10.1042/bj0251267.

Daly, M. J. 2009. A new perspective on radiation resistance based on Deinococcus radiodurans. Nat Rev Microbiol. 7:237–245. doi:10.1038/nrmicro2073.

Dancis, A., D. Haile, D. S. Yuan, and R. D. Klausner. 1994. The Saccharomyces cerevisiae copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. Journal of Biological Chemistry. 269:25660–25667. doi:10.1016/S0021-9258(18)47300-0.

Dardenne, M. 2002. Zinc and immune function. Eur J Clin Nutr. 56:S20–S23. doi:10.1038/sj.ejcn.1601479.

Das, S., B. Levinson, C. Vulpe, S. Whitney, J. Gitschier, and S. Packman. 1995. Similar splicing mutations of the Menkes/mottled copper-transporting ATPase gene in occipital horn syndrome and the blotchy mouse. Am J Hum Genet. 56:570–576.

Davidsson, L., A. Cederblad, B. Lönnerdal, and B. Sandström. 1991. The effect of individual dietary components on manganese absorption in humans. The American Journal of Clinical Nutrition. 54:1065–1070. doi:10.1093/ajcn/54.6.1065.

Davies, K. M., D. J. Hare, V. Cottam, N. Chen, L. Hilgers, G. Halliday, J. F. B. Mercer, and K. L. Double. 2013. Localization of copper and copper transporters in the human brain†. Metallomics. 5:43–51. doi:10.1039/c2mt20151h.

Davis, C. D., L. Zech, and J. L. Greger. 1993. Manganese metabolism in rats: an improved methodology for assessing gut endogenous losses. Proc Soc Exp Biol Med. 202:103–108. doi:10.3181/00379727-202-43518.

Davis, S. R., R. J. McMahon, and R. J. Cousins. 1998. Metallothionein Knockout and Transgenic Mice Exhibit Altered Intestinal Processing of Zinc with Uniform Zinc-Dependent Zinc Transporter-1 Expression. The Journal of Nutrition. 128:825–831. doi:10.1093/jn/128.5.825.

Dibb-Fuller, M. P., A. BEST, D. A. Stagg, W. A. Cooley, and M. J. Woodward. 2001. An in-vitro model for studying the interaction of Escherichia coli O157:H7 and other enteropathogens with bovine primary cell cultures. Journal of Medical Microbiology. 50:759–769. doi:10.1099/0022-1317-50-9-759.

Dick, A. T. 1954. Studies on the assimilation and storage of copper in crossbred sheep. Aust. J. Agric. Res. 5:511–555. doi:10.1071/ar9540511.

Dick, A. T., D. W. Dewey, and J. M. Gawthorne. 1975. Thiomolybdates and the copper–molybdenum–sulphur interaction in ruminant nutrition. The Journal of Agricultural Science. 85:567–568. doi:10.1017/S0021859600062468.

van Dijk, E. L., H. Auger, Y. Jaszczyszyn, and C. Thermes. 2014. Ten years of next-generation sequencing technology. Trends in Genetics. 30:418–426. doi:10.1016/j.tig.2014.07.001.

Doyle, J. J., and J. E. Spaulding. 1978. Toxic and Essential Trace Elements in Meat — a Review. Journal of Animal Science. 47:398–419. doi:10.2527/jas1978.472398x.

Dube, S. K., and K. A. Marcker. 1969. The Nucleotide Sequence of N-Formyl-Methionyl-Transfer RNA. European Journal of Biochemistry. 8:256–262. doi:10.1111/j.1432-1033.1969.tb00522.x.

Eckhert, C. D., and L. S. Hurley. 1977. Reduced DNA Synthesis in Zinc Deficiency: Regional Differences in Embryonic Rats. The Journal of Nutrition. 107:855–861. doi:10.1093/jn/107.5.855.

Egan, A. R. 1972. Reproductive responses to supplemental zinc and manganese in grazing Dorset Horn ewes. Aust. J. Exp. Agric. 12:131–135. doi:10.1071/ea9720131.

Eisses, J. F., and J. H. Kaplan. 2005. The Mechanism of Copper Uptake Mediated by Human CTR1: A Mutational Analysis. Journal of Biological Chemistry. 280:37159–37168. doi:10.1074/jbc.M508822200.

Engle, T. E., and J. W. Spears. 2000. Dietary copper effects on lipid metabolism, performance, and ruminal fermentation in finishing steers. Journal of Animal Science. 78:2452–2458. doi:10.2527/2000.7892452x.

Espinosa, C. D., and H. H. Stein. 2021. Digestibility and metabolism of copper in diets for pigs and influence of dietary copper on growth performance, intestinal health, and overall immune status: a review. J Animal Sci Biotechnol. 12:13. doi:10.1186/s40104-020-00533-3.

Everson, G. J., L. S. Hurley, and J. F. Geiger. 1959. Manganese Deficiency in the Guinea Pig. The Journal of Nutrition. 68:49–56. doi:10.1093/jn/68.1.49.

Farr, S. B., R. D'Ari, and D. Touati. 1986. Oxygen-dependent mutagenesis in Escherichia coli lacking superoxide dismutase. Proc. Natl. Acad. Sci. U.S.A. 83:8268–8272. doi:10.1073/pnas.83.21.8268.

Fay, J., G. E. Cartwright, and M. M. Wintrobe. 1949. Studies on Free Erythrocyte Protoporphyrin, Serum Iron, Serum Iron-Binding Capacity and Plasma Copper during Normal Pregnancy. J. Clin. Invest. 28:487–491. doi:10.1172/JCI102095.

Fedurco, M., A. Romieu, S. Williams, I. Lawrence, and G. Turcatti. 2006. BTA, a novel reagent for DNA attachment on glass and efficient generation of solid-phase amplified DNA colonies. Nucleic Acids Research. 34:e22. doi:10.1093/nar/gnj023.

Ferguson, W. S., A. H. Lewis, and S. J. Watson. 1943. The teart pastures of Somerset: I. The cause and cure of teartness. The Journal of Agricultural Science. 33:44–51. doi:10.1017/S002185960004836X.

Festa, R. A., and D. J. Thiele. 2011. Copper: An essential metal in biology. Current Biology. 21:R877–R883. doi:10.1016/j.cub.2011.09.040.

Fields, M., N. Craft, C. Lewis, J. Holbrook, A. Rose, S. Reiser, and J. C. Smith. 1986. Contrasting Effects of the Stomach and Small Intestine of Rats on Copper Absorption. The Journal of Nutrition. 116:2219–2228. doi:10.1093/jn/116.11.2219.

Fiers, W., R. Contreras, F. Duerinck, G. Haegeman, D. Iserentant, J. Merregaert, W. Min Jou, F. Molemans, A. Raeymaekers, A. Van den Berghe, G. Volckaert, and M. Ysebaert. 1976. Complete nucleotide sequence of bacteriophage MS2 RNA: primary and secondary structure of the replicase gene. Nature. 260:500–507. doi:10.1038/260500a0.

Finley, J. W., P. E. Johnson, and L. K. Johnson. 1994. Sex affects manganese absorption and retention by humans from a diet adequate in manganese. The American Journal of Clinical Nutrition. 60:949–955. doi:10.1093/ajcn/60.6.949.

Flohr, J. R. 2016. A survey of current feeding regimens for vitamins and trace minerals in the US swine industry. JSHAP. 24:290–303.

Flusberg, B. A., D. R. Webster, J. H. Lee, K. J. Travers, E. C. Olivares, T. A. Clark, J. Korlach, and S. W. Turner. 2010. Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods. 7:461–465. doi:10.1038/nmeth.1459.

Fogel, S., and J. W. Welch. 1982. Tandem gene amplification mediates copper resistance in yeast. Proceedings of the National Academy of Sciences. 79:5342–5346. doi:10.1073/pnas.79.17.5342.

Fosmire, G. J. 1990. Zinc toxicity. The American Journal of Clinical Nutrition. 51:225–227. doi:10.1093/ajcn/51.2.225.

Fridovich, I. 1995. Superoxide Radical and Superoxide Dismutases. Annual Review of Biochemistry. 64:97–112. doi:10.1146/annurev.bi.64.070195.000525.

Fry, R. S., J. W. Spears, K. E. Lloyd, A. T. O'Nan, and M. S. Ashwell. 2013. Effect of dietary copper and breed on gene products involved in copper acquisition, distribution, and use in Angus

and Simmental cows and fetuses1,2. Journal of Animal Science. 91:861–871. doi:10.2527/jas.2011-3888.

Funel, N. 2014. Chapter 2 - Advances in Primary Cell Culture of Pancreatic Cancer. In: A. S. Azmi, editor. Molecular Diagnostics and Treatment of Pancreatic Cancer. Academic Press, Oxford. p. 11–38. Available from: https://www.sciencedirect.com/science/article/pii/B9780124081031000029

Gavin, C. E., K. K. Gunter, and T. E. Gunter. 1999. Manganese and calcium transport in mitochondria: implications for manganese toxicity. Neurotoxicology. 20:445–453.

Genther-Schroeder, O. N., M. E. Branine, and S. L. Hansen. 2016. The effects of increasing supplementation of zinc-amino acid complex on growth performance, carcass characteristics, and inflammatory response of beef cattle fed ractopamine hydrochloride. Journal of Animal Science. 94:3389–3398. doi:10.2527/jas.2015-0209.

Geszvain, K., C. Butterfield, R. E. Davis, A. S. Madison, S.-W. Lee, D. L. Parker, A. Soldatova, T. G. Spiro, G. W. Luther, and B. M. Tebo. 2012. The molecular biogeochemistry of manganese(II) oxidation. Biochemical Society Transactions. 40:1244–1248. doi:10.1042/BST20120229.

Gilabert, E. R., E. Ruiz, C. Osorio, and E. Ortega. 1996. Effect of dietary zinc deficiency on reproductive function in male rats: Biochemical and morphometric parameters. The Journal of Nutritional Biochemistry. 7:403–407. doi:10.1016/S0955-2863(96)00063-0.

Goodwin, F. E. 2012. Zinc and Zinc Alloys. In: Kirk-Othmer Encyclopedia of Chemical Technology. John Wiley & Sons, Ltd. p. 1–47. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/0471238961.2609140307151504.a01.pub3

Gooneratne, S. R., W. T. Buckley, and D. A. Christensen. 1989. Review of copper deficiency and metabolism in ruminants. Can. J. Anim. Sci. 69:819–845. doi:10.4141/cjas89-096.

Gooneratne, S. R., J. M. C. Howell, and J. M. Gawthorne. 1985. Distribution of copper in the kidney of sheep after thiomolybdate administration. Nutrition Research. 5:S-546.

Gould, L., and N. R. Kendall. 2011. Role of the rumen in copper and thiomolybdate absorption. Nutrition Research Reviews. 24:176–182. doi:10.1017/S0954422411000059.

Grahn, B. H., P. G. Paterson, K. T. Gottschall-Pass, and Z. Zhang. 2001. Zinc and the Eye. Journal of the American College of Nutrition. 20:106–118. doi:10.1080/07315724.2001.10719022.

Gross, J. B., B. M. Myers, L. J. Kost, S. M. Kuntz, and N. F. LaRusso. 1989. Biliary copper excretion by hepatocyte lysosomes in the rat. Major excretory pathway in experimental copper overload. J Clin Invest. 83:30–39. doi:10.1172/JCI113873.

Gulec, S., and J. F. Collins. 2014. Molecular Mediators Governing Iron-Copper Interactions. Annual Review of Nutrition. 34:95–116. doi:10.1146/annurev-nutr-071812-161215.

Gummow, B., C. J. Botha, A. T. Basson, and S. S. Bastianello. 1991. Copper Toxicity in Ruminants: Air Pollution as a Possible Cause. Onderstepoort J. Vet. Res. 58:33–39.

Gunshin, H., B. Mackenzie, U. V. Berger, Y. Gunshin, M. F. Romero, W. F. Boron, S. Nussberger, J. L. Gollan, and M. A. Hediger. 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature. 388:482–488. doi:10.1038/41343.

Guthrie, G. J., T. B. Aydemir, C. Troche, A. B. Martin, S.-M. Chang, and R. J. Cousins. 2015. Influence of ZIP14 (slc39A14) on intestinal zinc processing and barrier function. American Journal of Physiology-Gastrointestinal and Liver Physiology. 308:G171–G178. doi:10.1152/ajpgi.00021.2014.

Haaranen, S. 1962. The effect of zinc on itching tail root eczema in cattle. Nordisk Veterinaermedicin. 14:265–269.

Hahn, J. D., and D. H. Baker. 1993. Growth and plasma zinc responses of young pigs fed pharmacologic levels of zinc. Journal of Animal Science. 71:3020–3024. doi:10.2527/1993.71113020x.

HaMai, D., A. Campbell, and S. C. Bondy. 2001. Modulation of oxidative events by multivalent manganese complexes in brain tissue. Free Radical Biology and Medicine. 31:763–768. doi:10.1016/S0891-5849(01)00639-6.

Hansen, S. L., M. S. Ashwell, A. J. Moeser, R. S. Fry, M. D. Knutson, and J. W. Spears. 2010. High dietary iron reduces transporters involved in iron and manganese metabolism and increases intestinal permeability in calves. Journal of Dairy Science. 93:656–665. doi:10.3168/jds.2009-2341.

Harless, E. 1847. Das blaue Blut einiger wirbellosen Tiere und dessen Kupfergehalt. Arch. Anat. Physiol. 148.

Harris, E. D. 1991. Copper Transport: An Overview. Proceedings of the Society for Experimental Biology and Medicine. 196:130–140. doi:10.3181/00379727-196-43171B.

Harris, E. D. 2000. Cellular Copper Transport and Metabolism. Annual Review of Nutrition. 20:291–310. doi:10.1146/annurev.nutr.20.1.291.

Harrison, R. G. 1906. Observations on the living developing nerve fiber. Proceedings of the Society for Experimental Biology and Medicine. 4:140–143. doi:10.3181/00379727-4-98.

Hassett, R., and D. J. Kosman. 1995. Evidence for Cu(II) Reduction as a Component of Copper Uptake by Saccharomyces cerevisiae(∗). Journal of Biological Chemistry. 270:128–134. doi:10.1074/jbc.270.1.128.

Haycock, J. W. 2011. 3D Cell Culture: A Review of Current Approaches and Techniques. In: J. W. Haycock, editor. 3D Cell Culture: Methods and Protocols. Humana Press, Totowa, NJ. p. 1–15. Available from: https://doi.org/10.1007/978-1-60761-984-0_1

Heather, J. M., and B. Chain. 2016. The sequence of sequencers: The history of sequencing DNA. Genomics. 107:1–8. doi:10.1016/j.ygeno.2015.11.003.

Hellman, N. E., and J. D. Gitlin. 2002. Ceruloplasmin Metabolism and Function. Annual Review of Nutrition. 22:439–458. doi:10.1146/annurev.nutr.22.012502.114457.

Hepburn, J. J., J. D. Arthington, S. L. Hansen, J. W. Spears, and M. D. Knutson. 2009. Technical note: Copper chaperone for copper, zinc superoxide dismutase: A potential biomarker for copper status in cattle1. Journal of Animal Science. 87:4161–4166. doi:10.2527/jas.2009-1978.

Hidiroglou, M., and D. A. Shearer. 1976. Concentration of manganese in the tissues of cycling and anestrous ewes. Can J Comp Med. 40:306–309.

Hiers, J. M., W. J. Miller, and D. M. Blackmon. 1968. Endogenous Secretion and Reabsorption of 65Zinc in Ruminants as Affected by Zinc Deficiency and Feeding of Ethylenediaminetetraacetate or Cadmium1. Journal of Dairy Science. 51:730–736. doi:10.3168/jds.S0022-0302(68)87063-8.

Higuchi, R., G. Dollinger, P. S. Walsh, and R. Griffith. 1992. Simultaneous Amplification and Detection of Specific DNA Sequences. Nat Biotechnol. 10:413–417. doi:10.1038/nbt0492-413.

Higuchi, R., C. Fockler, G. Dollinger, and R. Watson. 1993. Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions. Nat Biotechnol. 11:1026–1030. doi:10.1038/nbt0993-1026.

Hinskens, B., J. C. Philcox, P. Coyle, and A. M. Rofe. 2000. Increased zinc absorption but not secretion in the small intestine of metallothionein-null mice. Biol Trace Elem Res. 78:231–240. doi:10.1385/BTER:78:1-3:231.

Hoadley, J. E., A. S. Leinart, and R. J. Cousins. 1988. Relationship of 65Zn Absorption Kinetics to Intestinal Metallothionein in Rats: Effects of Zinc Depletion and Fasting. The Journal of Nutrition. 118:497–502. doi:10.1093/jn/118.4.497.

Hocquette, J.-F., I. Cassar-Malek, A. Scalbert, and F. Guillou. 2009. Contribution of Genomics to the understanding of physiological functions. Journal of physiology and pharmacology : an official journal of the Polish Physiological Society. 60 Suppl 3:5–16.

Hogan, K. G., D. F. L. Money, and A. Blayney. 1968. The effect of a molybdate and sulphate supplement on the accumulation of copper in the livers of penned sheep. New Zealand Journal of Agricultural Research. 11:435–444. doi:10.1080/00288233.1968.10431440.

Holley, R. W., J. Apgar, G. A. Everett, J. T. Madison, M. Marquisee, S. H. Merrill, J. R. Penswick, and A. Zamir. 1965. Structure of a Ribonucleic Acid. Science. 147:1462–1465. doi:10.1126/science.147.3664.1462.

Holley, R. W., J. Apgar, S. H. Merrill, and P. L. Zubkoff. 1961. Nucleotide and Oligonucleotide Compositions of the Alanine-, Valine-, and Tyrosine-Acceptor “Soluble” Ribonucleic Acids of Yeast. J. Am. Chem. Soc. 83:4861–4862. doi:10.1021/ja01484a040.

Horn, N., and Z. Tümer. 2002. Menkes Disease and the Occipital Horn Syndrome. In: Connective Tissue and Its Heritable Disorders. John Wiley & Sons, Ltd. p. 651–685. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/0471221929.ch14

Hostetler, C. E., R. L. Kincaid, and M. A. Mirando. 2003. The role of essential trace elements in embryonic and fetal development in livestock. The Veterinary Journal. 166:125–139. doi:10.1016/S1090-0233(02)00310-6.

Huang, L., and J. Gitschier. 1997. A novel gene involved in zinc transport is deficient in the lethal milk mouse. Nat Genet. 17:292–297. doi:10.1038/ng1197-292.

Huang, L., and S. Tepaamorndech. 2013. The SLC30 family of zinc transporters – A review of current understanding of their biological and pathophysiological roles. Molecular Aspects of Medicine. 34:548–560. doi:10.1016/j.mam.2012.05.008.

Huang, L., Y. Y. Yu, C. P. Kirschke, E. R. Gertz, and K. K. C. Lloyd. 2007. Znt7 (Slc30a7)-deficient mice display reduced body zinc status and body fat accumulation. J Biol Chem. 282:37053–37063. doi:10.1074/jbc.M706631200.

Huch, M., and B.-K. Koo. 2015. Modeling mouse and human development using organoid cultures. Development. 142:3113–3125. doi:10.1242/dev.118570.

Humbert, W., M. Aprahamian, C. Stock, and J. F. Grenier. 1982. Copper accumulation in primary biliary cirrhosis. Histochemistry. 74:85–93. doi:10.1007/BF00495054.

Hung, I. H., M. Suzuki, Y. Yamaguchi, D. S. Yuan, R. D. Klausner, and J. D. Gitlin. 1997. Biochemical Characterization of the Wilson Disease Protein and Functional Expression in the Yeast Saccharomyces cerevisiae. Journal of Biological Chemistry. 272:21461–21466. doi:10.1074/jbc.272.34.21461.

Hurley, L. S., C. L. Keen, and D. L. Baly. 1984. Manganese deficiency and toxicity: effects on carbohydrate metabolism in the rat. Neurotoxicology. 5:97–104.

Huster, D., M. Hoppert, S. Lutsenko, J. Zinke, C. Lehmann, J. Mössner, F. Berr, and K. Caca. 2003. Defective cellular localization of mutant ATP7B in Wilson's disease patients and hepatoma cell lines. Gastroenterology. 124:335–345. doi:10.1053/gast.2003.50066.

Irie, S., and M. Tavassoli. 1986. Liver endothelium desialates ceruloplasmin. Biochemical and Biophysical Research Communications. 140:94–100. doi:10.1016/0006-291X(86)91062-4.

Jackson, D. A., R. H. Symons, and P. Berg. 1972. Biochemical Method for Inserting New Genetic Information into DNA of Simian Virus 40: Circular SV40 DNA Molecules Containing Lambda Phage Genes and the Galactose Operon of Escherichia coli. Proceedings of the National Academy of Sciences. 69:2904–2909. doi:10.1073/pnas.69.10.2904.

Jackson, K. A., R. M. Helston, J. A. McKay, E. D. O'Neill, J. C. Mathers, and D. Ford. 2007. Splice Variants of the Human Zinc Transporter ZnT5 (SLC30A5) Are Differentially Localized

and Regulated by Zinc through Transcription and mRNA Stability *. Journal of Biological Chemistry. 282:10423–10431. doi:10.1074/jbc.M610535200.

Jackson, M. J. 1989. Physiology of Zinc: General Aspects. In: C. F. Mills, editor. Zinc in Human Biology. Springer, London. p. 1–14. Available from: https://doi.org/10.1007/978-1-4471-3879-2_1

Jenkitkasemwong, S., C.-Y. Wang, B. Mackenzie, and M. D. Knutson. 2012. Physiologic implications of metal-ion transport by ZIP14 and ZIP8. Biometals. 25:643–655. doi:10.1007/s10534-012-9526-x.

Jensen, C., and Y. Teng. 2020. Is It Time to Start Transitioning From 2D to 3D Cell Culture? Frontiers in Molecular Biosciences. 7. Available from: https://www.frontiersin.org/articles/10.3389/fmolb.2020.00033

Ji, F., X. G. Luo, L. Lu, B. Liu, and S. X. Yu. 2006. Effect of Manganese Source on Manganese Absorption by the Intestine of Broilers1 1Supported by the National Basic Research Program of China (project no. 2004CB117501), National Natural Science Foundation of China (project no. 30270968), and Chinese Academy of Agricultural Sciences Foundation for First-Place Outstanding Scientists. Poultry Science. 85:1947–1952. doi:10.1093/ps/85.11.1947.

Johnson, K. S. 2006. Manganese Redox Chemistry Revisited. Science. 313:1896–1897. doi:10.1126/science.1133496.

Johnson, M. A., M. M. Smith, and J. T. Edmonds. 1998. Copper, iron, zinc, and manganese in dietary supplements, infant formulas, and ready-to-eat breakfast cereals. The American Journal of Clinical Nutrition. 67:1035S-1040S. doi:10.1093/ajcn/67.5.1035S.

Jou, W. M., G. Haegeman, M. Ysebaert, and W. Fiers. 1972. Nucleotide Sequence of the Gene Coding for the Bacteriophage MS2 Coat Protein. Nature. 237:82–88. doi:10.1038/237082a0.

Kaler, S. G., C. R. Ferreira, and L. S. Yam. 2020. Estimated birth prevalence of Menkes disease and ATP7A-related disorders based on the Genome Aggregation Database (gnomAD). Molecular Genetics and Metabolism Reports. 24:100602. doi:10.1016/j.ymgmr.2020.100602.

Kapałczyńska, M., T. Kolenda, W. Przybyła, M. Zajączkowska, A. Teresiak, V. Filas, M. Ibbs, R. Bliźniak, Ł. Łuczewski, and K. Lamperska. 2018. 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. Arch Med Sci. 14:910–919. doi:10.5114/aoms.2016.63743.

Karcioglu, Z. A. 1982. Zinc in the eye. Survey of Ophthalmology. 27:114–122. doi:10.1016/0039-6257(82)90195-3.

Katouli, M., L. Melin, M. Jensen-Waern, P. Wallgren, and R. Möllby. 1999. The effect of zinc oxide supplementation on the stability of the intestinal flora with special reference to composition of coliforms in weaned pigs. Journal of Applied Microbiology. 87:564–573. doi:10.1046/j.1365-2672.1999.00853.x.

Keen, C. L., J. G. Bell, and B. Lönnerdal. 1986. The Effect of Age on Manganese Uptake and Retention from Milk and Infant Formulas in Rats. The Journal of Nutrition. 116:395–402. doi:10.1093/jn/116.3.395.

Keen, C. L., J. L. Ensunsa, M. H. Watson, D. L. Baly, S. M. Donovan, M. H. Monaco, and M. S. Clegg. 1999. Nutritional aspects of manganese from experimental studies. Neurotoxicology. 20:213–223.

Keilin, D., and T. Mann. 1939. Carbonic Anhydrase. Nature. 144:442–443. doi:10.1038/144442b0.

Kelleher, C. A., M. Ivan, M. Lamand, and J. Mason. 1983. The absorption of labelled molybdenum compounds in sheep fitted with re-entrant cannulae in the ascending duodenum. Journal of Comparative Pathology. 93:83–92. doi:10.1016/0021-9975(83)90045-2.

Kelm, J. M., N. E. Timmins, C. J. Brown, M. Fussenegger, and L. K. Nielsen. 2003. Method for generation of homogeneous multicellular tumor spheroids applicable to a wide variety of cell types. Biotechnology and Bioengineering. 83:173–180. doi:10.1002/bit.10655.

Kendall, N. R., H. R. Holmes-Pavord, P. A. Bone, E. L. Ander, and S. D. Young. 2015. Liver copper concentrations in cull cattle in the UK: are cattle being copper loaded? Veterinary Record. 177:493–493. doi:10.1136/vr.103078.

Kilian, K. A., B. Bugarija, B. T. Lahn, and M. Mrksich. 2010. Geometric cues for directing the differentiation of mesenchymal stem cells. Proceedings of the National Academy of Sciences. 107:4872–4877. doi:10.1073/pnas.0903269107.

Kim, A., M. P. Murphy, and T. D. Oberley. 2005. Mitochondrial redox state regulates transcription of the nuclear-encoded mitochondrial protein manganese superoxide dismutase: a proposed adaptive response to mitochondrial redox imbalance. Free Radic Biol Med. 38:644–654. doi:10.1016/j.freeradbiomed.2004.10.030.

Kim, H., H.-Y. Son, S. M. Bailey, and J. Lee. 2009. Deletion of hepatic Ctr1 reveals its function in copper acquisition and compensatory mechanisms for copper homeostasis. American Journal of Physiology-Gastrointestinal and Liver Physiology. 296:G356–G364. doi:10.1152/ajpgi.90632.2008.

Kimura, E. 1993. Roles of Zinc(II) ion in zinc enzymes. Pure and Applied Chemistry. 65:355–359. doi:10.1351/pac199365030355.

Kirschke, C. P., and L. Huang. 2003. ZnT7, a novel mammalian zinc transporter, accumulates zinc in the Golgi apparatus. J Biol Chem. 278:4096–4102. doi:10.1074/jbc.M207644200.

Klein, D., P. Scholz, G. A. Drasch, J. Müller-Höcker, and K. H. Summer. 1991. Metallothionein, copper and zinc in fetal and neonatal human liver: changes during development. Toxicology Letters. 56:61–67. doi:10.1016/0378-4274(91)90090-S.

Kleinman, H. K., D. Philp, and M. P. Hoffman. 2003. Role of the extracellular matrix in morphogenesis. Curr Opin Biotechnol. 14:526–532. doi:10.1016/j.copbio.2003.08.002.

Klevay, L. M. 2013. Myelin and traumatic brain injury: The copper deficiency hypothesis. Medical Hypotheses. 81:995–998. doi:10.1016/j.mehy.2013.09.011.

Kline, R. D., V. W. Hays, and G. L. Cromwell. 1971. Effects of Copper, Molybdenum and Sulfate on Performance, Hematology and Copper Stores of Pigs and Lambs. Journal of Animal Science. 33:771–779. doi:10.2527/jas1971.334771x.

Klomp, L. W. J., S.-J. Lin, D. S.Yuan, R. D. Klausner, V. C. Culotta, and J. D. Gitlin. 1997. Identification and Functional Expression of HAH1, a Novel Human Gene Involved in Copper Homeostasis *. Journal of Biological Chemistry. 272:9221–9226. doi:10.1074/jbc.272.14.9221.

Knudsen, E., B. Sandström, and P. Solgaard. 1996. Zinc, Copper and Magnesium Absorption from a Fibre-rich Diet. Journal of Trace Elements in Medicine and Biology. 10:68–76. doi:10.1016/S0946-672X(96)80014-9.

Kolb, D. 1988. Oxidation states of manganese. J. Chem. Educ. 65:1004. doi:10.1021/ed065p1004.2.

Kozarewa, I., Z. Ning, M. A. Quail, M. J. Sanders, M. Berriman, and D. J. Turner. 2009. Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes. Nat Methods. 6:291–295. doi:10.1038/nmeth.1311.

Krebs, N. F. 2000. Overview of Zinc Absorption and Excretion in the Human Gastrointestinal Tract. The Journal of Nutrition. 130:1374S-1377S. doi:10.1093/jn/130.5.1374S.

Kreuder, J., A. Otten, H. Fuder, Z. Tümer, T. Tønnesen, N. Horn, and D. Dralle. 1993. Clinical and biochemical consequences of copper-histidine therapy in Menkes disease. Eur J Pediatr. 152:828–832. doi:10.1007/BF02073380.

Kulski, J. K. 2016. Next-Generation Sequencing — An Overview of the History, Tools, and "Omic" Applications. In: J. K. Kulski, editor. Next Generation Sequencing - Advances, Applications and Challenges. InTech. Available from: http://www.intechopen.com/books/next-generation-sequencing-advances-applications-and-challenges/next-generation-sequencing-an-overview-of-the-history-tools-and-omic-applications

Kumaratilake, J. S., and J. McC. Howell. 1989. Intravenously administered tetra-thiomolybdate and the removal of copper from the liver of copper-loaded sheep. Journal of Comparative Pathology. 101:177–199. doi:10.1016/0021-9975(89)90065-0.

Kumari, R. 2020. How Are Organoids Different from 3D Primary Cell Cultures? Available from: https://blog.crownbio.com/how-are-organoids-different-from-3d-primary-cell-cultures

Kuska, B. 1998. Beer, Bethesda, and Biology: How "Genomics" Came Into Being. JNCI: Journal of the National Cancer Institute. 90:93. doi:10.1093/jnci/90.2.93.

La Fontaine, S., and J. F. B. Mercer. 2007. Trafficking of the copper-ATPases, ATP7A and ATP7B: Role in copper homeostasis. Archives of Biochemistry and Biophysics. 463:149–167. doi:10.1016/j.abb.2007.04.021.

Lancaster, M. A., and J. A. Knoblich. 2014. Organogenesis in a dish: modeling development and disease using organoid technologies. Science. 345:1247125. doi:10.1126/science.1247125.

Langhans, S. A. 2018. Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. Frontiers in Pharmacology. 9. Available from: https://www.frontiersin.org/articles/10.3389/fphar.2018.00006

Lee, C. H., A. Singla, and Y. Lee. 2001. Biomedical applications of collagen. International Journal of Pharmaceutics. 221:1–22. doi:10.1016/S0378-5173(01)00691-3.

Lee, D.-Y., N. F. Shay, and R. J. Cousins. 1992. Altered Zinc Metabolism Occurs in Murine Lethal Milk Syndrome. The Journal of Nutrition. 122:2233–2238. doi:10.1093/jn/122.11.2233.

Lee, H. H., A. S. Prasad, G. J. Brewer, and C. Owyang. 1989. Zinc absorption in human small intestine. American Journal of Physiology-Gastrointestinal and Liver Physiology. 256:G87–G91. doi:10.1152/ajpgi.1989.256.1.G87.

Lee, J., M. J. Cuddihy, and N. A. Kotov. 2008. Three-dimensional cell culture matrices: state of the art. Tissue Eng Part B Rev. 14:61–86. doi:10.1089/teb.2007.0150.

Lee, S.-H., H. A. Jouihan, R. C. Cooksey, D. Jones, H. J. Kim, D. R. Winge, and D. A. McClain. 2013. Manganese Supplementation Protects Against Diet-Induced Diabetes in Wild Type Mice by Enhancing Insulin Secretion. Endocrinology. 154:1029–1038. doi:10.1210/en.2012-1445.

Lefever, S., J. Hellemans, F. Pattyn, D. R. Przybylski, C. Taylor, R. Geurts, A. Untergasser, J. Vandesompele, and on behalf of the RDML consortium. 2009. RDML: structured language and reporting guidelines for real-time quantitative PCR data. Nucleic Acids Research. 37:2065–2069. doi:10.1093/nar/gkp056.

Leyva-Illades, D., P. Chen, C. E. Zogzas, S. Hutchens, J. M. Mercado, C. D. Swaim, R. A. Morrisett, A. B. Bowman, M. Aschner, and S. Mukhopadhyay. 2014. SLC30A10 Is a Cell Surface-Localized Manganese Efflux Transporter, and Parkinsonism-Causing Mutations Block Its Intracellular Trafficking and Efflux Activity. J. Neurosci. 34:14079–14095. doi:10.1523/JNEUROSCI.2329-14.2014.

Li, L., and X. Yang. 2018. The Essential Element Manganese, Oxidative Stress, and Metabolic Diseases: Links and Interactions. Oxidative Medicine and Cellular Longevity. 2018:e7580707. doi:10.1155/2018/7580707.

Li, Z., and T. J. Deming. 2010. Tunable hydrogel morphology via self-assembly of amphiphilic pentablock copolypeptides. Soft Matter. 6:2546–2551. doi:10.1039/B927137F.

Lin, W., D. R. Vann, P.-T. Doulias, T. Wang, G. Landesberg, X. Li, E. Ricciotti, R. Scalia, M. He, N. J. Hand, and D. J. Rader. 2017. Hepatic metal ion transporter ZIP8 regulates manganese

homeostasis and manganese-dependent enzyme activity. J Clin Invest. 127:2407–2417. doi:10.1172/JCI90896.

Linder, M. C., and C. A. Goode. 1991. Biochemistry of Copper. Springer Science+Business Media New York. Available from: https://link.springer.com/book/10.1007/978-1-4757-9432-8

Linder, M. C., and M. Hazegh-Azam. 1996. Copper biochemistry and molecular biology. The American Journal of Clinical Nutrition. 63:797S-811S. doi:10.1093/ajcn/63.5.797.

Little, A. G., G. Lau, K. E. Mathers, S. C. Leary, and C. D. Moyes. 2018. Comparative biochemistry of cytochrome c oxidase in animals. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 224:170–184. doi:10.1016/j.cbpb.2017.11.005.

Liu, Q., S. Barker, and M. D. Knutson. 2021. Iron and manganese transport in mammalian systems. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1868:118890. doi:10.1016/j.bbamcr.2020.118890.

Loh, H. Y., J. W. Spears, O. Guimaraes, A. C. Miller, M. P. Thorndyke, T. A. Thomas, and T. E. Engle. 2024. Trace Mineral Source Influences Trace Mineral Solubility in Water and Mineral Binding Strength to Ruminal Digesta. Biol Trace Elem Res. doi:10.1007/s12011-024-04318-x. Available from: https://doi.org/10.1007/s12011-024-04318-x

Lönnerdal, B. 1996. Bioavailability of copper. The American Journal of Clinical Nutrition. 63:821S-829S. doi:10.1093/ajcn/63.5.821.

Lönnerdal, B. 2000. Dietary Factors Influencing Zinc Absorption. The Journal of Nutrition. 130:1378S-1383S. doi:10.1093/jn/130.5.1378S.

Lönnerdal, B., A. Cederblad, L. Davidsson, and B. Sandström. 1984. The effect of individual components of soy formula and cows' milk formula on zinc bioavailability. Am J Clin Nutr. 40:1064–1070. doi:10.1093/ajcn/40.5.1064.

López-Alonso, M., and M. Miranda. 2020. Copper Supplementation, A Challenge in Cattle. Animals. 10:1890. doi:10.3390/ani10101890.

Luza, S. C., and H. C. Speisky. 1996. Liver copper storage and transport during development: implications for cytotoxicity. The American Journal of Clinical Nutrition. 63:812S-820S. doi:10.1093/ajcn/63.5.812.

Maares, M., and H. Haase. 2020. A Guide to Human Zinc Absorption: General Overview and Recent Advances of In Vitro Intestinal Models. Nutrients. 12:762. doi:10.3390/nu12030762.

Mackenzie, B., and M. D. Garrick. 2005. Iron Imports. II. Iron uptake at the apical membrane in the intestine. Am J Physiol Gastrointest Liver Physiol. 289:G981-986. doi:10.1152/ajpgi.00363.2005.

Madejczyk, M. S., and N. Ballatori. 2012. The iron transporter ferroportin can also function as a manganese exporter. Biochimica et Biophysica Acta (BBA) - Biomembranes. 1818:651–657. doi:10.1016/j.bbamem.2011.12.002.

Malecki, E. A., A. G. Devenyi, J. L. Beard, and J. R. Connor. 1999. Existing and emerging mechanisms for transport of iron and manganese to the brain. Journal of Neuroscience Research. 56:113–122. doi:10.1002/(SICI)1097-4547(19990415)56:2<113::AID-JNR1>3.0.CO;2-K.

Malecki, E. A., G. M. Radzanowski, T. J. Radzanowski, D. D. Gallaher, and J. L. Greger. 1996. Biliary Manganese Excretion in Conscious Rats Is Affected by Acute and Chronic Manganese Intake but Not by Dietary Fat. The Journal of Nutrition. 126:489–498. doi:10.1093/jn/126.2.489.

Malm, E. K., V. Srivastava, G. Sundqvist, and V. Bulone. 2014. APP: an Automated Proteomics Pipeline for the analysis of mass spectrometry data based on multiple open access tools. BMC Bioinformatics. 15:441. doi:10.1186/s12859-014-0441-8.

Maret, W. 2015. Analyzing free zinc(ii) ion concentrations in cell biology with fluorescent chelating molecules. Metallomics. 7:202–211. doi:10.1039/c4mt00230j.

Marguerat, S., and J. Bähler. 2010. RNA-seq: from technology to biology. Cell. Mol. Life Sci. 67:569–579. doi:10.1007/s00018-009-0180-6.

Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y.-J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. I. Alenquer, T. P. Jarvie, K. B. Jirage, J.-B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley, and J. M. Rothberg. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature. 437:376–380. doi:10.1038/nature03959.

Marioni, J. C., C. E. Mason, S. M. Mane, M. Stephens, and Y. Gilad. 2008. RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. Genome Res. 18:1509–1517. doi:10.1101/gr.079558.108.

Marklund, S. L., N. G. Westman, E. Lundgren, and G. Roos. 1982. Copper- and Zinc-containing Superoxide Dismutase, Manganese-containing Superoxide Dismutase, Catalase, and Glutathione Peroxidase in Normal and Neoplastic Human Cell Lines and Normal Human Tissues1. Cancer Research. 42:1955–1961.

Marston, H. R., and H. J. Lee. 1948. Nutritional Factors Involved in Wool Production By Merino Sheep II. The Influence of Copper Deficiency on the Rate of Wool Growth and on the Nature of the Fleece. Aust. Jnl. Of Bio. Sci. 1:376–387. doi:10.1071/bi9480376.

Martin, J. A., and Z. Wang. 2011. Next-generation transcriptome assembly. Nat Rev Genet. 12:671–682. doi:10.1038/nrg3068.

Maryon, E. B., S. A. Molloy, and J. H. Kaplan. 2013. Cellular glutathione plays a key role in copper uptake mediated by human copper transporter 1. American Journal of Physiology-Cell Physiology. 304:C768–C779. doi:10.1152/ajpcell.00417.2012.

Masuoka, J., and P. Saltman. 1994. Zinc(II) and copper(II) binding to serum albumin. A comparative study of dog, bovine, and human albumin. Journal of Biological Chemistry. 269:25557–25561. doi:10.1016/S0021-9258(18)47285-7.

Matthews, J. C., and K. E. Webb Jr. 1995. Absorption of L-carnosine, L-methionine, and L-methionylglycine by isolated sheep ruminal and omasal epithelial tissue1. Journal of Animal Science. 73:3464–3475. doi:10.2527/1995.73113464x.

Maverakis, E., M. A. Fung, P. J. Lynch, M. Draznin, D. J. Michael, B. Ruben, and N. Fazel. 2007. Acrodermatitis enteropathica and an overview of zinc metabolism. Journal of the American Academy of Dermatology. 56:116–124. doi:10.1016/j.jaad.2006.08.015.

McArdle, H. J., S. M. Gross, I. Creaser, A. M. Sargeson, and D. M. Danks. 1989. Effect of chelators on copper metabolism and copper pools in mouse hepatocytes. American Journal of Physiology-Gastrointestinal and Liver Physiology. 256:G667–G672. doi:10.1152/ajpgi.1989.256.4.G667.

McHargue, J. S. 1925. The Occurrence of Copper, Manganese, Zinc, Nickel, and Cobalt in Soils, Plants, and Animals, and their Possible Function as Vital Factors. Journal of Agricultural Research. 4.

McMahon, R. J., and R. J. Cousins. 1998a. Mammalian Zinc Transporters. The Journal of Nutrition. 128:667–670. doi:10.1093/jn/128.4.667.

McMahon, R. J., and R. J. Cousins. 1998b. Regulation of the zinc transporter ZnT-1 by dietary zinc. Proceedings of the National Academy of Sciences. 95:4841–4846. doi:10.1073/pnas.95.9.4841.

Meissner, W. 1816. Versuche iiber den Kupfer-Gehalt einiger Pflanzeaschen. Schweigger’s Jahrb.

Messerschmidt, A. 2010. 8.14 - Copper Metalloenzymes. In: H.-W. (Ben) Liu and L. Mander, editors. Comprehensive Natural Products II. Elsevier, Oxford. p. 489–545. Available from: https://www.sciencedirect.com/science/article/pii/B9780080453828001805

Messersmith, E. M., D. T. Smerchek, and S. L. Hansen. 2021. The Crossroads between Zinc and Steroidal Implant-Induced Growth of Beef Cattle. Animals. 11:1914. doi:10.3390/ani11071914.

Metzker, M. L. 2010. Sequencing technologies — the next generation. Nat Rev Genet. 11:31–46. doi:10.1038/nrg2626.

Miller, J. K., and W. J. Miller. 1960. Development of zinc deficiency in Holstein calves fed a purified diet. Journal of Dairy Science. 43:1854–1856. doi:10.3168/jds.S0022-0302(60)90426-4.

Miller, J. K., and W. J. Miller. 1962. Experimental Zinc Deficiency and Recovery of Calves. The Journal of Nutrition. 76:467–474. doi:10.1093/jn/76.4.467.

Miller, R. C., T. B. Keith, M. A. Mccarty, and W. T. S. Thorp. 1940. Manganese as a Possible Factor Influencing the Occurrence of Lameness in Pigs. Proceedings of the Society for Experimental Biology and Medicine. 45:50–51. doi:10.3181/00379727-45-11578P.

Miller, W. J. 1969. Absorption, Tissue Distribution, Endogenous Excretion, and Homeostatic Control of Zinc in Ruminants. The American Journal of Clinical Nutrition. 22:1323–1331. doi:10.1093/ajcn/22.10.1323.

Miller, W. J. 1970. Zinc Nutrition of Cattle: A Review1. Journal of Dairy Science. 53:1123–1135. doi:10.3168/jds.S0022-0302(70)86355-X.

Miller, W. J., Y. G. Martin, R. P. Gentry, and D. M. Blackmon. 1968. 65Zn and Stable Zinc Absorption, Excretion and Tissue Concentrations as Affected by Type of Diet and Level of Zinc in Normal Calves. The Journal of Nutrition. 94:391–401. doi:10.1093/jn/94.3.391.

Miller, W. J., and J. K. Miller. 1963. Photomicrographs of Skin from Zinc-Deficient Calves. Journal of Dairy Science. 46:1285–1287. doi:10.3168/jds.S0022-0302(63)89261-9.

Miller, W. J., J. D. Morton, W. J. Pitts, and C. M. Clifton. 1965a. Effect of zinc deficiency and restricted feeding on wound healing in the bovine. Proceedings of the Society for Experimental Biology and Medicine. 118:427–430. doi:10.3181/00379727-118-2986.

Miller, W. J., M. W. Neathery, R. P. Gentry, D. M. Blackmon, J. W. Lassiter, and F. M. Pate. 1972. Distribution and Turnover Rates of Radioactive Manganese in Various Tissues after Duodenal Dosing in Holstein Calves Fed a Practical-Type Diet2. Journal of Animal Science. 34:460–464. doi:10.2527/jas1972.343460x.

Miller, W. J., W. J. Pitts, C. M. Clifton, and J. D. Morton. 1965b. Effects of Zinc Deficiency per se on Feed Efficiency, Serum Alkaline Phosphatase, Zinc in Skin, Behavior, Greying, and other Measurements in the Holstein Calf1. Journal of Dairy Science. 48:1329–1334. doi:10.3168/jds.S0022-0302(65)88458-2.

Miret, S., R. J. Simpson, and A. T. McKie. 2003. Physiology and Molecular Biology of Dietary Iron Absorption. Annual Review of Nutrition. 23:283–301. doi:10.1146/annurev.nutr.23.011702.073139.

Montgomery, M. L., G. E. Sheline, and I. L. Chaikoff. 1943. The Elimination of Administered Zinc in Pancreatic Juice, Duodenal Juice, and Bile of the Dog as Measured by its Radioactive Isotope (Zn65). J Exp Med. 78:151–159. doi:10.1084/jem.78.3.151.

Mores, N., J. Cristani, I. A. Piffer, W. Barioni, and G. M. M. Lima. 1998. Effects of zinc oxide on postweaning diarrhea control in pigs experimentally infected with E. coli. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 50:513–523.

Moynahan, E. J. 1974. Acrodermatitis Enteropathica: A Lethal Inherited Human Zinc-Deficiency Disorder. The Lancet. 304:399–400. doi:10.1016/S0140-6736(74)91772-3.

Mullis, K. B. 1990. The Unusual Origin of the Polymerase Chain Reaction. Scientific American. 262:56–65.

Mullis, K. B., and F. A. Faloona. 1987. [21] Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. In: Methods in Enzymology. Vol. 155. Academic Press. p. 335–350. Available from: https://www.sciencedirect.com/science/article/pii/0076687987550236

Myint, Z. W., T. H. Oo, K. Z. Thein, A. M. Tun, and H. Saeed. 2018. Copper deficiency anemia: review article. Ann Hematol. 97:1527–1534. doi:10.1007/s00277-018-3407-5.

NASEM, (National Academies of Sciences, Engineering, and Medicine). 2016. Nutrient Requirements of Beef Cattle, 8th Revised Edition. National Academies Press, Washington, D.C. Available from: http://www.nap.edu/catalog/19014

Negri, S., C. Fila, S. Farinato, A. Bellomi, and P. P. Pagliaro. 2007. Tissue engineering: chondrocyte culture on type 1 collagen support. Cytohistological and immunohistochemical study. Journal of Tissue Engineering and Regenerative Medicine. 1:158–159. doi:10.1002/term.15.

Nelson, C. M., and M. J. Bissell. 2006. Of Extracellular Matrix, Scaffolds, and Signaling: Tissue Architecture Regulates Development, Homeostasis, and Cancer. Annual Review of Cell and Developmental Biology. 22:287–309. doi:10.1146/annurev.cellbio.22.010305.104315.

Nevitt, T., H. Öhrvik, and D. J. Thiele. 2012. Charting the travels of copper in eukaryotes from yeast to mammals. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1823:1580–1593. doi:10.1016/j.bbamcr.2012.02.011.

Nyrén, P. 1987. Enzymatic method for continuous monitoring of DNA polymerase activity. Analytical Biochemistry. 167:235–238. doi:10.1016/0003-2697(87)90158-8.

Nyrén, P., and A. Lundin. 1985. Enzymatic method for continuous monitoring of inorganic pyrophosphate synthesis. Analytical Biochemistry. 151:504–509. doi:10.1016/0003-2697(85)90211-8.

O'dell, B. L. 1969. Effect of Dietary Components upon Zinc Availability123: A Review with Original Data. The American Journal of Clinical Nutrition. 22:1315–1322. doi:10.1093/ajcn/22.10.1315.

Orent, E. R., and E. V. Mccollum. 1931. Effects of deprivation of manganese in the rat. Journal of Biological Chemistry. 92:651–678.

Ott, E. A., W. H. Smith, M. Stob, and W. M. Beeson. 1964. Zinc Deficiency Syndrome in the Young Lamb. The Journal of Nutrition. 82:41–50. doi:10.1093/jn/82.1.41.

Ott, E. A., W. H. Smith, M. Stob, H. E. Parker, and W. M. Beeson. 1965a. Zinc Deficiency Syndrome in the Young Calf. Journal of Animal Science. 24:735–741. doi:10.2527/jas1965.243735x.

Ott, E. A., W. H. Smith, M. Stob, H. E. Parker, R. B. Harrington, and W. M. Beeson. 1965b. Zinc Requirement of the Growing Lamb Fed a Purified Diet. The Journal of Nutrition. 87:459–463. doi:10.1093/jn/87.4.459.

Ozsolak, F., and P. M. Milos. 2011. RNA sequencing: advances, challenges and opportunities. Nat Rev Genet. 12:87–98. doi:10.1038/nrg2934.

Padmanabhan, R., Raji Padmanabhan, and R. Wu. 1972. Nucleotide sequence analysis of DNA: IX. Use of oligonucleotides of defined sequence as primers in DNA sequence analysis. Biochemical and Biophysical Research Communications. 48:1295–1302. doi:10.1016/0006-291X(72)90852-2.

Pajarillo, E. A. B., E. Lee, and D.-K. Kang. 2021. Trace metals and animal health: Interplay of the gut microbiota with iron, manganese, zinc, and copper. Animal Nutrition. 7:750–761. doi:10.1016/j.aninu.2021.03.005.

Pal, P. K., A. Samii, and D. B. Calne. 1999. Manganese neurotoxicity: a review of clinical features, imaging and pathology. Neurotoxicology. 20:227–238.

Palmiter, R. D., T. B. Cole, and S. D. Findley. 1996a. ZnT-2, a mammalian protein that confers resistance to zinc by facilitating vesicular sequestration. The EMBO Journal. 15:1784–1791. doi:10.1002/j.1460-2075.1996.tb00527.x.

Palmiter, R. D., T. B. Cole, C. J. Quaife, and S. D. Findley. 1996b. ZnT-3, a putative transporter of zinc into synaptic vesicles. Proceedings of the National Academy of Sciences. 93:14934–14939. doi:10.1073/pnas.93.25.14934.

Palmiter, R. D., and S. D. Findley. 1995. Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. The EMBO Journal. 14:639–649. doi:10.1002/j.1460-2075.1995.tb07042.x.

Pampaloni, F., E. G. Reynaud, and E. H. K. Stelzer. 2007. The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol. 8:839–845. doi:10.1038/nrm2236.

Papavasiliou, P., S. Miller, and G. Cotzias. 1966. Role of liver in regulating distribution and excretion of manganese. American Journal of Physiology-Legacy Content. 211:211–216. doi:10.1152/ajplegacy.1966.211.1.211.

Park, J. D., N. J. Cherrington, and C. D. Klaassen. 2002. Intestinal Absorption of Cadmium Is Associated with Divalent Metal Transporter 1 in Rats. Toxicological Sciences. 68:288–294. doi:10.1093/toxsci/68.2.288.

Payne, A. S., and J. D. Gitlin. 1998. Functional Expression of the Menkes Disease Protein Reveals Common Biochemical Mechanisms Among the Copper-transporting P-type ATPases *. Journal of Biological Chemistry. 273:3765–3770. doi:10.1074/jbc.273.6.3765.

Peehl, D. M. 2005. Primary cell cultures as models of prostate cancer development. Endocr Relat Cancer. 12:19–47. doi:10.1677/erc.1.00795.

Perry, D. L. 2016. Handbook of Inorganic Compounds. CRC Press.

Petersen, O. W., L. Rønnov-Jessen, A. R. Howlett, and M. J. Bissell. 1992. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proceedings of the National Academy of Sciences. 89:9064–9068. doi:10.1073/pnas.89.19.9064.

Peterson, M. G., and J. F. B. Mercer. 1988. Differential expression of four linked sheep metallothionein genes. European Journal of Biochemistry. 174:425–429. doi:10.1111/j.1432-1033.1988.tb14115.x.

Pfeiffer, R. F. 2007. Wilson's Disease. Semin Neurol. 27:123–132. doi:10.1055/s-2007-971173.

Phillippo, M., W. R. Humphries, T. Atkinson, G. D. Henderson, and P. H. Garthwaite. 1987. The effect of dietary molybdenum and iron on copper status, puberty, fertility and oestrous cycles in cattle. The Journal of Agricultural Science. 109:321–336. doi:10.1017/S0021859600080758.

Phillippo, M., W. R. Humphries, C. B. Lawrence, and J. Price. 1982. Investigation of the effect of copper status and therapy on fertility in beef suckler herds. The Journal of Agricultural Science. 99:359–364. doi:10.1017/S0021859600030148.

Plumlee, M. P., D. M. Thrasher, W. M. Beeson, F. N. Andrews, and H. E. Parker. 1956. The Effects of a Manganese Deficiency upon the Growth, Development, and Reproduction of Swine. Journal of Animal Science. 15:352–367. doi:10.2527/jas1956.152352x.

Polishchuk, E. V., and R. S. Polishchuk. 2016. The emerging role of lysosomes in copper homeostasis. Metallomics. 8:853–862. doi:10.1039/c6mt00058d.

Poulsen, H. D. 1995. Zinc Oxide for Weanling Piglets. Acta Agriculturae Scandinavica, Section A — Animal Science. 45:159–167. doi:10.1080/09064709509415847.

Prasad, A. S. 1995. Zinc: an overview. Nutrition. 11:93–99.

Price, J., A. M. Will, G. Paschaleris, and J. K. Chesters. 1987. Identification of thiomolybdates in digesta and plasma from sheep after administration of 99Mo-labelled compounds into the rumen. British Journal of Nutrition. 58:127–138. doi:10.1079/BJN19870076.

Prohaska, J. R. 2008. Role of copper transporters in copper homeostasis. The American Journal of Clinical Nutrition. 88:826S-829S. doi:10.1093/ajcn/88.3.826S.

Quadri, M., A. Federico, T. Zhao, G. J. Breedveld, C. Battisti, C. Delnooz, L.-A. Severijnen, L. Di Toro Mammarella, A. Mignarri, L. Monti, A. Sanna, P. Lu, F. Punzo, G. Cossu, R. Willemsen, F. Rasi, B. A. Oostra, B. P. van de Warrenburg, and V. Bonifati. 2012. Mutations in SLC30A10 Cause Parkinsonism and Dystonia with Hypermanganesemia, Polycythemia, and Chronic Liver Disease. The American Journal of Human Genetics. 90:467–477. doi:10.1016/j.ajhg.2012.01.017.

Quail, M. A., M. Smith, P. Coupland, T. D. Otto, S. R. Harris, T. R. Connor, A. Bertoni, H. P. Swerdlow, and Y. Gu. 2012. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics. 13:341. doi:10.1186/1471-2164-13-341.

Rahman, I., and S. K. Biswas. 2006. OXIDANTS AND ANTIOXIDANTS | Antioxidants, Enzymatic. In: G. J. Laurent and S. D. Shapiro, editors. Encyclopedia of Respiratory Medicine. Academic Press, Oxford. p. 258–266. Available from: https://www.sciencedirect.com/science/article/pii/B0123708796002830

Ramos, D., D. Mar, M. Ishida, R. Vargas, M. Gaite, A. Montgomery, and M. C. Linder. 2016. Mechanism of Copper Uptake from Blood Plasma Ceruloplasmin by Mammalian Cells. PLOS ONE. 11:e0149516. doi:10.1371/journal.pone.0149516.

Rani, M., A. Devi, S. P. Singh, R. Kumari, and A. Kumar. 2023. 3D Cell Culture Techniques. In: S. Mani, M. Singh, and A. Kumar, editors. Animal Cell Culture: Principles and Practice. Springer International Publishing, Cham. p. 197–212. Available from: https://doi.org/10.1007/978-3-031-19485-6_14

Raulin, J. 1869. Études chimiques sur la végétation ... Ann. Sci. Natl. Botan. Biol. Ser.5. 11:93.

Ravi, M., V. Paramesh, S. r. Kaviya, E. Anuradha, and F. D. P. Solomon. 2015. 3D Cell Culture Systems: Advantages and Applications. Journal of Cellular Physiology. 230:16–26. doi:10.1002/jcp.24683.

Reyes, J. G. 1996. Zinc transport in mammalian cells. American Journal of Physiology-Cell Physiology. 270:C401–C410. doi:10.1152/ajpcell.1996.270.2.C401.

Riordan, J. F. 1976. Biochemistry of Zinc. Medical Clinics of North America. 60:661–674. doi:10.1016/S0025-7125(16)31851-X.

Rodrigues, M. T., P. P. Carvalho, M. E. Gomes, and R. L. Reis. 2015. Chapter 11 - Biomaterials in Preclinical Approaches for Engineering Skeletal Tissues. In: A. Atala and J. G. Allickson, editors. Translational Regenerative Medicine. Academic Press, Boston. p. 127–139. Available from: https://www.sciencedirect.com/science/article/pii/B9780124103962000116

Rodríguez-Rodríguez, E., L. M. Bermejo, A. M. López-Sobaler, and R. M. Ortega. 2011. [An inadequate intake of manganese may favour insulin resistance in girls]. Nutr Hosp. 26:965–970. doi:10.1590/s0212-16112011000500007.

Roelofsen, H., H. Wolters, M. J. A. Van Luyn, N. Miura, F. Kuipers, and R. J. Vonk. 2000. Copper-induced apical trafficking of ATP7B in polarized hepatoma cells provides a mechanism for biliary copper excretion. Gastroenterology. 119:782–793. doi:10.1053/gast.2000.17834.

Rojas, M. A., I. A. Dyer, and W. A. Cassatt. 1965. Manganese Deficiency in the Bovine. Journal of Animal Science. 24:664–667. doi:10.2527/jas1965.243664x.

Rolfs, A., and M. A. Hediger. 1999. Metal ion transporters in mammals: structure, function and pathological implications. J Physiol. 518:1–12. doi:10.1111/j.1469-7793.1999.0001r.x.

Ronaghi, M., S. Karamohamed, B. Pettersson, M. Uhlén, and P. Nyrén. 1996. Real-Time DNA Sequencing Using Detection of Pyrophosphate Release. Analytical Biochemistry. 242:84–89. doi:10.1006/abio.1996.0432.

Roskelley, C. D., and M. J. Bissell. 1995. Dynamic reciprocity revisited: a continuous, bidirectional flow of information between cells and the extracellular matrix regulates mammary epithelial cell function. Biochem. Cell Biol. 73:391–397. doi:10.1139/o95-046.

Roskelley, C. D., P. Y. Desprez, and M. J. Bissell. 1994. Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction. Proceedings of the National Academy of Sciences. 91:12378–12382. doi:10.1073/pnas.91.26.12378.

Roth, J. A., C. Horbinski, D. Higgins, P. Lein, and M. D. Garrick. 2002. Mechanisms of Manganese-Induced Rat Pheochromocytoma (PC12) Cell Death and Cell Differentiation. NeuroToxicology. 23:147–157. doi:10.1016/S0161-813X(01)00077-8.

Roth, J., S. Ponzoni, and M. Aschner. 2013. Manganese Homeostasis and Transport. In: L. Banci, editor. Metallomics and the Cell. Vol. 12. Springer Netherlands, Dordrecht. p. 169–201. Available from: https://link.springer.com/10.1007/978-94-007-5561-1_6

Rothschild, M. A., M. Oratz, and S. S. Schreiber. 1977. Albumin Synthesis. In: V. M. Rosenoer, M. Oratz, and M. A. Rothschild, editors. Albumin: Structure, Function and Uses. Pergamon. p. 227–253. Available from: https://www.sciencedirect.com/science/article/pii/B978008019603950017X

Rothschild, M. A., M. Oratz, and S. S. Schreiber. 1988. Serum albumin. Hepatology. 8:385. doi:10.1002/hep.1840080234.

Sah, S. K., G. Agrahari, and T.-Y. Kim. 2020. Insights into superoxide dismutase 3 in regulating biological and functional properties of mesenchymal stem cells. Cell & Bioscience. 10:22. doi:10.1186/s13578-020-00386-3.

Sahagian, B. M., I. Harding-Barlow, and H. M. Perry. 1967. Transmural Movements of Zinc, Manganese, Cadmium and Mercury by Rat Small Intestine. The Journal of Nutrition. 93:291–300. doi:10.1093/jn/93.3.291.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. Science. 239:487–491. doi:10.1126/science.2448875.

Sandström, B., B. Arvidsson, A. Cederblad, and E. Björn-Rasmussen. 1980. Zinc absorption from composite meals. I. The significance of whest extraction rate, zinc, calcium, and protein content in meals based on bread. Am J Clin Nutr. 33:739–745. doi:10.1093/ajcn/33.4.739.

Sandström, B., and A. Cederblad. 1980. Zinc absorption from composite meals. II. Influence of the main protein source. Am J Clin Nutr. 33:1778–1783. doi:10.1093/ajcn/33.8.1778.

Sandström, B., L. Davidsson, R. Eriksson, and M. Alpsten. 1988. Effect of Long-Term Trace Element Supplementation on Blood Trace Element Levels and Absorption of 75Se, 54Mn and 65Zn. In: L. S. Hurley, C. L. Keen, B. Lönnerdal, and R. B. Rucker, editors. Trace Elements in Man and Animals 6. Springer US, Boston, MA. p. 687–688. Available from: https://doi.org/10.1007/978-1-4613-0723-5_253

Sanger, F., G. M. Air, B. G. Barrell, N. L. Brown, A. R. Coulson, J. C. Fiddes, C. A. Hutchison, P. M. Slocombe, and M. Smith. 1977a. Nucleotide sequence of bacteriophage φX174 DNA. Nature. 265:687–695. doi:10.1038/265687a0.

Sanger, F., G. G. Brownlee, and B. G. Barrell. 1965. A two-dimensional fractionation procedure for radioactive nucleotides. Journal of Molecular Biology. 13:373-IN4. doi:10.1016/S0022-2836(65)80104-8.

Sanger, F., J. E. Donelson, A. R. Coulson, H. Kössel, and D. Fischer. 1973. Use of DNA Polymerase I Primed by a Synthetic Oligonucleotide to Determine a Nucleotide Sequence in Phage f1 DNA. Proceedings of the National Academy of Sciences. 70:1209–1213. doi:10.1073/pnas.70.4.1209.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977b. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences. 74:5463–5467. doi:10.1073/pnas.74.12.5463.

Santamaria, A. B. 2008. Manganese exposure, essentiality & toxicity. Indian Journal of Medical Research. 128:484.

Scheiber, I. F., Y. Wu, S. E. Morgan, and N. Zhao. 2019. The intestinal metal transporter ZIP14 maintains systemic manganese homeostasis. Journal of Biological Chemistry. 294:9147–9160. doi:10.1074/jbc.RA119.008762.

Schroeder, H. A., J. J. Balassa, and I. H. Tipton. 1966. Essential trace metals in man: Manganese: A study in homeostasis. Journal of Chronic Diseases. 19:545–571. doi:10.1016/0021-9681(66)90094-4.

Scott, B. J., and A. R. Bradwell. 1983. Identification of the serum binding proteins for iron, zinc, cadmium, nickel, and calcium. Clinical Chemistry. 29:629–633. doi:10.1093/clinchem/29.4.629.

Shendure, J., and H. Ji. 2008. Next-generation DNA sequencing. Nat Biotechnol. 26:1135–1145. doi:10.1038/nbt1486.

Shin, Y. A. 1973. Interaction of metal ions with polynucleotides and related compounds. XXII. Effect of divalent metal ions on the conformational changes of polyribonucleotides. Biopolymers. 12:2459–2475. doi:10.1002/bip.1973.360121103.

Shin, Y. Ae., and G. L. Eichhorn. 1968. Interactions of metal ions with polynucleotides and related compounds. XI. The reversible unwinding and rewinding of deoxyribonucleic acid by zinc(II) ions through temperature manipulation. Biochemistry. 7:1026–1032. doi:10.1021/bi00843a022.

Shuster, T. A., F. Martin, and A. K. Nagy. 1996. Zinc causes an apparent increase in rhodopsin phosphorylation. Current Eye Research. 15:1019–1024. doi:10.3109/02713689609017650.

Sigel, H. 2000. Metal Ions in Biological Systems: Volume 37: Manganese and Its Role in Biological Processes. CRC Press.

Silva, J. J. R. F. da, and R. J. P. Williams. 2001. The Biological Chemistry of the Elements: The Inorganic Chemistry of Life. OUP Oxford.

Skrajnowska, D., and B. Bobrowska-Korczak. 2019. Role of Zinc in Immune System and Anti-Cancer Defense Mechanisms. Nutrients. 11:2273. doi:10.3390/nu11102273.

Sloot, W. N., J. Korf, J. F. Koster, L. E. A. de Wit, and J. B. P. Gramsbergen. 1996. Manganese-Induced Hydroxyl Radical Formation in Rat Striatum Is Not Attenuated by Dopamine Depletion or Iron Chelationin Vivo. Experimental Neurology. 138:236–245. doi:10.1006/exnr.1996.0062.

Søli, N. E., and J. P. Rambæk. 1978. Excretion of Intravenously Injected Copper-64 in Sheep. Acta Pharmacologica et Toxicologica. 43:205–210. doi:10.1111/j.1600-0773.1978.tb02256.x.

Solomons, N. W., and R. A. Jacob. 1981. Studies on the bioavailability of zinc in humans: effects of heme and nonheme iron on the absorption of zinc. Am J Clin Nutr. 34:475–482. doi:10.1093/ajcn/34.4.475.

Spears, J. W., and E. B. Kegley. 2002. Effect of zinc source (zinc oxide vs zinc proteinate) and level on performance, carcass characteristics, and immune response of growing and finishing steers1,2. Journal of Animal Science. 80:2747–2752. doi:10.1093/ansci/80.10.2747.

Standish, J. F., and C. B. Ammerman. 1971. Effect of Excess Dietary Iron as Ferrous Sulfate and Ferric Citrate on Tissue Mineral Composition of Sheep. Journal of Animal Science. 33:481–484. doi:10.2527/jas1971.332481x.

Stern, B. R., M. Solioz, D. Krewski, P. Aggett, T.-C. Aw, S. Baker, K. Crump, M. Dourson, L. Haber, R. Hertzberg, C. Keen, B. Meek, L. Rudenko, R. Schoeny, W. Slob, and T. Starr. 2007. Copper and Human Health: Biochemistry, Genetics, and Strategies for Modeling Dose-response Relationships. Journal of Toxicology and Environmental Health, Part B. 10:157–222. doi:10.1080/10937400600755911.

Stirn, F. E., C. A. Elvehjem, and E. B. Hart. 1935. THE INDISPENSABILITY OF ZINC IN THE NUTRITION OF THE RAT. Journal of Biological Chemistry. 109:347–359. doi:10.1016/S0021-9258(18)75241-1.

Studer, J. M., W. P. Schweer, N. K. Gabler, and J. W. Ross. 2022. Functions of manganese in reproduction. Animal Reproduction Science. 238:106924. doi:10.1016/j.anireprosci.2022.106924.

Suttle, N. F. 1975. The role of organic sulphur in the copper-molybdenum-S interrelationship in ruminant nutrition. British Journal of Nutrition. 34:411–420. doi:10.1017/S0007114575000475.

Suttle, N. F. 1977. Reducing the potential copper toxicity of concentrates to sheep by the use of molybdenum and sulphur supplements. Animal Feed Science and Technology. 2:235–246. doi:10.1016/0377-8401(77)90027-X.

Suttle, N. F. 1991. The Interactions Between Copper, Molybdenum, and Sulphur in Ruminant Nutrition. Annual Review of Nutrition. 11:121–140. doi:10.1146/annurev.nu.11.070191.001005.

Suttle, N. F. 2012. Copper Imbalances in Ruminants and Humans: Unexpected Common Ground. Advances in Nutrition. 3:666–674. doi:10.3945/an.112.002220.

Suttle, N. F., and A. C. Field. 1983. Effects of dietary supplements of thiomolybdates on copper and molybdenum metabolism in sheep. Journal of Comparative Pathology. 93:379–389. doi:10.1016/0021-9975(83)90025-7.

Suzuki, T., K. Ishihara, H. Migaki, W. Matsuura, A. Kohda, K. Okumura, M. Nagao, Y. Yamaguchi-Iwai, and T. Kambe. 2005. Zinc Transporters, ZnT5 and ZnT7, Are Required for the Activation of Alkaline Phosphatases, Zinc-requiring Enzymes That Are Glycosylphosphatidylinositol-anchored to the Cytoplasmic Membrane *. Journal of Biological Chemistry. 280:637–643. doi:10.1074/jbc.M411247200.

Tan, J., T. Zhang, L. Jiang, J. Chi, D. Hu, Q. Pan, D. Wang, and Z. Zhang. 2011. Regulation of Intracellular Manganese Homeostasis by Kufor-Rakeb Syndrome-associated ATP13A2 Protein*. Journal of Biological Chemistry. 286:29654–29662. doi:10.1074/jbc.M111.233874.

Tandy, S., M. Williams, A. Leggett, M. Lopez-Jimenez, M. Dedes, B. Ramesh, S. K. Srai, and P. Sharp. 2000. Nramp2 Expression Is Associated with pH-dependent Iron Uptake across the Apical Membrane of Human Intestinal Caco-2 Cells*. Journal of Biological Chemistry. 275:1023–1029. doi:10.1074/jbc.275.2.1023.

Tanzi, R. E., K. Petrukhin, I. Chernov, J. L. Pellequer, W. Wasco, B. Ross, D. M. Romano, E. Parano, L. Pavone, L. M. Brzustowicz, M. Devoto, J. Peppercorn, A. I. Bush, I. Sternlieb, M. Pirastu, J. F. Gusella, O. Evgrafov, G. K. Penchaszadeh, B. Honig, I. S. Edelman, M. B. Soares, I. H. Scheinberg, and T. C. Gilliam. 1993. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nat Genet. 5:344–350. doi:10.1038/ng1293-344.

Tavassoli, M., T. Kishimoto, and M. Kataoka. 1986. Liver endothelium mediates the hepatocyte's uptake of ceruloplasmin. Journal of Cell Biology. 102:1298–1303. doi:10.1083/jcb.102.4.1298.

Tavelin, S., J. Gråsjö, J. Taipalensuu, G. Ocklind, and P. Artursson. 2002. Applications of Epithelial Cell Culture in Studies of Drug Transport. In: C. Wise, editor. Epithelial Cell Culture Protocols. Humana Press, Totowa, NJ. p. 233–272. Available from: https://doi.org/10.1385/1-59259-185-X:233

Taylor, S., M. Wakem, G. Dijkman, M. Alsarraj, and M. Nguyen. 2010. A practical approach to RT-qPCR—Publishing data that conform to the MIQE guidelines. Methods. 50:S1–S5. doi:10.1016/j.ymeth.2010.01.005.

Tchernitchko, D., M. Bourgeois, M.-E. Martin, and C. Beaumont. 2002. Expression of the two mRNA isoforms of the iron transporter Nrmap2/DMTI in mice and function of the iron responsive element. Biochemical Journal. 363:449–455. doi:10.1042/bj3630449.

Tibbitt, M. W., and K. S. Anseth. 2009. Hydrogels as extracellular matrix mimics for 3D cell culture. Biotechnology and Bioengineering. 103:655–663. doi:10.1002/bit.22361.

Tillquist, N. M., M. P. Thorndyke, T. A. Thomas, S. J. Coleman, and T. E. Engle. 2022. Impact of Cell Culture and Copper Dose on Gene Expression in Bovine Liver. Biol Trace Elem Res. 200:2113–2121. doi:10.1007/s12011-021-02829-5.

Timmins, N. E., and L. K. Nielsen. 2007. Generation of Multicellular Tumor Spheroids by the Hanging-Drop Method. In: H. Hauser and M. Fussenegger, editors. Tissue Engineering. Humana Press, Totowa, NJ. p. 141–151. Available from: https://doi.org/10.1007/978-1-59745-443-8_8

Todd, J. R. 1969. Chronic copper toxicity of ruminants. Proc. Nutr. Soc. 28:189–198. doi:10.1079/PNS19690037.

Tompsett, S. L. 1940. Factors influencing the absorption of iron and copper from the alimentary tract. Biochem J. 34:961–969. doi:10.1042/bj0340961.

Tong, K. K., and H. J. McArdle. 1995. Copper uptake by cultured trophoblast cells isolated from human term placenta. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1269:233–236. doi:10.1016/0167-4889(95)00123-6.

Trinder, D., P. S. Oates, C. Thomas, J. Sadleir, and E. H. Morgan. 2000. Localisation of divalent metal transporter 1 (DMT1) to the microvillus membrane of rat duodenal enterocytes in iron deficiency, but to hepatocytes in iron overload. Gut. 46:270–276. doi:10.1136/gut.46.2.270.

Tümer, Z., and L. B. Møller. 2010. Menkes disease. Eur J Hum Genet. 18:511–518. doi:10.1038/ejhg.2009.187.

Tuschl, K., P. T. Clayton, S. M. Gospe, S. Gulab, S. Ibrahim, P. Singhi, R. Aulakh, R. T. Ribeiro, O. G. Barsottini, M. S. Zaki, M. L. Del Rosario, S. Dyack, V. Price, A. Rideout, K. Gordon, R. A. Wevers, W. K. "Kling" Chong, and P. B. Mills. 2012. Syndrome of Hepatic

Cirrhosis, Dystonia, Polycythemia, and Hypermanganesemia Caused by Mutations in SLC30A10 , a Manganese Transporter in Man. The American Journal of Human Genetics. 90:457–466. doi:10.1016/j.ajhg.2012.01.018.

Tuschl, K., P. B. Mills, H. Parsons, M. Malone, D. Fowler, M. Bitner-Glindzicz, and P. T. Clayton. 2008. Hepatic cirrhosis, dystonia, polycythaemia and hypermanganesaemia--a new metabolic disorder. J Inherit Metab Dis. 31:151–163. doi:10.1007/s10545-008-0813-1.

Underwood, E. 1977. Trace Elements in Human and Animal Nutrition. 4th ed. Elsevier.

Underwood, E. J. 1971. Trace elements in human and animal nutrition. Ed. 3. Trace elements in human and animal nutrition. Ed. 3. Available from: https://www.cabdirect.org/cabdirect/abstract/19721492550

Underwood, E. J., and M. Somers. 1969. Studies of zinc nutrition in sheep. I. The relation of zinc to growth, testicular development, and spermatogenesis in young rams. Aust. J. Agric. Res. 20:889–897. doi:10.1071/ar9690889.

Underwood, E. J., and N. F. Suttle. 1999. The mineral nutrition of livestock. 3rd ed. CABI Pub, Wallingford, Oxon, UK ; New York, NY, USA.

Valasek, M. A., and J. J. Repa. 2005. The power of real-time PCR. Advances in Physiology Education. 29:151–159. doi:10.1152/advan.00019.2005.

Valentine, R. A., K. A. Jackson, G. R. Christie, J. C. Mathers, P. M. Taylor, and D. Ford. 2007. ZnT5 Variant B Is a Bidirectional Zinc Transporter and Mediates Zinc Uptake in Human Intestinal Caco-2 Cells *. Journal of Biological Chemistry. 282:14389–14393. doi:10.1074/jbc.M701752200.

Vallee, B. L. 1959. Biochemistry, Physiology and Pathology of Zinc. Physiological Reviews. 39:443–490. doi:10.1152/physrev.1959.39.3.443.

Vallee, B. L. 1977. Zinc biochemistry in normal and neoplastic growth processes. Experientia. 33:600–601. doi:10.1007/bf01946521.

Vallee, B. L., and H. Neurath. 1954. CARBOXYPEPTIDASE, A ZINC METALLOPROTEIN. J. Am. Chem. Soc. 76:5006–5007. doi:10.1021/ja01648a088.

VanWouwe, J. P. 1989. Clinical and laboratory diagnosis of acrodermatitis enteropathica. Eur J Pediatr. 149:2–8. doi:10.1007/BF02024322.

Victery, W., J. M. Smith, and A. J. Vander. 1981. Renal tubular handling of zinc in the dog. American Journal of Physiology-Renal Physiology. 241:F532–F539. doi:10.1152/ajprenal.1981.241.5.F532.

Voelkerding, K. V., S. A. Dames, and J. D. Durtschi. 2009. Next-Generation Sequencing: From Basic Research to Diagnostics. Clinical Chemistry. 55:641–658. doi:10.1373/clinchem.2008.112789.

Von Der Mark, K., V. Gauss, H. Von Der Mark, and P. Müller. 1977. Relationship between cell shape and type of collagen synthesised as chondrocytes lose their cartilage phenotype in culture. Nature. 267:531–532. doi:10.1038/267531a0.

Vulpe, C., B. Levinson, S. Whitney, S. Packman, and J. Gitschier. 1993. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper–transporting ATPase. Nat Genet. 3:7–13. doi:10.1038/ng0193-7.

Wada, Y., W. Kajiwara, and K. Kato. 1995. Wilson's disease-like lesion in a calf. Vet Pathol. 32:538–539. doi:10.1177/030098589503200513.

Wang, C., Y. Guan, M. Lv, R. Zhang, Z. Guo, X. Wei, X. Du, J. Yang, T. Li, Y. Wan, X. Su, X. Huang, and Z. Jiang. 2018. Manganese Increases the Sensitivity of the cGAS-STING Pathway for Double-Stranded DNA and Is Required for the Host Defense against DNA Viruses. Immunity. 48:675-687.e7. doi:10.1016/j.immuni.2018.03.017.

Wang, C.-Y., S. Jenkitkasemwong, S. Duarte, B. K. Sparkman, A. Shawki, B. Mackenzie, and M. D. Knutson. 2012. ZIP8 Is an Iron and Zinc Transporter Whose Cell-surface Expression Is Up-regulated by Cellular Iron Loading. Journal of Biological Chemistry. 287:34032–34043. doi:10.1074/jbc.M112.367284.

Wang, F., B.-E. Kim, M. J. Petris, and D. J. Eide. 2004. The Mammalian Zip5 Protein Is a Zinc Transporter That Localizes to the Basolateral Surface of Polarized Cells *. Journal of Biological Chemistry. 279:51433–51441. doi:10.1074/jbc.M408361200.

Wang, F., V. M. Weaver, O. W. Petersen, C. A. Larabell, S. Dedhar, P. Briand, R. Lupu, and M. J. Bissell. 1998. Reciprocal interactions between β1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: A different perspective in epithelial biology. Proceedings of the National Academy of Sciences. 95:14821–14826. doi:10.1073/pnas.95.25.14821.

Wang, K., B. Zhou, Y.-M. Kuo, J. Zemansky, and J. Gitschier. 2002. A Novel Member of a Zinc Transporter Family Is Defective in Acrodermatitis Enteropathica. The American Journal of Human Genetics. 71:66–73. doi:10.1086/341125.

Wang, X., and B. Zhou. 2010. Dietary zinc absorption: A play of Zips and ZnTs in the gut. IUBMB Life. 62:176–182. doi:10.1002/iub.291.

Wang, Z., M. Gerstein, and M. Snyder. 2009. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 10:57–63. doi:10.1038/nrg2484.

Wapnir, R. A. 1998. Copper absorption and bioavailability. The American Journal of Clinical Nutrition. 67:1054S-1060S. doi:10.1093/ajcn/67.5.1054S.

Ward, J. D., and J. W. Spears. 1997. Long-term effects of consumption of low-copper diets with or without supplemental molybdenum on copper status, performance, and carcass characteristics of cattle. Journal of Animal Science. 75:3057–3065. doi:10.2527/1997.75113057x.

Watson, J. D., and F. H. C. Crick. 1953. Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. Nature. 171:737–738. doi:10.1038/171737a0.

Weaver, V. M., O. W. Petersen, F. Wang, C. A. Larabell, P. Briand, C. Damsky, and M. J. Bissell. 1997. Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and In Vivo by Integrin Blocking Antibodies. Journal of Cell Biology. 137:231–245. doi:10.1083/jcb.137.1.231.

Webb, S. M., G. J. Dick, J. R. Bargar, and B. M. Tebo. 2005. Evidence for the presence of Mn(III) intermediates in the bacterial oxidation of Mn(II). Proceedings of the National Academy of Sciences. 102:5558–5563. doi:10.1073/pnas.0409119102.

Weiss, K. C., and M. C. Linder. 1985. Copper transport in rats involving a new plasma protein. American Journal of Physiology-Endocrinology and Metabolism. 249:E77–E88. doi:10.1152/ajpendo.1985.249.1.E77.

Weston, R. H., and J. Kastelic. 1967. The Transfer of Zinc from the Blood to the Rumen in Sheep. Aust. Jnl. Of Bio. Sci. 20:975–982. doi:10.1071/bi9670975.

Wijmenga, C., and L. W. J. Klomp. 2004. Molecular regulation of copper excretion in the liver. Proceedings of the Nutrition Society. 63:31–39. doi:10.1079/PNS2003316.

Wilson, J. G. 1952. Herd functional infertility, with reference to nutrition and mineral intake. Veterinary Record. 64:621–623.

Wilson, J. G. 1966. Bovine functional infertility in Devon and Cornwall: response to manganese therapy. Veterinary Record. 79:562–566.

Winge, D. R., and R. K. Mehra. 1990. Host Defenses against Copper Toxicity. In: G. W. Richter and K. Solez, editors. International Review of Experimental Pathology. Vol. 31. Academic Press. p. 47–83. Available from: https://www.sciencedirect.com/science/article/pii/B9780123649317500070

Wintrobe, M. M., G. E. Cartwright, and C. J. Gubler. 1953. Studies on the Function and Metabolism of Copper ,. The Journal of Nutrition. 50:395–419. doi:10.1093/jn/50.4.395.

Witholt, R., R. H. Gwiazda, and D. R. Smith. 2000. The neurobehavioral effects of subchronic manganese exposure in the presence and absence of pre-parkinsonism. Neurotoxicology and Teratology. 22:851–861. doi:10.1016/S0892-0362(00)00108-2.

Wright, C. L., J. W. Spears, and K. E. Webb Jr. 2008. Uptake of zinc from zinc sulfate and zinc proteinate by ovine ruminal and omasal epithelia1,2. Journal of Animal Science. 86:1357–1363. doi:10.2527/jas.2006-650.

Wu, L. J., A. G. M. Leenders, S. Cooperman, E. Meyron-Holtz, S. Smith, W. Land, R. Y. L. Tsai, U. V. Berger, Z.-H. Sheng, and T. A. Rouault. 2004. Expression of the iron transporter ferroportin in synaptic vesicles and the blood–brain barrier. Brain Research. 1001:108–117. doi:10.1016/j.brainres.2003.10.066.

Wu, R., and A. D. Kaiser. 1968. Structure and base sequence in the cohesive ends of bacteriophage lambda DNA. Journal of Molecular Biology. 35:523–537. doi:10.1016/S0022-2836(68)80012-9.

Yamaji, S., J. Tennant, S. Tandy, M. Williams, S. K. Singh Srai, and P. Sharp. 2001. Zinc regulates the function and expression of the iron transporters DMT1 and IREG1 in human intestinal Caco-2 cells. FEBS Letters. 507:137–141. doi:10.1016/S0014-5793(01)02953-2.

Yin, Z., H. Jiang, E.-S. Y. Lee, M. Ni, K. M. Erikson, D. Milatovic, A. B. Bowman, and M. Aschner. 2010. Ferroportin is a manganese-responsive protein that decreases manganese cytotoxicity and accumulation. Journal of Neurochemistry. 112:1190–1198. doi:10.1111/j.1471-4159.2009.06534.x.

Zallen, D. T. 2003. Despite Franklin's work, Wilkins earned his Nobel. Nature. 425:15–15. doi:10.1038/425015b.

Zhan, K., M. Lin, M.-M. Liu, Y.-N. Sui, and G.-Q. Zhao. 2017. Establishment of primary bovine intestinal epithelial cell culture and clone method. In Vitro Cell.Dev.Biol.-Animal. 53:54–57. doi:10.1007/s11626-016-0082-5.

Zhan, K., T. Y. Yang, Y. Chen, M. C. Jiang, and G. Q. Zhao. 2020. Propionate enhances the expression of key genes involved in the gluconeogenic pathway in bovine intestinal epithelial cells. Journal of Dairy Science. 103:5514–5524. doi:10.3168/jds.2019-17309.

Zhang, W., R. Wang, X. Zhu, D. O. Kleemann, C. Yue, and Z. Jia. 2007. Effects of Dietary Copper on Ruminal Fermentation, Nutrient Digestibility and Fibre Characteristics in Cashmere Goats. Asian-Australasian Journal of Animal Sciences. 20:1843–1848. doi:10.5713/ajas.2007.1843.

Zhang, Zhixiong, R. Zhang, J. Yin, S. Zhao, X. Qin, F. Chen, Y. Yang, L. Bai, Z. Guo, Y. Wu, Y. Li, and Zhidong Zhang. 2023. Antiviral Effect of Manganese against Foot-and-Mouth Disease Virus Both in PK15 Cells and Mice. Viruses. 15:390. doi:10.3390/v15020390.

Zhou, B., and J. Gitschier. 1997. hCTR1: A human gene for copper uptake identified by complementation in yeast. Proceedings of the National Academy of Sciences. 94:7481–7486. doi:10.1073/pnas.94.14.7481.

Zhou, Y., D. W. Hutmacher, S.-L. Varawan, T. M. Lim, Y. Zhou, D. W. Hutmacher, S.-L. Varawan, and T. M. Lim. 2006. Effect of Collagen-I Modified Composites on Proliferation and Differentiation of Human Alveolar Osteoblasts. Aust. J. Chem. 59:571–578. doi:10.1071/CH06165.

Zlotkin, S. H., S. Atkinson, and G. Lockitch. 1995. Trace Elements in Nutrition for Premature Infants. Clinics in Perinatology. 22:223–240. doi:10.1016/S0095-5108(18)30310-5.

Zudenigo, D., and M. Relja. 1990. Hepatolenticular degeneration. Neurologija. 39:115–127.

# CHAPTER 2 - TRACE MINERAL SOURCE INFLUENCES TRACE MINERAL SOLUBILITY IN WATER AND MINERAL BINDING STRENGTH TO RUMINAL DIGESTA


## Summary

Two experiments were conducted to examine the impact of trace mineral (TM) source on in vitro and in vivo solubility characteristics. Experiment 1: Hydroxy TM (HTM) and sulfate TM (STM) sources of Cu, Mn, and Zn were incubated separately in water for 24 h. Immediately after mixing, initial pH of each solution was greater ($P < 0.03$) for HTM compared to STM for all elements. Final pH tended to be greater for Cu ($P = 0.09$) and Zn ($P = 0.07$) from HTM compared to STM. Water solubility of Cu, Mn, and Zn from STM was greater ($P < 0.01$) than HTM sources. Experiment 2: Eight steers fitted with rumen cannula were blocked by body weight and randomly assigned to treatments. Treatments consisted of 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either STM or HTM sources. Steers were individually fed a cracked corn-corn silage-based diet. Treatments were top-dressed daily. Rumen contents were collected at 0, 2, and 4 h post-feeding on d 1 and 14. On d 15, strained ruminal fluid and particle-associated microorganisms were obtained. Zinc was more tightly bound ($P = 0.01$) to the digesta in HTM-supplemented steers compared to STM on d 14. These data indicate that TM source influences pH and solubility of Cu, Mn, and Zn in water and may affect rumen soluble Cu concentrations and binding strength of Zn to solid digesta.

## Introduction

It has been well established that hydroxy trace minerals (HTM) sources of copper (Cu) and zinc (Zn) are relatively insoluble in water, whereas sulfate trace minerals (STM) sources of Cu and Zn are highly soluble in water (Cao et al., 2000; Spears et al., 2004; Arthington, 2015). When the pH of the aqueous solution is reduced below 4.0 with hydrochloric acid (HCl), Cu and Zn from both HTM and STM are highly soluble (Cao et al., 2000; Spears et al., 2004). In 2017, Faulkner and Weiss (2017) were the first to report that Cu, manganese (Mn), and Zn from HTM improved fiber digestibility in lactating dairy cows compared to iso-amounts of supplemental Cu, Mn, and Zn from STM sources. High soluble concentrations of Cu and Zn in the ruminal environment can negatively impact rumen fermentation (Durand and Kawashima, 1980). Therefore, the authors hypothesized that high rumen solubility of Cu, Mn, and Zn from STM sources may have impaired rumen microbial fermentation.

Recent research has confirmed the findings of Faulkner and Weiss (2017). Using a rumen pulse dose technique in canulated steers, Cu and Zn from HTM were less soluble in the rumen than Cu and Zn from STM sources when steers received a corn silage-steam-flaked based diet (Caldera et al., 2019), a medium-quality hay diet (Guimaraes et al., 2021), and a diet formulated for a high producing dairy cow (Guimaraes et al., 2022). Furthermore, Cu and Zn from HTM were less tightly bound to ruminal solid digesta than Cu and Zn from STM (Caldera et al., 2019; Guimaraes et al., 2021). Lactating dairy cows (Daniel et al., 2020; Miller et al., 2020) and steers (Guimaraes et al., 2021; Guimaraes et al., 2022) supplemented with STM also had lower fiber digestion than those receiving HTM.

Solubility experiments comparing different trace mineral (TM) sources have used in vitro techniques or in vivo bolus dose models to investigate TM ruminal solubility. Limited research

has investigated the impact of TM sources on these parameters through dietary inclusion. Therefore, the objectives of this study were to: 1) determine the effect of TM source (HTM vs STM) on pH and solubility of Cu, Mn, and Zn after incubation in water (pH 7.0); and 2) compare the effect of dietary TM source in vivo on: a) ruminal soluble TM concentrations, b) TM binding strength toward rumen digesta, and c) TM concentration in rumen bacteria and protozoa after feeding a diet supplemented with Cu, Mn, and Zn from STM or HTM sources. We hypothesized that STM forms of Cu, Mn, and Zn would have greater solubility in water, reduce water pH, have a greater solubility and binding strength to rumen digesta within the rumen, and bioaccumulate in protozoa and bacteria to a greater extent than HTM sources of Cu, Mn, and Zn.

## Materials and methods

### *Ethical Statement*

Prior to the initiation of this experiment, all animal care, handling, and procedures described herein were approved by the Colorado State University Animal Care and Use Committee (IACUC approval #1555 and #3574)

### *Mineral sources and composition*

Two different sources of minerals were used throughout the entire experiment: 1) sulfate sources of trace minerals (STM): Cu sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), Mn sulfate monohydrate ($MnSO_4 \cdot H_2O$), and Zn sulfate monohydrate ($ZnSO_4 \cdot H_2O$), and 2) hydroxy sources of trace minerals (HTM): IntelliBond C ($Cu_2(OH)_3Cl_2$; Selko USA), IntelliBond M ($Mn_2(OH)_3Cl$; Selko USA), IntelliBond Z ($Zn_5(OH)_8Cl_2 \cdot H_2O$; Selko USA).

### *Experiment 1 (In vitro solubility study)*

The solubility of Cu, Mn, and Zn from STM and HTM sources in this experiment was analyzed as described by Spears et al. (2004) with slight modification. Briefly, approximately 0.025g of Cu, Zn, and Mn from STM and HTM were brought to a final volume of 50 mL with

deionized (DI) water in 50 mL conical tubes (n = 4 replicates/element/source, N = 24). Tubes were capped and swirled to mix the solution, and initial pH was measured. Samples were incubated for 24 h at 39°C with agitation at 200 rpm. After 24 h, samples were filtered through ashless filter paper (Whatman plc, Maidstone, UK), and the final pH of the filtrates was measured. The filtrate was then analyzed for mineral concentration.

### *Experiment 2*

#### *Animals and Diets*

Eight crossbred Angus steers (509 kg ± 10 kg; approximately 1.5 years of age) fitted with ruminal cannula were utilized in this study. Before TM supplementation, all steers were transitioned to a diet formulated for a high-producing lactating dairy cow without Cu, Mn, and Zn supplementation for 28 d (Table 1). After the 28 d acclimation period, steers were stratified by body weight (BW) to treatments and individually housed where they received their respective trace mineral treatments: 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg dry matter (DM) from either STM or HTM sources (n = 4/treatment). Each steer received approximately 11.6 kg of the basal diet (as-fed basis) per day for 14 d and individual intakes were recorded and measured daily. Dry distiller's grain (DDG) was used as the carrier for the treatment. Treatment mixtures were top-dressed onto the feed and mixed thoroughly by hand after basal diet delivery. The basal diet contained 7, 57, and 35 mg/kg of DM of Cu, Mn, and Zn, respectively. Trace mineral supplements used in the experiment were analyzed and TM concentration ranged between 335-368 mg Cu, 1120-1580 mg Mn, and 2030-2340 mg Zn/kg DM of DDG, which was included as 2% of the diet. With the addition of TM supplementation, steers received approximately 16 mg Cu, 92 mg Mn, and 91 mg Zn/kg of DM.

Rumen contents were thoroughly mixed by hand and approximately 150 g rumen samples were collected in three 50 mL conical tubes from the geometric center of the rumen at 0, 2, and 4 h post-feeding on d 1 and d 14 of the experiment. The conical tubes were centrifuged at 1500 x *g* for 15 minutes immediately after collection and the supernatant was separated from the pellet. Both the supernatant and pellet were stored separately at -20°C until analyzed for Cu, Mn, and Zn concentrations. Copper, Mn, and Zn concentrations within the supernatant were considered to be soluble whereas the Cu, Mn, and Zn concentrations in the digest were considered to be insoluble.

***Dialysis***

Dried digesta (pellet) samples from experiment 2 were exposed to dialysis for the investigation of the binding strength of minerals to the digesta. Dialysis tubing (regenerated cellulose, MWCO: 12-14kD, width: 45 mm, vol/length: 6.4mL/cm; Spectrum™ Spectra/Por™, Fisher Scientific, Waltham, MA, USA) was prepared as described by Caldera et al. (2019). Briefly, dialysis tubing was cut into 15 cm segments and treated with 1mM ethylenediaminetetraacetate (EDTA) in 2% sodium bicarbonate solution to remove metal contamination. Treated dialysis tubing was rinsed with DI water, and stored in a solution containing 50% ethanol and 1mM EDTA at 4°C. Dialysis tubing was rinsed with DI water three times before use. Dialysis procedures were conducted as described by Jones et al. (1985) and Guimaraes et al. (2021) with slight modifications. Briefly, 0.25 g of samples were dialysis against 0.01 M EDTA in 0.05 M Tris (EDTA-Tris) buffer, adjusted to a pH of 6.8 using 6 M hydrochloric acid (HCl) and 10 M sodium hydroxide (NaOH). Samples were mixed with 10 mL of EDTA-Tris buffer and transferred into rinsed dialysis tubing. Samples were then dialyzed against 1.0 L of Tris-EDTA buffer for 16 h at 4°C with continuous stirring. The buffer was

replaced with another 1.0 L of EDTA-Tris buffer and the dialysis continued for another 6 h at 4°C. After dialysis, samples were removed from the dialysis bags and placed into a pre-weighed acid-washed crucible. Samples were dried at 60 °C for 24 h, dry ashed at 600°C  or 12 h, reconstituted in 1.2 N HCl, and then analyzed for Cu, Mn, and Zn.

***Protozoa and Bacteria mineral concentration***

Approximately 250 g of rumen samples were collected from the geometric center of the rumen from each steer 2 h post-feeding 14 d after treatment initiation. Strained ruminal fluid (SRF) and particle-associated organisms (PAO) collection methods were adapted from methods described by Craig et al. (1984) with slight modifications. Collected rumen samples were squeezed through 8 layers of cheesecloth into three 50 mL conical tubes to obtain SRF. Half of the remaining digesta (approximately 125g) was placed in a bag and washed with phosphate-buffered saline (PBS) to obtain PAO. Protozoa and bacteria fractions were extracted through procedures described by Olubobokun et al. (1988) and De Mulder et al. (2017) with slight modifications. Briefly, SRF and PAO samples were centrifuged at 650 x g for 15 minutes to obtain protozoa pellet. The supernatant from the tubes was transferred into a new set of 50 mL conical tubes for bacteria extraction. The remaining pellet was washed again with PBS and centrifuged at 650 x g for 15 minutes. The pellet was the protozoa fraction of the samples and was analyzed for protozoal Cu, Mn, and Zn concentrations. The supernatant was then centrifuged at 25,000 x g for 30 minutes, the pellet obtained represented the bacteria fraction of the samples. The supernatant was discarded, and the bacteria pellet was washed with PBS and recentrifuged at 25,000 x g for 30 minutes. The supernatant was discarded, and the bacterial pellets were analyzed for bacterial Cu, Mn, and Zn concentration.

***Mineral analysis***

Dry matter analysis was conducted on all solid samples prior to mineral analysis. All solid samples were analyzed using a dry ashing method according to the official methods of analysis (AOAC, 2016). In brief, approximately 1g of sample was weighed into an acid-washed crucible and ashed in a Thermo-Fisher Thermolyne muffle furnace at 600°C for 12 h. After ashing, samples were weighed and resuspended in a total of 5 mL of boiling 1.2 M HCl for mineral analysis. Samples were reconstituted with DI water to a total volume of 10 mL.

Liquid samples were digested using an open vessel digestion method adapted from Edgell (1989) with slight modification. Briefly, 1 mL of samples were mixed with 70% nitric acid (Trace Element grade; ThermoFisher, Waltham, MA) at a 1:1 ratio. Sample-acid mixtures were left to react at room temperature under a fume hood for 10 minutes. After 10 minutes, samples-acid mixtures were placed into 70 °C water bath for 4 h with occasional swirling. Samples were removed from the water bath and allowed to cool to room temperature. After cooling, 1 mL of 30% hydrogen peroxide was added to the sample-acid mixture and allowed to react for 10 minutes. Samples were transferred back into the 70°C water bath for 2 h. Samples were then diluted with DI water to fit within a linear range of a standard curve generated by linear regression of known TM concentrations and analyzed for Cu, Mn, and Zn via Atomic Absorption Spectroscopy (PinAAcle 500, PerkinElmer, Waltham, MA).

***Statistical Analysis***

All analyses in this experiment were performed for a randomized block design using R Statistical software version 4.3.2 and packages within R ( R Core Team, 2023). Initial pH, final pH, TM (Cu, Mn, Zn) concentration, and solubility percentage from experiment 1 and protozoal and bacterial TM concentrations from experiment 2 were analyzed using a student's t-test. Trace mineral concentrations in the digesta and soluble fractions from experiment 2 and TM

contractions of the digesta post-dialysis were analyzed using a linear mixed-effects model (lme4 ver. 1.1-32; Bates et al., 2015; Kuznetsova et al., 2017). A three-way interaction between treatment x day x hour was analyzed using type III Analysis of Variance (car ver. 3.1-1; John and Sanford, 2019), with animal being the random effect in the statistical model. A two-way interaction (treatment x day) was investigated using a linear mixed-effects model with animal being the random variable. Least-squares means between treatment groups were estimated using emmeans package (ver. 1.8.5; Lenth et al., 2024). For all response variables, a P-value of less than or equal to 0.05 was considered significant, and tendencies were determined with a P-value greater than 0.05 but less than or equal to 0.10.

**Results**

***Experiment 1 (In vitro solubility study)***

The influence of trace mineral source on pH and TM solubility is shown in Table 2. Initial pH, at the time of mixing each TM source with water, was lesser for STM sources of Cu ($P = 0.02$), Mn ($P = 0.006$), and Zn ($P = 0.002$) compared to HTM sources of Cu, Mn, and Zn. Final pH following a 24-h incubation in DI water at 39°C tended to be lesser for Cu ($P = 0.09$) and Zn ($P = 0.07$) from STM sources compared to Cu and Zn from HTM sources. Final pH was similar for both Mn sources ($P = 0.16$). At the end of the incubation, percent solubility of Cu, Mn, and Zn was greater ($P < 0.01$) for STM compared to HTM sources.

***Experiment 2***

Table 3 describes the influence of TM source on soluble and insoluble Cu, Mn, and Zn concentrations in ruminal fluid and solid digesta fractions. There were no treatment x hour ($P > 0.40$) or treatment x day x hour ($P > 0.30$) interactions. Therefore, only main effects and the treatment x day interactions are shown. Data are compared by day within treatment and/or by

treatment within day. There was a tendency (P = 0.087) for a treatment x day interaction for digesta Mn concentrations. Overall, Mn concentrations in digesta tended (P = 0.098) to be greater in STM supplemented steers on d 14 compared to HTM supplemented steers. Furthermore, Mn concentrations in digesta increased (P = 0.004) by 44.4% from day 1 to day 14 in STM supplemented steers. There was also a tendency (P = 0.055) for a treatment x day interaction for ruminal soluble Cu concentration. Ruminal soluble Cu concentrations increased (P = 0.002) by 27 % from day 1 to day 14 in STM supplemented steers.

***Dialysis***

The influence of dialyzing rumen solid digesta against EDTA-Tris buffer on the remaining Cu, Mn, and Zn concentrations of rumen solid digesta on day 1 and day 14 of the experiment are shown in Table 4. There were no treatment x hour (P > 0.40) or treatment x day x hour (P > 0.10) interactions for any response variables. Therefore, data were compared by day within treatment and/or by treatment within day. A treatment x day interaction was detected (P = 0.024) for Zn remaining in the digesta post-dialysis. Zinc concentrations in rumen solid digesta were similar on d 1 for both treatments. On d 14, Zn concentration in the digesta of steers supplemented with HTM was greater (P = 0.01) than steers supplemented with STM. Post dialysis, the digesta collected from STM (P < 0.001) and HTM (P = 0.02) supplemented steers on day 1 had lesser Zn concentration compared to day 14.

***Protozoa and Bacteria mineral concentration***

The concentration of Cu, Mn, and Zn in protozoal and bacterial fractions in SRF and PAO are shown in Table 5. Trace mineral source had no impact (P > 0.10) on Cu, Mn, and Zn concentrations of particle-associated or strained ruminal fluid bacteria or protozoa.

**Discussion**

The present study indicates that Cu, Mn, and Zn from HTM have a lower solubility in water when compared to STM sources. The solubility of Cu from the current study is similar to the findings from Spears et al. (2004), where Cu from HTM was approximately 70% less soluble in water compared to Cu from STM. The solubility of Mn and Zn from the current study also follows the same trends as Cu, where Mn and Zn from HTM had a lower solubility compared to Mn and Zn from STM sources (Cao et al., 2000). Water is a dipole molecule, with a strong electronegative oxygen that attracts electrons from hydrogens, causing the hydrogens to have a partial positive charge (Murray et al., 2009). Due to the electrostatic forces of the dipole property of water, the sulfate molecule and the metal ion disassociate in water. In contrast, the covalent bond strength between hydroxychloride molecule and a particular element (e.g., Cu) within the crystalline matrix is greater than the electrostatic forces of water making the hydroxychloride-element molecule less soluble in water (Arthington, 2015). Manganese from HTM had a greater solubility when compared to Cu and Zn from HTM. The reason for the greater solubility is not well understood. Perhaps the reason for greater solubility in Mn from HTM in water compared to Cu and Zn from HTM sources is due to the difference in the number of protons between Mn (~25), Cu (~29), and Zn (~30). As the strength of a covalent bond depends on the extent of overlap of orbitals involved, the lower proton number in Mn may lead to a weaker positive force attraction towards the orbiting electrons compared to Cu and Zn, which may result in greater disassociation and solubility of HTM Mn in an aqueous environment.

When dissolved in water, STM sources of Cu, Mn, and Zn reduced pH to a greater extent than Cu, Mn, and Zn from HTM. This observed reduction in initial and final pH is due to the disassociation of metals (M; Cu, Mn, and Zn) and $SO_4^{2-}$ by the surrounding water molecules,

which are dipolar molecules that can hydrolyze weak ionic bond within $MSO_4$. The reaction between metals and water, $M^{2+} + 2H_2O \leftrightarrow MOH^+ + H_3O^+$, increases the concentration of $H_3O^+$, which leads to the reduction of pH. Initial pH values were greater for HTM vs STM of Cu, Mn, and Zn by 2.62, 1.51, and 1.15 pH units, respectively. After 24h incubation at 39°C, the solution containing Cu and Zn from HTM tended to have a greater final pH compared to STM. In general, the addition of Cu, Mn, and Zn from STM to water created a lower pH environment.

The rumen environment is very complex compared to controlled in vitro environments due to the presence of feed, microorganism populations, enzymes, ligands, etc. which can lead to mineral reduced mineral solubility and bioavailability. For example, high concentrations of soluble sulfur and molybdenum in the rumen can interact with soluble Cu and form insoluble Cu thiomolybdate complexes, which reduces Cu bioavailability (Dick et al., 1975; Suttle and Field, 1983). The diet used in the current study was low in TM antagonists, so the solubility characteristic may differ with higher TM antagonists in the diet. The in vitro rumen fermentation model can serve as a good indicator of what might be happening in the rumen, however, due to the buffering agents used, lower liquid-to-TM ratio, and the lack of removal of VFA from the system, interpretation of results is limited (Clarkson et al., 2021).

In the current study, Mn concentration in the digesta of steers fed HTM tended ($P = 0.098$) to be lower compared to STM on day 14, which may indicate that more Mn was released from HTM, but soluble Mn concentration was not different between treatments. A study conducted by Caldera et al. (2019) observed a lesser soluble Mn concentration at 4 and 8 h post-dosing, and higher soluble Mn concentration in ruminal fluid 24 h post-dosing in HTM-supplemented steers compared to STM-supplemented steers. Guimaraes et al. (2021) observed similar results when steers were fed medium-quality grass hay, where the soluble Mn

concentration in the rumen was greater at 4 and 6 h post-dosing, but lesser at 18 h post-dosing in steers receiving STM than HTM. In addition, higher soluble Zn and Cu concentrations were observed in steer dosed with STM compared to HTM (Caldera et al., 2019; Guimaraes et al., 2021). In the present study, soluble concentrations of Zn and Cu were not affected by TM source. The potential reason that contributes to the difference between the current study and previous studies may be how the TM was delivered to the animals. In this study, TM supplementation was added to the total mixed ration, while studies conducted by Caldera et al. (2019) and Guimaraes et al. (2021) utilized direct bolus dosing of TM treatments into the rumen. Genther and Hansen (2015) utilized dietary TM supplementation and observed lower rumen soluble Zn and Cu when expressed as a percent of whole rumen fluid concentration in HTM compared to STM supplemented cattle.

The binding strength of the mineral towards the digesta can also influence the bioavailability of TM. Although no difference was observed in Zn concentration in the digesta, Zn concentration in the digesta post-dialysis was greater in steers supplemented with HTM compared to STM on d 14, which indicates that Zn from HTM had a stronger binding strength toward the digesta compared to Zn from STM. The binding strength of Zn toward digesta changed over time. Post-dialysis, Zn concentration in the digesta was greater on day 14 compared to day 1 for both treatments, which may indicate that the binding strength increased over time. The amount of Cu and Mn not released following dialysis was not affected by TM source. In contrast, steers pulse dosed with HTM had a greater release of Cu and Zn from solid digesta than those dosed with STM following dialysis (Caldera et al., 2019).

Mineral concentration in protozoal and bacterial fractions of rumen contents was not influenced by trace mineral source. The methods used in this study were adapted from

Olubobokun et al. (1988) and De Mulder et al. (2017). Olubobokun et al. (1988) and De Mulder et al. (2017) utilized diaminopimelic acid analysis, differential interference microscopy, and 16S sequencing to determine the purity of their protozoa and bacteria fractions. The authors reported that after fractionation of ruminal contents via filtration and centrifugation, the protozoal fraction contained bacteria associated with small feed particles. Although not measured in the current study, it is likely that the protozoa fraction contained bacteria, which most likely contributed to the Cu, Mn, and Zn concentrations of the protozoal fraction. A study conducted by Gresakova et al. (2018) observed a higher Mn bacterial concentration in sheep fed Mn glycine hydrate compared to Mn sulfate. They also observed variations in Cu, Mn, and Zn concentrations in rumen protozoal and bacterial fractions when different Mn concentrations were supplemented. A potential reason for the discrepancies between studies may be due to the length of the feeding period. In the current experiment, the feeding period was 14 days compared to the study conducted by Gresakova et al. (2018), which was 16 weeks. A longer feeding period may be required to change the mineral concentration in the protozoa and bacteria population residing in the rumen.

**Conclusion**

Results from the in-vitro study of this experiment indicate that HTM is less soluble than STM in water and that STM Cu, Mn, and Zn decrease the solute pH at neutral pH environments. Hydroxy TM also contributed to a higher initial pH for all elements and tended to have a higher final 24 h pH for Zn and Cu compared to STM. Dietary intake of different mineral sources can potentially influence Mn solubility and Zn binding strength toward the digesta. This study did not observe changes in TM concentration in the protozoal and bacterial fraction of rumen fluid.

Longer term studies to investigate the interaction of TM source with rumen microorganisms are warranted.

**Table 2.1.** Ingredient and nutrient composition of the basal diet. (Experiment 2)

| Item | |
|---|---|
| Ingredient, % dry matter basis | |
| Corn silage | 27.64 |
| Cracked corn | 34.40 |
| Distiller's grains | 12.78 |
| Alfalfa hay | 10.91 |
| Wheat straw | 8.44 |
| Liquid supplement[1] | 5.00 |
| Limestone | 0.67 |
| Salt | 0.17 |
| Analyzed nutrient composition (DM basis) | |
| DM, % as fed | 59.8 |
| Crude protein, % | 16.3 |
| Acid detergent fiber, % | 18.6 |
| Neutral detergent fiber, % | 31.1 |
| Ether extract, % | 6.4 |
| NEg, Mcal/kg[2] | 1.08 |
| NEm, Mcal/kg[3] | 1.73 |
| Calcium, % | 0.95 |
| Magnesium, % | 0.27 |
| Phosphorus, % | 0.31 |
| Potassium, % | 0.89 |
| Sodium, % | 0.21 |
| Sulfur, % | 0.20 |
| Copper, mg/kg | 6.97 |
| Manganese, mg/kg | 56.51 |
| Zinc, mg/kg | 34.52 |
| Iron, mg/kg | 111.26 |
| Molybdenum, mg/kg | 0.64 |

[1]Liquid supplement provided in a molasses suspension: 3.72% NPN (urea), 14.07% Calcium, 0.11% Phosphorus, 1.81 % Potassium, 110,000 IU/kg vitamin A, 9.4 IU/kg vitamin E, and 440 g/metric ton of monensin (Rumensin 90, Elanco Animal Health, Greenfield, IN, USA).
[2]NEg = Net energy for gain.
[3]NEm = Net energy for maintenance.

**Table 2.2.** Influence of trace mineral source on in vitro initial and final pH and trace mineral solubility after 24 hours of incubation in deionized water at 39°C. (Experiment 1)

| Item | Treatment | | SEM[3] | P < |
|---|---|---|---|---|
| | HTM[1] | STM[2] | | |
| Initial pH | | | | |
| Cu | 8.16 | 5.54 | 0.5 | 0.03 |
| Mn | 7.60 | 6.10 | 0.3 | 0.01 |
| Zn | 7.32 | 6.17 | 0.2 | 0.01 |
| Final pH | | | | |
| Cu | 5.82 | 4.18 | 0.3 | 0.10 |
| Mn | 6.75 | 4.98 | 0.4 | 0.16 |
| Zn | 6.16 | 4.55 | 0.3 | 0.07 |
| Solubility (%)[4] | | | | |
| Cu | 1.34 | 70.35 | 13.1 | 0.01 |
| Mn | 13.88 | 93.75 | 15.1 | 0.01 |
| Zn | 3.13 | 98.23 | 17.4 | 0.01 |

[1]HTM = 0.025g of Cu from CuOHCl, 0.025g of Mn from MnOHCl, and 0.025g of Zn from ZnOHCl were incubated in 50mL of deionized water for 24 hours at 39°C.
[2]STM = 0.016g of Cu from $CuSO_4$, 0.022g of Mn from $MnSO_4$, and 0.039g of Zn from $ZnSO_4$ were incubated in 50mL of deionized water for 24 hours at 39°C.
[3]SEM = standard error of the mean.
[4]Solubility of each element following a 24-hour incubation in deionized water at 39°C

**Table 2.3.** Influence of trace mineral source on copper, manganese, and zinc concentrations of ruminal fluid and digesta fractions on day 1 and day 14 of the experiment. (Experiment 2)

| Item[4] | Day | | | | SEM[3] | P= | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 1 | | Day 14 | | | | | | |
| | HTM[1] | STM[2] | HTM[1] | STM[2] | | Trt | Day | Hour | Trt*Day |
| Digesta, mg/kg | | | | | | | | | |
| Cu | 29.70 | 30.02 | 22.41 | 25.70 | 2.6 | 0.611 | 0.085 | 0.152 | 0.650 |
| Mn | 127.10 | 162.06$^{x}$ | 141.21$^{a}$ | 234.04$^{b,y}$ | 21.7 | 0.217 | 0.013 | 0.924 | 0.087 |
| Zn | 153.91 | 153.98 | 187.62 | 210.20 | 20.1 | 0.724 | 0.058 | 0.757 | 0.625 |
| Soluble, mg/L | | | | | | | | | |
| Cu | 0.78 | 0.71$^{x}$ | 0.83 | 0.98$^{y}$ | 0.05 | 0.684 | 0.007 | 0.543 | 0.055 |
| Mn | 2.80 | 2.74 | 1.89 | 2.27 | 0.26 | 0.769 | 0.002 | <0.001 | 0.294 |
| Zn | 1.93 | 1.90 | 3.11 | 3.70 | 0.31 | 0.637 | <0.001 | 0.038 | 0.124 |

[1]HTM = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl.
[2]STM = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.
[3] SEM = standard error of the mean.
[4]The interaction of hour post-feeding (0, 2, and 4 h) or the interaction of hour post-feeding x treatment x day were not significant sources of variation in the model. Therefore, mineral concentrations were averaged for hour of sampling within a day.
[a,b]Different superscripts indicate a significant difference between treatments within day; P-value < 0.10.
[x,y]Different superscripts indicate a significant difference between days within the same treatment; P-value < 0.05.

**Table 2.4.** Influence of dialyzing rumen solid contents against EDTA-Tris buffer for 22 hours on the remaining copper, manganese, and zinc content of rumen solid digesta collected, averaged across 0, 2, and 4 hours post-feeding, on day 1 and day 14 of the experiment. (Experiment 2)

| Item[4] | Day | | | | SEM[3] | P= | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 1 | | Day 14 | | | | | | |
| | HTM[1] | STM[2] | HTM[1] | STM[2] | | Trt | Day | Hour | Trt*Day |
| Digesta, mg/kg | | | | | | | | | |
| Cu | 15.92 | 14.51 | 22.08 | 10.94 | 3.5 | 0.181 | 0.755 | 0.447 | 0.245 |
| Mn | 17.80 | 18.63 | 21.96 | 25.15 | 1.6 | 0.579 | 0.016 | 0.026 | 0.560 |
| Zn | 9.61$^{x}$ | 10.17$^{x}$ | 20.06$^{a,y}$ | 14.74$^{b,y}$ | 1.5 | 0.102 | <0.001 | 0.402 | 0.024 |

[1]HTM = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl.
[2]STM = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.
[3] SEM = standard error of the mean.
[4] The interaction of hour post-feeding (0, 2, and 4 h) or the interaction of hour post-feeding x treatment x day were not significant sources of variation in the model. Therefore, mineral concentrations were averaged for hour of sampling within a day.
$^{a,b}$Different superscripts indicate a significant difference between treatments within day; P-value < 0.05.
$^{x,y}$Different superscripts indicate a significant difference between days within the same treatment; P-value < 0.05.

**Table 2.5.** Influence of trace mineral source on copper, manganese, and zinc concentrations (mg/kg DM) in protozoa and bacteria associated with the liquid and solid phases of ruminal contents. (Experiment 2)

| Item | Treatment | | SEM[3] | P = |
|---|---|---|---|---|
| | HTM[1] | STM[2] | | |
| **Protozoa** | | | | |
| SRF[4] | | | | |
| Cu | 82.91 | 95.42 | 4.1 | 0.149 |
| Mn | 401.71 | 358.97 | 42.8 | 0.654 |
| Zn | 484.17 | 458.72 | 18.2 | 0.666 |
| PAO[5] | | | | |
| Cu | 100.08 | 101.01 | 3.5 | 0.906 |
| Mn | 330.76 | 337.35 | 27.1 | 0.915 |
| Zn | 602.86 | 580.25 | 26.1 | 0.589 |
| **Bacteria** | | | | |
| SRF[4] | | | | |
| Cu | 80.51 | 105.77 | 17.8 | 0.526 |
| Mn | 312.35 | 313.34 | 25.4 | 0.986 |
| Zn | 456.95 | 570.88 | 85.6 | 0.555 |
| PAO[5] | | | | |
| Cu | 75.68 | 73.55 | 6.0 | 0.879 |
| Mn | 321.35 | 288.84 | 19.4 | 0.445 |
| Zn | 547.08 | 480.49 | 30.7 | 0.318 |

[1]HTM = Rumen contents were obtained from steers supplemented with 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl for at least 14 days.
[2]STM = Rumen contents were obtained from steers supplemented with 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$ for at least 14 days.
[3]SEM = standard error of the mean.
[4]Strained ruminal fluid.
[5]Particle-associated microorganisms

**References**

AOAC, I. 2016. Official Methods of Analysis of AOAC International - 20th Edition, 2016. 20th Edition. (G. W. Latimer, editor.). AOAC International, Rockville, Maryland, USA. Available from: https://www.techstreet.com/standards/official-methods-of-analysis-of-aoac-international-20th-edition-2016?product_id=1937367

Arthington, J. 2015. New Concepts in Trace Mineral Supplementation of Grazing Cattle. 26th Annual Florida Ruminant Nutrition Symposium. 104–117.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software. 67:1–48. doi:10.18637/jss.v067.i01.

Caldera, E., B. Weigel, V. N. Kucharczyk, K. S. Sellins, S. L. Archibeque, J. J. Wagner, H. Han, J. W. Spears, and T. E. Engle. 2019. Trace mineral source influences ruminal distribution of copper and zinc and their binding strength to ruminal digesta1,2,3. J Anim Sci. 97:1852–1864. doi:10.1093/jas/skz072.

Cao, J., P. R. Henry, R. Guo, R. A. Holwerda, J. P. Toth, R. C. Littell, R. D. Miles, and C. B. Ammerman. 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. J Anim Sci. 78:2039–2054. doi:10.2527/2000.7882039x.

Clarkson, A. H., S. W. Paine, and N. R. Kendall. 2021. Evaluation of the solubility of a range of copper sources and the effects of iron & sulphur on copper solubility under rumen simulated conditions. Journal of Trace Elements in Medicine and Biology. 68:126815. doi:10.1016/j.jtemb.2021.126815.

Craig, W. M., B. J. Hong, G. A. Broderick, and R. J. Bula. 1984. In Vitro Inoculum Enriched with Particle-Associated Microorganisms for Determining Rates of Fiber Digestion and Protein Degradation. Journal of Dairy Science. 67:2902–2909. doi:10.3168/jds.S0022-0302(84)81653-7.

Daniel, J. B., S. K. Kvidera, and J. Martín-Tereso. 2020. Total-tract digestibility and milk productivity of dairy cows as affected by trace mineral sources. J Dairy Sci. 103:9081–9089. doi:10.3168/jds.2020-18754.

De Mulder, T., K. Goossens, N. Peiren, L. Vandaele, A. Haegeman, C. De Tender, T. Ruttink, T. V. de Wiele, and S. De Campeneere. 2017. Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. FEMS Microbiology Ecology. 93:fiw251. doi:10.1093/femsec/fiw251.

Dick, A. T., D. W. Dewey, and J. M. Gawthorne. 1975. Thiomolybdates and the copper–molybdenum–sulphur interaction in ruminant nutrition. The Journal of Agricultural Science. 85:567–568. doi:10.1017/S0021859600062468.

Durand, M., and R. Kawashima. 1980. Influence of minerals in rumen microbial digestion. In: Y. Ruckebusch and P. Thivend, editors. Digestive Physiology and Metabolism in Ruminants: Proceedings of the 5th International Symposium on Ruminant Physiology, held at Clermont —

Ferrand, on 3rd–7th September, 1979. Springer Netherlands, Dordrecht. p. 375–408. Available from: https://doi.org/10.1007/978-94-011-8067-2_18

Edgell, K. 1989. USEPA method study 37 SW-846 method 3050 acid digestion of sediments, sludges, and soils. Available from: https://cfpub.epa.gov/si/si_public_record_Report.cfm?Lab=ORD&dirEntryId=30330

Faulkner, M. J., and W. P. Weiss. 2017. Effect of source of trace minerals in either forage- or by-product-based diets fed to dairy cows: 1. Production and macronutrient digestibility. J Dairy Sci. 100:5358–5367. doi:10.3168/jds.2016-12095.

Genther, O. N., and S. L. Hansen. 2015. The effect of trace mineral source and concentration on ruminal digestion and mineral solubility. Journal of Dairy Science. 98:566–573. doi:10.3168/jds.2014-8624.

Gresakova, L., K. Venglovska, and K. Cobanova. 2018. Nutrient digestibility in lambs supplemented with different dietary manganese sources. Livestock Science. 214:282–287. doi:10.1016/j.livsci.2018.07.001.

Guimaraes, O., S. Jalali, J. J. Wagner, J. W. Spears, and T. E. Engle. 2021. Trace mineral source impacts rumen trace mineral metabolism and fiber digestion in steers fed a medium-quality grass hay diet. Journal of Animal Science. 99:skab220. doi:10.1093/jas/skab220.

Guimaraes, O., J. J. Wagner, J. W. Spears, V. L. N. Brandao, and T. E. Engle. 2022. Trace mineral source influences digestion, ruminal fermentation, and ruminal copper, zinc, and manganese distribution in steers fed a diet suitable for lactating dairy cows. Animal. 16:100500. doi:10.1016/j.animal.2022.100500.

John, F., and W. Sanford. 2019. An R Companion to Applied Regression. Third edition. Available from: https://us.sagepub.com/en-us/nam/an-r-companion-to-applied-regression/book246125

Jones, A. O. L., M. R. S. Fox, and B. E. Jr. Fry. 1985. In vitro assessment of zinc binding to protein foods as a potential index of zinc bioavailability. Comparison of in vitro and in vivo data. J. Agric. Food Chem. 33:1123–1128. doi:10.1021/jf00066a026.

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen. 2017. lmerTest Package: Tests in Linear Mixed Effects Models. Journal of Statistical Software. 82:1–26. doi:10.18637/jss.v082.i13.

Lenth, R. V., B. Bolker, P. Buerkner, I. Giné-Vázquez, M. Herve, M. Jung, J. Love, F. Miguez, H. Riebl, and H. Singmann. 2024. emmeans: Estimated Marginal Means, aka Least-Squares Means. Available from: https://cran.r-project.org/web/packages/emmeans/index.html

Miller, M. D., J. S. Lanier, S. K. Kvidera, H. M. Dann, C. S. Ballard, and R. J. Grant. 2020. Evaluation of source of corn silage and trace minerals on rumen characteristics and passage rate of Holstein cows. Journal of Dairy Science. 103:8864–8879. doi:10.3168/jds.2020-18490.

Murray, R. K., D. A. Bender, K. M. Botham, P. J. Kennelly, V. W. Rodwell, and A. P. Weil. 2009. Harper's Illustrated Biochemistry 28th edition. 28th Edition. The McGraw-Hill Companies, Inc. Available from: https://awesomechem.files.wordpress.com/2016/10/harpers-illustrated-biochemistry-28th-ed-robert-k-murray-et-al-mcgraw-hill-2009.pdf

Olubobokun, J. A., W. M. Craig, and W. A. Nipper. 1988. Characteristics of Protozoal and Bacterial Fractions from Microorganisms Associated with Ruminal Fluid or Particles2. Journal of Animal Science. 66:2701–2710. doi:10.2527/jas1988.66102701x.

R Core Team. 2023. R: A language and environment for statistical computing. Available from: https://www.R-project.org/

Spears, J. W., E. B. Kegley, and L. A. Mullis. 2004. Bioavailability of copper from tribasic copper chloride and copper sulfate in growing cattle. Animal Feed Science and Technology. 116:1–13. doi:10.1016/j.anifeedsci.2004.06.002.

Suttle, N. F., and A. C. Field. 1983. Effects of dietary supplements of thiomolybdates on copper and molybdenum metabolism in sheep. Journal of Comparative Pathology. 93:379–389. doi:10.1016/0021-9975(83)90025-7.

# CHAPTER 3 - TRACE MINERAL SOURCE INFLUENCES IN VITRO FERMENTATION CHARACTERISTICS AND TRACE MINERAL SOLUBILITY


## Summary

Two experiments were conducted to determine: 1) the impact of strained rumen fluid (SRF) alone or SRF with particle-associated microorganisms (PAO) included and dilution on in vitro dry matter digestibility (DMD) and 2) the impact of trace mineral (TM) source on in vitro fermentation characteristics and TM solubility under simulated abomasal and intestinal conditions. In Experiment 1, three cannulated steers were adapted to a diet formulated to meet the nutrient requirements for lactating dairy cows. Strained RF was obtained by straining rumen content through 2 layers of cheesecloth. Half of the remaining digesta was washed with McDougall's buffer and filtered through 2 layers of cheesecloth to obtain PAO. Both SRF and PAO were filtered again through 8 layers of cheesecloth. Strained RF was mixed with either McDougall's buffer (SRF) or PAO (SRF+PAO) at a ratio of 1:2 or 1:4 and incubated at 39°C for 12 h using the ground basal diet as the substrate. Digestibility of DM was greater in digestion tubes containing SRF and SRF+PAO at a 1:2 ratio. In Experiment 2, eight steers fitted with ruminal cannula were blocked by body weight and assigned to one of two treatment groups. Treatments consisted of 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either: 1) sulfate (STM) or 2) hydroxychloride (HTM) sources. Steers were housed in individual pens and fed the same diet as described in experiment 1. Dietary TM treatments were mixed with dried distillers grains and mixed in the diet, by hand, immediately after basal diet delivery. Dietary treatments were fed for 14 d. On day 15, SRF+PAO was collected from each steer (STM-RF and HTM-RF) and used in a series of in vitro crossover experiments. In vitro substrates (S) used were the ground diets consumed by the animals on each treatment (STM-S and HTM-S). Incubations

containing HTM-S had greater ($P < 0.01$) total VFA concentration and propionic acid molar proportions, but lesser ($P < 0.01$) acetic acid molar proportions than STM-S. Rumen fluid from steers supplemented with HTM had a greater ($P < 0.03$) total VFA than STM-RF at 24h post incubation. After 12 h post incubation, the molar proportion of propionic acid in HTM-RF was lesser ($P = 0.04$) than STM-RF. After simulated abomasal digestion, soluble Mn concentration in HTM-S was greater ($P < 0.01$) than STM-S. These data indicate that the source of trace minerals can influence in vitro rumen fermentation characteristics and Mn solubility under simulated abomasal conditions.

**Introduction**

Ruminal in vitro techniques are a widely used methodology in animal nutrition research for studying ruminal digestion and fermentation characteristics (Craig et al., 1984; Vinyard and Faciola, 2022). The ruminal in vitro method was first introduced by Tilley and Terry (1963), where a mixture of 10 mL of strained rumen fluid (SRF) and 40 mL of McDougall's solution (McDougall, 1948) was used for incubation. This method is still widely used by many investigators examining rumen fermentation characteristics (Mabjeesh et al., 2000; Gifford et al., 2021; Tangredi et al., 2023). However, particle-associated microorganisms (PAO) make up a major proportion of total rumen microorganisms. When PAO was combined with SRF microorganisms, greater fiber digestion, protein digestion, and enzyme activity were observed when compared to SRF and McDougall's buffer mixture (Brock et al., 1982; Craig et al., 1984; Craig et al., 1987). Therefore, understanding the impact of PAO in combination with SRF is important when using an in vitro system to study the impact of dietary supplements on rumen fermentation characteristics.

Copper (Cu), zinc (Zn), and manganese (Mn) are essential trace minerals (TM), but excess amounts of these minerals can have negative impacts on rumen fermentation (Slyter and Wolin, 1967; Forsberg, 1978; Durand and Kawashima, 1980). Trace mineral source can influence TM solubility in the rumen and a higher concentration of soluble Cu and Mn has been reported to decrease in vitro rumen fermentation (Slyter and Wolin, 1967; Forsberg, 1978; Cao et al., 2000). Faulker and Weiss (2017) were the first to observe a greater fiber digestibility when hydroxy trace mineral (HTM) sources of Cu, Mn, and Zn were supplemented to lactating dairy cows compared to sulfate trace mineral (STM) sources. Furthermore, steers that received Cu, Zn, and Mn in STM had lesser fiber digestibility and total ruminal volatile fatty acid (VFA) concentrations compared to steers that received iso-concentrations of Cu, Zn, and Mn from HTM (Guimaraes et al., 2021). However, the mechanism(s) of the influence of soluble TM on rumen fermentation characteristics is unknown. Using in vitro techniques to investigate fermentation characteristics in the rumen and mimic different portions of the gastrointestinal tract to study mineral solubility is common practice and helps to provide some insight into the impact of trace mineral source on fermentation characteristics and mineral solubility. Historically, treatments of interest are typically added to the substrate used for in vitro fermentation, and rumen fluid from common donor animals, not receiving the treatments of interest is used. Therefore, two experiments were conducted to determine: 1) the impact of PAO inclusion in SRF and the ratio with McDougall's buffer on in vitro dry matter disappearance (DMD) and 2) the impact of TM source on in vitro rumen fermentation characteristics and TM solubility in simulated abomasal and intestinal environments. We hypothesized that SRF containing PAO organisms with a lower dilution ratio with McDougall's buffer would have greater in vitro DMD compared to RF without PAO and RF with a higher dilution ratio with McDougall's buffer. For experiment 2, we

hypothesized that HTM sources of Cu, Mn, and Zn would have improved in vitro DMD, fiber digestibility, total VFA, and higher solubility in simulated abomasal and intestinal conditions compared to STM sources of Cu, Mn, and Zn.

## Materials and methods

### *Ethical Statement*

Prior to the initiation of this experiment, all animal care, handling, and procedures described herein were approved by the Colorado State University Animal Care and Use Committee (CSU IACUC approval #1555 and #3574)

### *Animals and Diets*

Eight crossbred Angus steers (509 kg ± 10 kg BW; approximately 1.5 years of age) fitted with ruminal cannula were acclimated to a corn-corn silage-based diet described by Loh et al. (2024; basal diet: DM: 59.8%, CP: 16.29%, ADF: 18.63%, NDF: 31.08%, NEg: 1.08 Mcal/kg, NEm: 1.73 Mcal/kg, TDN: 71.39%, calcium: 0.95%, phosphorus: 0.31%, sulfur: 0.2%, copper: 7 mg/kg, manganese: 56.5 mg/kg, and zinc: 34.5 mg/kg; Supplementary Table S1) for 21 days. The diet was formulated to meet or exceed NASEM (NASEM, 2021) requirements for lactating dairy cattle with the exception of Cu, Mn, and Zn.

### *Experiment 1: In Vitro Method Evaluation*

Three steers were randomly selected from the steers described above for ruminal in vitro method evaluation. Rumen fluid collection methods were adapted from procedures described by Craig et al. (1984) with slight modifications as described below. The basal diet, described above, was collected, dried, and ground through a 2 mm screen using a Wiley Mill. Rumen contents from 3 cannulated steers were collected 2h post-feeding, squeezed through 2 layers of cheesecloth, and mixed to obtain strained ruminal fluid (SRF). Strained rumen fluid was filtered through 8 layers of cheesecloth and mixed with McDougall's buffer at 1:2 and 1:4 ratios (SRF:

McDougall's buffer) to generate 2 digestion solutions: SRF 1:2 and SRF 1:4. Half of the remaining rumen contents (digesta) from obtaining SRF were placed in thermoses containing pre-warmed (39°C) McDougall's buffer. Pre-warmed McDougall buffer was used to rinse and flush the digesta to remove PAO from the solid digesta. The McDougall's buffer-PAO solution (abbreviated as PAO) was mixed with SRF at 1:2 and 1:4 ratios (SRF: McDougall's buffer-PAO solution). The mixtures were then filtered through 8 layers of cheesecloth to generate other 2 digestion solutions: SRF+PAO 1:2 and SRF+PAO 1:4. A total of 4 different digestion solutions were used in this experiment: 1) SRF 1:2, 2) SRF 1:4, 3) SRF+PAO 1:2, and 4) SRF+PAO 1:4 (**Figure 1**). Thirty milliliters of digestion solution were added into 50 mL conical tube containing 0.5 g of ground basal diet and incubated at 39°C for 12h (3 replicates/digestion solution; n = 12). After incubation, conical tubes were centrifuged at 2000 x *g* for 20 minutes and the supernatant was discarded. The pellet was dried at 60°C for 48h and DMD was determined.

***Experiment 2: Animals and Trace Mineral Supplementation***

Animals, trace mineral supplementation, and total Cu, Mn, and Zn intake are described in Loh et al. (2024). In brief, eight steers fitted with ruminal cannula were blocked by BW and assigned randomly to one of two treatment groups. Treatments consisted of 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either 1) sulfate (STM) or 2) hydroxychloride (HTM). Steers were housed in individual pens equipped with automatic waterers and concrete feed bunks. Steers were fed the corn-corn silage-based diet, described in Experiment 1, once daily in the morning. Dietary TM treatments were mixed with dried distillers grains (200g) and mixed in the diet, by hand, immediately after basal diet delivery. Dietary treatments were fed for 14 d. On day 15, rumen fluid was collected from each steer and used in a series of in vitro experiments.

***In vitro incubations - rumen***

Prior to initiating the in vitro experiments, the basal diet was ground using a Wiley Mill fitted with a 2 mm screen. The ground basal diet was mixed with 10 mg Cu, 40 mg Mn, and 60 mg Zn/kg DM from either: 1) STM (STM-S) or 2) HTM (HTM-S) and was used as the substrate for in vitro fermentation. Rumen fluid was collected from each steer (n=4/treatment) using the SRF+PAO method at a 1:2 (SRF:PAO) ratio on d 15 of the experiment as outlined in experiment 1. Total volume of the digestion solution and substrate amount were modified to allow for DMD and NDF analysis. Briefly, 150 mL of SRF+PAO at 1:2 ratio (SRF: McDougall's buffer-PAO solution) from each steer receiving STM (STM-RF) or HTM (HTM-RF) was added into 250 mL Erlenmeyer flasks containing 5.0 g of either STM-S or HTM-S in triplicate, using a cross over design (3 replicates/time point (0, 12 and 24 h)/substrate (STM-S or HTM-S)/8 steers (STM-RF or HTM-RF), n = 144; Figure 2). The digestion vessels were incubated for 12 and 24 h at 39°C with digestion vessel being hand swirled every 4 h. After each incubation time, the contents in 250 mL Erlenmeyer were centrifuged at 2000 x *g* for 15 minutes. After centrifugation, 1.6 mL of supernatant was transferred into microcentrifuge tubes containing 0.4 mL of 25% metaphosphoric acid. The microcentrifuge tubes were then stored at -20°C until VFA analysis could be conducted. The remaining contents in each digestion tube were dried at 60°C and used to determine the digestibility of DM (DMD) and neutral detergent fiber (NDFD). DMD was calculated using the following equation: $\frac{(initial\ weight - remaining\ weight)}{initial\ weight} \times\ 100\%$. Dried samples were then ground using a pestle and mortar before NDF analysis using Ankom 200 Fiber analyzer (ANKOM Technology, NY, USA).

***In vitro incubations - abomasum and intestine***

At the same time rumen contents were collected for in vitro incubation, a second sampling was conducted to determine the impact of TM source on Cu, Mn, and Zn solubility using a sequence of in vitro pH changes and enzyme additions to mimic the abomasal and duodenal environments. Diet preparation and rumen fluid collection were similar as previously described for the rumen in vitro incubation, with slight modifications. Briefly, after obtaining rumen fluid from the steers (HTM-RF or STM-RF; n=4/treatment), 30 mL of SRF+PAO at 1:2 (SRF:PAO) ratio was added into 50 mL conical tubes containing 0.5 g of diet + substrate treatment (HTM-S or STM-S) and incubated at 39°C for 24 h (3 replicates/substrate source/8 steers, n = 48). After the 24 h incubation, the 50 mL digestion tubes were centrifuged at 2000 x g, and then abomasal and intestinal digestion was mimicked by using procedures described by Ward and Spears (1993) with slight modifications. Briefly, abomasal digestion was simulated by adding a mixture of 1.5% pepsin (CAS 9001-75-6; Acros Organics) in 0.97 M HCl to fermentation vessels to lower the pH to 2.5. The acidified in vitro vessels were incubated for 2 h at 39°C. After the 2 h incubation, tubes were centrifuged at 2000 x *g* for 15 minutes and 2 mL of the supernatant was removed to determine abomasal soluble Cu, Mn, and Zn concentrations. The pH of the remaining solution in the digestion tube was then increased to approximately 6.5 by the addition of 3.5% pancreatin (8 x USP; CAS: 8049-47-6; Sigma-Aldrich) in a 1 M $NaHCO_3$ solution. The mixture was mixed and incubated for another 2 h at 39°C. Following incubations, the mixture was centrifuged at 2000 x g to obtain supernatant fraction for mineral analysis.

***Mineral analysis***

Liquid samples were digested using an open vessel digestion method adapted from Edgell (1989) with slight modification. Briefly, 2 mL of samples and 2 mL of 70% nitric acid (trace element grade; ThermoFisher, Waltham, MA) were added into 15 mL conical tubes. The tubes

were left to stand at room temperature for 10 min and then incubated in a 70°C water bath for 4 h with occasional mixing. Two mL of 30% hydrogen peroxide was then added to each tube, and the tubes were left to react at room temperature for 10 minutes before being placed in a 70°C water bath for another 2h. All tubes were brought to a total volume of 10 mL with deionized water before analysis for Cu, Mn, and Zn using an atomic absorption spectrometer (PinAAcle 500, PerkinElmer, Waltham, MA).

***Statistical analysis***

Power calculation was reconducted with data obtained from experiment 1 using Russ Lenth's power calculator (Lenth, 2006). All analyses in this experiment were performed using R Statistical software version 4.3.2 and packages within R (R Core Team, 2023). For experiment 1, a linear model was fitted. A Type III Analysis of Variance was conducted using Tukey HSD methods to investigate the interaction between method (SRF vs SRF+PAO) and ratio (1:2 vs 1:4) on in vitro DMD. For experiment 2, a linear mixed model was fitted with the restricted maximum likelihood method to investigate the effects of all two and three-way interactions with animals considered as the random variable (package lme4 ver. 1.1-32; Bates et al., 2015; Kuznetsova et al., 2017). Models from experiment 2 were analyzed with Type III Analysis of Variance (car ver. 3.1-1; John and Sanford, 2019) conducted using the Kenward-Roger method. Estimated marginal means between groups were estimated and pairwise comparisons were conducted using the emmeans package (ver. 1.8.5; Lenth et al., 2024). For all response variables, a p-value less than or equal to 0.05 was considered significant.

## Results

### *Experiment 1 (In Vitro Method Evaluation)*

The power of this study is 0.86, by using n = 6, levels of treatment = 2, SD = 1.48, alpha = 0.05, and detectable contrast = 2.5. The influence of SRF and SRF+PAO and dilution ratio on in vitro dry matter disappearance is shown in Figure 3 (means, standard error of the means, and p-values for main effects and interaction are provided in Supplementary Table S2). There was a two-way interaction ($P < 0.001$) between method (SRF or SRF+PAO) and ratio (1:2 or 1:4). Following a 12 h digestion, SRF+PAO at a 1:4 ratio had a lesser ($P < 0.001$) DMD compared to SRF+PAO with 1:2 ratio and SRF with a 1:2 and 1:4 ratio. Strained rumen fluid at a 1:4 ratio with McDougall's buffer has a lesser ($P < 0.03$) DMD compared to SRF and SRF+PAO at a 1:2 ratio. Strained rumen fluid and SRF+PAO at a 1:2 ratio had similar ($P = 0.94$) DMD after incubation at 39°C for 12 h.

### *Experiment 2*

The influence of rumen fluid source and substrate trace mineral source of Cu, Mn, and Zn on in vitro DMD and NDFD is shown in Table 1. Dry matter disappearance and NDFD were not influenced ($P > 0.2$) by rumen fluid source, substrate treatment, or their interaction.

The influence of rumen fluid source and substrate trace mineral source of Cu, Mn, and Zn on in vitro VFA composition is shown in Table 2. There were no three-way interactions ($P > 0.05$) detected between rumen fluid source, substrate treatment, and time. Therefore, only two-way interactions and main effects are reported. There was a rumen fluid source x substrate source interaction ($P < 0.02$) for total VFA molar concentrations and molar proportion of acetic acid and propionic acid. Fermentation vessels containing HTM-S had greater ($P < 0.01$) total VFA and molar proportions of propionic acid, but lesser ($P < 0.01$) molar proportions of acetic

acid than STM-S, averaged across time when rumen fluid from HTM-supplemented steers (HTM-RF) was used for incubation. There was also a rumen fluid x hour interaction observed for total VFA and molar proportion of acetic acid, propionic acid, and butyric acid. Steers receiving dietary HTM supplementation had greater ($P = 0.0246$) total VFA molar concentrations than STM supplemented group at 24 h post incubation, averaged over substrate treatment. The molar proportion of acetic acid at 0h was greater ($P < 0.001$) than at 12h and 24h in both STM-RF and HTM-RF, and greater ($P = 0.001$) at 12h compared to at 24h in HTM-RF. At 12 h post-incubation, molar proportions of propionic acid in vessels incubated with HTM-RF were lower ($P = 0.037$) than molar proportions of propionic acid in vessels incubated with STM-RF. The molar proportion of propionic acid at 12h and 24h were greater ($P < 0.001$) than 0h in both STM-RF and HTM-RF. The molar proportion of butyric acid at 0h was lesser ($P < 0.001$) than 12h and 24h in both STM-RF and HTM-RF. The molar proportion of butyric acid at 12h was lesser ($P = 0.001$) than at 24 h in HTM-RF. The molar proportion of isobutyric acid was greater ($P = 0.009$) in digestion vessels containing HTM-RF compared to digestion vessels containing STM-RF.

The effect of rumen fluid source and substrate treatment on in vitro simulated abomasal and intestinal TM solubility are shown in Table 3. There were no 2-way interactions between the rumen fluid source and substrate treatment ($P > 0.20$), therefore, only main effects are reported. Following in vitro simulated abomasal digestion of solid digesta, soluble Mn concentration in HTM-S was greater ($P < 0.001$) than in STM-S. Intestinal soluble Cu, Mn, and Zn concentrations and abomasal soluble Zn and Cu were not influenced ($P > 0.05$) by treatment.

**Discussion**

The present study compared in vitro DMD when incubated with SRF or SRF+PAO at a 1:2 vs 1:4 ratio with McDougall's buffer for 12 h. In general, a greater DMD was observed when

a 1:2 ratio of SRF with McDougall's buffer or SRF+PAO was used compared to a 1:4 ratio dilution, and both 1:2 ratio of digestion solutions produced similar DMD. Dry matter disappearance was lower in SRF and SRF+PAO at a 1:4 ratio, with SRF+PAO having the lowest DMD. The improvement in dry matter digestibility observed in SRF or SRF+PAO with a 1:2 ratio may be due to an increase in microorganism numbers and/or species that aid with digestion (Senshu et al., 1980; Craig et al., 1984; Craig et al., 1987). Although VFA was not analyzed in experiment 1, the utilization of PAO+SRF was suggested to generate a similar VFA pattern compared to in vivo experiments, which provides a better prediction of VFA composition (Senshu et al., 1980). In addition, a higher in vitro rate of fiber digestion was observed when PAO was incorporated (Craig et al., 1984). With the findings from current study along with previous literature, SRF+PAO at 1:2 ratio was used for experiment 2.

In vitro DMD and NDFD were not influenced by TM sources in the current study, which is similar to the findings by Genther and Hansen (2015). Genther and Hansen (2015) supplemented different concentrations (low or high) and sources (STM or HTM) of Cu, Mn, and Zn through dietary inclusion for 12 d. They measured in situ DMD and NDF at 6, 12, 24, and 36h and did not observe an effect of TM source on in situ DMD and NDFD. In contrast, lower NDFD was observed in lactating dairy cows (Faulkner and Weiss, 2017) and steers (Caldera et al., 2019; Guimaraes et al., 2021; Guimaraes et al., 2022) supplemented with STM compared to HTM-supplemented cattle. The discrepancies in findings between studies may be due to differences in TM supplementation concentration (5/10/20/25mg Cu, 15/20/32/40/60mg Mn, and 30/35/60/12mg Zn/DM), technique used (in-situ degradability, total tract digestibility, or in vitro degradability), and diet composition (varies in fiber and starch proportions; Genther and Hansen, 2015; Faulkner and Weiss, 2017; Caldera et al., 2019; Guimaraes et al., 2021; Guimaraes et al.,

2022). In addition, the discrepancies in results may be due to the difference in the liquid-to-solid ratio between in vivo studies and the in vitro model used in this study. Studies conducted by Reynolds et al. (2004) and Park et al. (2011) reported a liquid-to-solid ratio of approximately 6:1 in early lactation dairy cows. The liquid-to-solid ratio in the current study was 30:1, which was 5 times more diluted than the rumen environment of lactating dairy cows. A more diluted liquid-to-solid ratio may impact in vitro response variables. Future in vitro experiments should take into consideration the rumen liquid-to-solid ratio.

Total VFA molar concentrations were lesser by approximately 20% in STM-S compared to HTM-S when HTM-RF was used for incubation. The total VFA molar concentration in the digestion vessel incubated with HTM-RF is greater than digestion vessels incubated with STM-RF by approximately 40% at 24h post incubation. The in vitro data from the current experiment suggest that both long-term (rumen fluid source) and short-term (substrate treatment) HTM supplementation improved total VFA production. Previous research reported greater total VFA in the rumen of steers supplemented with HTM compared to STM when fed a dairy-type diet (Guimaraes et al., 2022) and medium-quality grass hay diet (Guimaraes et al., 2021). This study also observed a lower acetic acid molar proportion and a higher molar proportion of propionic acid in HTM-S. This suggests an increase in starch digestion was occurring in HTM-S (Bannink et al., 2006). The molar proportion of butyric acid was not influenced by TM treatment in this study, which is similar to previous findings reported by Guimaraes et al. (2022). In contrast, studies conducted by Guimaraes et al. (2021) and Daniel et al. (2020) reported a greater molar proportion of butyric acid in the rumen fluid of steers supplemented with STM.

Soluble Mn concentration was greater in HTM-S by about 7% than STM-S following simulated abomasal digestion, which may indicate more Mn from HTM may be more available

for absorption by the small intestine after immediate release from the abomasum into the duodenum. However, in vitro soluble Mn concentration did not differ across treatments following simulated intestinal digestion. This suggests that Mn from both sources may be similarly soluble and available for absorption after the increase in pH in the later portions of the small intestine. In contrast, soluble Cu and Zn concentrations were not affected by the treatment following simulated abomasal digestion. The solubilities of Cu and Zn from this study are supported by previous in vitro studies conducted by Spears et al. (2004) and Cao et al. (2000). These researchers reported similar solubility for Cu and Zn from different sources when HCl was used to lower the pH of the solution in vitro.

**Conclusion**

Factors impacting Cu, Mn, and Zn solubility in different portions of the gastrointestinal tract of beef cattle are extremely complex and challenging to study. The current experiment utilized rumen fluid from animals that had received dietary treatments of interest prior to extracting the rumen fluid for in vitro use. Data indicate that rumen fluid obtained from cattle receiving different trace mineral sources prior to collection impacted fermentation characteristics and solubility as indicated by a rumen fluid x substrate interaction. Furthermore, these data also indicate that using different dilutions of rumen fluid to buffer ratio and the microbial composition of the rumen fluid used for in vitro analysis can alter DMD. Future research is needed to investigate the mechanisms by which trace minerals are solubilized, absorbed, and metabolized in the digestive tract of beef cattle. A better understanding of trace mineral solubility in the gastrointestinal tracts will allow for more accurate trace mineral supplementation to ruminants.

## Tables and Figures

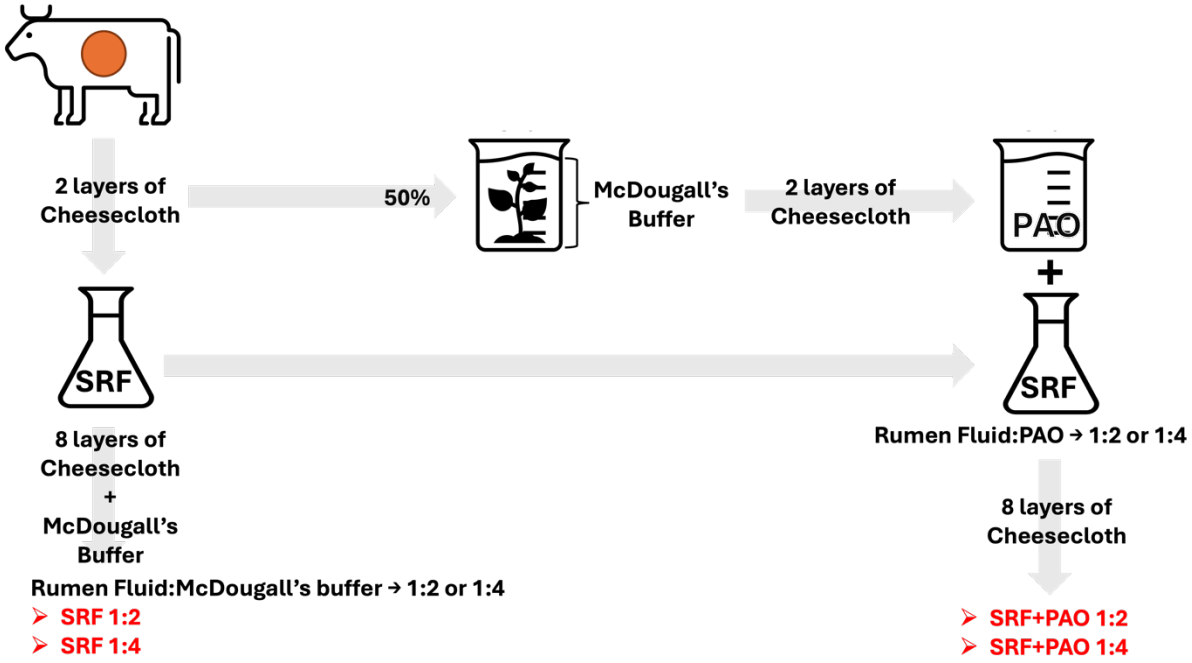


Figure 3.1. Experimental design for experiment 1.

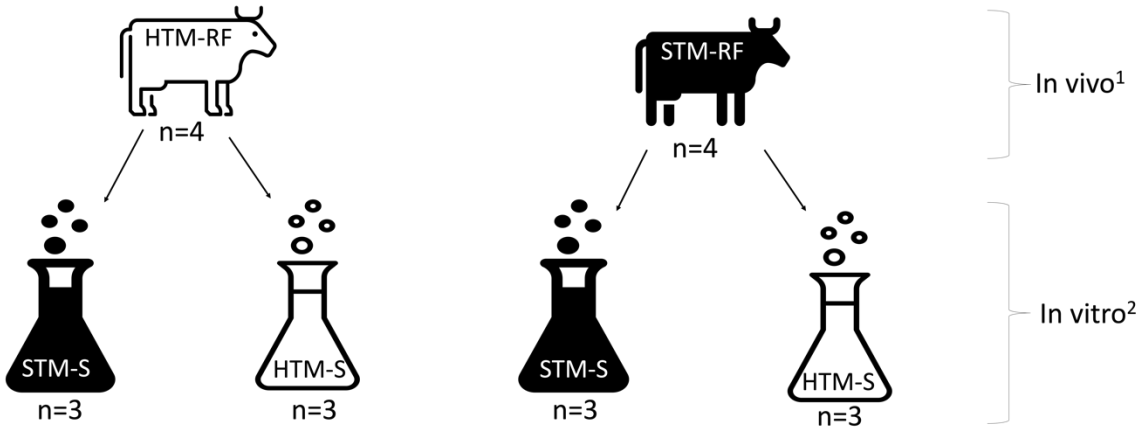


Figure 3.2. Experimental design for experiment 2. [1]Rumen fluid was collected from steers that received the following dietary TM treatment for 14 days: HTM-RF = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl; STM-RF = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$. Rumen fluid was used as the digestion solution for the in vitro experiment. [2]Total mixed rations were mixed with the following treatment and used as the substrate for in vitro fermentation vessels: HTM-S = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl; STM-S = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

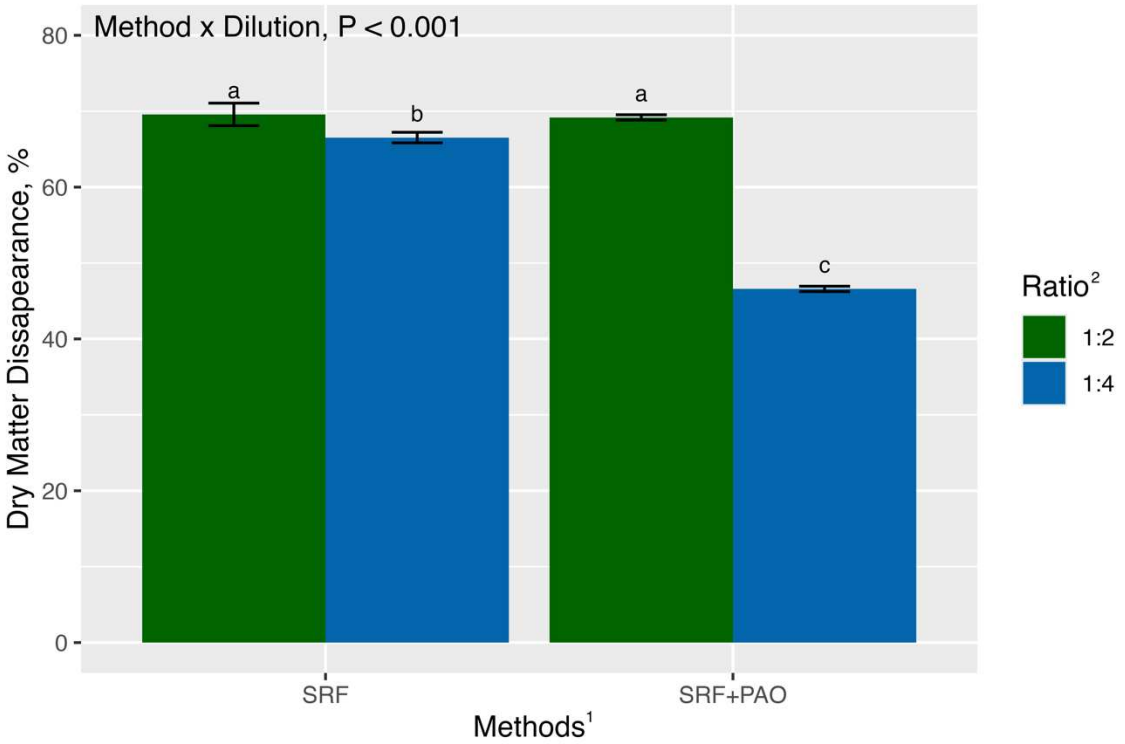


Figure 3.3. Influence of strained rumen fluid (SRF) or SRF with particle-associated microorganisms (PAO) and dilution ratio on in vitro dry matter disappearance after incubation at 39°C for 12 hours. [1]Methods: SRF = Strained Rumen fluid; or SRF + PAO = Strained Rumen fluid + particle-associated microorganisms. [2]Ratio: 1:2 = Strained Rumen fluid: McDougall's buffer or Strained Rumen fluid: particle-associated microorganisms, 1:2 ratio; or 1:4 = Strained Rumen fluid: McDougall's buffer or Strained Rumen fluid: particle-associated microorganisms, 1:4 ratio. [a,b,c] = Means with different superscript differ; p-value < 0.05.

Table 3.1. Influence of trace mineral source on dry matter (DM) and neutral detergent fiber (NDF) digestibility from rumen fluid obtained from canulated beef steers receiving either hydroxy or sulfate trace mineral sources of copper, manganese, and zinc (Experiment 2).

| Item | Rumen Fluid[1] | | | | SEM[3] | P = | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HTM-RF | | STM-RF | | | | | | | |
| | Substrate[2] | | | | | | | | | |
| | HTM-S | STM-S | HTM-S | STM-S | | Rumen fluid source | Time | Substrate source | Rumen fluid source x substrate source | Rumen fluid source x Time |
| N[4] = | 24 | 24 | 24 | 24 | | | | | | |
| DM Digestibility, % | | | | | | | | | | |
| 12h | 49.36 | 49.92 | 50.19 | 52.98 | 1.2 | 0.893 | <0.001 | 0.414 | 0.323 | 0.079 |
| 24h | 61.15 | 62.82 | 58.92 | 60.08 | 1.3 | | | | | |
| NDF Digestibility, % | | | | | | | | | | |
| 12h | 38.75 | 39.67 | 40.52 | 46.04 | 1.7 | 0.387 | <0.001 | 0.447 | 0.402 | 0.487 |
| 24h | 56.76 | 55.48 | 56.17 | 54.14 | 1.3 | | | | | |

[1]Rumen fluid collected from steers that received the following dietary TM treatment for 14 days: HTM-RF = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl; STM-RF = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

[2]Total mixed ration were mixed with the following treatment and used as the substrate for in vitro fermentation vessels: HTM-S = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl; STM-S = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

[3]SEM = standard error of the mean.

[4]N = 24 (n = 12/Time (12h and 24h))

Table 3.2. Influence of trace mineral source on volatile fatty acid (VFA) from rumen fluid obtained from canulated beef steers receiving either hydroxy or sulfate trace mineral sources of copper, manganese, and zinc (Experiment 2).

| Item | Rumen Fluid[1] | | | | SEM[3] | P = | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | HTM-RF | | STM-RF | | | | | | | |
| | Substrate[2] | | | | | | | | | |
| | HTM-S | STM-S | HTM-S | STM-S | | Rumen fluid source | Time | Substrate source | Rumen fluid source x substrate source | Rumen fluid source x Time |
| N[4] = | 36 | 36 | 36 | 36 | | | | | | |
| Total VFA, mM | | | | | | | | | | |
| 0h | 103.90 | 75.99 | 81.15 | 84.24 | 6.8 | 0.233 | <0.001 | 0.016 | 0.029 | <0.001 |
| 12h | 245.74 | 162.29 | 184.24 | 179.44 | 14.3 | | | | | |
| 24h | 282.44 | 267.57 | 170.34 | 165.69 | 16.9 | | | | | |
| VFA, mM/100mM | | | | | | | | | | |
| Acetic Acid | | | | | | | | | | |
| 0h | 59.51 | 61.12 | 59.84 | 59.93 | 0.55 | 0.372 | <0.001 | 0.188 | 0.013 | 0.015 |
| 12h | 52.85 | 56.75 | 52.26 | 50.32 | 0.64 | | | | | |
| 24h | 51.53 | 51.81 | 51.37 | 51.40 | 0.35 | | | | | |
| Propionic Acid | | | | | | | | | | |
| 0h | 27.75 | 27.05 | 27.11 | 27.19 | 0.39 | 0.217 | <0.001 | 0.358 | 0.014 | <0.001 |
| 12h | 30.32 | 28.64 | 33.17 | 34.60 | 0.55 | | | | | |
| 24h | 30.71 | 30.35 | 33.27 | 33.17 | 0.41 | | | | | |
| Isobutyric Acid | | | | | | | | | | |
| 0h | 0.92 | 0.76 | 0.66 | 0.68 | 0.04 | 0.009 | <0.001 | 0.105 | 0.096 | 0.485 |
| 12h | 0.65 | 0.54 | 0.48 | 0.48 | 0.02 | | | | | |
| 24h | 0.70 | 0.72 | 0.53 | 0.52 | 0.02 | | | | | |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Butyric Acid | | | | | | | | | | |
| 0h | 11.82 | 11.07 | 12.38 | 12.20 | 0.34 | 0.635 | <0.001 | 0.265 | 0.127 | <0.001 |
| 12h | 16.18 | 14.07 | 14.09 | 14.59 | 0.44 | | | | | |
| 24h | 17.06 | 17.12 | 14.83 | 14.91 | 0.37 | | | | | |

[1]Rumen fluid collected from steers that received the following dietary TM treatment for 14 days: HTM-RF = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl; STM-RF = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

[2]Total mixed ration were mixed with the following treatment and used as the substrate for in vitro fermentation vessels: HTM-S = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl; STM-S = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

[3]SEM = standard error of the mean.

[4]N = 36 (n = 12/Time (0h, 12h and 24h))

Table 3.3. Influence of trace mineral source on the solubility of trace mineral following simulated abomasal and intestinal digestion

| Item | Rumen Fluid[1] | | | | SEM[3] | P = | | |
|---|---|---|---|---|---|---|---|---|
| | HTM-RF | | STM-RF | | | | | |
| | Substrate treatment[2] | | | | | | | |
| | HTM-S | STM-S | HTM-S | STM-S | | Rumen fluid source | Substrate source | Rumen fluid x Substrate |
| n = | 12 | 12 | 12 | 12 | | | | |
| Abomasal, mg/L | | | | | | | | |
| Cu | 0.18 | 0.17 | 0.23 | 0.20 | 0.01 | 0.568 | 0.163 | 0.435 |
| Mn | 7.69 | 7.06 | 7.83 | 7.42 | 0.17 | 0.807 | <0.001 | 0.407 |
| Zn | 2.06 | 1.83 | 2.17 | 2.11 | 0.08 | 0.427 | 0.389 | 0.562 |
| Intestinal, mg/L | | | | | | | | |
| Cu | 0.30 | 0.32 | 0.38 | 0.39 | 0.01 | 0.067 | 0.059 | 0.563 |
| Mn | 1.21 | 1.34 | 1.46 | 1.45 | 0.04 | 0.132 | 0.409 | 0.382 |
| Zn | 1.99 | 2.00 | 2.11 | 2.04 | 0.04 | 0.712 | 0.642 | 0.455 |

[1]Rumen fluid was collected from steers that received the following dietary TM treatment for 14 days: HTM-RF = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl; STM-RF = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

[2]Total mixed ration were mixed with the following treatment and used as the substrate for in vitro fermentation vessels: HTM-S = 10 mg Cu/kg DM from CuOHCl; 40 mg Mn/ kg DM from MnOHCl; 60 mg Zn/kg DM from ZnOHCl; STM-S = 10 mg Cu/kg DM from $CuSO_4$; 40 mg Mn/ kg DM from $MnSO_4$; 60 mg Zn/kg DM from $ZnSO_4$.

[3] SEM = standard error of the mean.

## References


Bannink, A., J. Kogut, J. Dijkstra, J. France, E. Kebreab, A. M. Van Vuuren, and S. Tamminga. 2006. Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. Journal of Theoretical Biology. 238:36–51. doi:10.1016/j.jtbi.2005.05.026.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software. 67:1–48. doi:10.18637/jss.v067.i01.

Brock, F. M., C. W. Forsberg, and J. G. Buchanan-Smith. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl Environ Microbiol. 44:561–569. doi:10.1128/aem.44.3.561-569.1982.

Caldera, E., B. Weigel, V. N. Kucharczyk, K. S. Sellins, S. L. Archibeque, J. J. Wagner, H. Han, J. W. Spears, and T. E. Engle. 2019. Trace mineral source influences ruminal distribution of copper and zinc and their binding strength to ruminal digesta1,2,3. J Anim Sci. 97:1852–1864. doi:10.1093/jas/skz072.

Cao, J., P. R. Henry, R. Guo, R. A. Holwerda, J. P. Toth, R. C. Littell, R. D. Miles, and C. B. Ammerman. 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. J Anim Sci. 78:2039–2054. doi:10.2527/2000.7882039x.

Craig, W. M., G. A. Broderick, and D. B. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. J Nutr. 117:56–62. doi:10.1093/jn/117.1.56.

Craig, W. M., B. J. Hong, G. A. Broderick, and R. J. Bula. 1984. In Vitro Inoculum Enriched with Particle-Associated Microorganisms for Determining Rates of Fiber Digestion and Protein Degradation. Journal of Dairy Science. 67:2902–2909. doi:10.3168/jds.S0022-0302(84)81653-7.

Daniel, J. B., S. K. Kvidera, and J. Martín-Tereso. 2020. Total-tract digestibility and milk productivity of dairy cows as affected by trace mineral sources. J Dairy Sci. 103:9081–9089. doi:10.3168/jds.2020-18754.

Durand, M., and R. Kawashima. 1980. Influence of minerals in rumen microbial digestion. In: Y. Ruckebusch and P. Thivend, editors. Digestive Physiology and Metabolism in Ruminants: Proceedings of the 5th International Symposium on Ruminant Physiology, held at Clermont — Ferrand, on 3rd–7th September, 1979. Springer Netherlands, Dordrecht. p. 375–408. Available from: https://doi.org/10.1007/978-94-011-8067-2_18

Edgell, K. 1989. USEPA method study 37 SW-846 method 3050 acid digestion of sediments, sludges, and soils. Available from: https://cfpub.epa.gov/si/si_public_record_Report.cfm?Lab=ORD&dirEntryId=30330

Faulkner, M. J., and W. P. Weiss. 2017. Effect of source of trace minerals in either forage- or by-product-based diets fed to dairy cows: 1. Production and macronutrient digestibility. J Dairy Sci. 100:5358–5367. doi:10.3168/jds.2016-12095.

Forsberg, C. W. 1978. Effects of heavy metals and other trace elements on the fermentative activity of the rumen microflora and growth of functionally important rumen bacteria. Can J Microbiol. 24:298–306. doi:10.1139/m78-050.

Genther, O. N., and S. L. Hansen. 2015. The effect of trace mineral source and concentration on ruminal digestion and mineral solubility. Journal of Dairy Science. 98:566–573. doi:10.3168/jds.2014-8624.

Gifford, R. J., M. P. Thorndyke, O. Guimaraes, H. Hallmark, S. Crane, T. A. Thomas, R. Goodall, J. J. Wagner, and T. E. Engle. 2021. The influence of Propionibacteria on in vivo rumen fermentation characteristics and in vitro lactic acid clearance rate in fistulated steers fed moderately high concentrate diets. Translational Animal Science. 5:S115–S119. doi:10.1093/tas/txab148.

Guimaraes, O., S. Jalali, J. J. Wagner, J. W. Spears, and T. E. Engle. 2021. Trace mineral source impacts rumen trace mineral metabolism and fiber digestion in steers fed a medium-quality grass hay diet. Journal of Animal Science. 99:skab220. doi:10.1093/jas/skab220.

Guimaraes, O., J. J. Wagner, J. W. Spears, V. L. N. Brandao, and T. E. Engle. 2022. Trace mineral source influences digestion, ruminal fermentation, and ruminal copper, zinc, and manganese distribution in steers fed a diet suitable for lactating dairy cows. Animal. 16:100500. doi:10.1016/j.animal.2022.100500.

John, F., and W. Sanford. 2019. An R Companion to Applied Regression. Third edition. Available from: https://us.sagepub.com/en-us/nam/an-r-companion-to-applied-regression/book246125

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen. 2017. lmerTest Package: Tests in Linear Mixed Effects Models. Journal of Statistical Software. 82:1–26. doi:10.18637/jss.v082.i13.

Lenth, R. V. 2006. Java Applets for Power and Sample Size [Computer software]. Available from: http://www.stat.uiowa.edu/~rlenth/Power.

Lenth, R. V., B. Bolker, P. Buerkner, I. Giné-Vázquez, M. Herve, M. Jung, J. Love, F. Miguez, H. Riebl, and H. Singmann. 2024. emmeans: Estimated Marginal Means, aka Least-Squares Means. Available from: https://cran.r-project.org/web/packages/emmeans/index.html

Loh, H. Y., J. W. Spears, O. Guimaraes, A. C. Miller, M. P. Thorndyke, T. A. Thomas, and T. E. Engle. 2024. Trace Mineral Source Influences Trace Mineral Solubility in Water and Mineral Binding Strength to Ruminal Digesta. Biol Trace Elem Res. doi:10.1007/s12011-024-04318-x. Available from: https://doi.org/10.1007/s12011-024-04318-x

Mabjeesh, S. J., M. Cohen, and A. Arieli. 2000. In Vitro Methods for Measuring the Dry Matter Digestibility of Ruminant Feedstuffs: Comparison of Methods and Inoculum Source. Journal of Dairy Science. 83:2289–2294. doi:10.3168/jds.S0022-0302(00)75115-0.

McDougall, E. I. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem J. 43:99–109.

NASEM, N. A. of S., and Medicine. 2021. Nutrient Requirements of Dairy Cattle: Eighth Revised Edition. The National Academies Press, Washington, DC. Available from: https://nap.nationalacademies.org/catalog/25806/nutrient-requirements-of-dairy-cattle-eighth-revised-edition

Park, A. F., J. E. Shirley, E. C. Titgemeyer, J. M. DeFrain, R. C. Cochran, E. E. Wickersham, T. G. Nagaraja, and D. E. Johnson. 2011. Characterization of ruminal dynamics in Holstein dairy cows during the periparturient period. Journal of Animal Physiology and Animal Nutrition. 95:571–582. doi:10.1111/j.1439-0396.2010.01085.x.

R Core Team. 2023. R: A language and environment for statistical computing. Available from: https://www.R-project.org/

Reynolds, C. K., B. Dürst, B. Lupoli, D. J. Humphries, and D. E. Beever. 2004. Visceral Tissue Mass and Rumen Volume in Dairy Cows During the Transition from Late Gestation to Early Lactation. Journal of Dairy Science. 87:961–971. doi:10.3168/jds.S0022-0302(04)73240-3.

Senshu, T., K. Nakamura, A. Sawa, H. Miura, and T. Matsumoto. 1980. Inoculum for In Vitro Rumen Fermentation and Composition of Volatile Fatty Acids. Journal of Dairy Science. 63:305–312. doi:10.3168/jds.S0022-0302(80)82931-6.

Slyter, L. L., and M. J. Wolin. 1967. Copper Sulfate-induced Fermentation Changes in Continuous Cultures of the Rumen Microbial Ecosystem1. Appl Microbiol. 15:1160–1164.

Spears, J. W., E. B. Kegley, and L. A. Mullis. 2004. Bioavailability of copper from tribasic copper chloride and copper sulfate in growing cattle. Animal Feed Science and Technology. 116:1–13. doi:10.1016/j.anifeedsci.2004.06.002.

Tangredi, B. V., H. Y. Loh, M. P. Thorndyke, O. Guimaraes, and T. E. Engle. 2023. The Effects of Olive Cake Supplementation on Feedlot Performance and Longissimus Muscle Fatty Acid Composition of American Wagyu Steers and In Vitro Rumen Fermentation Characteristics. Ruminants. 3:246–254. doi:10.3390/ruminants3030023.

Tilley, J. M. A., and R. A. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. Journal of the British Grassland Society. 18:104–111.

Vinyard, J. R., and A. P. Faciola. 2022. Unraveling the pros and cons of various in vitro methodologies for ruminant nutrition: a review. Translational Animal Science. 6:txac130. doi:10.1093/tas/txac130.

Ward, J. D., and J. W. Spears. 1993. Comparison of Copper Lysine and Copper Sulfate as Copper Sources for Ruminants Using In Vitro Methods1, 2. Journal of Dairy Science. 76:2994–2998. doi:10.3168/jds.S0022-0302(93)77638-9.

# CHAPTER 4 – APPLICATION AND FUTURE DIRECTIONS

## Application

Copper (Cu), manganese (Mn), and zinc (Zn) are essential microminerals, as known as trace minerals (TM), which are required in milligram amounts in the diet and assist with maintenance, development, and growth in cattle (NASEM, 2016). Supplementation of TM is critical for cattle in grazing, feedlot, and dairy operations to prevent TM deficiencies. However, over-supplementation of TM can lead to toxicities, so proper TM supplementation programs are important for the health and productivity of animals. Various forms of TM are available in the market, each with different chemical properties at different sections of the gastrointestinal (GI) tract, leading to variations in their bioavailability. There are many forms of TM available to be fed to cattle, which are categorized into organic (OTM) and inorganic TM (ITM; Clarkson et al., 2021). The commonly used TM sources are in inorganic form, where the metal ion is ionically bound to non-metal such as carbonate, chloride, oxide, and sulfate (Arthington and Ranches, 2021; Clarkson et al., 2021). Inorganic TM is frequently used in livestock diets and the formulations of mineral supplements as it is cheaper and more available on the market (Greene, 2000; Arthington and Ranches, 2021). However, ITM is generally less bioavailable and less stable compared to other forms of TM (Arthington and Ranches, 2021). Across various forms of ITM, sulfate and chloride have the highest bioavailability, followed by carbonates, and oxides being the least bioavailable form for cattle (Arthington and Ranches, 2021). Organic trace minerals generally refer to OTM that are covalently bound to an organic ligand, such as amino acid. Organic TM offers higher bioavailability in nonruminants, but its advantages in ruminants

are still unclear as the effect of supplementing OTM to cattle on grazing systems has been highly variable (Kabaija and Smith, 1988; Ahola et al., 2004; Marques et al., 2016).

Hydroxychloride TM (HTM) is a relatively new form of ITM, but unlike traditional ITM, HTM complexes are formed by covalent bonds, which contribute to OTM-like properties (Arthington, 2015). The differences in chemical properties of different TM sources can influence their bioavailability and efficacy as supplements. Solubility plays a big role in determining bioavailability as it allows the minerals to be released from a more complex compound into its ionic form, which could be absorbed into the bloodstream of the animals (Ibrahim et al., 1990; Underwood and Suttle, 1999; Clarkson et al., 2021). Studies conducted by Spears et al. (2004) and Cao et al. (2000) showed that Cu and Zn from Cu sulfate were soluble in water while Cu and Zn from tribasic Cu chloride (hydroxy) were almost insoluble in water. To our knowledge, no in vitro study was conducted to compare the solubility of Mn from different sources. In order to understand the impact of different sources on the solubility of Cu, Zn, and Mn in water, experiment 1 in chapter 2 of this dissertation incubated Cu, Mn, and Zn from sulfate (STM) and hydroxychloride (HTM) in water for 24 hours to compare the solubility of Cu, Zn, and Mn from different sources. The result from this study indicated that Cu, Mn, and Zn had lower solubility when incubated in water, which can be the indicator of the solubility of these elements in the rumen. Although TM solubility in the rumen is lower than in the in vitro model due to the complexity of the rumen environment, in-vitro solubility is still a good indicator of the solubility of TM in the rumen (Clarkson et al., 2021; Vigh et al., 2023). Additionally, we observed that Mn from HTM had a greater solubility in water when compared to Cu and Zn from HTM, which we think is due to the difference in the number of protons between Mn (~25), Cu (~29), and Zn (~30). The solubility of TM in the rumen can play a big role in determining the bioavailability of

the elements. Mineral interactions, specifically antagonistic interaction, can reduce the retention and bioavailability of minerals. Iron (Fe), molybdenum (Mo), and sulfur (S) are the commonly known elements that are antagonistic to Cu, where they can reduce the availability of Cu for absorption and/or the availability of Cu for metabolism. Copper bioavailability will primarily be affected by the three-way interaction between Cu, molybdenum, and sulfur (Dick, 1954; Hogan et al., 1968; Kline et al., 1971; Suttle, 1977; Suttle, 1991). The presence of microorganisms in the rumen rapidly reduces sulfate (from consumption of water, soil, or diet) into sulfide, which can then react with molybdate to form thiomolybdates (TMD; Dick et al., 1975; Gooneratne et al., 1989; Gould and Kendall, 2011). Thiomolybdates can then combine with Cu to form insoluble Cu-TMD in the rumen, which will limit the absorption of Cu (Dick et al., 1975). Thiomolybdates before binding to Cu are soluble and can be absorbed into the body. Thiomolybdates in the bloodstream are "Cu hungry." They will bind to any free Cu ion available and form insoluble Cu-TMD, hence leading to the reduction in Cu uptake by cells or binding to Cu-containing enzymes for their functionality (Dick et al., 1975; Gould and Kendall, 2011; López-Alonso and Miranda, 2020). In addition, TMD will also increase the secretion of Cu in sheep through bile and kidney, and remove Cu from Cu metalloenzymes (Gooneratne et al., 1985; Kumaratilake and Howell, 1989; Suttle, 1991). In addition, high dietary iron greatly reduces Cu concentration in the liver and plasma (Bremner et al., 1987; Phillippo et al., 1987). One potential mechanism by which iron could decrease Cu bioavailability is by reacting with sulfide and Cu in the rumen and forming Cu complexes that are not absorbable by the animals, but no evidence is available to support this hypothesis (Standish and Ammerman, 1971; Gould and Kendall, 2011; López-Alonso and Miranda, 2020).

Experiments to compare TM solubility in the rumen from different TM sources have used in vitro techniques or in vivo bolus dose models to investigate TM ruminal solubility. Limited research has investigated the impact of TM sources on these parameters through dietary inclusion. The current study utilized dietary inclusion for TM supplementation to simulate real-life applications and investigated solubility and fermentation characteristics using a cross-over design to have a deeper understanding of the relationship between rumen microbes and TM. By analyzing the solubility and the binding strength of TM in the rumen in experiment 2, we have a better understanding and estimation of the amount of soluble TM in the rumen which can potentially negatively impact microbial fermentation in the rumen. We also analyzed the TM concentration in the protozoa and bacteria to better understand the distribution and the role of the TM in the rumen. We observed Zn and Mn have different solubility coefficients and binding strengths in the rumen of steers being fed different source of TM, which is similar to previous published studies.

In experiment 4, we utilized the same set of steers from experiment 2 and conducted an in vitro experiment by obtaining rumen fluid from the same steers after 14 days of supplementation with sulfate or hydroxy source of TM. Before the initiation of the in vitro project, we conducted a pilot study to test a different in vitro digestion method. In this method, we incorporated the particle-associated microorganisms (PAO) in our strained rumen fluid (SRF) and used either 1:2 or 1:4 dilution (SRF or SRF+PAO: McDougall's buffer) with McDougall's buffer. Since SRF at 1:4 dilution (SRF: McDougall's buffer) was first proposed by Tilley and Terry (1963), many studies are still utilizing this method (Mabjeesh et al., 2000; Gifford et al., 2021). However, when PAO was combined with SRF microorganisms, greater fiber digestion, protein digestion, and enzyme activity were observed when compared to SRF and McDougall's buffer mixture

(Brock et al., 1982; Craig et al., 1984; Craig et al., 1987). In the current study, a greater dry matter disappearance (DMD) was observed when a 1:2 ratio of SRF or SRF+PAO to McDougall's buffer was used compared to a 1:4 ratio dilution (SRF or SRF+PAO: McDougall's buffer). Strained rumen fluid + PAO: McDougall's buffer at a 1:4 ratio had the lowest DMD in the current study. Although we did not analyze VFA, the utilization of PAO+SRF was suggested to generate a similar VFA pattern compared to in vivo experiments (Senshu et al., 1980). With these findings, we decided that SRF+PAO at a 1:2 ratio would be the best approach for the subsequent in vitro experiments. These data could serve as a reference for future research in deciding which type of in vitro digestion solution to use when examining fiber digestion in rumen fluid.

In the experiments in Chapter 3, we utilized a cross-over study design to investigate the dietary effect (long-term effect) and short-term effect of TM source on solubility and fermentation characteristics. Trace mineral supplementation via dietary inclusion was considered the long-term effect, which can potentially change the rumen environment, while the short-term effect was the addition of the TM supplement into the in vitro digestion tube at the time of incubation (Figure 3.1). Historically, treatments of interest were typically added to the substrate used for in vitro fermentation and incubated with rumen fluid from common donor animals that were not receiving the treatments of interest. The current experiment utilized rumen fluid from animals that had received the dietary treatments of interest prior to extracting the rumen fluid for in vitro use. In current study, total VFA molar concentrations were lesser by approximately 30% in STM-S compared to HTM-S when HTM-RF was used for incubation. The in vitro data from the current experiment suggest that both long-term (rumen fluid source) and short-term (substrate treatment) HTM supplementation improved total VFA production. The molar proportion of VFA

also changed when different sources of TM were used. Current studies suggest that short-term HTM could lead to a higher proportion of carbohydrate fermentation. We also evaluated the TM solubility in the abomasum and intestine by using the in vitro models. We observed a higher soluble Mn under simulated abomasal conditions which suggests Mn may be more available for absorption by the small intestine after immediate release from the abomasum into the duodenum. However, future researchers should pay attention to the liquid-to-solid ratio. In current study, the liquid-to-solid ratio was 30:1, which was 5 times more diluted than the rumen environment of lactating dairy cows (6:1).

**Future Directions**

The results from current study are inconsistent when compared to previous literature published on this topic. Future research should be conducted to investigate the relationship between TM source, rumen digesta, metal solubility, and bioavailability. Longer term studies to investigate the interaction of TM source with rumen environments are needed to understand the distribution of TM within the microbial community, solubility, redox potential, and fermentation characteristics. Furthermore, more research is also needed to investigate the mechanisms by which TM is solubilized, absorbed, and metabolized in the digestive tract of beef cattle. A better understanding of TM solubility in the gastrointestinal tracts will allow for more accurate TM supplementation to ruminants. There are a few models that we can utilize to study TM absorption, storage, and metabolism in live ruminants. As the knowledge we currently have on mineral metabolism is based on yeast, mice, and human models, more study in ruminants is required in order to properly supplement the animals. The interaction between feed particles, microorganisms, minerals, and their antagonist should be investigated to understand the variability that exists.

Trace element supplementation in ruminants is still challenging, especially Cu supplementation, due to the narrow window between toxicity and deficiency (Strickland et al., 2019; López-Alonso and Miranda, 2020; Abramowicz et al., 2021; Borobia et al., 2022). The optimum level of TM supplementation is still widely discussed to maximize animal performance without causing toxicity and deficiency, especially when performance-maximization technologies (direct-fed microbial, beta-agonists, steroids, feed additives, and more) are utilized. As previous research suggests, there is an interaction of TM with those compounds (Messersmith et al., 2021; Henderson et al., 2024; Hong et al., 2024). Without detailed knowledge of TM absorption, storage, and metabolism understanding, the interpretation of these research data is limited. Utilizing the yeast, mice, and human models to explain TM in ruminants is not appropriate, as ruminants have a unique digestive system (reducing environment in the rumen), which can contribute to alterations in TM availability to the animal. There are multiple technologies that can be used to investigate TM metabolism, which were discussed in Chapter 1.

Cell culture can serve as a great tool to investigate nutrient absorption and/or metabolism on a cellular level, as we will have the ability to isolate single cell out from the whole tissues. However, the variation between cell cultures is something worth paying attention to, as there are always some differences in permeability between Caco-2 cells from different laboratories (Artursson and Borchardt, 1997). For ruminants, there is no commercially available cell line such as Caco-2 cells. In order to investigate nutrient metabolism accurately, we need to use enterocytes and hepatocytes harvested from ruminants (bovid or ovis) to understand the mechanism of nutrient absorption, storage, and metabolism in ruminants. After establishing the stable cell lines, different doses of TM can be added into the media, and we can measure viable cells, TM concentration in the media and cell after incubation, and also harvest the cell to

examine the "-omics". There are limited studies that have successfully cultured enterocytes and hepatocytes from bovid and ovis tissues, and the reproducibility of the procedure is challenging in different laboratories (Loret et al., 2009; Hamilton et al., 2018; Zhang et al., 2021).

Other than cell culture, there are other methods that are considered more "traditional" and relatively more expensive. One of the methods is feeding trials, where we separate the animals into different groups, and feed them different levels of TM for a certain period. After the feeding period, intestinal, liver, and muscle tissues can be harvested during slaughter to investigate the differential expression of genes related to TM absorption. The differential expression of mRNA and different protein concentrations of transport-related proteins in those tissues along with TM concentration analysis could provide us with information on the mechanism of TM metabolism. This type of trial could be costly depending on sample size, feeding duration, and animal housing and care.

Utilizing a Ussing chamber is another method that we can apply to investigate nutrient absorption at the tissue level, specifically intestinal tissue. Ussing chambers allow the researcher to maintain the viability of the tissues (and monitor viability over time) after harvest to measure transports of nutrients across multiple layers of tissues (Clarke, 2009). To utilize a Ussing chamber, we need to "clip" the harvested intestinal tissues as a flat sheet between 2 chambers, with one chamber facing the mucosal membrane and another chamber facing the serosal membrane (Figure 4.1; Clarke, 2009). By using Ussing chambers, we will be able to measure the amount and rate of nutrient uptake through the intestinal tissues and the rate of release from the intestine. After noticing the reduction in electric signal, which is an indication of the loss of tissue integrity, we could harvest the tissues and conduct transcriptomic and/or proteomic analysis. With all the information combined, we should be able to gain insight into TM

absorption in the small and large intestines. The challenges of using Ussing chambers are tissue viability and feed contamination, which can hinder the accuracy in predicting the amount and rate of nutrient absorption.

One of my interests is also to investigate the mechanism of Cu, Mn, and Zn uptake and utilization in kidney, heart, brain, muscle, and other organs in ruminants. Among these elements, Cu has the ability to induce cell death when the concentrations exceed the threshold set in the cell. Some researchers have investigated the mechanism of Cu-induced cell death and suggest the potential of using Cu as a cancer treatment (Tsvetkov et al., 2022). Although cancer is not a concern in ruminant animals, the knowledge we gained in ruminant research (that was not widely researched in human or mouse models), can contribute to the pool of knowledge that not only benefits the food animal production industry but also contributes to other fields.

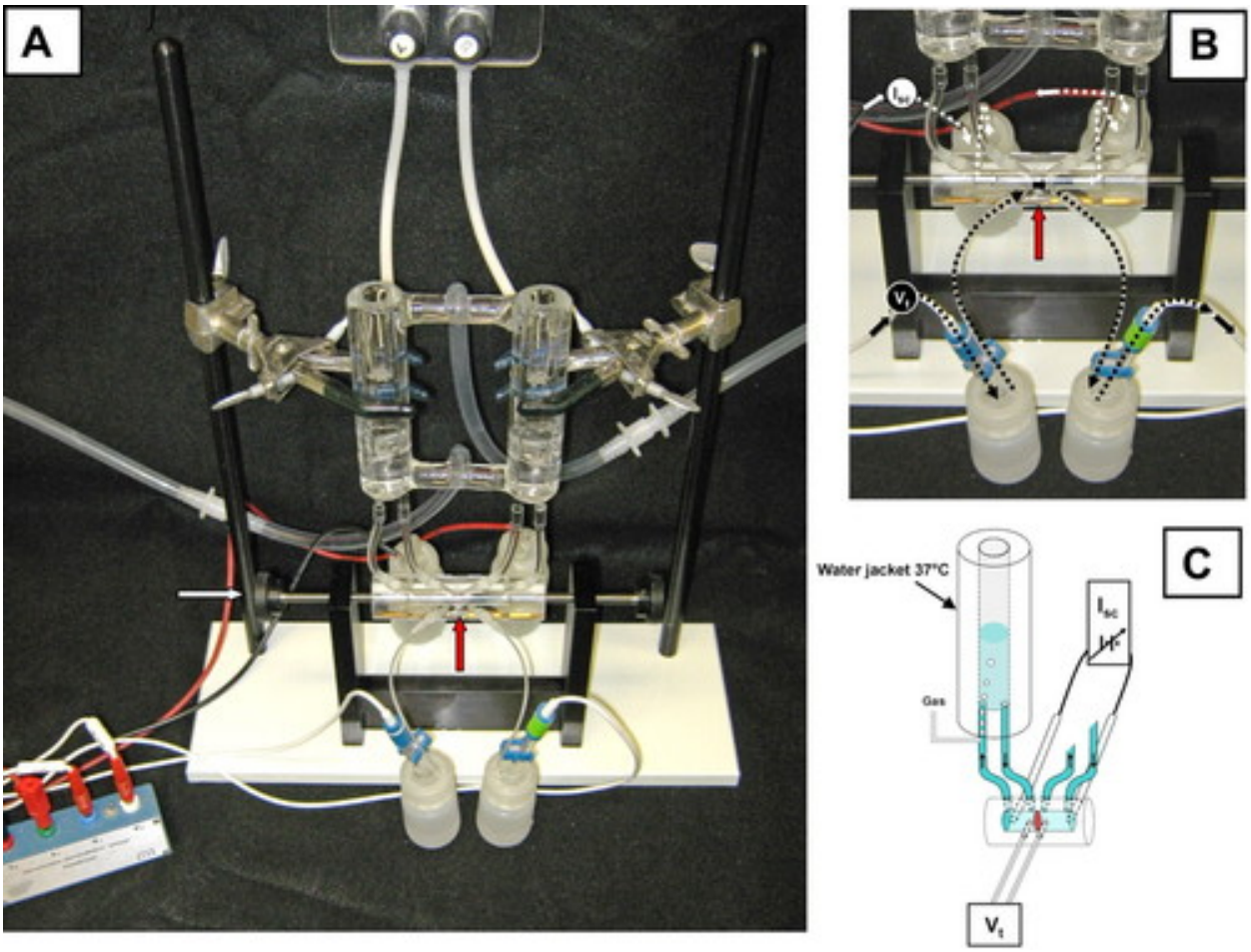


Figure 4.1. Ussing chamber design. Figure obtained from "A guide to Ussing chamber studies of mouse intestine" by Lane L. Clarke (2009)

## References


Abramowicz, B., L. Kurek, A. Chalabis-Mazurek, and K. Lutnicki. 2021. Copper and iron deficiency in dairy cattle. Journal of Elementology. 26. doi:10.5601/jelem.2020.25.4.2093. Available from: http://agro.icm.edu.pl/agro/element/bwmeta1.element.agro-52cd867d-e635-4b4c-98c4-eb665941c1c3

Ahola, J. K., D. S. Baker, P. D. Burns, R. G. Mortimer, R. M. Enns, J. C. Whittier, T. W. Geary, and T. E. Engle. 2004. Effect of copper, zinc, and manganese supplementation and source on reproduction, mineral status, and performance in grazing beef cattle over a two-year period123. Journal of Animal Science. 82:2375–2383. doi:10.2527/2004.8282375x.

Arthington, J. 2015. New Concepts in Trace Mineral Supplementation of Grazing Cattle. 26th Annual Florida Ruminant Nutrition Symposium. 104–117.

Arthington, J. D., and J. Ranches. 2021. Trace Mineral Nutrition of Grazing Beef Cattle. Animals. 11:2767. doi:10.3390/ani11102767.

Artursson, P., and R. T. Borchardt. 1997. Intestinal Drug Absorption and Metabolism in Cell Cultures: Caco-2 and Beyond. Pharm Res. 14:1655–1658. doi:10.1023/A:1012155124489.

Borobia, M., S. Villanueva-Saz, M. Ruiz de Arcaute, A. Fernández, M. T. Verde, J. M. González, T. Navarro, A. A. Benito, J. L. Arnal, M. De las Heras, and A. Ortín. 2022. Copper Poisoning, a Deadly Hazard for Sheep. Animals. 12:2388. doi:10.3390/ani12182388.

Bremner, I., W. R. Humphries, M. Phillippo, M. J. Walker, and P. C. Morrice. 1987. Iron-induced copper deficiency in calves: dose-response relationships and interactions with molybdenum and sulphur. Animal Science. 45:403–414. doi:10.1017/S0003356100002890.

Brock, F. M., C. W. Forsberg, and J. G. Buchanan-Smith. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl Environ Microbiol. 44:561–569. doi:10.1128/aem.44.3.561-569.1982.

Cao, J., P. R. Henry, R. Guo, R. A. Holwerda, J. P. Toth, R. C. Littell, R. D. Miles, and C. B. Ammerman. 2000. Chemical characteristics and relative bioavailability of supplemental organic zinc sources for poultry and ruminants. J Anim Sci. 78:2039–2054. doi:10.2527/2000.7882039x.

Clarke, L. L. 2009. A guide to Ussing chamber studies of mouse intestine. American Journal of Physiology-Gastrointestinal and Liver Physiology. 296:G1151–G1166. doi:10.1152/ajpgi.90649.2008.

Clarkson, A. H., S. W. Paine, and N. R. Kendall. 2021. Evaluation of the solubility of a range of copper sources and the effects of iron & sulphur on copper solubility under rumen simulated conditions. Journal of Trace Elements in Medicine and Biology. 68:126815. doi:10.1016/j.jtemb.2021.126815.

Craig, W. M., G. A. Broderick, and D. B. Ricker. 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. J Nutr. 117:56–62. doi:10.1093/jn/117.1.56.

Craig, W. M., B. J. Hong, G. A. Broderick, and R. J. Bula. 1984. In Vitro Inoculum Enriched with Particle-Associated Microorganisms for Determining Rates of Fiber Digestion and Protein Degradation. Journal of Dairy Science. 67:2902–2909. doi:10.3168/jds.S0022-0302(84)81653-7.

Dick, A. T. 1954. Studies on the assimilation and storage of copper in crossbred sheep. Aust. J. Agric. Res. 5:511–555. doi:10.1071/ar9540511.

Dick, A. T., D. W. Dewey, and J. M. Gawthorne. 1975. Thiomolybdates and the copper–molybdenum–sulphur interaction in ruminant nutrition. The Journal of Agricultural Science. 85:567–568. doi:10.1017/S0021859600062468.

Gifford, R. J., M. P. Thorndyke, O. Guimaraes, H. Hallmark, S. Crane, T. A. Thomas, R. Goodall, J. J. Wagner, and T. E. Engle. 2021. The influence of Propionibacteria on in vivo rumen fermentation characteristics and in vitro lactic acid clearance rate in fistulated steers fed moderately high concentrate diets. Translational Animal Science. 5:S115–S119. doi:10.1093/tas/txab148.

Gooneratne, S. R., W. T. Buckley, and D. A. Christensen. 1989. Review of copper deficiency and metabolism in ruminants. Can. J. Anim. Sci. 69:819–845. doi:10.4141/cjas89-096.

Gooneratne, S. R., J. M. C. Howell, and J. M. Gawthorne. 1985. Distribution of copper in the kidney of sheep after thiomolybdate administration. Nutrition Research. 5:S-546.

Gould, L., and N. R. Kendall. 2011. Role of the rumen in copper and thiomolybdate absorption. Nutrition Research Reviews. 24:176–182. doi:10.1017/S0954422411000059.

Greene, L. W. 2000. Designing mineral supplementation of forage programs for beef cattle. Journal of Animal Science. 77:1–9. doi:10.2527/jas2000.00218812007700ES0046x.

Hamilton, C. A., R. Young, S. Jayaraman, A. Sehgal, E. Paxton, S. Thomson, F. Katzer, J. Hope, E. Innes, L. J. Morrison, and N. A. Mabbott. 2018. Development of in vitro enteroids derived from bovine small intestinal crypts. Vet Res. 49:54. doi:10.1186/s13567-018-0547-5.

Henderson, J. A., J. McGill, and S. L. Hansen. 2024. 100 Evaluating the effect of liver copper concentration on vaccine response in lightweight dairy-beef steers. Journal of Animal Science. 102:27–28. doi:10.1093/jas/skae102.034.

Hogan, K. G., D. F. L. Money, and A. Blayney. 1968. The effect of a molybdate and sulphate supplement on the accumulation of copper in the livers of penned sheep. New Zealand Journal of Agricultural Research. 11:435–444. doi:10.1080/00288233.1968.10431440.

Hong, S., E. L. Rients, C. E. Franco, S. L. Hansen, and J. L. McGill. 2024. Impact of an Injectable Trace Mineral Supplement on the Immune Response and Outcome of Mannheimia

haemolytica Infection in Feedlot Cattle. Biol Trace Elem Res. doi:10.1007/s12011-024-04251-z. Available from: https://doi.org/10.1007/s12011-024-04251-z

Ibrahim, M. N. M., A. Van Der Kamp, G. Zemmelink, and S. Tamminga. 1990. Solubility of mineral elements present in ruminant feeds. J. Agric. Sci. 114:265–274. doi:10.1017/S0021859600072658.

Kabaija, E., and O. B. Smith. 1988. Trace element kinetics in the digestive tract of sheep fed diets with graded levels of dietary fibre. Journal of Animal Physiology and Animal Nutrition. 59:218–224. doi:10.1111/j.1439-0396.1988.tb00068.x.

Kline, R. D., V. W. Hays, and G. L. Cromwell. 1971. Effects of Copper, Molybdenum and Sulfate on Performance, Hematology and Copper Stores of Pigs and Lambs. Journal of Animal Science. 33:771–779. doi:10.2527/jas1971.334771x.

Kumaratilake, J. S., and J. McC. Howell. 1989. Intravenously administered tetra-thiomolybdate and the removal of copper from the liver of copper-loaded sheep. Journal of Comparative Pathology. 101:177–199. doi:10.1016/0021-9975(89)90065-0.

López-Alonso, M., and M. Miranda. 2020. Copper Supplementation, A Challenge in Cattle. Animals. 10:1890. doi:10.3390/ani10101890.

Loret, S., D. Rusu, B. E. Moualij, B. Taminiau, E. Heinen, G. Dandrifosse, and J. Mainil. 2009. Preliminary characterization of jejunocyte and colonocyte cell lines isolated by enzymatic digestion from adult and young cattle. Research in Veterinary Science. 87:123–132. doi:10.1016/j.rvsc.2008.12.002.

Mabjeesh, S. J., M. Cohen, and A. Arieli. 2000. In Vitro Methods for Measuring the Dry Matter Digestibility of Ruminant Feedstuffs: Comparison of Methods and Inoculum Source. Journal of Dairy Science. 83:2289–2294. doi:10.3168/jds.S0022-0302(00)75115-0.

Marques, R. S., R. F. Cooke, M. C. Rodrigues, B. I. Cappellozza, R. R. Mills, C. K. Larson, P. Moriel, and D. W. Bohnert. 2016. Effects of organic or inorganic cobalt, copper, manganese, and zinc supplementation to late-gestating beef cows on productive and physiological responses of the offspring1. Journal of Animal Science. 94:1215–1226. doi:10.2527/jas.2015-0036.

Messersmith, E. M., D. T. Smerchek, and S. L. Hansen. 2021. The Crossroads between Zinc and Steroidal Implant-Induced Growth of Beef Cattle. Animals. 11:1914. doi:10.3390/ani11071914.

NASEM, (National Academies of Sciences, Engineering, and Medicine). 2016. Nutrient Requirements of Beef Cattle, 8th Revised Edition. National Academies Press, Washington, D.C. Available from: http://www.nap.edu/catalog/19014

Phillippo, M., W. R. Humphries, T. Atkinson, G. D. Henderson, and P. H. Garthwaite. 1987. The effect of dietary molybdenum and iron on copper status, puberty, fertility and oestrous cycles in cattle. The Journal of Agricultural Science. 109:321–336. doi:10.1017/S0021859600080758.

Senshu, T., K. Nakamura, A. Sawa, H. Miura, and T. Matsumoto. 1980. Inoculum for In Vitro Rumen Fermentation and Composition of Volatile Fatty Acids. Journal of Dairy Science. 63:305–312. doi:10.3168/jds.S0022-0302(80)82931-6.

Spears, J. W., E. B. Kegley, and L. A. Mullis. 2004. Bioavailability of copper from tribasic copper chloride and copper sulfate in growing cattle. Animal Feed Science and Technology. 116:1–13. doi:10.1016/j.anifeedsci.2004.06.002.

Standish, J. F., and C. B. Ammerman. 1971. Effect of Excess Dietary Iron as Ferrous Sulfate and Ferric Citrate on Tissue Mineral Composition of Sheep. Journal of Animal Science. 33:481–484. doi:10.2527/jas1971.332481x.

Strickland, J. M., T. H. Herdt, D. G. Sledge, and J. P. Buchweitz. 2019. Short communication: Survey of hepatic copper concentrations in Midwest dairy cows. Journal of Dairy Science. 102:4209–4214. doi:10.3168/jds.2018-15566.

Suttle, N. F. 1977. Reducing the potential copper toxicity of concentrates to sheep by the use of molybdenum and sulphur supplements. Animal Feed Science and Technology. 2:235–246. doi:10.1016/0377-8401(77)90027-X.

Suttle, N. F. 1991. The Interactions Between Copper, Molybdenum, and Sulphur in Ruminant Nutrition. Annual Review of Nutrition. 11:121–140. doi:10.1146/annurev.nu.11.070191.001005.

Tilley, J. M. A., and R. A. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. Journal of the British Grassland Society. 18:104–111.

Tsvetkov, P., S. Coy, B. Petrova, M. Dreishpoon, A. Verma, M. Abdusamad, J. Rossen, L. Joesch-Cohen, R. Humeidi, R. D. Spangler, J. K. Eaton, E. Frenkel, M. Kocak, S. M. Corsello, S. Lutsenko, N. Kanarek, S. Santagata, and T. R. Golub. 2022. Copper induces cell death by targeting lipoylated TCA cycle proteins. Science. 375:1254–1261. doi:10.1126/science.abf0529.

Underwood, E. J., and N. F. Suttle. 1999. The mineral nutrition of livestock. 3rd ed. CABI Pub, Wallingford, Oxon, UK ; New York, NY, USA.

Vigh, A., A. D. Criste, N. Corcionivoschi, and C. Gerard. 2023. Rumen Solubility of Copper, Manganese and Zinc and the Potential Link between the Source and Rumen Function: A Systematic Review. Agriculture. 13:2198. doi:10.3390/agriculture13122198.

Zhang, J., L. Deng, L. He, Y. Zhao, Y. Shi, Z. Zhang, and B. Zhao. 2021. Morphology and Transcriptome Analysis of Intestinal Organoids in Bovine fetus. In Review. Available from: https://www.researchsquare.com/article/rs-940188/v1

Supplementary Table S3.1. Ingredient and nutrient composition of the basal diet.

| Item | |
|---|---|
| Ingredient, % dry matter basis | |
| Corn silage | 27.64 |
| Cracked corn | 34.40 |
| Distiller's grains | 12.78 |
| Alfalfa hay | 10.91 |
| Wheat straw | 8.44 |
| Liquid supplement[1] | 5.00 |
| Limestone | 0.67 |
| Salt | 0.17 |
| Analyzed nutrient composition (DM basis) | |
| DM, % as fed | 59.8 |
| Crude protein, % | 16.3 |
| Acid detergent fiber, % | 18.6 |
| Neutral detergent fiber, % | 31.1 |
| Ether extract, % | 6.4 |
| NEg, Mcal/kg[2] | 1.08 |
| NEm, Mcal/kg[3] | 1.73 |
| Calcium, % | 0.95 |
| Magnesium, % | 0.27 |
| Phosphorus, % | 0.31 |
| Potassium, % | 0.89 |
| Sodium, % | 0.21 |
| Sulfur, % | 0.20 |
| Copper, mg/kg | 6.97 |
| Manganese, mg/kg | 56.51 |
| Zinc, mg/kg | 34.52 |
| Iron, mg/kg | 111.26 |
| Molybdenum, mg/kg | 0.64 |

[1]Liquid supplement provided in a molasses suspension: 3.72% NPN (urea), 14.07% Calcium, 0.11% Phosphorus, 1.81 % Potassium, 110,000 IU/kg vitamin A, 9.4 IU/kg vitamin E, and 440 g/metric ton of monensin (Rumensin 90, Elanco Animal Health, Greenfield, IN, USA).
[2]NEg = Net energy for gain.
[3]NEm = Net energy for maintenance.

Supplementary Table S3.2. Influence of strained rumen fluid (SRF) or SRF with particle-associated microorganisms (PAO) and dilution ratio on in vitro dry matter disappearance after incubation at 39°C for 12 hours.

| Item | Method[1] | | SEM[3] | P < | | |
|---|---|---|---|---|---|---|
| | SRF | SRF+PAO | | Method | Dilution | Method x Dilution |
| N[4] = | 6 | 6 | | | | |
| Ratio[2] | | | | | | |
| 1:2 | 69.58 | 69.17 | 2.9 | 0.001 | 0.001 | 0.001 |
| 1:4 | 66.53 | 46.59 | | | | |

[1]Methods: SRF = Strained Rumen fluid; or SRF + PAO = Strained Rumen fluid + particle-associated microorganisms.
[2]Ratio: 1:2 = Strained Rumen fluid: McDougall's buffer or Strained Rumen fluid: particle-associated microorganisms, 1:2 ratio; or 1:4 = Strained Rumen fluid: McDougall's buffer or Strained Rumen fluid: particle-associated microorganisms, 1:4 ratio.
[3] SEM = standard error of the mean.
[4]N = 6 (n = 3/ratio (1:2 and 1:4))