Effects of Dietary Cu, Zn and Mn on Bovine Neutrophil Function

Thesis

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ABSTRACT

The effects of supplementing diets with inorganic and organic Cu, Zn and Mn on blood neutrophil function were examined in 27 mid-lactation Holstein cows. Cows were assigned to 9 blocks of 3 and were grouped by parity, milk production and days in milk. Cows within each block were randomly assigned to one of three treatments: 1) control diet devoid of supplemental Cu, Zn and Mn, 2) diet supplemented with Cu, Zn and Mn via sulfates; 3) diet supplemented with Cu, Zn and Mn via organic form. All cows were initially fed a pre-treatment total mixed ration with a target of 8 ppm Cu, 35 ppm Zn and 35 ppm Mn for 30 d. During the treatment period, cows fed diets with mineral supplementation via sulfates and organic forms had target daily intakes of 18 ppm Cu, 60 ppm Zn and 60 ppm Mn for 30 d. Control cows were fed the pre-treatment devoid diet for an additional 30 d. In vitro neutrophil functions were measured after 30 d on experimental or control diets. Percentage of neutrophils phagocytizing, intracellular kill and phagocytic index did not differ among treatments. Serum Cu, Zn and Mn were also not affected by treatment after 30 d. Milk production, milk concentrations of fat, protein and lactose, milk urea nitrogen and somatic cell counts did not differ among treatments. Dry matter intake also did not vary among experimental groups. Results from this study demonstrated that dietary Cu, Zn and Mn supplemented either as sulfates or organic form
for 30 d had no effect on in vitro blood neutrophil function or other measured parameters in mid-lactation Holstein cows.
DEDICATION

Dedicated to my grandparents, mother and sister for the opportunity to be involved on my family dairy farm, allowing a passion to develop for the dairy industry and animal health.

I am forever grateful for your love, patience and guidance.
I would like to sincerely thank my advisor, Dr. Joe Hogan, for being my mentor, teacher, and supporter during this incredible opportunity. Without your patience and guidance throughout the past two years, I would not be the successful student I am today, and for that I am very appreciative. Thank you also for the numerous laughs in the office and plenty of ice cream trips, as they were a great de-stressor. Thank you to Dr. Ramesh Selvaraj and Dr. Bill Weiss for your immunology and nutrition expertise and for serving as my committee members. Also, to Dr. Weiss and Matt Faulkner for helping design a work plan for my trial, formulating a ration and aiding with lab and farm work. A special thank you goes to Janet McCormick for educating me on laboratory techniques in the mastitis lab and being the “know all” person to discuss every day trials. In addition, being someone to vent to when my trial went through its frustrations. In addition, I would like to thank Katie Cole for being my office “partner in crime”. I don’t know how I could have made it through this chapter in my academic career without you. You’ve been a great friend and someone to laugh and share frustrations with. Thank you for assisting with moving cows and working in the lab as well during my trial. I would also like to thank Kevin Miller and the rest of the Krauss Dairy Crew for their dedication and time spent every day to make my research possible. Also, for helping get stubborn cows into
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# TABLE OF CONTENTS

ABSTRACT..............................................................................................................ii
DEDICATION..............................................................................................................iv
ACKNOWLEDGEMENTS............................................................................................v
VITA...........................................................................................................................vii
LIST OF TABLES.........................................................................................................x
LIST OF FIGURES.......................................................................................................xi

INTRODUCTION........................................................................................................1

REVIEW OF LITERATURE........................................................................................3

- Mastitis..................................................................................................................3
- Immune System Overview.....................................................................................6
- Role of Neutrophil in Mammary Gland Defense..................................................9
- Nutrition and Trace Minerals Role in Immunity...................................................14
- Organic vs Inorganic Supplementation................................................................19
- Trace Minerals and Bovine Mammary Health.......................................................20
- Thesis Statement..................................................................................................24

MATERIALS AND METHODS..................................................................................25

- Experimental Design............................................................................................25
- Bacteria..................................................................................................................26
- Preparation of Blood Neutrophils.......................................................................27
- Neutrophil Assay...................................................................................................28
- Serum Mineral Concentrations..........................................................................29
- Diet and Milk Parameters....................................................................................30
- Statistical Analysis...............................................................................................32
RESULTS.........................................................................................................................33
Neutrophil Parameters....................................................................................................33
Blood Serum Concentrations of Cu, Zn and Mn.............................................................35
Milk Production and Components..................................................................................35
Dry Matter Intake.............................................................................................................38

DISCUSSION....................................................................................................................40

THESIS SUMMARY.........................................................................................................48

LIST OF REFERENCES.....................................................................................................49

APPENDIX: TABLES.........................................................................................................58
LIST OF TABLES

TABLE

1. Ingredient composition of diet.................................................................31

2. Nutrient composition of diet and ingredients...............................................31

3. Mean Cu, Zn and Mn blood serum concentrations on d 30 (beginning of Treatment diets) and d 60 (end of treatment diets) of trial period for cows fed diets unsupplemented control diets, sulfate or organic supplemented Cu, Zn and Mn supplemented diets.................................................................35

4. Least squares mean values for milk components in control diet, sulfate supplementation of Cu, Zn and Mn and organic supplementation of Cu, Zn and Mn.................................................................................................37

5. Summary of body weight and BCS for all cows..............................................58

6. Correlation coefficients for neutrophil parameters and serum Cu, Zn and Mn concentrations........................................................................................................59
LIST OF FIGURES

FIGURE

1. The percentage of neutrophils phagocytizing (% positive) for neutrophils from cows fed control diet (n=9), sulfate supplementation of Cu, Zn and Mn (n=9) and organic supplementation of Cu, Zn and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means.................................................................33

2. Mean intracellular kill for neutrophils from cows fed control diet (n=9), sulfate supplementation of Cu, Zn and Mn (n=9) and organic supplementation of Cu, Zn and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means ..................................................................................34

3. Phagocytic index for neutrophils from cows fed control diet (n=9), sulfate supplementation of Cu, Zn and Mn (n=9) and organic supplementation of Cu, Zn and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means ..................................................................................34

4. Mean daily milk production tested weekly for cows fed control diet (n =9), sulfate supplementation of Cu, Zn and Mn (n =9) and organic supplementation of Cu, Zn and Mn (n =9) for 30 d. Dispersion bars represent standard error of the means ..................................................................................36

5. Mean milk urea nitrogen (MUN) values of milk tested weekly from cows fed control diet (n = 9), sulfate supplementation of Cu, Zn and Mn (n = 9) and organic supplementation of Cu, Zn and Mn (n=9) for 30d. Dispersion bars represent standard error of the means ..................................................................................37

6. Mean SCC of milk tested weekly for cows fed control diet (n =9), sulfate supplementation of Cu, Zn, and Mn (n =9) and organic supplementation of Cu, Zn and Mn (n =9) for 30 d. Dispersion bars represent standard error of the means ..................................................................................38
7. Mean daily DMI for cows fed control diet (n=9), sulfate supplementation of Cu, Zn and Mn (n = 9) and organic supplementation of Cu, Zn and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means.
INTRODUCTION

Cellular defenses are important in the protection of the mammary gland against intramammary infections (IMI). Neutrophils are essential in mammary gland resistance and ability to fight infection. In fact, neutrophils become the most prevalent leukocyte, contributing greater than 90% of the total mammary gland leukocyte population during infection (Sordillo and Streicher, 2002). Neutrophils are pro-inflammatory and kill by phagocytosis, stimulating an increased uptake of oxygen and hexose monophosphate activity, which is called the respiratory burst (Fantone and Ward, 1982). The respiratory burst is vital in killing bacteria, however, the process releases reactive oxygen species (ROS) which can damage neutrophils and reduce intracellular kill by the cells if the antioxidant systems of the neutrophil are lacking (Sordillo and Aitken, 2009).

Many factors contribute to mammary gland host defenses and prevention of mastitis. Nutritional status of the cow influences host defenses and effects the duration and severity of disease (Scaletti et al., 2003; Heinrichs et al., 2008; Goff, 2006). Micro minerals such as Cu, Zn and Mn have been shown to be beneficial in improving udder health by reducing SCC, and decreasing the incidence of mastitis (Scaletti et al., 2003, Siciliano-Jones et al., 2008; Hogan et al., 1990). Copper, Zn and Mn are essential for the functionality of many enzymes and structural and cellular proteins (Nocek et al., 2006).
Copper and Zn are a part of the cytosolic superoxide dismutase (SOD) enzyme which aids in neutralizing and removing ROS following respiratory bursts of neutrophils (Arthur and Boyne, 1985; Nockels, 1996). As a result, SOD aids intracellular kill and oxygen metabolite neutralization by neutrophils.

Traditionally, micro minerals have been supplemented via sulfates. However, organic supplementation has gained popularity due to the likelihood of increased bioavailability and absorption in the gut (Andrieu, 2008). Studies have shown that organic supplementation of micro minerals has been associated with improvements in milk yield, clinical udder score, and *Escherichia coli* counts in milk compared with inorganic and unsupplemented groups following an *E. coli* challenge (Scaletti and Harmon, 2012). Despite Cu, Zn and Mn influences on several health parameters, little is known about their direct effect on neutrophil function in combination and if differences exist between inorganic and organic sources. Enhancing mineral absorption may have a positive effect on neutrophil function due to increased accessibility to minerals in blood. Therefore, the objective of the current study was to examine the effects of inorganic and organic supplementation of dietary Cu, Zn and Mn on in vitro bovine neutrophil phagocytosis and intracellular kill.
REVIEW OF LITERATURE

Mastitis

Mastitis is the most costly common disease in the dairy industry today, the profit loss estimated at $185 per cow per case (Bramley et al., 1996). Mastitis is one of the diseases with the highest economic impact and is considered a major animal welfare concern (Contreras and Rodriguez, 2011). Mastitis not only affects milk quality, but also production of the cow (Pyorala, 2003). Losses are a result of discarded milk from clinical infections, antibiotic therapy, cull cow replacement costs, increased labor, veterinary service and treatment services, and reduced milk production in subclinically infected cows (Akers and Nickerson, 2011). The broad definition of mastitis is inflammation of the mammary gland, which includes both secretory tissue and non-secretory tissue (Contreras and Rodriguez, 2011). Mastitis can be classified into subcategories that coincide with the following criteria: lactation stage (lactation versus dry period), clinical manifestations (clinical versus subclinical signs) and the course of infection (acute, chronic, recurrent) (Contreras and Rodriguez, 2011). Disease status is usually monitored by changes in somatic cell count (SCC) of milk, appearance of udder and foremilk and measurement of milk electrical conductivity and lactose concentration (Akers and Nickerson, 2011). In addition, mastitis can be detected through several ancillary tests: N-
acetyl-beta-D-glucosaminidase activity, pH and quantification of acute phase proteins (Pyorala, 2003). Bacteriological sampling can also be beneficial, however it is not considered a routine test to detect mastitis. Tests for indicators of inflammation are often performed first to screen cows that are suspected with IMIs in one or more quarters (Pyorala, 2003), and then milk samples are cultured.

Mastitis can be divided into clinical and subclinical infections. Clinical mastitis is characterized by visual signs; inflammation of the mammary gland including pain, increased size of the gland and density. Systemic signs such as depression and fever may also occur (Contreras and Rodriguez, 2011). Moreover, the physical consistency of milk from clinical mastitis may change including color and presence of flakes or clots in milk. Mastitis causing pathogens are classified depending on their epidemiological patterns as either contagious or environmental (Zadoks et al., 2011). Clinical mastitis is most often associated with environmental pathogens (Contreras and Rodriguez, 2011). The reservoir for environmental pathogens is the environment or surroundings in which the cow lives (i.e. stalls, bedding, pasture). Common environmental pathogens include Steptococcus uberis, Steptococcus dysgalactiae, Klebsiella spp., and Escherichia coli (NMC, 1999). In contrast, subclinical mastitis cannot be detected visually, due to the gland and milk consistency appearing normal. In fact, subclinical mastitis cases are characterized by the absence of visual inflammatory changes, reduced milk secretion, increase in sodium/potassium ratio and a high bacteria count in a milk sample (Willumsen, et al., 2003; Filteau, et al., 1999). Subclinical mastitis is usually detected through an increase in number of somatic cells, which include macrophages, neutrophils and lymphocytes.
(Akers and Nickerson, 2011). A somatic cell count higher than 200,000 cells/ml often defines the diagnosis of subclinical mastitis (NMC, 2001). In contrast to clinical mastitis and environmental pathogens, subclinical mastitis is most often associated with contagious pathogens such as \textit{Staphylococcus aureus}, \textit{Streptococcus agalactiae} and \textit{Mycoplasma spp.} (NMC, 1999). The main reservoir for contagious pathogens is the mammary gland. Contagious pathogens residing on the skin of the teat or in the udder spread from cow to cow or between quarters of the same animal during the milking process (Contreras and Rodriguez, 2011). The milking equipment or hand of the milker can act as fomites in spreading contagious pathogens (Bruno, 2010).

The incidence of mastitis on dairy herds is influenced by many factors. Various genetic, physiological and environmental factors can compromise host defense mechanisms (Hopster, et al., 1998; Waller, 2000). Through many generations of careful selection for certain genetic traits, (ex. milk production), improved management, nutrition and technological advances in milk removal, the bovine mammary gland can produce more milk than past generations, including volume and higher components (Sordillo and Streicher, 2002). This increase in milk production is thought to place metabolic stress associated with milk synthesis and secretion, and a negative correlation is present between milk production capacity and resistance to mastitis (Detilleux et al., 1995). Mastitis most frequently occurs in the early stages of lactation, with a higher incidence during the first four weeks following parturition and with 74-95\% of the incidences observed in the first three months (Michie et al., 2003). The milking process can additionally cause damage to teat end and tissues, making the mammary gland more
susceptible to pathogen invasion and colonization (Sordillo and Streicher, 2002).

Furthermore, housing influences the prevalence of mastitis. Excess numbers of animals in a limited space and use of organic bedding (i.e. straw, sawdust) that support bacterial growth and survival are examples of this (NMC, 1996). Overcrowding puts the cow at risk due to heightened stress and overexposure, challenging their immune defenses (Hogan and Smith, 2003).

**Immune System: Overview**

The immune system’s function is to protect the body against disease caused by bacteria, viruses and other parasites. The body’s cells dedicated to fighting infection are classified as white blood cells, or leukocytes. Leukocytes are produced in the bone marrow and are then moved to the peripheral blood and lymph systems (Staples et al., 2008). Immune defenses can be broken down into two different systems: innate and adaptive immunity. Skin, mucous membranes and other secretions, such as saliva, are part of the innate response. These components react in a nonspecific way against harmful agents that may affect the body. Innate defenses include neutrophils, eosinophils, basophils, and macrophages, the complement system and several cytokines that are all associated with an immune response (Minatel and Carfagnini, 2000). Monocytes migrate from the circulation to the tissue, where they mature into macrophages. Macrophages are the primary cell to encounter pathogens and kill by phagocytosis, or engulfment (Staples et al., 2008). Phagocytosis is the process of recognition, ingestion and digestion of foreign particles (bacteria, tissue, etc.) by these cells (Paape et al., 1979). Phagocytosis
can be divided into several stages. The first stage involves the contact between the macrophage (or neutrophil) and foreign particle and adherence of this particle to the surface of the leukocyte. The second stage involves the movement of pseudopods around the particle. Third, the particle is pulled into the interior of the cell as a phagosome that is limited by a membrane. Next, the cytoplasmic granules migrate toward and fuse their membranes with that of the phagosome to form the phagolysosome. Lastly, the particle is then digested in the phagolysosome, which prevents self-destruction of the cell during killing of the pathogen (Paape et al., 1979). Macrophages use nitric oxide, superoxide anion and hydrogen peroxide in this method of killing (Staples et al., 2008). Additionally, they secrete cytokines including tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and several interleukins (IL). Cytokines regulate inflammation in the tissue to aid against pathogens (Staples et al., 2008). Characteristics of inflammation include, but are not limited to, swelling, redness, heat and pain (Staples et al., 2008). During inflammation, blood vessels dilate, causing blood flow to decrease while allowing neutrophils and fluid to flood the tissue. Neutrophils are the first leukocyte to arrive to the site of infection in large numbers. They are able to recognize, ingest and destroy pathogens in a similar fashion to macrophages (Staples et al., 2008). Innate immunity responds to pathogenic invasion immediately, without requiring any prior exposure to that pathogen. Innate immunity is considered the “non-specific” branch of immunity and is the predominant defense during early stages of infection. However, it does not have memory and is not increased by repeated exposure to the same insult (Bruno, 2010).
Adaptive immunity contains components like lymphocytes and antibodies that are specific to particular antigens. Adaptive immunity, also called specific immunity, requires previous exposure to become activated, is highly specific and involves memory (Minatel and Carfagnini, 2000). Adaptive immunity may take 4 to 7 days to respond (Staples et al., 2008). Specific antibodies or immunoglobulins are produced against specific pathogens, allowing the recognition of these pathogens if or when reinfection occurs. Lymphocytes are the main cell type involved in adaptive immunity. Similar to the innate immune cells such as neutrophils and macrophages, lymphocytes are produced in the bone marrow. The two major types of lymphocytes are B-lymphocytes and T-lymphocytes. Lymphocytes differ in both function and where they mature; B-lymphocytes are responsible for humoral immunity and mature in the bone marrow, whereas T-lymphocytes take part in cell-mediated immunity and mature in the thymus (Minatel and Carfagnini, 2000). Lymphocytes are able to recognize antigens through specific membrane receptors which define unique characteristics of specificity, diversity, memory and self versus non-self recognition (Sordillo and Streicher, 2002). Each lymphocyte is unique, meaning it is pre-programmed during maturation to recognize a single antigen. During infection with a particular microorganism, there is a clonal expansion of lymphocytes specific for the antigens of the foreign microorganism (Ringler, 1996). Unlike macrophages and neutrophils, B cells depend on their cell surface receptors to recognize specific pathogens (Minatel and Carfagnini, 2000). B-lymphocytes have the capability to produce antibodies against invading pathogens. Bovine antibodies (immunoglobulins, Ig), can be classified into several classes: IgM, IgG1, IgG2, IgA, and
IgE (Sordillo and Streicher, 2002). T-lymphocytes can be further classified into αβ T-cells and γδ T-cells. αβ T-cells can be divided into two subsets based on their surface expression of molecules and binding of the major histocompatibility complex (MHC) molecules: CD4+ and CD8+ T-cells (Minatel and Carfagnini, 2000). CD4+ and CD8+ T-cells are known as T-helper cells, which secrete cytokines and other signals that help regulate the immune response, and cytotoxic T-cells, that aid in killing cells that express foreign antigens, respectively (Minatel and Carfagnini, 2000).

Role of Neutrophil in Mammary Gland Defenses

Mammary gland immunity is defined as the protection and resistance to infectious disease (Sordillo and Streicher, 2002). The immune response depends on ability of host cells to recognize and discriminate between foreign and self-molecules (Sordillo and Streicher, 2002). The incidence of mastitis increases when mammary gland defense mechanisms are compromised. Several factors can contribute to decreased host immunity and increased mastitis. These include genetic, physiological or environmental factors (Hopster et al., 1998; Waller, 2000). The severity of the mastitis is dependent on the animal’s response to the entrance and multiplication of the pathogens inside the gland, and on virulence factors specific for different species of bacteria (Bruno, 2010). However, the mammary gland is protected by the two distinct categories of defense: innate and adaptive immunity. Innate serves as the predominant defense during early stages of infection, where responses are mediated by physical barriers such as the teat end (Sordillo and Streicher, 2002). This non-specific defense is initiated at the teat end, and
continues with host cells such as macrophages, neutrophils and cytokines (Sordillo and Streicher, 2002). Adaptive immunity involves the acquired immune system that is programmed to recognize specific antigens through memory from previous exposure. This recognition is carried out by antibodies, macrophages and several types of lymphocytes (Sordillo, 2005). Protection from IMIs requires that both innate and adaptive defenses are in synch.

Due to the highly integrated nature of mammary gland immunity, defense mechanisms can be further broken down into anatomical, cellular and soluble constituents (Sordillo and Streicher, 2002). Intramammary infections can only occur when the agent is physically present in the gland. Anatomical defenses are responsible for preventing bacterial entrance through the teat canal and subsequent invasion further into the gland. The teat end contains sphincter muscles that remain constricted between milkings to prevent infection. The teat canal is lined with keratin, which is a waxy substance derived from epithelium that has antimicrobial qualities and can prevent bacterial migration into the teat cistern (Sordillo, 2005). Antimicrobial agents have been identified within the keratin, including esterified and non-esterified fatty acids such as myristic, palmitoleic and linoleic acids (Treece et al., 1966). Keratin acts as a physical barrier to prevent any further upward movement into the mammary gland. However, failure in the physical barriers’ normal function can result in infection. For example, abrasion and cracks on teat skin favor bacterial colonization, increasing the risk of bacterial invasion, mainly between milking procedures when the duct is dilated (Bruno, 2010). Intramammary infection results once bacteria are able to pass through the teat
duct, multiply in the teat and gland cistern, and travel dorsally to the milk-producing tissues and structures (Akers and Nickerson, 2011). Once the agent surpasses the physical barriers, it will have to successfully evade cellular defense in order to cause disease.

When anatomical defenses are overcome, protection is dependent upon cellular defenses. Leukocytes that aid in mediating immune responses are neutrophils, macrophages, and lymphocytes (Sordillo and Streicher, 2002). Neutrophils are non-specific and are recruited actively to the site of infection during early stages of inflammation associated with infection (Sordillo, 2005). Neutrophils form the first line of cellular defense against invading pathogens (Paape et al., 2003). Neutrophils are characterized by a polymorphic-segmented nucleus, plentiful cytoplasmic granules that provide constituents for killing bacteria, large stores of glycogen for energy and a highly convoluted surface that is used for phagocytosis of bacteria and formation of intracellular phagocytic vacuoles (Paape et al., 2003). The multi-lobed shape of the nucleus allows the neutrophil to move through small openings so that cells easily can enter tissue to perform their specialized roles of phagocytosis (Paape et al., 1979; Herbert and Bardossi, 1969). Numerous receptors are also located on the neutrophil’s surface (Paape et al., 2003). These receptors can bind immunoglobulins, primarily IgG₂ and IgM, and complement which allows the neutrophil to recognize bacteria that have become opsonized or coated with immunoglobulins or complement (Paape et al., 1979). Some receptors are used to recognize chemoattractants that allow the neutrophil to migrate to the area of inflammation (Paape et al., 2003). As neutrophils phagocytize microorganisms, they have bactericidal effects that are mediated through a respiratory burst that produces
hydroxyl and oxygen radicals (Heyneman et al., 1990). Respiratory bursts are characterized by an increase in oxygen consumption, activation of the hexose-monophosphate shunt, and generation of reactive oxygen–derived free radicals and their metabolic products (Fantone and Ward, 1982). The respiratory burst originates at a site on the plasma membrane where phagocytosis has taken place by the activation of a membrane-bound oxidase that uses NADPH to reduce two molecules of oxygen into two superoxide (\(O_{2}^{-}\)) molecules. Reactive oxygen species or free radicals assist in killing bacteria, however it can lead to tissue damage and even cell death (Bruno, 2010). Once neutrophils engulf and kill pathogens, their fate is terminal (Paape et al, 2002). When blood and milk neutrophils were compared according to their phagocytic ability, it was found that milk neutrophils were poorer phagocytes than those in blood (Paape et al., 1979; Wisniowski et al., 1965). The margination and migration into tissues takes energy. For example, when neutrophils are recruited to the mammary gland, they have to pass through alveolar or ductal epithelium (Jain, 1976), utilizing energy reserves of the neutrophil. In addition, Naidu and Newbould (1973) found that milk neutrophils contained 38% less glycogen than blood neutrophils. Reduced phagocytic ability of milk neutrophils has also been related to the interference from components in the milk. Neutrophils encounter and ingest components such as fat and casein that interfere with phagocytosis (Paape and Guidry, 1977). Microscopic observation showed that 68% of neutrophils on average had phagocytized fat globules (Paape et al., 1979; Wergin and Paape, 1978).
Macrophages are another important phagocytic cell in the body. In a lactating dairy cow, macrophages are the primary leukocyte in a healthy, non-infected gland, whereas neutrophils are generally lower in number (Sordillo and Streicher, 2002). During intramammary infections, macrophages detect invading pathogens and help recruit neutrophils from the vasculature into the site of infection (Akers and Nickerson, 2011). Neutrophils become the most numerous leukocyte, comprising greater than 90% of the mammary gland leukocyte population during infection in order to phagocytize and kill pathogens (Sordillo and Streicher, 2002; Persson et al., 2003). Although similar in function, the change in leukocyte type and number between macrophages and neutrophils is thought to be due to macrophages having fewer Fc receptors. This could decrease their rate of phagocytosis when compared to neutrophils (Niemialtowski et al., 1988). Macrophages, however, have the ability to secrete substances, such as chemoattractants, that aid in the migration and bactericidal capabilities of neutrophils and this is believed to be of importance to the immune defense (Sordillo and Streicher, 2002). Macrophages also play a role in specific immune responses through antigen processing and presentation (Politis et al, 1992).

Mammary gland cellular defense can also be mediated through B and T-lymphocytes. B and T-cells are able to recognize specific antigens through cell surface receptors. The primary role of B-cells is the production of antibodies (Sordillo and Streicher, 2002). T-cells (αβ subgroup) can be classified into T-helper cells (CD4+) and cytotoxic T-cells (CD8+). During mastitis, CD4+ T-cells prevail and are activated in response to recognition of antigen-MHC class II complexes on antigen-presenting cells.
such as B-cells or macrophages (Sordillo and Streicher, 2002). T-helper cells activate other lymphocytes and macrophages by their ability to secrete certain cytokines. Based on cytokine repertoire, T-helper cells can facilitate cell-mediated or a humoral immune response (Brown et al., 1998). Cytotoxic T-cells can have a cytotoxic or suppressor function, meaning they can eliminate host cells that express foreign antigen, or they control response by suppressing activation of these cells during infection (Sordillo and Streicher, 2002). Cytotoxic cells may also have the ability to clean up damaged secretory cells, which could decrease the susceptibility of the mammary gland to infections (Taylor et al., 1994).

**Nutrition and Trace Minerals’ Role in Immunity**

Evidence has shown that nutritional factors are associated with mastitis in cows and heifers (Heinrichs et al., 2009). Vitamins and trace minerals are essential in the cow’s diet to support overall health and longevity. A dairy cow’s requirements for vitamins and minerals are affected by a variety of factors such as age, pregnancy, and production level or for the heifer, rate of growth (NRC, 2001). When the consumption of vitamins and minerals are not optimal, it can have negative effects on the cow’s immunity. They may be more susceptible to disease, such as mastitis, due to a depressed immune system and not being able to fight off the bacteria that invade the mammary gland (Bruno, 2010). Preventing deficiencies in trace minerals has been recognized for many years as important for the maintenance of production, reproduction and health (Nemec, et al.,
2012). As the demand of productivity increases, interest heightens for improving feeding strategies, feed efficiency and utilization of nutrients.

Trace minerals play a large role in immunity and host defense against pathogens as well as regulating cell functions throughout the body. Copper, Mn and Zn are required to support immune function and overall health. Deficiencies, whether marginal or severe, can lead to problems associated with innate and adaptive immunity (Nemec, 2012). Copper, Mn and Zn affect the immune system in many ways, including their presence in antioxidant pathways and in maintaining structure of epithelial barriers against infection (Nemec, 2012). Antioxidants can be defined, in simple terms, as any substance that delays, prevents or removes oxidative damage to a target molecule (Halliwell and Gutteridge, 2007). Antioxidants and associated enzymes play an important role in neutralizing oxygen metabolites after the neutrophil kills invading bacteria through respiratory bursts. In addition, they prevent damage of tissues and cells in the host, including protecting the neutrophil from self destruction or damage prior to bacterial kill (Arthur and Boyne, 1985). According to Sordillo and Aitken (2009), accumulation of ROS causes damage to mammalian tissues with main biological targets including lipids, proteins, DNA and other macromolecules. Important free radicals present in biological systems, that antioxidants help in removing, include superoxide, hydrogen peroxide, hydroxyl radical and fatty acid radicals (Bruno, 2010). Three enzymes in particular are responsible for controlling these compounds: SOD, glutathione peroxidase and catalase (Oldham and Bowen, 1998). Superoxide can be removed by dismutation, which may occur spontaneously in aqueous solution or catalyzed by this cytoplasmic enzyme which
protects cells from superoxide induced injury (Minatel and Carfanini, 2000). Superoxide dismutase can be found in two different forms: copper-zinc containing SOD that is present in the cytosol or a manganese containing SOD present in mitochondria (Minatel and Carfanini, 2000). The body’s antioxidant system allows for the conversion of superoxide to hydrogen peroxide by this superoxide dismutase (Bruno, 2010). The hydrogen peroxide product is very important for the formation of two potent antimicrobial agents: oxidized halide and hydroxyl radical. Hydrogen peroxide is then converted to water by glutathione peroxidase, an enzyme that depends on Se to function properly, and catalase. By these two processes, these enzymes help control most free radicals in the cells’ cytosol.

Copper (Cu) and zinc (Zn), which are incorporated with proteins and enzymes, are involved in the antioxidant defense system. Together they form the copper-zinc superoxide dismutase (SOD), as mentioned above, which converts superoxide to hydrogen peroxide in the cytoplasm of the cell (Nockels, 1996). A similar enzyme, manganese superoxide dismutase, is present in the mitochondria. These enzyme functions may be affected by the quantity of mineral and protein availability. Copper and zinc can also be bound to metallothionein and ceruloplasmin, which are extracellular proteins that have antioxidant capabilities (Nockels, 1996). Ceruloplasmin is an essential Cu transport protein that exhibits oxidase activity and accounts for the majority of Cu present in circulating plasma (Spears and Weiss, 2008; Harris, 1993). In addition, ceruloplasmin and metallothionein exhibit anti-inflammatory activity and may play critical roles in preventing oxidative tissue damage from infection and inflammation (Stabel et al., 1993).
Copper’s role is not limited to antioxidant defense. Copper is also important in cellular respiration, cardiac function, bone formation, connective tissue development and myelination of the spinal cord (Andrieu, 2008). Copper is a component of enzymes such as cytochrome oxidase, which is necessary for electron transport in aerobic respiration; lysyl oxidase, which aids in the formation of desmosine cross links in collagen and elastin which is essential for bone and connective tissues (NRC, 2001). Determining the role of Cu in ruminant diets, regarding immune responses and disease resistance, is made difficult because of numerous interactions that can occur between Cu and other minerals (Spears, 2000). Deficiency is observed in the presence of dietary antagonists such as sulfur, iron and molybdenum which reduce copper’s bioavailability (Spears and Weiss, 2008). A three way interaction occurs between copper, molybdenum and sulfur, leading to the formation of thiomolybdates in the rumen (Spears, 2003). Phagocytic activity of neutrophils from ruminants with Cu deficiency has been studied in various experiments; however results have varied due to these interactions. For example, Boyne and Arthur found that neutrophils from heifers with Mo or Fe-induced Cu deficiency showed impaired phagocytosis of *Candida albicans* (Minatel and Carfagnini, 2000; Boyne and Arthur, 1986). Niederman et al. reported a reduced phagocytic activity in neutrophils from gestating beef cattle supplemented with Fe and Cu sulfate, assuming an unknown interaction between both. In addition, these animals were not Cu-deficient according to liver levels of copper (Minatel and Carfagnini, 2000; Niederman et al., 1994). Other studies have reported no impairment in phagocytic activity of neutrophils from Cu-deficient ruminants (Boyne and Arthur, 1981; Gengelbach et al., 1997). Jones and Suttle
(1981) found that administration of Cu to Cu-depleted calves increased the ability of isolated peripheral blood granulocytes, primarily neutrophils, to kill ingested Candida albicans by over 2-fold. Both primary and induced Cu-deficiency impairs the neutrophils in ruminants by decreasing microbiocidal activity, O$_{2}^-$ production and SOD activity. Neutrophils have a rapid turnover rate and are terminally differentiated in the blood, therefore they are very susceptible to low Cu levels and are a reliable cell to use as a Cu status indicator (Minatel and Carfagnini, 2000).

The micronutrient Zn is an essential component of the dairy cow’s diet for maintaining health and performance (Cope et al., 2009). Zinc is the second most abundant trace element in mammals and birds and makes up a structural component of over 300 enzymes (Andrieu, 2008), including those involved in DNA and RNA synthesis (Spears and Weiss, 2008). Zinc plays a role in maintaining health and integrity of skin due to its role in cellular repair and replacement (Sordillo et al., 1997). Zinc is a component of thymosin, a hormone produced by thymic cells that regulates cell-mediated immunity (NRC, 2001). In addition to the antioxidant role in combination with copper, zinc may contribute to the reduction of somatic cell count due to its role in keratin formation. Zinc deficiency has been shown to impair immune responses and reduce resistance against disease (Spears and Weiss, 2008).

Manganese is essential in the body, besides being a part of the superoxide dismutase, due to its role in metabolism. Manganese is an important part of a range of enzymes that are involved in antioxidant protection, bone growth, carbohydrate and lipid metabolism, reproduction and immune and nervous function (Andrieu, 2008).
Organic versus Inorganic Supplementation

Traditionally, trace minerals have been supplemented in animal diets as inorganic salts, copper sulfates, zinc oxide or sodium selenite (Spears, 1996; Andrieu, 2008). However, there has been an interest in the use of chelated or organic trace minerals in place of inorganic forms due to increased bioavailability as measured by mineral absorption by and/or retention within the animal (Gressley, 2009). Organic trace minerals should undergo less dissociation in the reticulo-rumen, omasum and abomasum than their inorganic counterparts (Gressley, 2009). Other reasons for investigating organic trace minerals include increased absorption in the gut, negative interactions between ingested metal ions and dietary factors when inorganic trace minerals are fed, and environmental impacts regarding undigested mineral compounds (Andrieu, 2008).

Inorganic trace minerals are associated with a dry form of the sulfate and quickly dissociate from the sulfate when they come in contact with water in the gastrointestinal tract. Once dissociated, trace minerals can interact with digesta in the stomachs to form insoluble or indigestible compounds that get excreted through urine and feces (Nemec et al., 2012). Utilization of these inorganic trace minerals is dependent on the animal’s ability to convert them to organic biologically active forms (Spears, 1996). Due to low bioavailability, nutritionists recommend increased quantities of inorganic trace minerals in an attempt to guarantee uptake in the gut (Andrieu, 2008).

In contrast, organic trace minerals supplements vary in regard to the type of ligand or ligands used to form the metal complex or chelate (Spears, 1996). Chelation refers to a special type of complex formed between a ligand and a metal ion. To be
classified as such, it must contain a minimum of two functional groups (oxygen, nitrogen, amino etc.) that are capable of donating a pair of electrons to combine with a metal and form a heterocyclic ring structure with the metal (Spears, 1996; Kratzer and Vohra, 1986). Organic forms of trace minerals including metal amino acid chelates, metal complexes, metal methionine hydroxyl analog chelates, metal proteinates and metal propionates have been developed and researched to increase absorption and bioavailability (Nemec et al., 2012). The development and marketing of organic trace minerals has depended upon the theory that they are more similar than inorganic sources to forms that occur in the body (Spears, 1996). Trace minerals exist in the body and function almost completely as organic complexes or chelates and not as free inorganic ions (Spears, 1996). Physiological function of chelated and complexed metals is dependent upon the degree to which the ligands remain bound to the metal under physiological pH conditions. In other words, with organic ligands, metals would be protected from forming complexes with other dietary components, thus allowing for greater absorption (Cao et al., 2000). Research has also shown that a lower amount of organic trace mineral needs to be fed in comparison to inorganic forms due to this increased intestinal absorbency. The quantity of the mineral may not be as important as the form (Cope et al., 2009; Spears, 1996).

**Trace Minerals and Bovine Mammary Health**

Assessments of different trace mineral supplements is somewhat difficult because no standard test exists to predict availability of one trace mineral source relative to
another (Cao et al., 2000). Comparisons are often conducted using tissue culture or animal feeding trials (Gressley, 2009). According to Gressley (2009), one of the most accurate ways to measure bioavailability is by performing feeding trials in which animals are fed different sources of trace minerals and indices of mineral availability are measured. Blood or liver mineral concentrations, blood or liver concentration of mineral-containing proteins, activity of mineral-containing enzymes, or mineral retention calculated as difference between consumed and excreted minerals are examples of indices of mineral availability (Gressley, 2009). Results among individual feed trials vary due to their dependence on test conditions (i.e. composition of diet, stage of lactation, environmental conditions) and because of this variation, conclusions from one trial to another cannot be used to assess bioavailability values to a given mineral supplement (Cao et al., 2000).

Research has shown variable effects of feeding Zn, Cu and Mn in the organic form. Siciliano-Jones et al. (2008) saw an increase in milk production from diets supplemented with amino acid complexes versus sulfate supplementation of Cu, Zn, Mn and Co. Hoof health and fertility parameters were also improved, however bioavailability measured by liver mineral concentrations was not affected between treatments. In contrast, Nocek et al. (2006) found that liver Cu and Zn concentrations were higher in animals fed organic trace minerals at 100% NRC (2001) requirements compared with inorganic trace minerals at 100% NRC requirements. Supplementation of organic Cu and Zn had no effect on retained placenta, metritis, displaced abomasum, ketosis, mastitis (Nocek et al. 2006). Despite variability among studies, results generally point to
improved animal production and health responses for organic trace mineral supplementation versus inorganic (Gressley, 2009).

Although dietary supplementation of Cu, Zn and Mn may influence several health parameters, little is known about their effect directly on neutrophil function in combination and if that differs between inorganic and organic sources. Past research has shown Se has an essential role in regulating immune functions (Ibeagha et al., 2009). For dairy cows, Se influences both innate and adaptive immunity (Larsen, 1993; Ndiweni and Finch, 1995). Boyne and Arthur (1981) discovered a decrease in neutrophils’ ability to kill phagocytized Candida albicans in cows suffering from Se deficiency. A study by Ibeagha and colleagues (2009) looked at Se supplementation via inorganic and organic sources in periparturient dairy cows. Results showed that the organic supplemented Se treatment had significantly higher respiratory burst activities compared with inorganic and control groups. Higher activities could lead to increased intracellular kill of bacteria. Cows supplemented with inorganic Se had increased intracellular kill of bacteria, enhanced viability and reduced extracellular hydrogen peroxide concentration compared with non-supplemented cows (Gyang et al., 1984; Grasso et al., 1990; Hogan et al., 1990). Organic supplementation of Se had enhanced effects on several neutrophil functions, although phagocytosis did not differ between organic and inorganic treatments (Ibeagha et al., 2009). Weiss and Hogan (2005) also reported that phagocytosis and intracellular kill did not differ among organic and inorganic supplemented Se in their study. Weiss and Hogan (2005) suggested that the lack of intracellular kill differences between organic and inorganic supplemented animals were due to cows having a similar
Se status, even though serum concentrations were significantly higher in Se for the organic supplemented animals. Serum concentrations of Se were considered above adequate in both inorganic and organic Se supplemented cows.

Nutritional intervention of micronutrients, such as Se, Cu, and Zn has been shown to be effective in improving udder health and reducing SCC (Smith et al., 1984, 1997; Erskine, 1993; Harmon and Torre; 1997). Copper supplementation has been shown to lower bacterial counts, lower SCC, lower clinical udder scores and lower peak rectal temperatures than responses in control animals after intramammary challenge with *E. coli* (Scaletti et al., 2003). Knowing Cu is an essential micronutrient, it is also important to consider its source in the diet and what effects bioavailability may have on immune defenses. Scaletti and Harmon (2012) were the first to investigate effects of organic Cu supplementation on experimentally induced mastitis compared with an inorganic source. Scaletti and Harmon (2012), found that supplementation of Cu-proteinate was associated with improvements in milk yield, clinical udder score, and *E. coli* counts compared with Cu sulfate and unsupplemented groups following an *E. coli* challenge.
Nutritional supplementation of specific vitamins and minerals can affect the cellular defenses important in the protection against bovine IMIs. Neutrophils play an important role in mammary defenses by engulfing and killing bacteria that invade the udder. Neutrophils comprise the largest leukocyte cell population in the mammary gland during infection. Supplementation with micro minerals such as Cu, Zn, Mn and Se has been shown to be beneficial in improving udder health by reducing SCC and incidence of mastitis. Copper, Zn and Mn play important antioxidant roles essential for functionality of many enzymes and structural proteins in neutrophils.

Micro minerals have often been supplemented via sulfate form, however supplementation via organic sources may increase bioavailability in the gastrointestinal tract, ultimately enhancing intestinal absorption and utilization by the animal. Despite Cu, Zn and Mn influences on multiple health parameters, little is known about their direct effect on neutrophil phagocytosis and intracellular kill.

The hypothesis of the current study was supplementation of Cu, Zn and Mn will result in increased in vitro neutrophil function compared with unsupplemented diets and organic supplementation of Cu, Zn and Mn will result in increased in vitro neutrophil function compared with inorganic supplementation.
MATERIALS AND METHODS

All procedures using animals were approved through The Ohio State Institutional Animal Use and Care Committee (Columbus, OH), Animal Use Protocol: 2014A00000059.

Experimental Design

Thirty mid-lactation Holstein cows in the Ohio Agriculture Research and Development Center Krauss Dairy Herd were used to measure the effects of Cu, Zn and Mn supplementation on bovine neutrophil function. Cows were assigned to 10 blocks of 3 based on parity, milk production and DIM. Four blocks were composed of primiparous cows, and six blocks were composed of multiparous cows. Average (± SD) milk production was 31.4 ± 6.3 kg during pre-treatment period and DIM ranged from 205-383 at time of neutrophil assays. Cows were fed a TMR once daily through pre-treatment and treatment period with a target of 3 to 5% refusal. During pre-treatment period, all cows were placed on an un-supplemented diet (8.0 mg/kg of DM of Cu, 35.0 mg/kg of DM of Zn, and 35.0 mg/kg of DM of Mn) for 30 d and housed in free stalls. After this period, cows were moved into tie-stalls and placed on one of the three treatment diets for an additional 30 d. Cows within blocks were randomly assigned to one of three treatments by pulling slips of paper out of a container: 1) devoid of supplemental Cu, Zn and Mn; 2)
supplemented with Cu, Zn and Mn via sulfates; 3) supplemented with Cu, Zn and Mn via organic form (B-Traxim®; Pancosma, Geneva, Switzerland). The un-supplemented treatment cows, which served as the control, remained on pre-treatment Cu, Zn and Mn concentrations. The sulfate treatment was supplemented with a target of 18.0 mg/kg of DM of Cu, 60.0 mg/kg of DM of Zn and 60.0 mg/kg of DM of Mn. The organic (Cu as Cu complex of Cu sulfate and glycine, Zn as Zn glycine complex of Zn sulfate and glycine and Mn as Mn chelate of glycine, hydrate, B-Traxim® 2C, Pancosma) treatment was also supplemented with a target of 18.0 mg/kg of DM of Cu, 60.0 mg/kg of DM Zn, and 60.0 mg/kg of DM of Mn. Of the 30 Holstein cows, one block of multiparous cows were removed from the study due to two cows within the block having abnormally high or low blood neutrophil counts, leaving nine observations per treatment.

**Bacteria**

*Escherichia coli* 487, originally isolated from clinical mastitis, was the bacterial strain used for the bacterial phagocytosis assay. An overnight culture of *E. coli* 487 was prepared by inoculating 0.1 ml of stock culture into 12 ml of tryptic soy broth and incubated overnight in a gyratory shaker at 100 rpm and at 37°C. The bacteria were then centrifuged (Sorvall® RC-5B Refrigerated Superspeed Centrifuge, SS-34 rotor; DuPont Instruments, Wilmington, DE) for 20 min at 4°C and 5049 x g. The pellet was re-suspended into 12 ml of Hanks balanced salts solution (HBSS) (modified, without phenol red and sodium bicarbonate; Sigma and Aldrich, St. Louis, MO) and diluted to a 70% transmission at 540 nm (GENESYS™ 6 Spectrophotometer; Thermo Electron)
Corporation; Madison, WI), corresponding to approximately 8.0 x 10^6 cfu. Bacteria were serial diluted and spotted (10 µl x 4) on MacConkey (Becton Dickinson Microbiology Systems, Cockeysville, MD) agar plates and incubated at 37°C for 24 h to confirm the total count of *E. coli*. Bacteria were opsonized with 10% heat inactivated bovine serum for 20 min at 20°C and placed on ice. Bovine serum used for opsonization was collected and pooled from 12 lactating cows (Hogan et al., 1991).

**Preparation of Blood Neutrophils**

Blood (50 ml) was collected from each animal in a block on the same day via jugular vein with a 16 gauge, 38.1 mm needle. Neutrophils were isolated according to Carlson and Kaneko (1973). Blood was collected in sterile 60 ml BD Luer-Lok™ syringes (Becton Dickinson Microbiology Systems, Cockeysville, MD) containing 5 ml of EDTA anticoagulant (0.7% NaCl, 1.5% EDTA disodium salt: dehydrate, 0.0132 M KH₂PO₄ and 0.0132 M Na₂HPO₄; adjusted to pH 6.6). Forty ml of blood was dispensed into 50 ml conical bottom centrifuge tubes (Corning Incorporated, Corning NY) and centrifuged (Sorvall® RT7, RTH-750 rotor; Newtown, CT) for 45 min at 4°C and 1,294 x g. The plasma, buffy coat, and upper 2 ml of red blood cells were discarded, leaving approximately 8 ml remaining. Remaining red blood cells were lysed by adding 30 ml of 0.03 M NaCl to the tube and mixed for 90 s by hand. Eight ml of 0.63 M NaCl was added and mixed to bring saline solution back to equilibrium. After lysis, cells were centrifuged for 15 min at 4°C and 748 x g. The supernatant was removed and neutrophil pellet was washed with 5 ml of HBSS and then centrifuged again for 15 min at 4°C and 748 x g.
Again, the supernatant was removed and 1 ml of HBSS was added to re-suspend the pellet. Viability was measured using 0.05% trypan blue (Sigma-Aldrich, St. Louis, MO) dye in the amount of 450 µl of trypan to 10 µl of neutrophil suspension. One hundred cells were counted using 40X objective. Blue and clear cells were counted as dead and live, respectively. Total cell count was determined by counting all cells in four fields on a hemocytometer (Bright-line® hemocytometer; Reichert, Buffalo, NY). A differential stain (Protocol® HEMA 3® Stain Set; Fisher Scientific Company L.L.C; Middletown, VA) was completed to determine percentage of neutrophils in the preparation. This was measured by counting first 100 cells observed. The number of viable neutrophils was determined by total number of cells multiplied by viability and percentage of neutrophils [(Total cells) X (Viability) X (%neutrophil)]. Cell preparations averaged (± SD) 96.9 ± 1.5% viable cells and 94.2 ± 3.4% neutrophils. Concentrations of viable PMNs were adjusted to 4.0 x 10^6 viable neutrophils/ml of HBSS.

**Neutrophil Assays**

Neutrophil assays were prepared in a 4:1 [Average (± SD) 3.78 ± 1.1:1] bacteria to neutrophil ratio (Weiss and Hogan, 2005). Assays were completed in duplicate for each cow within each block. One ml of opsonized bacteria was mixed with 0.5 ml of neutrophil suspension in sterile 12 x 75 mm culture tubes with closures. Assays were incubated at 37°C for 1.5 h at 100 rpm on a gyratory shaker. After incubation, samples were diluted 2:1:1 as 50 µl of assay suspension, 25 µl of acridine orange (1.4 mg/10 ml PBS, Sigma-Aldrich; St. Louis, MO) and 25 µl of crystal violet (5 mg/10 ml PBS, Fisher
Scientific; Fair Lawn, NJ). Wet mount slides were prepared and the number of live (green bacterial cells), dead (red bacterial cells) bacterial cells were counted for the first 25 neutrophils visible, using the 1,000X oil immersion lens under UV light (Nikon Fluorescence Microscope; Nikon Inc.; Garden City, NY). Neutrophils were counted by moving the microscope stage left to right and observation fields were not repeated. Neutrophil count was completed in duplicate for each cow within each block. Assays were completed within the same day for each individual block to minimize bacterial variability within each block. Assays were blinded so that the individual counting the neutrophils and bacteria had no knowledge of treatments and respective cows. Variables measured were percent neutrophils phagocytizing, neutrophil intracellular kill and phagocytic index. Percent neutrophils phagocytizing was determined by number of neutrophils with internalized bacteria divided by the sum of neutrophils with internalized bacteria and neutrophils without internalized bacteria. Neutrophil intracellular kill was determined by number of dead intracellular bacteria divided by number of dead and live intracellular bacteria. Lastly, phagocytic index was determined by total number of phagocytized bacteria divided by the sum of number of neutrophils with internalized bacteria and neutrophils without internalized bacteria.

Serum Mineral Concentration

Six ml of blood was taken via tail vein on the first and last days of treatment period from each cow and centrifuged for 10 min at 4°C and 5,049 x g. Serum was then removed in 1 ml aliquots and placed in plastic snap vials. Vials were frozen at -20°C and
stored until all serum samples were collected. Blood serum was sent to a commercial diagnostic lab (Diagnostic Center for Population and Animal Health; Lansing, MI) to be analyzed for trace mineral (Cu, Zn, Mn) concentrations in the blood. Elemental analysis was completed by methods of Wahlen et al. (2005), using an Agilent 7500ce Inductively Coupled Plasma-Mass Spectrometer (ICP/MS).

**Diet and Milk Parameters**

Feed component samples were collected weekly and DM (100°C oven for 24 h) was calculated and recorded. Remaining feed samples were kept frozen at -20 °C until trial was complete. Once completed, composite samples were made for each feed component: corn silage, alfalfa silage, dry hay, and three treatment grain mixes. Composite samples were composed of 2-4 consecutive sample dates and sent to a commercial analytic lab (Cumberland Valley Analytical Services; Hagerstown, MD) for analysis of DM (Forage: Goering and Van Soest, 1970 and dried at 103°C for 3 hrs, Grain: method 930:15; AOAC, 2000), CP (AOAC, 1990), NDF (Van Soest et al., 1991), ash (method 942.05; AOAC, 2000), and minerals (Ca, P, Mg, K, Na, Fe, Mn, Zn, Cu and S; method 985.91; AOAC, 2000).
**Table 1. Ingredient composition of diet.**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%DM</th>
<th>Sulfate</th>
<th>Organic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>29.0</td>
<td>29.0</td>
<td>29.0</td>
</tr>
<tr>
<td>Alfalfa silage</td>
<td>29.0</td>
<td>29.0</td>
<td>29.0</td>
</tr>
<tr>
<td>Alfalfa hay</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Ground corn</td>
<td>19.5</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>Dried distillers grains</td>
<td>9.1</td>
<td>9.1</td>
<td>9.1</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Fat</td>
<td>0.37</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Iodized salt</td>
<td>0.37</td>
<td>0.37</td>
<td>0.37</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.59</td>
<td>0.59</td>
<td>0.59</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>Copper sulfate¹</td>
<td>-</td>
<td>0.003</td>
<td>-</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>-</td>
<td>0.006</td>
<td>-</td>
</tr>
<tr>
<td>Manganese sulfate</td>
<td>-</td>
<td>0.005</td>
<td>-</td>
</tr>
<tr>
<td>Traxim Cu-240²</td>
<td>-</td>
<td>-</td>
<td>0.003</td>
</tr>
<tr>
<td>Traxim Zn-260</td>
<td>-</td>
<td>-</td>
<td>0.008</td>
</tr>
<tr>
<td>Traxim Mn-220</td>
<td>-</td>
<td>-</td>
<td>0.009</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.26</td>
<td>0.26</td>
<td>0.26</td>
</tr>
<tr>
<td>Vitamin premix³</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>

¹Sulfate mineral supplement contained 252,000 ppm Cu, 355,000 ppm Zn and 315,000 ppm Mn
² Traxim mineral supplement contained 240,000 ppm Cu, 260,000 ppm Zn and 220,000 ppm Mn of supplement
³ Premix contained 2,775 IU/kg Vitamin A, 999 IU/kg Vitamin D, 16 IU/kg Vitamin E

**Table 2. Nutrient composition of diet and ingredients.**

<table>
<thead>
<tr>
<th>Total</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>DM, %</td>
<td>59.3</td>
</tr>
<tr>
<td>OM, %</td>
<td>92.4</td>
</tr>
<tr>
<td>NDF, %</td>
<td>28.4</td>
</tr>
<tr>
<td>CP, %</td>
<td>17.0</td>
</tr>
<tr>
<td>Ca, %</td>
<td>0.97</td>
</tr>
<tr>
<td>P, %</td>
<td>0.45</td>
</tr>
<tr>
<td>Mg, %</td>
<td>0.29</td>
</tr>
<tr>
<td>K, %</td>
<td>1.89</td>
</tr>
<tr>
<td>S, %</td>
<td>0.28</td>
</tr>
<tr>
<td>Na, %</td>
<td>0.24</td>
</tr>
<tr>
<td>Fe, mg/kg</td>
<td>225</td>
</tr>
<tr>
<td>Cu, mg/kg</td>
<td>9</td>
</tr>
<tr>
<td>Zn, mg/kg</td>
<td>41</td>
</tr>
<tr>
<td>Mn, mg/kg</td>
<td>41</td>
</tr>
</tbody>
</table>
Refusals for cows in tie stalls during treatment period were measured and recorded daily to determine dry matter intake (DMI). Dry matter intake was not determined during pre-treatment period due to cows being fed the same diet and housed in same free stall pen.

Cows were milked twice daily at 1:00 and 13:00 h. Test day milk and milk components (protein, fat, lactose, milk urea nitrogen, and SCC) were measured and recorded once a week using Dairy Herd Improvement Services (DHI Cooperative; Columbus, OH).

Statistical Analysis

A randomized block design was used to group cows into respective treatments. The two factors observed were treatment, which served as the fixed factor, and block as the random factor. Correlation analysis was used to determine correlation between variables. MANOVA (Hoteling) was used to analyze neutrophil parameters (% neutrophils phagocytizing, % intracellular kill and phagocytic index), blood serum concentrations for Cu, Zn and Mn, milk production, milk components (% fat, % protein, % solids, % lactose, MUN and SCC) and DMI. Statistical analysis were performed using the CORR, GLM procedures in SAS® Version 9.4 (SAS® Institute, 2013) with a significance level set at 0.05.
RESULTS

Neutrophil Parameters

Neutrophil responses did not differ among treatments. Mean percentage of neutrophils phagocytizing (Figure 1) were 73.4%, 73.2% and 76.2% for cows fed CON, SUL, and ORG diets, respectively (P=0.49; SEM ± 0.89). Mean intracellular kill (Figure 2) values were 55.6% for cows fed CON diet, 60.7% for cows fed SUL diets and 55.8% for cows fed ORG diets (P=0.63; SEM ± 1.9). Mean phagocytic index by treatment group (Figure 3) were 6.81 CON, 6.51 SUL and 7.08 ORG (P=0.83; SEM ± 0.30).

Figure 1. The percentage of neutrophils phagocytizing (% positive) for neutrophils from cows fed control diet (n=9), sulfate supplementation of Cu, Zn and Mn (n=9) and organic supplementation of Cu, Zn and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means. Means did not differ among treatments (P=0.49).
Figure 2. Mean intracellular kill for neutrophils from cows fed control diet (n=9), sulfate supplementation of Cu, Zn and Mn (n=9) and organic supplementation of Cu, Zn and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means. Means did not differ among treatments (P=0.63).

Figure 3. Phagocytic index for neutrophils from cows fed control diet (n=9), sulfate supplementation of Cu, Zn and Mn (n=9) and organic supplementation of Cu, Zn and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means. Means did not differ among treatments (P =0.83).
Blood Serum Concentrations of Cu, Zn and Mn

Blood serum concentrations of Cu, Zn and Mn did not differ among treatments on d 30 or d 60 (Table 3). Serum concentrations of Cu for CON, SUL and ORG treatments were each lower (P < 0.05) within treatment at the end of the treatment period compared with the start of the treatment period (d 60 versus d 30). Serum Zn and Mg did not differ within treatment between d 30 and d 60 (P > 0.05).

Table 3. Mean Cu, Zn and Mn blood serum concentrations on d 30 (beginning of treatment diets) and d 60 (end of treatment diets) of trial period for cows fed diets unsupplemented control diets, sulfate or organic supplemented Cu, Zn and Mn supplemented diets.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Day</th>
<th>Unit</th>
<th>Treatment</th>
<th>Treatment P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Control</td>
<td>Sulfate</td>
</tr>
<tr>
<td>Cu</td>
<td>30</td>
<td>µg/ml</td>
<td>0.77</td>
<td>0.70</td>
</tr>
<tr>
<td>Cu</td>
<td>60</td>
<td>µg/ml</td>
<td>0.62</td>
<td>0.60</td>
</tr>
<tr>
<td>Zn</td>
<td>30</td>
<td>µg/ml</td>
<td>1.08</td>
<td>1.12</td>
</tr>
<tr>
<td>Zn</td>
<td>60</td>
<td>µg/ml</td>
<td>1.01</td>
<td>1.08</td>
</tr>
<tr>
<td>Mn</td>
<td>30</td>
<td>ng/ml</td>
<td>7.06</td>
<td>2.12</td>
</tr>
<tr>
<td>Mn</td>
<td>60</td>
<td>ng/ml</td>
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<td>1.77</td>
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</tbody>
</table>

Milk Production and Components

Milk production did not differ among treatments. Mean milk production (Figure 4) by treatment was 28.7 kg CON, 28.5 kg SUL and 30.7 kg ORG (P=0.54; SEM ± 2.5). Milk urea nitrogen values did not differ among treatments. Mean MUN (Figure 5) by treatment was 10.1 mg/dl CON, 11.3 mg/dl SUL and 11.1 mg/dl ORG (P=0.30; SEM ± 0.60). Milk concentrations of fat, protein, and lactose did not differ among treatments (P
> 0.05; Table 4). Milk SCC did not differ among treatments. Mean SCC (Figure 6) by
treatment was 4.49 log_{10} cells/ml CON, 4.47 log_{10} cells/ml SUL and 3.80 log_{10} cells/ml
ORG (P=0.08; SEM ± 0.23).

Figure 4. Mean daily milk production tested weekly for cows fed control diet (n =9),
sulfate supplementation of Cu, Zn and Mn (n =9) and organic supplementation of Cu, Zn
and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means. Means did
not differ among treatments (P= 0.54).
Figure 5. Mean milk urea nitrogen (MUN) values of milk tested weekly from cows fed control diet (n = 9), sulfate supplementation of Cu, Zn and Mn (n = 9) and organic supplementation of Cu, Zn and Mn (n=9) for 30d. Dispersion bars represent standard error of the means. Means did not differ among treatments (P=0.30).

Table 4. Least squares mean values for milk components in control diet, sulfate supplementation of Cu, Zn and Mn and organic supplementation of Cu, Zn and Mn.

<table>
<thead>
<tr>
<th>Component</th>
<th>Treatment</th>
<th>Treatment</th>
<th>Treatment</th>
<th>SEM</th>
<th>P-value</th>
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</thead>
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<tr>
<td>Fat (%)</td>
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<td>Sulfate</td>
<td>Organic</td>
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<td></td>
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<tr>
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<td>3.53</td>
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<td>0.33</td>
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<tr>
<td>Protein (%)</td>
<td>Control</td>
<td>Sulfate</td>
<td>Organic</td>
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<td></td>
</tr>
<tr>
<td></td>
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<td>3.25</td>
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<td>0.07</td>
<td>0.56</td>
</tr>
<tr>
<td>Lactose (%)</td>
<td>Control</td>
<td>Sulfate</td>
<td>Organic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.77</td>
<td>4.69</td>
<td>4.77</td>
<td>0.08</td>
<td>0.17</td>
</tr>
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</table>
Figure 6. Mean SCC of milk tested weekly for cows fed control diet (n = 9), sulfate supplementation of Cu, Zn, and Mn (n = 9) and organic supplementation of Cu, Zn and Mn (n = 9) for 30 d. Dispersion bars represent standard error of the means. Means did not differ among treatments (P = 0.08).

DMI

Dry matter intake for cows did not differ among treatments. Mean DMI (Figure 7) by treatment was 22.3 kg for cows fed CON diet, 21.8 kg for cows fed SUL diet and 23.1 kg for cows fed ORG diet (P = 0.23; SEM ± 0.74).
Figure 7. Mean daily DMI for cows fed control diet (n=9), sulfate supplementation of Cu, Zn and Mn (n=9) and organic supplementation of Cu, Zn and Mn (n=9) for 30 d. Dispersion bars represent standard error of the means. Means did not differ among treatments (P=0.23).
Neutrophils play an important role in host defense regarding resistance to bovine mastitis. Neutrophils are non-specific leukocytes that serve as the primary line of cellular defense for the mammary gland once bacterial invasion has occurred at the teat end (Paape et al., 1979). Neutrophils are phagocytic and are able to kill pathogens by engulfment and digestion through granules and bactericidal effects in the cytosol (Sordillo and Streicher, 2002). Bactericidal effects are mediated through a respiratory burst that produces hydroxyl and oxygen radicals (Heyneman et al., 1990). The respiratory burst is very effective, however free radicals can damage surrounding tissues and cells of the host. Neutrophils become the most numerous leukocyte, comprising greater than 90% of the mammary gland leukocyte population during infection in order to phagocytize and kill pathogens (Sordillo and Streicher, 2002; Persson et al., 2003). Neutrophil function, immune health and resistance to mastitis are dependent on many factors, including an association between mastitis and nutrition. Nutritional interactions, regarding immune function and mastitis, have been most commonly related to supplementation of trace minerals and vitamins in the diet. Trace mineral research related to mastitis resistance has primarily focused on Cu, Zn, Mn and Se (Heinrichs et al., 2008). Zinc, Cu, and Mn are required for the functionality of
several structural proteins, enzymes and cellular proteins (Nocek et al., 2006). Requirements for micronutrients are affected by age, pregnancy, production level or for the heifer, rate of growth (NRC, 2001). Improvement of trace mineral feeding strategies is necessary to meet the demands of increased productivity in dairy cows. In the past, trace minerals have been fed as inorganic compounds, mostly sulfate salts (Nemec et al., 2012). In the inorganic form, trace minerals dissociate and can interact with digesta components to form indigestible compounds, thus preventing optimal absorption and utilization by the intestines. Organic forms, such as amino acid chelates, metal complexes, and metal proteinates, have been studied more recently to increase intestinal absorption and bioavailability (Predieri et al., 2005; Wright et al., 2008).

The purpose of the current trial was to determine if supplementation of mid-lactation dairy cow diets with Cu, Zn, and Mn for 30 d affected blood neutrophil function. Supplementation of Cu, Zn and Mn via SUL or ORG form had no effect on percentage of neutrophils phagocytizing, intracellular kill or phagocytic index. Previous studies have shown positive effects of micronutrient supplementation of diets on bovine host defenses. Scaletti et al. (2003) investigated neutrophil function in diets supplemented with Cu versus Cu fed at basal levels. Copper-supplemented animals had lower bacterial counts, lower SCC and lower clinical udder scores than the unsupplemented animals after \textit{E. coli} intramammary challenge. Boyne and Arthur (1981) reported Se and Cu supplementation had no effect on the ability of neutrophils from beef steers to phagocytize \textit{Candida albicans} compared with neutrophils from
steers fed a diet devoid of supplemental Se and Cu. However, intracellular kill by neutrophils was enhanced by supplementing Se and Cu. Nemec et al. (2012) investigated differences in neutrophil function between inorganic and organic sources of Cu, Mn and Zn. Similar to results of the current trial, Nemec et al. (2012) found no differences between inorganic and organic forms relating to neutrophil chemotaxis, phagocytosis or oxidative burst.

Differences in bovine neutrophil function related to supplementation of micronutrients in diets of dairy cows have been demonstrated using the assays employed in the current study (Grasso et al., 1990; Hogan et al., 1990; 1992). Each of these trials reported supplementation of diets or parental injection of Se, vitamin E, or both Se and vitamin E increased intracellular kill of bacteria by neutrophils. Phagocytic ability was not affected by supplementation of with vitamin E and Se. Weiss and Hogan (2005) also investigated neutrophil function differences between cows fed inorganic and organic sources of Se. Neutrophils from cows fed either selenate or Se-yeast supplemented diets did not differ regarding phagocytosis or intracellular kill. In previous trials intracellular kill by neutrophils was related to the blood concentrations of the micronutrients being tested (Hogan et al., 1990; 1992).

Blood serum concentrations for Cu did not differ among treatments after 30 d on experimental diets. In fact, serum Cu values in supplemented cows decreased during the 30 d of supplementation at a rate comparable to that in unsupplemented cows. The similar trend was observed for Zn and Mn with blood values not differing between cows fed the two forms of supplemental minerals or between supplemented and negative
control cows. Normal ranges for serum Cu is 0.6 to 1.1 µg/ml and serum Zn is 0.6 to 1.9 µg/ml (Herdt and Hoff, 2011). Manganese reference values vary but were estimated as 0.9 to 6.0 ng/ml (Herdt and Hoff, 2011). In the current trial, serum concentrations fell within these normal ranges for Cu, Zn and Mn in cows fed unsupplemented and supplemented diets. Previous studies found similar results regarding the relationship of unchanging plasma mineral concentrations and neutrophil function when fed organic Cu or Zn (Nemec et al., 2012). Blood measures are frequently used in assessment of mineral status because they are significantly correlated to nutritional status of some trace elements and are more reliable than assessing mineral status from diet evaluation (Herdt and Hoff, 2011).

In the current study, Cu, Zn and Mn concentrations were measured in serum. Copper and Zn absorption take place in the small intestine, but Cu can also be absorbed from the stomach (Cousins, 1985). Copper and Zn are transported across the brush border surface of the small intestine and bound to absorbable ligands such as albumin or amino acid complexes (Cousins, 1985). Copper is transported via blood to the liver, where Cu is placed back into plasma in the form of ceruloplasmin. The liver serves as the main storage pool for Cu and reflects long-term availability of dietary Cu to the animal (Herdt and Hoff, 2011). Ceruloplasmin accounts for 70 to 95% of plasma Cu (Harris, 1993). Copper is imported into the eukaryotic cell via Ctr1 receptor, which allows the delivery of Cu via permease or endocytosis (Prohaska and Gybina, 2004). Intracellular distribution of Cu is mediated through chaperones, such as copper chaperone for SOD (CCS), which allows the delivery of Cu specifically to SOD (Prohaska and Gybina, 2004;
Leitch et al., 2009). Copper and Cu proteins are distributed throughout the cell and in all cellular organelles, including the cytosol where SOD is present (Vulpe and Packman, 1995). Assessing Cu and Zn statuses are important due to their role in the superoxide dismutase (SOD) enzyme complex, which is located in the cytosol of eukaryotic cells. The SOD enzyme aids in neutralizing free radicals, such as those produced during the respiratory burst of neutrophils (Minatel and Carfagnini, 2000). Due to the toxicity of the reactive oxygen species (ROS) to a variety of cells, SOD aids in protecting cells and surrounding tissues from injury. Copper serves as the catalyst for simultaneous reduction and oxidation of superoxide, whereas Zn contributes to proper protein folding (Leitch et al., 2009). In contrast to Cu, Zn and Mn have no clear storage pools in the body. However, Zn is thought to have the highest concentration in muscle and bone (Cousins, 1985). Homeostasis of these minerals depends on a continual pool in lumen of the gut and intestinal epithelium (Herdt and Hoff, 2011). Intracellular distribution of Zn is thought to be mediated through albumin (Cousins, 1985). Intracellular distribution of Mn is not well understood.

The lack of change in serum concentrations in the current study during the treatment period may explain the observed similarity among treatments in neutrophil function. Serum concentrations for Cu, Zn and Mn did not differ among treatments. If no differences were observed among serum values, differences in neutrophil function among treatments were unlikely. Blood values (whole blood, plasma or serum) for trace minerals are important to measure because they reflect mineral status of transport pools and directly influence mineral availability to neutrophils and other circulating leukocytes.
Neutrophils are produced in the bone marrow and once mature, are able to cross the vascular endothelium into the peripheral blood (Jain, 1976). Once in the circulation, neutrophils have a very short life span, averaging a nine hour half-life (Jain, 1976). Neutrophils are presumably coming in contact and obtaining necessary trace minerals via blood as they are circulating (Leitch et al., 2009). This is especially true for minerals that do not have permeant stores in tissue, such as Zn and Mn. Although cows in the unsupplemented group had a 60 d depletion period, blood values of unsupplemented cows did not differ from those supplemented and were within normal ranges. With no differences among control and supplemented cows, 60 d may not have been long enough to observe differences in serum concentrations for Cu, Zn and Mn.

Milk production did not differ among treatment groups in the current study. Results of previous studies support these results regarding no increase in milk yield (Nemec et al., 2012; Scaletti et al., 2003). In contrast, some studies did observe increased milk production via supplementation of organic trace minerals (Siciliano-Jones et al., 2008; Nocek et al., 2006; Cope et al., 2009; Scaletti and Harmon, 2012). Milk components including fat, protein, and lactose concentrations did not differ among treatments. Siciliano-Jones et al. (2008) reported similar results for milk components when supplementation via inorganic and organic sources for Cu, Zn, Mn and Co was compared.

Corresponding to no differences in milk production, DMI did not differ among treatments. Previous studies observed no differences in DMI relative to trace mineral source (Cope et al., 2009; Wright and Spears, 2004). Related to DMI, the level of Cu, Zn
and Mn supplemented in SUL and ORG treatments was similar to CON. Analyzing the nutrient composition of the diet, SUL and ORG treatments averaged 9.5 ppm supplemented Cu, 21.5 ppm supplemented Zn and 15 ppm supplemented Mn above CON mineral levels. Scaletti et al. (2003) saw differences in cows’ plasma Cu when 20 ppm Cu was supplemented above control diet at calving and subsequent days of measurement.

Treatment effects were not observed for MUN levels. All treatment means fell within an acceptable range (10 to 14 mg/dl; Ferguson, 2000). MUN is a measure of the amount of nitrogen in the form of urea in milk. Urea is a major by-product of protein metabolism in ruminants and high concentrations in milk or blood can be an indication of poor utilization of dietary N (Godden et al., 2001). MUN levels higher than 14 mg/dl could indicate inefficiency of protein in the ration. MUN results have differed among studies comparing inorganic and organic sources of trace minerals. Siciliano-Jones et al. (2008) found no differences between organic and sulfate supplementation regarding MUN, however Hackbart et al. (2010) did observe a higher MUN level in organic versus inorganic supplementation of Cu, Zn, Mn and Co. Similar to milk production and MUN, SCC did not differ among treatments. Somatic cells in milk are primarily leukocytes, including neutrophils, macrophages and lymphocytes (Harmon, 1994). A SCC is therefore a quantitative measure of leukocytes in milk and is an important factor for evaluating infection status, milk quality and mammary gland health (Dohoo and Meek, 1982). A SCC of 200,000 cells/ml (5.30 log10 cells/ml) or less at the cow and quarter level is deemed normal or uninfected (Harmon, 1994). Somatic cell count means were below 100,000 cells/ml (5.00 log10 cells/ml) for each treatment. Regarding other studies,
Scaletti et al. (2003) found that SCC was lower in Cu supplemented cows versus Cu unsupplemented cows. However, Hackbart et al. (2010) observed no differences in SCC between inorganic and partial organic supplementation of Cu, Zn, Mn and Co.
SUMMARY

The positive impacts of trace mineral supplementation on dairy cattle health have been well documented, however there has been an interest in how different sources of minerals may enhance immune function and overall performance. Immune function, especially the ability of neutrophils to phagocytize and kill pathogens, is very important in mastitis resistance. In the current study, three diets were fed: 1) control, 2) sulfate supplementation of Cu, Zn and Mn, 3) organic supplementation of Cu, Zn and Mn. Neutrophils were isolated from the blood and phagocytizing ability and intracellular kill were examined. Organic supplementation of Cu, Zn and Mn did not affect in vitro neutrophil function compared to inorganic supplementation or control treatments. Serum concentrations for Cu, Zn and Mn did not differ among treatments, which may explain why no differences were observed among treatments for neutrophil phagocytosis or intracellular kill. Other parameters measured including dry matter intake, milk production, milk components (percentage of fat, protein and lactose), milk urea nitrogen and somatic cell count did not differ among treatments. In conclusion, dietary Cu, Zn and Mn supplemented either as sulfates or organic form for 30 d had no effect on in vitro blood neutrophil function or other measured parameters in mid-lactation Holstein cows for 30 d.
LIST OF REFERENCES


Ferguson, J.D. 2000. Milk Urea Nitrogen. Center for Animal Health and Productivity., New Bolton Center., 382 West Street Road, Kennett Square, PA 19348-1692, USA.


**APPENDIX: TABLES**

**Table 5.** Summary of body weight and BCS for all cows.

<table>
<thead>
<tr>
<th>Block</th>
<th>Cow</th>
<th>Treatment</th>
<th>Weight (kg)</th>
<th>BCS&lt;sup&gt;a&lt;/sup&gt;</th>
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<sup>a</sup> Body condition score (BCS) was measured using 1-5 scale.
Table 6. Correlation coefficients for neutrophil parameters and serum Cu, Zn and Mn concentrations.

<table>
<thead>
<tr>
<th></th>
<th>% Neut_Phago</th>
<th>% Intracell_Kill</th>
<th>PI</th>
<th>Serum Cu_30</th>
<th>Serum Cu_60</th>
<th>Serum Zn_30</th>
<th>Serum Zn_60</th>
<th>Serum Mn_30</th>
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* Green colored blocks denote correlation coefficients between parameters and white blocks denote P-values between parameters.