

**EFFECTS OF SUPPLEMENTATION OF DIETARY ANTIOXIDANTS  
AND CHELATED TRACE MINERALS IN PERIPARTURIENT  
DAIRY COWS AND SUBSEQUENT RESPONSE  
TO INTRAMAMMARY BACTERIAL  
CHALLENGE**

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of the Requirements for the Degree  
Master of Science**

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**by  
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled:

EFFECTS OF SUPPLEMENTATION OF DIETARY ANTIOXIDANTS  
AND CHELATED TRACE MINERALS IN PERIPARTURIENT  
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a candidate for the degree Master of Science,  
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## TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	ii
TABLE OF CONTENTS.....	iv
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABSTRACT.....	x
Chapter One: REVIEW OF LITERATURE.....	1
Introduction.....	1
The Role of Transition Period in Dairy Cows.....	2
Intramammary <i>Escherichia coli</i> Infection in Dairy Cows.....	6
Nutritional Strategies to Improve Immunity: Antioxidants and Microminerals... ..	12
Antioxidants.....	14
Microminerals.....	15
Conclusion.....	19
Chapter Two: HEALTH, METABOLISM, AND PRODUCTION OF PERIPARTURIENT DAIRY COWS SUPPLEMENTED WITH DIETARY ANTIOXIDANT AND CHELATED TRACE MINERALS.....	21
Abstract.....	21
Introduction.....	23
Material and Methods.....	25

Animals, Diets, Experimental Design, and Treatments .....	25
Health Monitoring .....	28
Blood Samples.....	29
Colostrum and Milk Samples .....	30
Energy Balance and Feed Efficiency .....	31
Laboratory Analyses.....	32
Statistical Analysis .....	36
Results.....	38
Dry Matter Intake .....	38
Colostrum .....	38
Milk .....	39
Feed Efficiency.....	40
Energy Status.....	41
Health .....	42
Metabolic Variables.....	43
Discussion .....	45
Conclusion .....	54

Chapter Three: RESPONSE OF DAIRY COWS SUPPLEMENTED WITH DIETARY ANTIOXIDANTS AND CHELATED TRACE MINERALS TO INTRAMAMMARY BACTERIAL CHALLENGE .....	73
Abstract.....	73
Introduction.....	75

Material and Methods .....	78
Animals, Diets, Experimental Design, and Treatments .....	78
Intramammary Bacterial Challenge.....	80
Milk Sampling and Analysis .....	81
Analysis.....	82
Blood Samples and Rectal Temperature .....	82
Statistical Analysis .....	86
Results.....	88
Bacteria.....	88
Dry Matter Intake .....	89
Milk .....	89
Feed Efficiency.....	93
Metabolic Variables.....	93
Discussion.....	95
Conclusion .....	101

Chapter Four: INTEGRATED OVERVIEW FROM THE STUDY OF PERIODS

OF DECLINED IMMUNE COMPETENCE IN DAIRY COWS

SUPPLEMENTED WITH DIETARY ANTIOXIDANT

AND CHELATED TRACE MINERALS .....

118

BIBLIOGRAPHY.....

122

VITA .....

140

## LIST OF TABLES

Table	Page
2.1. Composition of the top-dressed treatments.....	55
2.2. Ingredient composition of the basal diets .....	56
2.3. Chemical composition of the basal diets .....	57
2.4. Colostrum variables of periparturient dairy cows.....	59
2.5. Milk variables among different parities up to 27 days postpartum .....	60
2.6. Health issues of early postpartum cows until 14 days after parturition .....	68
2.7. Effect of treatments on comet variables in periparturient dairy cows .....	72
3.1. Composition of the treatments .....	103
3.2. Ingredient composition of the basal diets .....	104
3.3. Chemical composition of the basal diets .....	105

## LIST OF FIGURES

Figure	Page
2.1. Dry matter intake of periparturient dairy cows.....	58
2.2. Effects of treatment within parity on milk production of early lactation cows .....	61
2.3. Effect of time on energy- and 3.5% fat-corrected milk production, and milk composition of early lactation cows .....	62
2.4. Effect of treatment within parity on milk fat percent of early lactation cows .....	63
2.5. Effect of treatment within parity on solids non-fat percentage of early actation cows.....	64
2.6. Feed efficiency of early lactation cows .....	65
2.7. Effect of treatment body condition score of periparturient dairy cows .....	66
2.8. Calculated energy balance of periparturient dairy cows.....	67
2.9. Effect of treatment within parity on erythrocyte reduced glutathione concentrations in periparturient cows .....	69
2.10. Effect of treatment on erythrocyte oxidized glutathione concentrations in periparturient cows .....	70
2.11. Effect of treatment within parity on erythrocyte reduced over oxidized glutathione ratio in periparturient cows .....	71
3.1. Concentrations of <i>Escherichia coli</i> in milk of cows submitted to intramammary bacterial challenge.....	106
3.2. Effect of treatment within parity on dry matter intake in early lactation cows submitted to intramammary bacterial challenge.....	107
3.3. Effects of treatment within parity on dry matter intake in early lactation cows submitted to intramammary bacterial challenge.....	108

3.4.	Milk production of early lactation cows submitted to intramammary bacterial challenge .....	109
3.5.	Effect of quarter on milk composition of early lactation cows submitted to intramammary bacterial challenge.....	110
3.6.	Effects of treatment on percentage of solids non-fat in early lactation cows submitted to intramammary bacterial challenge.....	111
3.7.	Effect of treatment within parity on percentage of milk urea nitrogen in early lactation cows submitted to intramammary bacterial challenge .....	112
3.8.	Effect of parity within time on somatic cell score in early lactation cows submitted to intramammary bacterial challenge .....	113
3.9.	Effect of treatment within parity on somatic cell score in early lactation cows submitted to intramammary bacterial challenge.....	114
3.10.	Effect of treatment within parity on erythrocyte reduced glutathione in early lactation cows submitted to intramammary bacterial challenge .....	115
3.11.	Effect of treatment within parity on the ratio of erythrocyte reduced over oxidized glutathione in early lactation cows submitted to intramammary bacterial challenge .....	116
3.12.	Effect of time on comet variables in early lactation cows submitted to intramammary bacterial challenge .....	117

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**ABSTRACT**

Two experiments were conducted to investigate the effects of supplementation of synthetic dietary antioxidants and different sources of a blend of trace minerals on two distinct situations of immune dysfunction in dairy cows. In the first experiment, the effects of these supplements on health, metabolism, and production variables of periparturient dairy cows were examined. Supplementation over National Research Council requirements may not be beneficial to multiparous cows, although signs of antioxidant status and health improvement were seen when primiparous cows were supplemented. Treatments did not affect dry matter intake. Milk yield was not different over time between treatments in primiparous cows; however, the control group showed slightly increased milk production in multiparous cows. Animals fed organic trace

minerals had lower incidence of displaced abomasum and metritis compared to animals fed inorganic forms, even with higher incidence of clinical ketosis. However plasma concentrations of glucose, non-esterified fatty acids, and  $\beta$ -hydroxybutyrate were not affected by treatment. Animals fed organic trace minerals also had greater lymphocyte DNA damage than those fed inorganic trace minerals. Multiparous cows subjected to the combination of dietary antioxidants and organic trace minerals had increased rabies antibody titers in response to vaccination, suggesting an enhanced adaptive immune response. In the second experiment, we evaluated the response of supplemented early lactation dairy cows submitted to experimental *Escherichia coli* mastitis. Supplementation over National Research Council requirements may not be beneficial to primiparous cows, although production variables were improved when multiparous cows were supplemented with inorganic trace minerals. Dry matter intake and milk production were inversely related to somatic cells score. Milk quality was altered in the infected quarter and to a lesser extent, in control quarters. Feed efficiency declined after bacterial infusion as well as lymphocyte DNA damage. In conclusion, response of primiparous and multiparous animals varies among parities under different situations of stress. Interestingly, the effects of inorganic and organic trace minerals on animals are altered in these situations and the intensity of stress may affect the response.

# CHAPTER ONE

## REVIEW OF LITERATURE

### INTRODUCTION

The "transition period" in dairy cows is defined as the last 3 weeks of gestation until the first 3 weeks of lactation (Drackley, 1999; Grummer, 1995). This is a key point during the life of the cow, and it has been extensively studied due to its importance. During this period cows undergo a variety of metabolic and immunologic changes which increase the susceptibility to metabolic disorders and infectious diseases (Goff, 2006; Ingvarsten, 2006; Vangroenweghe et al., 2004b).

Mastitis costs the American Dairy Industry approximately two billion dollars every year (Sordillo, 2011). These enormous economic losses are due to decrease in milk production, decrease in milk quality, treatment of cases, extra labor, and sometimes even culling the animal. Nutritional strategies such as supplementation of different sources of microminerals and antioxidants (**AOX**) have been developed to strengthen the immune system mainly during the periparturient period. The goal of these strategies is to diminish the negative effects of inflammatory stress and improve the resistance of the host against intramammary infections (**IMI**).

## THE ROLE OF TRANSITION PERIOD IN DAIRY COWS

Metabolism is greatly altered during the transition period. The prepartum period is marked by accentuated fetal growth (Bauman and Currie, 1980) and the onset of lactation. However, the postpartum period is marked by an increase in milk production over time and deficit on intake of nutrients. This situation causes a negative balance of nutrients, when the intake of nutrients does not meet the requirements of the animal. More specifically concerning energy, transition dairy cows undergo a period of sustained negative energy balance (**NEB**) that is characterized by body weight (**BW**) and body condition score (**BCS**) loss. Bertics et al. (1992) observed that dry matter intake (**DMI**) is affected during the prepartum period, showing a decline of about 30% during the last couple of weeks before parturition. After parturition, DMI increases to support the demands of lactation, but this is still not enough to meet the animal requirements (NRC, 2001). Metabolic demands are increased to support lactation, and homeorhetic adaptations occur to partition nutrients to the mammary gland (**MG**). A good example is the metabolism of glucose, an important metabolite used as the main fuel for some cells and to synthesize lactose in the MG. Propionate, a product of rumen fermentation, is directly related to glucose concentration in plasma (Bell, 1995; Stocks and Allen, 2012) and is responsible for up to 60% of the glucose released by the liver during the transition period (Reynolds et al., 2003). Body requirements regulate hepatic glucose production (Reynolds, 1995) such that it is greatly increased to support lactation during the early

postpartum period (Bell, 1995; Overton and Waldron, 2004). While in NEB, adipose tissue is mobilized to form non-esterified fatty acids (**NEFA**; Overton and Waldron, 2004). In the bloodstream, NEFA become available to most of the body tissues as another fuel source for cells; this helps the body to maintain blood glucose concentrations (Herdt, 2000; Overton and Waldron, 2004). Bennink et al. (1972) observed decreased glucose oxidation in peripheral tissues. Glucose uptake is also decreased and NEFA is used as alternative fuel source (reviewed by Herdt, 2000). Evidence in rats shows that the hypothalamus senses fatty acids then modulates hepatic gluconeogenesis, signaling through the efferent hepatic branch of the vagus (Lam et al., 2005). Also, liver removal of substrates such as lactate, alanine, and glycerol is increased in early postpartum, suggesting increased gluconeogenesis (Reynolds et al., 2003). Although, when excessive amounts of body fat are mobilized, the metabolic capacity of the liver is overloaded causing accumulation of triglycerides (Emery et al., 1992; Herdt, 1988); this may lead to decreased gluconeogenic capacity (Cadórniga-Valiño et al., 1997). The incomplete oxidation of NEFA produces ketone bodies, with acetoacetate and  $\beta$ -hydroxybutyrate (**BHBA**) the main products of this reaction. High BHBA concentration in blood is associated with the appearance of metabolic diseases including displaced abomasum, clinical ketosis and metritis (Duffield et al., 2009) which could aggravate NEB. It is well established that elevated concentrations of NEFA and BHBA impair milk production (Duffield et al., 2009; Ospina et al., 2010; Rastani et al., 2005) and reproduction (Ospina et al., 2010; Walsh et al., 2007).

The immune dysfunction that happens during the transition period favors the incidence of mastitis cases that is linked to impaired polymorphonuclear leukocyte (PMN) function (Cai et al., 1994). The resolution of the disease is dependent on the ability of PMN in recognizing the pathogen, then migrating to the site of infection, phagocytizing the microorganisms, and eliminating them (Vangroenweghe et al., 2005). The processes to kill invading pathogens involve generation of reactive oxygen species (ROS; Heyneman et al., 1990), however generation of ROS is negatively affected by the physiological adaptations that occur during the transition period. The relationship between NEB and impaired immunity has been reported by many researchers. Wathes et al. (2009) reported that cows in severe NEB at early postpartum presented lower concentrations of IGF-1, glucose, and white blood cell count, and higher concentrations of NEFA and BHBA compared to cows under moderate negative energy balance. Cows in NEB show reduced PMN phagocytic capability compared to cows in positive energy balance (Moyes et al., 2009); this might be due to the negative effects of NEFA and BHBA on phagocytosis (Calder et al., 1990; Scalia et al., 2006; Suriyasathaporn et al., 2000). Elevated NEFA in blood has been associated with systemic inflammatory conditions (reviewed by Sordillo et al., 2009), reduced viability of PMN and increase ROS production (Scalia et al., 2006). In cows with metritis, neutrophil myeloperoxidase activity and NEFA levels in plasma were negatively correlated (Hammon et al., 2006). In vitro proliferation of peripheral blood mononuclear cells and production of ROS by PMN declined with concentrations of NEFA higher than 0.013 and 0.5 mM, respectively (Ster et al., 2012). Increase in BHBA impairs PMN migration to the mammary gland after a

lipopolysaccharide (**LPS**) infusion (Zarrin et al., 2014) and reduces phagocytic capability of *Escherichia coli* (Grinberg et al., 2008). On the other hand, glucose is the primary fuel source to phagocytic neutrophils and macrophages (Pithon-Curi et al., 2004) and yields positive effects in vitro and in vivo on lymphocyte proliferation, phagocytosis by macrophages, bactericidal activity and production of cytokines (Calder, 1995; Castell and Newsholme, 1998). Newbould (1973), while studying the in vitro effects of glucose on phagocytosis by milk leukocytes, found that increasing concentrations of glucose resulted in higher competence of the cells. The effects of glucose itself on the immune system, more specifically on cell function, are still not deeply understood, so more studies are warranted. It is been reported that modulation of immune cell function is also dependent on hormones. Mallard et al. (1998) reviewed that cortisol, GH, and IGF-1 are related to the variation in peripheral blood lymphocytes proliferative response to mitogen; also, GH is positively and IGF-1 is negatively associated to serum ovalbumin and whey antibody. The release of stored cells and activation of the colony-stimulating factors by the bone marrow in response to recombinant bovine somatotropin increased proliferative responses to leukocytes (Burvenich et al., 1999). At the farm level, Eppard et al. (1996) reported that bovine somatotropin administered to periparturient cows decreased the incidence of clinical cases of mastitis.

Based on these findings, it is suggestive that health in dairy cows may be improved by higher concentrations of glucose in blood during the transition period directly on cell function and indirectly by decreasing the mobilization of adipose tissue

and associated physiological signals. Furthermore, GH seems to be a key hormone for strengthening the immune system.

## **INTRAMAMMARY *ESCHERICHIA COLI* INFECTION IN DAIRY COWS**

During the transition period, cows are more vulnerable to bacterial IMI due to immunosuppression (Burvenich et al., 2007; Vangroenweghe et al., 2004b, 2005). *Escherichia coli*, a gram-negative bacteria, populate the gastrointestinal tract of warm blooded animals (Hogan and Smith, 2003), and being excreted with the feces, contaminate the environment. Nemeth et al. (1994) found similar *E. coli* isolates in feces and in mastitic milk. Environmental exposure is the main cause of IMI compared to the transfer of the pathogen from infected to uninfected animal (Hogan and Smith, 2003). Therefore, *E. coli* is considered an environmental mastitis pathogen. According to Hogan and Smith (2003), about 85% of coliform mastitis cases show clinical signs. *Escherichia coli*, and other gram-negative bacteria, present a LPS portion as part of the bacterial wall which gives them the endotoxic character (Burvenich et al., 2003; Hogan and Smith, 2003). Low doses of LPS are able to induce an immune response by the host while elevated doses may induce lethal shock when administered intravenously (Lohuis et al., 1988a,b). Clinical signs of *E. coli* mastitis include abnormal appearance of the milk, swelling of the udder, drop in intake, decrease in milk production, dehydration, and

diarrhea (Wilson and González, 2003). However, in severe cases, diarrhea, rumen stasis, paralysis, hypothermia, hypersalivation, very high pulse rate, and lethal shock may also occur (Burvenich et al., 2003; Wenz et al., 2006).

*Escherichia coli* mastitis is often associated with quick appearance of clinical signs of infections, followed by efficient clearance of the pathogen, although some strains of these bacteria may enable mechanisms to ensure successful and chronic proliferation. Adhesion of *E. coli* to mammary epithelial cells was not observed in vivo (Frost et al., 1980) and in vitro (Opdebeeck et al., 1988) for most of the strains tested. Dogan et al. (2006) showed different patterns of adherence and internalization when comparing acute and chronic strains of *E. coli*. Burvenich et al. (2003) stated that *E. coli* generally do not invade the mammary tissue; in accordance to Döpfer et al. (2001), these bacteria usually stay in the lumen of teat canal and lactiferous sinus. Pathogenicity of acute *E. coli* isolates has not been related to adherence and colonization of the MG. The capability and efficiency of adhesion suggests being strain-dependent (Döpfer et al., 2001; Lammers et al., 2001).

The innate immune system needs to recognize the invading pathogen as such in order to be activated against the threat. Toll-like receptors (**TLR**) are a specific class of receptors expressed on the surface of antigen-presenting cells, dendritic cells and macrophages, responsible for assimilating and binding conserved microbial components called pathogen-associated molecular patterns (Werling et al., 2006). Many studies have shown that activation of different TLR results in different gene expression patterns, leading to innate immune response, development of acquired immune response, and

generation of pro-inflammatory cytokines and chemokines. Akira and Takeda (2004) compiled data from the literature and associated TLR to their ligand and its origin. Once phagocytes and epithelial cells are activated, inflammation is initiated, then migration of leukocytes to the site of infection occur (Rainard and Riollot, 2006).

Macrophages and monocytes constitute the major somatic cell types in a non-infected mammary gland, but, when infected, neutrophils are the predominant type of somatic cells (Saad and Östensson, 1990). Migration of neutrophils occurs from the bloodstream to the infected mammary gland through the blood-milk barrier via a process called diapedesis (Paape et al., 2002). Interleukin (IL)-8 is an important cytokine/chemokine produced by a variety of cells such as endothelial and epithelial cells, cells of the monocytic lineage, and neutrophils (Matsukawa et al., 2000). It is involved on the recruitment and activation of neutrophils, enhancing respiratory burst activity, degranulation, and production of arachidonate metabolites (Mukaida et al., 1998). During *E. coli* mastitis, expression of IL-8 is caused directly by LPS and the bacteria themselves, but also indirectly by proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  and IL-1 $\beta$  (Matsukawa et al., 2000; Mukaida, 2003).

Once in the infected MG, neutrophils start to recognize the invading microorganism and its products. In order to facilitate this process and make it more efficient, opsonization of pathogens is common and highly useful, but not mandatory (Zhen et al., 2008). The same authors tested the in vitro activity of a specific egg yolk immunoglobulin (**IgY**) against six different strains of mastitis-causing *E. coli* and observed that specific IgY inhibited growth of the pathogens and also enhanced the

phagocytic activity of macrophages and PMN. Additionally, Hogan et al. (1992) showed, in both serum and colostrum, a positive correlation between IgM titers to *E. coli* J5 bacterin and number of intracellular bacteria per phagocytizing neutrophil.

Immunoglobulins G<sub>2</sub> and M are opsonic antibodies or opsonins which recognize the bacterium through Fab-regions and bind to PMN via Fc-receptors on the PMN plasma membrane (Burvenich et al., 2003). Nevertheless, even in the absence of opsonins, carbohydrates on the membrane of the PMN can bind to microbes mediating phagocytosis (Paape et al., 1996). The binding of *E. coli* to the receptors on PMN surface triggers phagocytosis and induces respiratory burst activity (Burvenich et al., 2003).

Neutrophils generate toxic compounds with powerful killing characteristics, the ROS, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide anions (O<sub>2</sub><sup>-</sup>), and hydroxyl radical (OH<sup>-</sup>). The PMN consumption of O<sub>2</sub> to generate ROS increases when phagocytosis is activated (Burvenich et al., 2003), and hexose monophosphate shunt is also activated leading to ROS generation (Shepherd, 1986). Impaired ROS production is related to severity of *E. coli* mastitis (Mehrzhad et al., 2005), and production of ROS seems to be the most important mechanisms against this organism (Staudinger et al., 2002; Swain et al., 2000).

Extra- and intracellular production of ROS have been associated to PMN bactericidal effects (Rinaldi et al., 2008), but presence of ROS in the extracellular matrix is also associated with tissue damage (Capuco et al., 1986; Zhao and Lacasse, 2008). Activation of NADPH-oxidase, an enzyme bound to the membrane, initiates the respiratory burst activity ( $2\text{O}_2 + \text{NADPH} \rightarrow 2\text{O}_2^- + \text{NADP}^+ + \text{H}^+$ ; Burvenich et al., 2003). Since oxygen is the main molecule in this reaction, anaerobic conditions are known to decrease

efficiency of PMN bactericidal capacity (Mandell, 1974). Myeloperoxidase also is part of the respiratory burst activity, catalyzing the reactions between  $H_2O_2$  and halides ions after being released from the azurophilic granules into the phagosome (Chapman, 2001). Rosen and Klebanoff (1976) suggest this system improves potency of  $O_2^-$  against *E. coli*. Other mechanisms, including production of reactive nitrogen species and non-oxidative defense, also play a role on controlling the infection and clearing the pathogen, but they are not within the scope of this review. Apoptosis of PMN occurs after phagocytosis and killing of multiple engulfed pathogens. Mammary gland macrophages then phagocytize these apoptotic neutrophils. The fast removal of PMN by macrophages is essential to minimize injuries to the mammary tissue (Paape et al., 2002).

The severity of *E. coli* mastitis is host dependent (Burvenich et al., 2003; Rainard and Riollet, 2006), and it can be influenced by parity, stage of lactation, SCC, genetic resistance, nutritional status, pre-existing diseases, and others (Paape et al., 2002). Decreased PMN count, function (Cai et al., 1994; Diez-Fraile et al., 2003), and viability (Ditcham et al., 1996) around early postpartum period are causes of increased incidence of mastitis, when most of the severe cases happen (Vandeputte-Van Messom et al., 1993). Likewise, the velocity in which PMN influx into the MG can alleviate the severity of mastitis (Vandeputte-Van Messom et al., 1993). After peak lactation, time the cows are entering positive energy balance and at lower risk of metabolic diseases, coinciding with the increase in dry matter intake, the symptoms are usually mild to moderate and the disease is self-cured, sometimes difficult to even notice the insult (Burvenich et al., 2003). The same authors also reviewed that, SCC is inversely related to incidence of

coliform mastitis, based on observations from several herds. The *E. coli* infection usually shows rapid progression due to the fast multiplication of the microorganism (Kornalijnslijper et al., 2004) and bacteria count in milk is related to severity (Shuster et al., 1996; Vandeputte-Van Messom et al., 1993). However, elevated pre-infection SCC may inhibit the initial growth of the pathogen (Shuster et al., 1996) and thus control the severity of infection. Döpfer et al. (1999) studied *E. coli* mastitis cases in 300 herds and observed that identical strains of the pathogen caused recurrent cases in different quarters relative to the one infected first. This fact was attributed to initial infection of multiple quarters, each at a different level and with variations in lesion development. Despite these previous facts, primiparous cows are known to be healthier than older animals, having regularly less severe cases and near 100% of the experimental mastitis cases self-cured (Vangroenweghe et al., 2004a). The PMN of primiparous animals produce more ROS and have higher viability compared to old cows (Mehrzaad et al., 2002). In contrast, older cows present higher SCC, but increased number of mastitis cases as well (Burvenich et al., 2003).

Loss of milk production is a characteristic of coliform mastitis. Depending of the severity, a lapse in milk production could accompany the cow until the end of the current lactation or subsequent lactations by partially damaging the mammary tissue or even causing loss of the affected quarter. According to Burvenich et al. (2003), epithelial lesions can be observed in experimentally-induced mastitis independent of the dose tested (200 to  $10^9$  CFU). Cows with *E. coli* mastitis have higher milk production loss than cows with intramammary challenged with LPS. Endotoxin is released from the bacteria at the

time of cell's death (Hogan and Smith, 2003), causing disruption of blood flow and then affecting secretory cells. Suppression of milk production during *E. coli* mastitis may be due the continuous release of endotoxin into the body compared to LPS mastitis in which toxin release is limited. Besides bacterial products, chemicals and reactive compounds produced by the body to combat the pathogen also damage the milk-producing cells leading to decreased milk production.

*Escherichia coli* are present in the environment and they use different mechanisms to get established inside the MG. The host organism needs to efficiently recognize, phagocyte and kill the invading pathogen through a variety of mechanisms. Unfortunately, *E. coli* mastitis results in many different clinical symptoms and loss of milk production. Severity of infection is mainly controlled by host factors. Healthy cows have a better response against the infection. Environmental cleanliness and herd health should be closely monitored to avoid or at least minimize the severity of cases.

## **NUTRITIONAL STRATEGIES TO IMPROVE IMMUNITY: ANTIOXIDANTS AND MICROMINERALS**

Cells produce free radicals as part of their normal metabolism, but accumulation of them is harmful to the body. Antioxidants play a role in neutralizing the free radicals, thereby not damaging lipid membrane, protein, and DNA of cells. However, when levels

of free radicals surpass the antioxidant capacity, oxidative stress occurs (Bowman et al., 2008). Periparturient and high-producing dairy cows, also animals with infections such as mastitis (Bernabucci et al., 2005; Bowman et al., 2008; Sordillo and Aitken, 2009) are known to undergo oxidative stress, mainly marked by the accumulation of ROS, which may lead to dysfunction of immunity and increased metabolic stress (Sordillo et al., 2009). Controlled production of ROS is not harmful; these compounds participate in the chemistry of diverse enzymes and are also used by phagocytes to kill invading microorganisms (Burvenich et al., 2003; Miller et al., 1993). They are primarily formed as end products of the mitochondrial electron transport chain or via activation of NADPH oxidase (Sordillo and Aitken, 2009). In addition, inflammatory processes can worsen the oxidative stress by stimulating ROS production (Burvenich et al., 2003; Sordillo et al., 2008) or decreasing the antioxidant capacity (O'Boyle et al., 2006). Reactive oxygen species cause peroxidation of lipids (Miller et al., 1993) that, under oxidative stress, greatly affects immune cells which possess high levels of polyunsaturated fatty acids in their membranes (Spears and Weiss, 2008). Valko et al. (2007) compiled data concerning free radicals and AOX in humans and reported significant effects of oxidative stress in DNA as transcription alterations, signal transduction and permanent genomic alterations. These authors associated oxidative stress with cancer and aging in humans. Several review papers (Miller et al., 1993; Sordillo and Aitken, 2009; Valko et al., 2007) describe the oxidative chain reactions; lipid peroxidation reaction produces potent ROS (i.e.  $H_2O_2$ ,  $O_2^-$ , and  $OH^\cdot$ ) in the mitochondria and in the cytosol of cells, and, in excess, aggravates oxidative stress.

## *Antioxidants*

The function of dietary AOX is to protect dietary lipids against oxidation, avoiding or reducing the negative effects of ROS accumulation. Feedstuffs with higher levels of unsaturated fatty acids, for example polyunsaturated fatty acids, are more prone to oxidation (Shiota et al., 1999). As reviewed by Bowman et al. (2008), a dietary AOX blend was able to prevent loss and oxidation of unsaturated fatty acids which stabilized the fat of wet distiller's grains. Ethoxyquin and tertiary butyl hydroquinone are some compounds used in the final diet to provide protection to fatty acids and vitamins. It is reported that dietary AOX improves carbohydrate and fiber digestibility, and microbial protein efficiency in vitro (Vázquez-Añón and Jenkins, 2007). They scavenge free radicals by binding to their unpaired electrons in the fatty acids or in the media then blocking oxidation (Bowman et al., 2008).

In poultry, oxidized fat was found to increase gut cell turnover and compromise the immune response. However, when ethoxyquin was added to the diet villus integrity and gut cell turnover was maintained (Dibner et al., 1996). Wang et al. (1997) found higher concentrations of reduced over oxidized forms of glutathione (**GSH:GSSG**) in the duodenum and ileum tissues of poultry supplemented with ethoxyquin. It is suggestive that this dietary AOX might spare the oxidation of glutathione by glutathione peroxidase, thereby clearing peroxides from the diet and digesta. Feedlot beef cattle have reduced incidence of liver abscess (Vázquez-Añón et al., 2005a) and improved BW gain (Vázquez-Añón et al., 2005b) with inclusion of dietary AOX. In dairy cattle, Vázquez-Añón et al. (2008) fed either fresh or oxidized fat with or without dietary AOX and

observed that cows fed AOX increased DMI, 3.5% fat-corrected milk, and milk fat yield, but decreased milk protein percentage and concentration of short-chain fatty acids in milk. Also, no differences in BCS and BW were reported. In contrast, Wang et al. (2010), when feeding either low or high saturation fatty acids with or without the same dietary AOX used in the previous experiment, found no difference in DMI, no effect of AOX in milk fat and protein concentrations, however increased milk yield in cows fed lower saturation fatty acids plus AOX; AOX decreased plasma NEFA but did not affect BHBA concentrations. Immunological variables were also measured in both experiments. Vázquez-Añón et al. (2008) reported that AOX could increase glutathione peroxidase, but the increase in antioxidant status and superoxide dismutase (**SOD**) was only seen when in combination with oxidized fat. In the second experiment, a decrease was observed in H<sub>2</sub>O<sub>2</sub> contents and total antioxidant capacity was increased for cows supplemented with AOX. Based on these results, the inclusion of AOX may have partially alleviated the detrimental effects of fat oxidation in dairy cows. The effects of dietary AOX on production variables require further investigation.

Dietary antioxidants work by scavenging free radicals from feedstuffs and final diets. Research regarding the absorption of these dietary antioxidants by the animal would be of great value, because if they are absorbed they may play a role in protecting the cells themselves against lipid peroxidation or in binding to extracellular free radicals produced by the cells, thereby improving the immune system of the animal through more intrinsic ways.

### ***Microminerals***

Trace minerals are essential microminerals needed for vital processes of the body, including functioning as antioxidants. According to Wright et al. (2008), the ruminal absorption of trace minerals is minimal, whereas they are effectively absorbed by the intestine. Inorganic forms of minerals, such as sulfates, oxides, and carbonates, might have lower bioavailability compared to organic sources as they can dissociate in the upper gastrointestinal tract forming indigestible compounds that are unavailable for absorption by the intestine and thus excreted in the manure (Gressley, 2009). In contrast, organic forms of trace minerals have higher bioavailability compared to inorganic sources (Spears, 2003). Feeding organic trace minerals has been shown in several studies to improve immune function, health, and production variables of diverse species of animals, but data are still inconclusive (Hackbart et al., 2010; Rabiee et al., 2010; Nemeč et al., 2012). Organic trace minerals are typically bound to amino acids or proteins through complex, chelation or proteination processes (Gressley, 2009). In these last two, minerals are covalently bound to organic molecules (Spears, 1996). Organic trace minerals become resilient enough to bypass the upper gastrointestinal tract, although dissociation still happens, thus remaining available for absorption by the gut tissues (Andrieu, 2008; Wright et al., 2008). Additionally, Andrieu (2008) reported that enterocytes are negatively charged to protect themselves against highly-charged or toxic ions, thus making absorption easier for organic than inorganic ions.

Trace minerals can help the immune system to scavenge free radicals thereby transforming them into non-hazardous compounds. Copper and Mn are integral components of SOD in the cytosol and in the mitochondria, respectively, and Zn acts as

cofactor for this enzyme in the cytosol (Andrieu, 2008; Weiss, 2002). Superoxide dismutase works as the first enzymatic line of defense against oxidative stress by converting  $O_2^-$  to  $H_2O_2$ . Thereafter, a Se-dependent enzyme, glutathione peroxidase, is responsible for converting  $H_2O_2$  to  $H_2O$  through oxidation of GSH to GSSG (Miller et al., 1993; Nelson et al., 2008). The reduced to oxidized glutathione reaction is reversible by the enzyme glutathione reductase that reduces equivalents from NADPH generated by the pentose phosphate pathway (Nelson et al., 2008). Glutathione peroxidase can be an indicator of oxidative stress (Tüzün et al., 2002), although erythrocyte glutathione can reflect better the antioxidant capacity of cells and glutathione activity in other tissues (Bernabucci et al., 2005). Andrieu (2008) discussed other functions performed by most of the trace minerals cited above such as: Zn – involvement in keratin generation, epithelial tissue integrity, synthesis of nucleic acids and cell division, bone formation, appetite control, bone formation, energy metabolism, and others; Cu – component of ceruplasmin that is responsible to regulate iron availability and oxidation-reduction reactions (Healy and Tipton, 2007) then possibly scavenging superoxide radicals (Broadley and Hoover, 1989), tissue keratinization, energy metabolism, cellular respiration, bone formation, etc.; Mn – tissue keratinization (Tomlinson et al., 2004), energy metabolism, bone formation, reproduction, and nervous functions.

The replacement of Cu, Mn, and Zn from inorganic to organic sources has shown to be an alternative that may benefit dairy cattle. Spears and Weiss (2008) cited an experiment in which heifers were fed for 120 days before parturition different sources of Cu. Animals fed Cu proteinate had a lower proportion of infected quarters at calving

compared to the ones fed basal diet or Cu sulfate. In another trial, heifers challenged with intramammary *E. coli* showed lower *E. coli* count and milk yield tended to be higher from two through five days after infection when fed organic Cu (Scaletti and Harmon, 2012). Supplementation of organic Zn was observed to reduce the incidence of intramammary infections (Spain et al., 1993), but no difference in SCC or milk production was reported. The influence of Zn on SCC are contradictory (Kellogg, 1990). Cows supplemented with organic Zn over one year experienced fewer foot issues (Andrieu, 2008). Studies evaluating the effects of Mn itself and different feeding strategies on health and production of dairy cows are still scarce. When evaluating the effects of feeding dairy cows an organic mix of Zn, Cu, and Mn, Siciliano-Jones et al. (2008) found increase in milk production and improvement on hoof health. In contrast, Chester-Jones et al., (2013) observed no difference in milk yield or in milk composition, but a decrease in the number of days open. An experiment on a commercial dairy farm fed dairy cows a mix of OTM from 21 days prepartum to 250 days postpartum and found no difference in milk yield, energy-corrected milk, and 3.5% fat-corrected milk, but these animals tended to have lower somatic cell count in milk. Also, organic trace mineral-fed animals showed lower incidence of heel erosion (DeFrain et al., 2009). In another study, feeding organic trace minerals had no effect on DMI, health, or embryo quality, but increased milk production in mid-lactation dairy cows (Hackbart et al., 2010).

Rabiee et al. (2010) considered 20 different studies in their meta-analysis where they were able to evaluate many variables affected by feeding organic forms of trace minerals. They found an increase of 0.93 kg in milk yield, 0.04 kg in milk fat, and 0.03

kg in milk protein, but milk solids percentage was not affected nor somatic cell count. This difference in fat and protein contents may be responsible for the increased energy-corrected milk and 3.5% fat-corrected milk, however, these data were highly heterogeneous. Reproductive data showed no evidence of heterogeneity in this study; days open decreased 13.5 days (weighted mean difference), services per conception also decreased, and the 150 days of lactation pregnancy risk was greater for cows fed organic trace minerals. Overall, results of feeding organic sources of trace minerals appear inconclusive for production and reproduction variables, but seem to improve health of dairy cows.

## CONCLUSION

Transition cows undergo a variety of changes in the energetic metabolism and dynamic of tissues. During periods of physiological imbalances and failed adaptations, cells produce and release excess ROS into the body; this can directly affect immunity, production, and in extreme cases, can lead to animal death. Susceptibility to metabolic disorders and infectious diseases are increased during this period and they can be explained through a cascade of events. Mastitis is the disease that widely affects herds in US. A decrease in milk production and quality combined with the cost and inconvenience of treating sick animals causes the net income of farms to decrease. The immune system

is greatly affected, and losses of milk production and milk quality are clear during mastitis. In general, primiparous cows are healthier than multiparous cows, but the severity of the disease depends mainly on the host and on the ability of the immune system to fight and clear the invading pathogen. The antioxidant capacity of the animal is also very important to keep the animal healthy. Nutritional approaches have been developed to strengthen the immune system and overall health throughout the transition period, thus resulting in improvement of productive and reproductive characteristics. Feeding dietary antioxidants and organic trace minerals seems a reasonable technique to improve health. However, based on the information available to date, it is still unclear the effects of AOX and OTM on production and reproduction.

**CHAPTER TWO**  
**HEALTH, METABOLISM, AND PRODUCTION OF PERIPARTURIENT**  
**DAIRY COWS SUPPLEMENTED WITH DIETARY**  
**ANTIOXIDANT AND CHELATED TRACE**  
**MINERALS**

**ABSTRACT**

The aim of this study was to evaluate the effects of dietary antioxidants and organic trace mineral supplementation on health, metabolism, and production variables of periparturient dairy cows. Sixty pregnant Holstein cows were blocked based on date of expected parturition, parity (primiparous vs. multiparous), and initial body weight to a randomized complete block design study. Cows were individually fed a basal diet appropriate for physiological state formulated to meet or exceed National Research Council recommendations from d 39.3 ± 4.6 prepartum to d 27 postpartum. Treatments were top-dressed to the basal diet at a rate of 200 g/d for prepartum and 250 g/d for postpartum animals, as: 1) no supplemental antioxidant nor trace minerals (Cu, Mn, and Zn; **CTL**), 2) trace minerals provided as 100% sulfates without antioxidant (**ITM**), 3) trace minerals provided as 100% sulfates with antioxidant (**ITMAOX**), 4) trace minerals provided as 50% of sulfates and 50% of chelates without antioxidant (**OTM**), and 5)

trace minerals provided as 50% of sulfates and 50% of chelates with antioxidant (OTMAOX). Treatments were iso-mineral except CTL; all were iso-methionine. Dry matter intake and milk yield were recorded daily. A colostrum sample was collected at the first milking after calving and milk samples for compositional analyses were collected weekly. Blood samples were collected at d  $-18 \pm 4.6$ ,  $-6 \pm 3.8$ , 0, 7, and 14 relative to parturition. Animals were vaccinated against *Escherichia coli* at drying-off,  $-27.3 \pm 4.6$ , and 14 d from calving, and also vaccinated for experimental purposes against rabies within 24 h from calving. Incidence of health cases were monitored for the first 14 days from calving. Milk production slightly decreased in multiparous under ITM as well as milk SNF percent, however fat percent was greater in these animals. Also, primiparous under ITM had lower fat percent in milk. Dry matter intake, feed efficiency, body weight, energy balance, glucose, NEFA, and BHBA were not affected by treatment. The reduced to oxidized glutathione ratio was affected differently by OTMAOX among parities. Animals fed organic trace minerals had lower incidence of displaced abomasum and metritis compared to animals fed inorganic forms, even though this group experienced a higher incidence of clinical ketosis. The OTM animals also presented higher Comet assay DNA damage. Multiparous cows subjected to OTMAOX treatment had increased rabies antibody titers, suggesting enhanced adaptive immune response. Results suggest that supplementation over requirements may not be beneficial to multiparous cows although signs of antioxidant status and health improvement were seen when primiparous cows were supplemented.

## INTRODUCTION

Dairy cows are exposed to a variety of physiologic and metabolic changes during the transition period, defined as last 3 weeks before to 3 weeks after parturition (Drackley, 1999; Sordillo et al., 2009). Cows lose body weight (**BW**) to compensate the deficit of energy due to accentuated increase in milk production accompanied by a slower increase in dry matter intake (**DMI**; NRC, 2001). It characterizes negative energy balance (**NEB**) that, among many other factors, metabolic health and the immune system of the animal are weakened (Sordillo et al., 2009), consequently increasing susceptibility to metabolic and infectious diseases (Goff, 2006; Sordillo and Aitken, 2009). Negative energy balance is marked by depletion in blood glucose with increased concentrations of non-esterified fatty acids (**NEFA**) that when partially oxidized, produce  $\beta$ -hydroxybutyrate (**BHBA**). Excessive circulating concentrations of NEFA and BHBA respectively cause metabolic diseases as fatty liver and ketosis what may influence the appearance of secondary issues (Duffield et al., 2009; Ospina et al., 2010). Production of reactive oxygen species (**ROS**) is part the normal metabolism of cells, but when homeostasis is disturbed and the antioxidant capacity of the animal is diminished, ROS are able to accumulate causing oxidative stress (Bowman et al., 2008; Sordillo et al., 2009). Several authors have shown oxidative stress is increased in periparturient dairy cows. Plasma ROS was positively related to NEFA and BHBA, while NEFA was negatively related to erythrocytes thiol groups and superoxide dismutase (**SOD**) activity

(Bernabucci et al., 2005). The same authors also observed that cows with more pronounced body condition score (**BCS**) loss in the transition period had higher plasma ROS and thiobarbituric acid-reactive substances with lower erythrocyte thiol groups and plasma SOD compared to cows with less pronounced BW loss. Reactive oxygen species cause peroxidation of lipids (Miller et al., 1993) that, under oxidative stress, greatly affects immune cells which possess high levels of polyunsaturated fatty acids in their membranes (Spears and Weiss, 2008). Valko et al. (2007) compiled data concerning free radicals and AOX in humans and reported significant effects of oxidative stress in DNA as transcription alterations, signal transduction and permanent genomic alterations. These authors associated oxidative stress with cancer and aging in humans.

Nutritional approaches have been studied in order to improve immunity and health. Dietary antioxidants protect dietary lipids against oxidation, this way reducing the load of ROS into the animal. They scavenge free radicals by binding to their unpaired electrons, thereby blocking further oxidation reactions (Bowman et al., 2008). In poultry, dietary supplementation with the antioxidant ethoxyquin maintained gut cell turnover and villus integrity (Dibner et al., 1996) as well as increased the reduced to oxidized glutathione ratio (**GSH:GSSG**) in the duodenal and ileal tissues (Wang et al., 1997). In dairy cows, dietary antioxidants were able to decrease plasma NEFA and hydrogen peroxide ( $H_2O_2$ ) concentrations, and increase total antioxidant capacity (Wang et al., 2010).

Trace minerals can help the immune system to scavenge free radicals thereby transforming them into non-hazardous compounds. Copper and Mn are integral

components of superoxide dismutase (**SOD**) in the cytosol and in the mitochondria, respectively, and Zn acts as cofactor for this enzyme (Andrieu, 2008; Weiss, 2002). Superoxide dismutase works as the first enzymatic line of defense against oxidative stress by converting  $O_2^-$  to  $H_2O_2$ . Use of organic trace minerals in dairy cows reduced the incidence of intramammary infection (Spain et al., 1993), improved hoof health and showed a tendency to lower somatic cell count (**SCC**) in milk (DeFrain et al., 2009), and improved reproduction by decreasing number of days open (Chester-Jones et al., 2013; Rabiee et al., 2010).

The objective of this experiment was to evaluate the effects of feeding dietary antioxidants and organic trace minerals on health, metabolism, and production variables of transition dairy cows. We hypothesized that animals supplemented with both dietary antioxidants and organic trace minerals in the prepartum and early postpartum period would improve transition to lactation due to a possible synergistic effect of the treatments.

## **MATERIAL AND METHODS**

### ***Animals, Diets, Experimental Design, and Treatments***

The use and care of all animals were approved by the University of Missouri Animal Care and Use Committee. This experiment was conducted as a randomized

complete block design with cow as experimental unit. Sixty pregnant Holstein cows housed at University of Missouri Foremost Dairy Center were blocked based on date of expected parturition, parity [primiparous (**PP**) and multiparous cows (**MP**;  $3.17 \pm 1.25$  parities)], and initial BW. All cows enrolled on this study had a current locomotion score lower than two (Sprecher et al., 1997). All multiparous cows had SCC lower than 150,000 cells/mL of milk for the last two months of the previous lactation, were dried-off at least 15 days prior to the beginning of the trial, and blocks were balanced for milk yield based on the previous lactation milk production. Cows within block were randomly assigned to receive to one of five treatments: 1) no supplemental dietary antioxidants or trace minerals (**CTL**); 2) trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants (**ITM**); 3) trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants (**ITMAOX**); 4) trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants (**OTM**); and 5) trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants (**OTMAOX**). The dietary antioxidant supplemented was Agrado Plus (Novus International, St. Charles, MO), and chelated Cu, Mn, and Zn were supplied as metal methionine hydroxy analog chelates (Mintrex, Novus International). Treatments were iso-mineral and provided at least 100% of the (NRC, 2001) requirements for Cu, Mn, and Zn, except CTL; all were made iso-methionine by supplemental Ca-salt of 2-hydroxy-4-methylthio-butanoic acid (MHA feed supplement, Novus International). Treatments were top-dressed and mixed into the top portion of the diet immediately prior

to morning feeding to prepartum and postpartum cows at a rate of 200 and 250 g/d, respectively, using ground corn as carrier (Table 2.1). Dry and lactating cows were fed a basal diet, formulated to meet or exceed predicted requirements for all nutrients (NRC, 2001). Basal diets were provided as TMR for ad libitum (intake to achieve about 10% refusals). Cows were fed once (0700 h) and twice (1400 and 0700 h) daily during the prepartum and postpartum periods respectively, from  $-39 \pm 4.6$  to 27 d relative to parturition via individual feeding system (American Calan Inc, Northwood, NH). Cows were trained how to use the feeding system at least one week prior the beginning of the trial. Intake was recorded daily throughout the experiment. Feeds were sampled weekly and dry matter (**DM**) content was determined by forced-air oven drying at 55°C for 72 h. Diets were adjusted each week for ingredient DM content. Weekly feed samples were ground through a 0.2-mm sieve and combined to monthly composites. Table 2.2 and Table **2.3**, respectively, show the ingredient and chemical composition of the diets. Water was available for ad libitum consumption throughout the experimental period. Lactating cows were milked twice daily (1800 and 0600 h) and production was recorded for each milking.

A 5-point scale was used to assign BCS (Wildman et al., 1982) during the experiment. Animals were independently assessed weekly by 2 individuals and the average of the scores was the assigned value. Animals were weighed and BW was recorded immediately prior to the morning feeding at the beginning of the trial,  $-18 \pm 4.6$ , 0, and 21 d from parturition.

All cows were intravenously administered 500 mL of 23% calcium gluconate at the day of parturition to minimize any problems associated with calcium metabolism or hypocalcaemia. Animals were vaccinated against *Escherichia coli* (Enviracor J-5, Pfizer Animal Health, Kalamazoo, MI) at dry-off,  $-27.3 \pm 4.6$ , and 14 d from parturition. To evaluate the effects of treatments on postpartum humoral immune response, a novel antigen was introduced to the cows via intramuscular injection of 2 mL of rabies vaccine (IMRAB, Merial Inc., Athens, GA) within 24 h from parturition.

### ***Health monitoring***

Cows were monitored for abnormal conditions and behavior during the entire experiment. Lameness score (**LS**) was assessed weekly, every Thursday, by 2 individuals, using a 5-point scale as describe by (Sprecher et al., 1997), and the average of the scores was the assigned values. Information on birth of twins, dystocia, clinical ketosis (**CK**), displaced abomasum (**DA**), retained placenta (**RP**), and metritis were recorded for the first 14 days of lactation as present or not. These data were collected through on-farm diaries, veterinary visit summaries, and daily technician recordings. Definitions for dystocia, CK, DA, RP, and metritis have been described previously (Duffield et al., 1999; LeBlanc, 2008; Mee, 2008). Clinical ketosis was defined based on visual score of a urine ketone body test (Ketostix, Bayer Corp., Elkhart, IN; (Chamberlin et al., 2013) equivalent to 80 mg/dL or higher of acetoacetate, metritis based on vaginal discharge with two or more consecutive days with rectal temperature higher than 39°C, DA was diagnosed by a veterinarian, and RP was defined as fetal membranes being retained by the dam for more than 24 h after parturition.

### ***Blood Samples***

Coccygeal blood samples were taken into evacuated tubes containing K<sub>3</sub>-EDTA (Monoject, Tyco Healthcare Group LP, Mansfield, MA), Na-heparin (BD Vacutainer, Franklin Lakes, NJ), and no anticoagulant (BD Vacutainer) at  $-39 \pm 4.6$ ,  $-18 \pm 4.6$ ,  $-6 \pm 3.8$ , 0, 7, and 14 from calving, between 3 to 4 h after the morning feeding. The d 0 sample was collected before the administration of calcium gluconate and vaccination against rabies. The blood sample collected on d 28 from parturition was collected immediately after the morning milking with the sole purpose to analyze response of the cows against the rabies vaccination. Blood samples without presence of an anticoagulant were left in room temperature for 2 h to allow clotting then centrifuged at 3000 x g for 10 min at 23°C; serum was separated and stored at -20°C for further analysis. Heparinized blood samples were centrifuged at 3000 x g for 10 min at 4°C within 30 min from the collection, plasma and red blood cells were separated and stored at -20°C and -80°C, respectively, for further analysis. Whole blood processing from EDTA tubes started within 30 min from collection. Isolation of lymphocytes from these samples were obtained using a 15-mL polypropylene conical tube (BD Falcon, Franklin Lakes, NJ), where 6 mL of whole blood was laid on 6 mL of Ficoll (GE Healthcare, Pittsburgh, PA) that had been previously warmed to room temperature. The tube was centrifuged at 800 x g for 15 min at 23°C, then plasma was removed by vacuum, buffy coat was transferred to a new 15-mL tube, and the rest of blood cells were discarded. In the tube containing the buffy coat, 10 ml Tris-NH<sub>4</sub>Cl was added, and contents were gently mixed with a pipette. The tube was centrifuged at 800 x g for 4 min at 4°C, and the supernatant was discarded.

The pellet was washed by addition and suspension with 10 mL of Ca<sup>2+</sup> - and Mg<sup>2+</sup> -free PBS (Sigma-Aldrich, St. Louis, MO). This wash procedure was repeated twice. After washing, 4.5 mL of the PBS was added, the pellet was re-suspended, and 1.5 mL of the suspension was transferred to snap cap tubes. These tubes were centrifuged at 800 x g for 4 min, the supernatant was discarded, and cells re-suspended in 200 µL of PBS. The concentration of lymphocytes was calculated using an automatic cell counter (Cellometer Vision, Nexcelom Bioscience LLC., Lawrence, MA), then adjusted to 3 x 10<sup>5</sup> cells/mL with a freezing media constituted of 10% DMSO (Fisher Scientific, Fair Lawn, NJ), 40% PBS, and 50% fetal calf serum (HyClone Laboratories Inc., Logan, UT), and finally, stored at -80°C until time of analysis.

#### ***Colostrum and Milk Samples***

Cows were individually milked at the next scheduled milking time following parturition (1800 or 0600 h) and colostrum yield was recorded. The entire amount of colostrum obtained was agitated to ensure even distribution of the constituents. Colostrum was then sampled into a 50-mL polypropylene tube (BD Falcon) and immediately frozen at -20°C for further analysis of IgA, IgG, and IgM concentrations. Another aliquot of colostrum was sampled and immediately refrigerated at 4°C with a preservative (Broad Spectrum Microtab, Advanced Instruments Inc., Norwood, MA) until analyzed (within 48 h) for fat, protein, solids non-fat (**SNF**), and SCC by a certified DHIA laboratory (Mid-South Dairy Records, Springfield, MO).

Milk samples were collected weekly from consecutive p.m. and a.m. milkings, then immediately refrigerated at 4°C with a preservative (Broad Spectrum Microtab,

Advanced Instruments Inc.) until analyzed (within 48 h; Mid-South Dairy Records) for fat, protein, SNF, milk urea nitrogen (**MUN**) and SCC. Weekly milk composition was a calculated composite of each milking corrected for milk yield.

Somatic cells score (**SCS**) was calculated using the equation of Jamrozik and Schaeffer (2012):  $SCS = [\log_2(SCC/100,000) + 3]$ . Also, energy-corrected milk (**ECM**) and 3.5% fat-corrected milk (**FCM**) were calculated as reviewed by (Davidson et al., 2008),  $ECM \text{ (kg/d)} = \{[\text{milk yield (kg/d)} \times 0.327] + [\text{milk fat (kg/d)} \times 12.86] + [\text{milk true protein (kg/d)} \times 7.65]\}$ , and  $3.5\% \text{ FCM (kg/d)} = \{[\text{milk yield (kg/d)} \times 0.4324] + [\text{milk fat (kg/d)} \times 16.2162]\}$ .

### ***Energy Balance and Feed Efficiency***

Energy balance (**EB**) was calculated for weeks -3, 0, and 3 from parturition. All the equations used were proposed by NRC (2001). Net energy provided daily through the diet (**NE<sub>i</sub>**) was calculated as  $NE_i = \text{energy content of the diet} \times \text{dry matter intake (DMI)}$ . Net energy required for body maintenance (**NE<sub>m</sub>**) was calculated using the equation  $NE_m = BW^{0.75} \times 0.08$  for multiparous cows, and  $NE_m = (0.96 \times BW)^{0.75} \times (0.1 \times BCS + 0.65)$  for primiparous cows. Net energy for pregnancy (**NE<sub>p</sub>**) was computed by  $NE_p = \{[(0.0318 \times \text{days pregnant} - 0.0353) \times (\text{calf BW}/45)]/0.14\} \times 0.64$ . Net energy for lactation (**NE<sub>l</sub>**) was calculated using the equation  $NE_l = \text{milk production} \times [(0.0929 \times \text{milk fat percent}) + (0.0588 \times \text{milk true protein percent}) + 0.192]$ . Only for first or second lactation animals, net energy for growth (**NE<sub>g</sub>**) was computed,  $NE_g = 13.29 \times (\text{NE}_g \text{ provided by the diet})^{0.9116} \times (0.701 \times BW)^{-0.6837}$ . Estimated EB prepartum was calculated by  $EB = NE_i - (NE_m + NE_p + NE_g)$ , and estimated EB postpartum was calculated using

the equation  $EB = NE_i - (NE_m + NE_l + NE_g)$ . Feed efficiency was evaluated by calculation of ratios of milk production variables over DMI, as milk yield:DMI, ECM:DMI, and 3.5% FCM:DMI.

### ***Laboratory Analyses***

***Glucose, BHBA, and NEFA.*** Plasma glucose, BHBA, and NEFA concentrations were determined via enzymatic colorimetric methods from commercially available kits that were modified for use of 96-well microplates (Costar, Corning Inc., Acton, MA). All assays were validated in our laboratory and read using a microplate reader spectrophotometer (BioTek, Winooski, VT). Plasma glucose was determined by glucose oxidase (Sigma-Aldrich, St. Louis, MO), plasma BHBA with the use of D-3-Hydroxybutyrate: NAD oxidoreductase (Roche Applied Science, Indianapolis, IN), and plasma NEFA using NEFA HR(2) reagents (WAKO Chemicals USA, Inc., Richmond, VA). Samples were assayed in triplicates. Inter- and intra-assay coefficients of variation were respectively 3.49 and 3.93% for glucose, 4.35 and 4.36% for BHBA, and 2.31 and 3.27% for NEFA.

***Glutathiones.*** Red blood cell total and oxidized (GSSG) glutathione were determined by enzymatic colorimetric methods using a commercial kit (Cayman Chemical, Ann Arbor, MI) Samples were analyzed in triplicate and all methods were validated in our laboratory. Reduced glutathione was mathematically calculated by subtracting GSSG from total glutathione, and the ratio of glutathiones was calculated by dividing the reduced by the oxidized glutathione. Inter- and intra-assay coefficients of

variation were 0.94 and 2.72% for total glutathione, and 1.70 and 1.69% for GSSG, respectively.

***Rabies antibody titers.*** Rabies antibody titers were quantified in serum by ELISA (Platelia Rabies II kit, Bio-Rad Laboratories Inc., Hercules, CA) using the methods employed by Nemeč et al. (2012). Samples were assayed in duplicates. The limit of detection for this assay was 0.125 EU/mL, therefore values below the limit of detection were considered 0.0001 EU/mL. Intra-assay coefficient of variation was 0.85%.

***Comet assay.*** Comet assay is able to detect DNA damage (Ersson and Moller, 2011) using electrophoresis for migration of the damaged DNA. The extent of migration correlated with the amount of DNA damage to a cell (Oliveira and Johansson, 2012). The most useful measurements are tail length, percent DNA in tail, and tail moment (product of tail length and percent DNA in tail) because it bears a linear relationship to the break frequency (Collins, 2004). A commercial kit (CometAssay, Trevigen Inc., Gaithersburg, MD) was used to run this assay. Processes were performed under dimmed light. First, lysis solution A was prepared by mixing 100 mL of lysis solution (part of the kit) with 1 mL of DMSO. Lysis solution A was chilled at 4°C for at least 20 min prior to use. The samples were removed from the -80°C freezer and 1 mL of PBS was added to the frozen media and mixed together. The samples were centrifuged for at 200 x g for 10 min at 4°C. One milliliter was pipette out while leaving the pellet at the bottom of the tube. Next, 200 µL of PBS was added to the sample in the tube. Low-melting agarose (LM Agarose, Trevigen Inc.) was melted in the microwave for no more than 10 s with the cap loosened. Then, the bottle with melted agarose was placed in a 37°C water bath for

20 min to cool. Tubes of 0.6 mL were placed in a tube heater at 37°C and then 300 mL of the molten agarose gel was added to the 0.6-mL tubes. Agarose gel remained at 37°C until applied to sample slide. After adding the agarose gel to the 0.6-mL tubes, the pellet was re-suspended from the sample tubes. Thirty mL of cells were added from the sample tube to the 0.6-mL tubes containing agarose gel, and mixed very well. Immediately following, 10 µL was added on to the 96-well comet slide (Trevigen Inc.). The pipette tip was used to spread the agarose gel containing cells over the sample area to ensure complete coverage. After all samples are applied to the 96-well comet slide, the slide was placed in the dark at 4°C for 30 min. Following this step, the slide was immersed in pre-chilled lysis solution A and left at 4°C for 14 h. After incubation, alkaline unwinding solution was prepared by combining 0.8 g of NaOH pellets, 500 µL of 200 mM EDTA (Trevigen Inc.), and 99.5 mL of nanopure water for every 100 mL made. It was ensured that the pH was greater than 13 and allowed chilling time prior to use. Next, the slide immersed in lysis solution A was removed and excess was drained off. The slide was then immersed in the freshly prepared and chilled alkaline unwinding solution and left the slide immersed for 20 min at room temperature in the dark. 500 mM EDTA stock solution was prepared by mixing 40 mL of 200 mM EDTA (pH = 10) with 60 mL of nanopure water for every 100 mL made. The pH was adjusted to 8 following the combination of listed ingredients. Next, 1 L of alkaline electrophoresis solution was created by mixing 8 g of NaOH pellets with 2 mL of 500 mM EDTA. After the pellets were dissolved, nanopure water was added to solution until final volume of solution reached 1 L. The pH was checked to be greater than 13. Next, the solution was chilled to

4°C prior to use. Nine hundred mL of pre-chilled alkaline electrophoresis solution were added to the Comet Assay ES TANK (Trevigen Inc.). The slide was placed in the slide tray and covered with the slide tray overlay. The power supply was set to 21 volts and applied to slide for 40 min. After the electrophoresis had ended, the slide was removed from the tank and drained any excess solution from the slide. Then, the slide was immersed twice in nanopure water for 5 min each time and then immersed in 70% ethanol for 5 min as well. The slide was dried at 37°C for 15 min and then stored in the dark at room temperature with controlled humidity. When all slides were processed and ready to be stained, TE buffer was prepared by adding 5 mL of 2 M Tris (Fisher Scientific, Fair Lawn, NJ), 2 mL of EDTA 500 mM pH = 8, and nanopure water completed up to 1 L. The pH was adjusted to 7.5. Next, SYBR Gold solution was prepared by diluting 1 µL of SYBR Gold Nucleic Acid Gel Stain (Life Technologies, Eugene, OR) in 30 mL of TE buffer, and stored in the dark at 4°C. Twenty µL of SYBR Gold Solution was added to each well of the 96-well comet slide and then the slide was stained for 30 min at room temperature in the dark. Following this step, the slide was gently tapped to remove excess SYBR Gold Solution and then immersed briefly in nanopure water. The slide was allowed to dry completely for approximately 15 min in the dark at 37°C. Next, the samples were viewed by epifluorescence microscopy at 494 nm excitation, 521 nm emission, and 100 ms of exposure time at 20 x magnification, and a picture was taken. Using CometScore software (TriTek Corp., Sumerduck, VA), no more than 10 cells per photo were selected and then the average of the measured values was assigned one for each sample.

***Immunoglobulins A, G, and M.*** Concentrations of immunoglobulins in colostrum were determined by the sandwich ELISA method (Bovine IgA, IgG, and IgM ELISA quantitation sets, Bethyl Laboratories Inc., Montgomery, TX). Samples were assayed in triplicates. Inter- and intra-assay coefficients of variation were respectively 4.66 and 4.17% for IgA, 5.01 and 5.05% for IgG, and 5.17 and 5.18% for IgM.

***Milk components.*** Milk fat, true protein, and SNF were determined by infrared spectroscopy (Bentley 2000, Bentley Instruments Inc., Chaska, MN), MUN by a modified Berthelot reaction (ChemSPec 150, Bentley Instruments Inc.), and SCC by flow cytometry (Somacount 300; Bentley Instruments Inc.) in a commercial Dairy Herd Improvement Association laboratory (Midwest Dairy Records, Springfield, MO).

### ***Statistical Analysis***

A multiparous cow assigned to the CTL treatment was found unable to use the feeding system at the beginning of the experiment and by consequence DA occurred. Her data was excluded from the statistical analyses. The data was analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC). The influence option in the model statement for MIXED procedure was used to detect extreme observations. Extreme observations were removed from the data set if they presented at least two of the following characteristics: standardized residual ( $r_i$ ) > 2.5 standard deviations, high leverage ( $h_{ii} \geq 2p/n$ , where  $n$  = number of observations and  $p$  = number of parameters), and cook's distance ( $D_i$ ) > 1 (Kaps and Lamberson, 2009). Results indicate that none of the observations were outliers. All the variables were evaluated for normality using the UNIVARIATE procedure and normalized before analyses if necessary. For normalization, BCS, BW,

immunoglobulin concentrations in colostrum, all milk composition variables except SCS, rabies antibody titers, and comet tail moment data were  $\log_{10}$ -transformed. For DMI, comet percentage of DNA in tail and tail length, square-root, and LS, -2 exponential transformations were used, respectively. *P*-values are shown based on normalized data, however means and SEM are presented as non-normalized values.

Data were analyzed as a randomized complete block design using the MIXED procedure with repeated statement for variables measured over time (DMI, BCS, BW, LS, glucose, BHBA, NEFA, glutathiones, rabies titers, comet assay, milk variables, EB, and feed efficiency). The statistical model included fixed effects as treatment, parity, and time as well as the resultant interactions. Block and block within treatment and lactation were the specified terms for the random statement. Time was the specific term for the repeated statement with block within treatment and parity as subject. Either autoregressive order one or compound symmetry were the covariance structures selected based on the lowest Akaike's information criterion (Littell et al., 1998). Pre-treatment values of BCS, BW, and LS were used as covariates for these same variables. For colostrum (yield, nutrient composition, and immunoglobulins) data, the repeated statement in the MIXED procedure was not used; additionally time effect and associated interactions were excluded from the model, and block within treatment and parity removed from the random statement. Three orthogonal contrasts were used to interpret effect of treatments: 1) effect of treatments containing dietary antioxidants (ITMAOX and OTMAOX) versus treatments not containing dietary antioxidants (ITM and OTM), excluding CTL (CAO); 2) effect of treatments containing organic trace minerals (OTM and OTMAOX) versus

treatments not containing organic trace minerals (ITM and ITMAOX), excluding CTL (CTM); and 3) effect of treatments containing any type of supplementation (ITM, ITMAOX, OTM and OTMAOX) versus control (CTL; CAS). The PDIFF option was used to separate least square means. Significance was declared at  $P \leq 0.05$ , and tendency was considered when  $0.05 < P \leq 0.10$ . Data are reported as least squares means  $\pm$  SEM.

Health events (twins, dystocia, CK, DA, metritis and RP) are reported, but they were not analyzed statistically because the experiment was not powered appropriately to detect statistical differences in clinical events of health disorders.

## RESULTS

### *Dry Matter Intake*

There were no treatment main effects ( $P = 0.70$ ), interactions ( $P \geq 0.52$ ), or contrast ( $P \geq 0.31$ ) differences for dry matter intake. However, effects of parity ( $11.49 \pm 0.43$  kg/d for PP vs.  $13.42 \pm 0.43$  kg/d for MP), time, and parity  $\times$  time (Figure 2.1) were highly significant ( $P < 0.001$ ). Primiparous and multiparous cows displayed an approximate 30% decline in DMI during the last 3 weeks of the dry period (time effect,  $P < 0.001$ ). After d 3, DMI was greater ( $P < 0.001$ ) for MP than PP until the end of the trial.

### *Colostrum*

Parity did not affect colostrum yield ( $6.32 \pm 0.75$  vs.  $6.84 \pm 0.75$  kg, for PP and MP, respectively), but affected ( $P < 0.03$ ) fat ( $7.88 \pm 0.62$  vs.  $5.74 \pm 0.62\%$ , for PP and MP, respectively) and IgA concentrations ( $0.48 \pm 0.09$ , PP vs.  $0.89 \pm 0.10$  mg/mL, MP). Also, colostrum of cows supplemented with dietary antioxidants showed decreased fat content (contrast CAO,  $P = 0.02$ ) compared to cows that did not receive this supplementation (Table 2.4)

### ***Milk***

Effects of parity and time on milk variables are shown in Table 2.5 and Figure 2.3, respectively. Milk production differed ( $P < 0.001$ ) between parities from d 1 until the end of the trial; overall, MP produced more milk ( $P < 0.001$ ) than PP. In addition, a trend for a different response of treatments was observed among parities over time (treatment by parity,  $P = 0.08$ ; Figure 2.2). Treatments did not affect milk production in PP. However, in MP, CTL had increased milk yield over time compared to other treatments ( $P \leq 0.10$ ), whereas ITM displayed lower milk yield relative to the other treatments ( $P \leq 0.10$ ). Energy- and 3.5% fat-corrected milk were not affected by treatment or its interactions (Table 2.5). Nevertheless, an effect of time was observed ( $P \leq 0.01$ ) for both variables, with milk yield during the study reaching the maximal values at wk 3 postpartum (Figure 2.3).

A parity  $\times$  treatment interaction was observed for fat percent ( $P = 0.05$ ; Figure 2.4). During the first two weeks of lactation, fat percent was not different between treatments within time in both of the parities. On the third week of lactation, PP subjected to CTL and OTM had elevated ( $P \leq 0.05$ ) concentrations of fat compared to ITM and

ITMAOX, and on the fourth week of lactation, only OTM kept elevated compared to ITM ( $P = 0.09$ ) and ITMAOX ( $P = 0.01$ ). For MP cows, CTL showed the lowest ( $P \leq 0.01$ ) fat content on the third week of lactation. At the fourth week, ITMAOX was higher ( $P < 0.05$ ) than CTL and OTM, and ITM was higher ( $P = 0.02$ ) than OTM. Fat yield in PP tended to be lower ( $P < 0.09$ ) for OTM than ITMAOX on week 1 of lactation, there were no differences between treatments within time on weeks 2 and 3, and at week 4, OTM was higher ( $P < 0.04$ ) than CTL and ITMAOX. Treatment did not affect fat yield for MP on weeks 1, 2, and 3 of lactation, but during the last week of the experiment, OTM tended to be lower ( $P = 0.06$ ) than ITM.

The treatment ITM showed a tendency for higher ( $P = 0.09$ ) protein content than ITMAOX in PP, but lower ( $P < 0.01$ ) content than CTL, ITMAOX, and OTM in MP. The only effect seen after the first week was when CTL showed lower ( $P = 0.07$ ) protein percentage than ITMAOX at week 4. Even with the differences in protein percentage, it was not translated to differences in protein yield of milk in any of the treatments.

Results of percentage of SNF are in agreement with milk production. No difference in SNF percent for PP, but ITM was lower ( $P \leq 0.03$ ) compared to CTL, OTM, and OTMAOX in MP (Figure 2.5). Milk urea nitrogen was only affected by time ( $P < 0.001$ ; Figure 2.3B). Contrasts were not significant for milk production or composition variables.

### ***Feed Efficiency***

There was a parity  $\times$  time effect ( $P < 0.01$ ) for the milk yield to DMI ratio (Figure 2.6A), such that PP had 18% lower feed efficiency compared to MP (parity effect,  $P <$

0.001). In early lactation cows up to 27 d postpartum, milk yield to DMI ratio was not affected by time ( $P = 0.28$ ). Primiparous cows had a 24% lower ECM to DMI ratio compared to MP (parity,  $P < 0.001$ ; parity  $\times$  time;  $P = 0.02$ ). Figure 2.6B represents the effects of time within parities of ECM feed efficiency. For ECM to DMI ratio, at the first week of parturition, ITM had the greatest ( $P < 0.001$ ) ratio compared to all other treatments, but this difference was not observed for the next weeks (time  $\times$  treatment,  $P < 0.01$ ). The treatment  $\times$  time effect was caused by two high values on the first week of lactation from MP under ITM.

The same behavior from ECM to DMI ratio was observed for the 3.5% FCM to DMI ratio. Parity, time, and parity  $\times$  time (Figure 2.6C) effects were significant ( $P < 0.05$ ). A time  $\times$  treatment effect ( $P = 0.003$ ) occurs because of a difference on ITM at the first week of lactation that showed greater ( $P \leq 0.001$ ) feed efficiency compared to other treatments. No difference between treatments was observed in PP, however, in MP, ITM tended to be greater ( $P < 0.09$ ) than the others (parity  $\times$  treatment,  $P = 0.09$ ). The same two readings described previously also influenced this dataset. Only effects of parity and time were observed ( $P \leq 0.01$ ). No differences on orthogonal contrasts were seen for any of the variables.

### ***Energy status***

Parity and its associated interactions did not affect BCS of periparturient dairy cows. Between wk -6 and -2 BCS did not change, slightly decreased at wk -1, and reached nadir at wk 3 from calving (time effect,  $P < 0.001$ ). No differences of treatments

or orthogonal contrasts were observed for this variable; however, treatment  $\times$  time tended to be significant ( $P = 0.098$ ; Figure 2.7).

In accordance with the results for BCS, animals lost BW over time ( $671.46 \pm 5.23$ ,  $615.95 \pm 5.12$ , and  $586.38 \pm 5.23$  kg at d  $-39 \pm 4.6$ , 0, and 21 from parturition, respectively; time effect,  $P < 0.001$ ). As expected, BW differed between parities ( $604.45 \pm 6.80$  kg for PP vs.  $644.75 \pm 6.89$  kg for MP, respectively; parity effect,  $P < 0.001$ ). No other effects were observed for this measurement.

Cows underwent light NEB at wk -3 relative to parturition, when PP tended ( $P = 0.087$ ) to have lower NEB than MP. Energy balance severely declined ( $P < 0.001$ ) for both of the parities at wk 1 compared to wk -3. Multiparous cows then showed a highly significant ( $P = 0.004$ ) recovery from NEB at wk 3 compared to wk 1, contrasting what occurred in PP, when no improvement in energy status was observed (Figure 2.8). In general, PP tended ( $P = 0.08$ ) to undergo more severe NEB than MP.

### ***Health***

Lameness score tended to be affected ( $P \leq 0.09$ ) by time and parity  $\times$  time, although was significantly different between PP and MP ( $1.000 \pm 0.000$  vs.  $1.025 \pm 0.008$ , respectively;  $P = 0.03$ ). Although statistically significant, these effects may not be of biological value because these scores were assigned in increments of 1.0 and only two MP presented LS higher than 2 for the entire experimental period in two distinct assessments. No other effects were observed.

Table 2.6 presents the health issues of postpartum cows until d 14 of calving. Primiparous had higher prevalence of metritis than multiparous cows (6 vs. 1 case,

respectively). However, birth of twins and DA were more prevalent in MP than PP. Animals using any type of supplementation seems to have elevated cases of clinical ketosis compared to not supplemented animals. In addition, supplementation with chelated trace minerals decreased the prevalence of DA and metritis compared to supplementation of inorganic forms.

### ***Metabolic Variables***

Concentrations of glucose were higher ( $P < 0.001$ ) for PP ( $64.71 \pm 0.85$  mg/mL) compared to MP ( $59.87 \pm 0.82$  mg/mL). After calving, concentration of glucose dropped significantly ( $P < 0.001$ ) compared to the day of calving and before. In contrast, overall concentrations of NEFA were higher ( $P < 0.001$ ) in MP ( $570.48 \pm 32.17$   $\mu$ Eq/L) than in PP ( $422.60 \pm 33.11$   $\mu$ Eq/L), such that NEFA concentrations peaked higher ( $P < 0.01$ ) and earlier for MP ( $814.97 \pm 57.74$   $\mu$ Eq/L at the day of calving) compared to PP ( $663.00 \pm 58.70$   $\mu$ Eq/L at d 7 postpartum). Concentrations of NEFA were lower ( $P \leq 0.01$ ) during the prepartum compared to the postpartum period. Similarly, BHBA concentrations were also more elevated ( $P < 0.001$ ) in MP ( $6.99 \pm 0.29$  mg/dL) than in PP ( $5.48 \pm 0.29$  mg/dL). Peak of BHBA was at d 0 for PP and d 7 for MP, and concentrations of BHBA at peak differed ( $8.37 \pm 0.45$  and  $6.63 \pm 0.46$  mg/dL, respectively;  $P < 0.01$ ). Postpartum concentrations of BHBA were higher ( $P < 0.001$ ) than the prepartum concentrations. A treatment  $\times$  time interaction was observed ( $P = 0.03$ ) for BHBA, but it was due a single observation at d 14 of MP under ITM.

Total glutathione was higher ( $P < 0.001$ ) for PP ( $183.50 \pm 3.17$   $\mu$ M) than MP ( $164.74 \pm 3.19$   $\mu$ M). Time tended to affect total glutathione ( $P = 0.08$ ), having the peak

at the day of calving ( $176.39 \pm 2.76 \mu\text{M}$ ) and the nadir on d 14 ( $171.91 \pm 2.76 \mu\text{M}$ ). Animals supplemented with dietary antioxidants had increased ( $P = 0.03$ ) total glutathione compared to animals not being supplemented with it. In concordance with total glutathione, GSH was also higher ( $P < 0.01$ ) in PP ( $88.25 \pm 5.02 \mu\text{M}$ ) than in MP ( $70.42 \pm 5.05 \mu\text{M}$ ). There tended to be a time effect ( $P = 0.06$ ) on GSH concentration, with peak ( $83.82 \pm 4.31 \mu\text{M}$ ) and nadir ( $76.82 \pm 4.31 \mu\text{M}$ ) on the day of calving and d 14, respectively. Effects of parity  $\times$  treatment for GSH ( $P = 0.03$ ; Figure 2.9) and for GSH to GSSG ratio ( $P = 0.03$ ; Figure 2.11), and time  $\times$  treatment for GSSG concentrations ( $P = 0.09$ ; Figure 2.10) are illustrated below. No other effects were seen for these variables.

Table 2.7 presents the results of the comet assay variables. Cows fed ITMAOX showed a smaller ( $P < 0.05$ ) percentage of DNA in the tail, comet tail length, and tail moment compared to CTL, OTM, and OTMAOX. Animals supplemented with inorganic sources of trace minerals had lower ( $P < 0.02$ ) comet tail, percentage of DNA in tail, and tail moment compared to animals supplemented with the organic sources. In addition, use of any type of supplementation tended ( $P \leq 0.09$ ) to decrease comet tail length and moment.

Rabies antibody titers tended to be greater ( $P = 0.06$ ) in PP ( $0.37 \pm 0.06 \text{ EU/mL}$ ) compared to MP ( $0.24 \pm 0.06 \text{ EU/mL}$ ). Antibody titers for rabies were extremely low on d 0 and 7 relative to parturition and did not differ from each other in any of the parities. On d 14, PP had 103% more antibody titers than MP ( $P < 0.001$ ). On d 28 the difference declined to 28%, but was still highly significant ( $P < 0.001$ ). Concentrations of rabies titers peaked on d 28 for both of the parities ( $0.75 \pm 0.11$  for PP vs.  $0.58 \pm 0.11$

EU/mL for MP), although no difference was detected between d 14 ( $0.72 \pm 0.11$  EU/mL) and 28 ( $0.75 \pm 0.11$  EU/mL) in PP, contrasting the findings in MP, for which d 14 ( $0.35 \pm 0.11$  EU/mL) was different ( $P < 0.001$ ) from d 28 ( $0.58 \pm 0.11$  EU/mL). Treatments behaved differently among parities (parity  $\times$  time  $\times$  treatment effect,  $P = 0.02$ ); within the same collection time, treatments did not differ for rabies titers in PP. In MP, treatments did not differ on d 0 and 7, however, OTMAOX was the highest on d 14 when it tended to be greater ( $P < 0.07$ ) than CTL and ITMAOX, but it was greater ( $P \leq 0.001$ ) than ITM and OTM. Also, CTL and ITMAOX concentrations of rabies titers were not different between each other, but both were higher ( $P < 0.04$ ) than ITM and OTM. By d 28, OTMAOX had still the highest concentrations, but was only significantly greater than CTL ( $P < 0.04$ ).

## DISCUSSION

Results of DMI for this experiment are consistent with findings of other authors. Before parturition, intake decreased by approximately 30% at the last three weeks prepartum (Bertics et al., 1992) for both of the parities. As expected, after parturition DMI was greater for MP than PP (Flis and Wattiaux, 2005; Janovick and Drackley, 2010).

Colostrum composites were observed to be in the range described by Kehoe et al. (2007) during a survey that included 55 Holstein dairy herds. Primiparous cows had higher fat percent in colostrum similar to observations by Nonaka and Oshita (2003). Concentrations of IgA were higher for MP than PP, contrasting findings from Devery-Pocius and Larson (1983) in dairy cows who observed no statistical difference in IgA concentrations in colostrum of primiparous and multiparous cows, but fourth lactation or greater animals showed numerically higher concentrations compared to first and second lactations animals. McGee et al. (2006) reported that in dairy × beef crossbred animals, there was no parity effect in IgA concentrations, but greater IgA mass in colostrum of MP cows. The cutoff point for colostrum quality is total immunoglobulin concentrations above 50 mg/mL (Heinrichs and Jones, 2011). Accordingly, our results suggest that PP can have as good of quality of colostrum as MP cows, contrasting common beliefs at the farm level. To our knowledge, there is no research on the effects of dietary antioxidants on colostrum composition of dairy cows, but we observed that the use of dietary antioxidants depress colostrum fat percent which contrasts the effects of dietary antioxidants on milk composition (Boerman et al., 2014; He and Armentano, 2011; Wang et al., 2010). Lower fat percent may improve the antioxidant capacity of the colostrum. The decline in macromolecules susceptible to peroxidative damage could lead to less production of ROS, thus diminishing the load of ROS to the newborn at a time when they are susceptible to stress due to changes in oxygen pressure and start of regular breathing as found in humans (Saugstad, 2005; Wiedemann et al., 2003).

The results of our milk yield and composition variables were consistent with other published results (Chamberlin et al., 2013; Hart et al., 2014; Kessel et al., 2008; Kessler et al., 2014; Rastani et al., 2005; De Vries and Veerkamp, 2000). Even with no observed significant effects of addition of any type of supplements used in this study on milk yield, the inclusion of supplements seems to be increase milk production in PP cows. Therefore, more studies to evaluate dose response of mineral supplementation in PP would be valuable. On the other hand, MP cows appeared to undergo a negative effect of mineral supplementation over NRC (2001) requirements on milk production, because all of the treatments except CTL, received extra amounts of minerals and had a negative effect on milk production. Feeding organic trace minerals increased milk yield (Rabiee et al., 2010), furthermore feeding blended (organic plus inorganic) trace minerals to primiparous and multiparous cows elevated milk production compared to only feeding either the inorganic or organic sources (Ballantine et al., 2002; Nocek et al., 2006)

Effects of parity on milk composition yield, but not percentage were caused by dilution factors due to difference in milk production between parities. Levels of milk fat percentage in this experiment are usually seen in Jersey cows (Smith et al., 2013; Weisbjerg et al., 2013), although similar results were found by Janovick and Drackley (2010) in Holstein cows. Fat content in PP was the lowest for ITM and the highest for OTM; conversely, MP animals had highest fat content under ITM and the lowest under OTM. Our data is in agreement with Hackbart et al. (2010) for MP animals and, also, fat yield was not different between treatments., However, our results contradict Nocek et al. (2006) who observed higher fat content for primiparous cows fed 100% inorganic

minerals compared to cows fed a blended form of trace minerals, but higher fat yield in cows fed blended trace minerals than primiparous cows fed only the inorganic form. In addition, in our study, fat percent in multiparous cows was not different but fat yield followed the similar pattern of primiparous cows. Results from the previous described findings are in accordance with other publications (Ballantine et al., 2002; Rabiee et al., 2010). The diminished milk protein content in MP under ITM agrees with some studies (Kincaid and Socha, 2004; Nemeč et al., 2012), yet disagrees with others (Ballantine et al., 2002; Hackbart et al., 2010). The lower protein content in our results was also likely the cause for reduced SNF content in the same animals. Similar to protein concentration, published reports concerning protein yield for cows fed treatments similar to those in this study have also been inconsistent (DeFrain et al., 2009; Nemeč et al., 2012; Nocek et al., 2006; Siciliano-Jones et al., 2008). Next, concentrations of MUN observed in this experiment are similar to the ones reported by Nemeč et al., (2012). Contrasting the literature (Burvenich et al., 2003; Hagnestam-Nielsen et al., 2009; Janovick and Drackley, 2010), SCS was lower in MP than PP, what may suggest higher incidence of subclinical mastitis in this primiparous animals.

The lack of treatment effect on feed efficiency observed in this study is in contrast to results from Nocek et al. (2006), but similar to the results of Kincaid and Socha (2004) and Nemeč et al. (2012). Overall, PP displayed lower feed efficiency than MP because they still require part of the nutrients ingested to be directed to growth, this way diminishing the amount of nutrients directed toward milk yield. Feed efficiency was consistently different between parities within time; however, during the last week of the

experiment, the milk yield to DMI ratio had declined for MP and the difference between parities was absent. Multiparous cows were observed to improve EB at week 3, and BCS was not different between weeks 3 and 4 from parturition. Therefore nutrients could be also directed toward body reserves replenishment once they are not mobilizing as much adipose tissue to support lactation, which may have caused decrease in feed efficiency at this time, also . Vallimont et al. (2011), while examining the relationship between feed efficiency and BCS, observed that BCS was higher in less efficient than in more efficient cows in late lactation.

Effects of parity and time on BCS and BW are in agreement with many published reports and anecdotal field observations. Nemeč et al. (2012) observed no difference in BCS between the experimental groups; however, Hackbart et al. (2010) found that the group under blended trace minerals had lower BCS at the second month of lactation. In the current experiment, ITM cows had slightly lower BCS than from cows on the other treatments.

According to the review published by Overton and Yasui (2014), healthier hoof tissue in dairy cows is one of the common benefits of feeding organic trace minerals. Similar to Formigoni et al. (2011) and Uchida et al. (2001), we did not observe treatment effects on LS, whereas other authors have reported improvements for this health indice (DeFrain et al., 2009; Nocek et al., 2006; Siciliano-Jones et al., 2008). However, it should noted that LS in all treatments were excellent, such that there was little room for any beneficial effects of treatment to be realized for this variable. Health variables were not different between treatments, agreeing with Hackbart et al. (2010) and Nocek et al.

(2006). However, this experiment was not designed with the statistical power to detect treatment differences in clinical disease. Results for glucose, NEFA, and BHBA concentrations followed tendencies that have been reported by others (Douglas et al., 2006; Guo et al., 2007; Janovick and Drackley, 2010).

The glutathione system is important in scavenging ROS from the body. Glutathiones metabolize  $H_2O_2$  to less harmful compounds such as  $H_2O$  by oxidizing GSH to GSSG in a reversible reaction (Nelson et al., 2008). Reduced glutathione is predominant during normal cellular conditions and GSSG increases under heavy oxidative loads (O'Boyle et al., 2006), such that under conditions of oxidative stress, the GSH to GSSG ratio decreases (Lykkesfeldt and Svendsen, 2007). When GSH was unable to saturate glutathione peroxidase, elimination of  $H_2O_2$  was linearly related with GSH concentrations (Michiels et al., 1994). Thus, elevation in GSH concentrations may strengthen the antioxidant defense of cells (Lykkesfeldt and Svendsen, 2007). A depletion of the antioxidant activity occurred after parturition as suggested by the decline in total glutathione and GSH concentrations on d 14 compared to d 0 from parturition. In addition, GSSG concentrations were lower at d 0 than d 14, suggesting that more of the reduced glutathione had been oxidized on d 14 postpartum. Furthermore, the addition of dietary antioxidants increased total glutathione in this study, perhaps due to the decreased  $H_2O_2$  in plasma as observed by Wang et al. (2010). In situations of stress such as the early lactation period, concentrations of total glutathione decline (Settivari et al., 2006). Among many factors, a decline in BCS and the associated metabolism might also play a role in decreasing the antioxidant capacity of the animals. Bernabucci et al. (2005) and

O'Boyle et al. (2006) respectively observed cows that lost more BCS decreased erythrocyte thiol groups and cows with higher BCS tended to have lower GSH:GSSG. These reports contrast Dobbelaar et al. (2010) who observed that heifers that underwent more accentuated BCS loss had higher blood concentrations of GSH, lower of GSSG, higher GSH:GSSG, but they tended to have higher ROS as well. Metabolism and the antioxidant capacity of cows are different between parities. Primiparous cows under ITM and OTMAOX were observed to have the lowest and the highest GSH:GSSG ratio, respectively. In MP, CTL and OTMAOX displayed the highest and the lowest ratio, respectively. One plausible explanation for these differences between parities could be that, in PP, sulfates, bound to trace minerals, may have increased the formation of thiosulfates that react with GSH oxidizing to GSSG (Kamoun, 2004), this way lowering the ratio GSH:GSSG. On the other hand, OTMAOX has lesser amount of sulfates than ITM and also has the addition of a dietary antioxidant that was reported to decrease plasma  $H_2O_2$  and tended to increase total antioxidant capacity without significant changes in SOD (Wang et al., 2010). This combination of activities would spare GSH and increase the ratio of the glutathiones. In MP, intake of minerals already contained in the basal diet might have been sufficient to meet requirements due to the greater DMI combined with lower amount of body tissue growth. Under these conditions, supplementation of trace minerals may lead to negative effects. It is possible that the slight increase in milk production for MP fed CTL may have come from a better antioxidant status showed by greater GSH:GSSG. However, the role of supplementing

organic trace minerals with dietary antioxidants on antioxidant defense among different parities would remain unclear.

Glutathione protects cells against harmful endogenous and exogenous agents. The glutathione system helps to protect against errors in DNA and protein synthesis and helps to maintain amino acid transport (Lafleur et al., 1994). According to the comet assay results, systemic lymphocyte DNA damage was greater in OTMAOX animals, suggesting a negative effect of supplementing organic trace minerals and dietary antioxidants together. Furthermore, any type of supplementation protected against DNA damage, although groups supplemented with inorganic forms of trace minerals may have offered better protection of the DNA against damage compared to groups supplemented with the organic forms. These findings together suggest different actions of minerals on the antioxidant system among parities. However, again, a more complete profile of the oxidative status would have helped with interpretation of these results.

We observed that the peak of antibody response against rabies occurred on d 28, 4 weeks from vaccination as observed by Nemec et al. (2012) and as proposed by Sun et al. (2008). The immune response and antioxidant status seem to vary more in MP than PP. Treatments did not influence rabies antibody titers concentrations in PP. Contradicting Nemec et al. (2012) in dairy cows, ITM and OTM did not differ for antibody titers throughout the experiment, although this agrees with reports in beef cattle (Nunnery et al., 2007; Spears and Kegley, 2002). The effect of trace minerals supplemented above NRC (2001) requirements on the adaptive immune system is still inconclusive in cattle. The effects of organic minerals on adaptive immune system were also studied in poultry

(Moghaddam and Jahanian, 2009) and swine (Richards et al., 2010), and it was observed that the use of organic minerals enhanced response to different types of vaccines. Interestingly, the fortification of organic trace minerals with dietary antioxidants (OTMAOX) improved response against rabies vaccination at d 14 compared to all other treatments, but this difference decreased at d 28, in which CTL group had the lowest concentrations of rabies antibody titers. Seifried et al. (2007), in their review article, stated that innate and adaptive immune systems are linked by ROS and their effects on cytokine production, and the shift in the GSH:GSSH ratio as well. At low levels of antigen, adaptive immunity is activated in order for T cells to build a stronger defense system against the pathogen; an up-regulation of the ROS production and the expression of inflammatory cytokines are necessary for this process. This mechanism of activation is necessary for the survival of the individual, mainly in situations of stress. Decreased antioxidant status demonstrated by a decline on GSH:GSSG ratio in OTMAOX is likely to explain the better response of this treatment against rabies vaccination. In contrast, research in mice revealed that administration of a superoxide scavenger did not change the ability of the animals to recover from Influenza H1N1 (Case et al., 2011); however, the drug used in that study only targeted the mitochondrial superoxide, whereas other parts of the system may also need to be targeted for the positive effects on immunity to be realized.

The study of more mechanistic effects on use of organic trace minerals, either blended with other minerals or blended with different forms of the compounds, and

dietary antioxidants would be very helpful to fully understand the physiology behind the processes, from increasing fat in milk to cellular development.

## **CONCLUSION**

The results of this study suggest that supplementation of copper, manganese, and zinc above NRC (2001) recommendations may not be beneficial to multiparous cows; however, signs of improved antioxidant status and health were observed when primiparous cows were supplemented. In addition, colostrum of primiparous animals showed equal quality as colostrum from multiparous cows. Animals fed organic trace minerals showed evidence of increased DNA damage in blood lymphocytes. Mechanisms of action of dietary antioxidants are not fully understood in dairy cattle, but based on the variability in responses to treatments in this study, antioxidants may play different roles between parities and may be influenced by trace mineral supplementation. Multiparous cows subjected to OTMAOX treatment had increased rabies antibody titers, suggesting enhanced adaptive immune response.

**Table 2.1.** Composition of the top-dressed treatments

Item, %	Treatments <sup>1,2</sup>				
	CTL	ITM	ITMAOX	OTM	OTMAOX
Cu, sulfate <sup>3</sup>	-	0.30	0.30	0.15	0.15
Mn, sulfate <sup>4</sup>	-	0.86	0.86	0.43	0.43
Zn, sulfate <sup>5</sup>	-	0.84	0.84	0.42	0.42
Cu, chelate <sup>6</sup>	-	-	-	0.21	0.21
Mn, chelate <sup>7</sup>	-	-	-	0.60	0.60
Zn, chelate <sup>8</sup>	-	-	-	0.83	0.83
Antioxidant <sup>9</sup>	-	-	2.01	-	2.01
HMTBa <sup>10</sup>	1.55	1.55	1.55	-	-
Corn, ground	98.45	96.45	94.44	97.36	95.35

<sup>1</sup>Prepartum and postpartum animals were supplemented 200 and 250 g/d of the mix, respectively.

<sup>2</sup>Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants.

<sup>3</sup>Copper sulfate contained 25.2% of Cu.

<sup>4</sup>Manganese sulfate contained 22.2% of Mn.

<sup>5</sup>Zinc sulfate contained 36% of Zn.

<sup>6</sup>Copper chelate contained 15% of Cu (Mintrex, Novus International, St. Charles, MO).

<sup>7</sup>Manganese chelate contained 13% of Mn (Mintrex, Novus International).

<sup>8</sup>Zinc chelated contained 16% of Zn (Mintrex, Novus International).

<sup>9</sup>Agrado Plus (Novus International).

<sup>10</sup>Ca-salt of 2-hydroxy-4-methylthio-butanoic acid (MHA feed supplement, Novus International).

**Table 2.2.** Ingredient composition of the basal diets

Item	Prepartum period	Postpartum period
DM, <sup>1</sup> %	61.49	47.61
Ingredients, % of DM		
Corn silage	21.21	35.40
Alfalfa haylage	-	7.08
Alfalfa hay	-	7.87
Brome hay	54.79	-
Corn, ground	9.90	24.55
Soybeans hulls, ground	10.61	4.13
Brewer's grain, wet	-	4.92
Soybean meal, 47.5% CP	-	6.29
Amino Plus <sup>2</sup>	-	3.44
Blood, meal	0.53	0.98
Sodium bicarbonate	-	0.79
Salt white	0.22	0.30
Limestone, ground	-	1.08
Calcium chloride dihydrate	1.24	-
Potassium carbonate	-	0.33
Magnesium oxide	0.21	0.18
Magnesium sulfate heptahydrate	0.88	-
Dynamate <sup>3</sup>	-	0.33
Trace mineral premix <sup>4</sup>	0.10	0.10
ADE premix	0.11	0.06
Vitamin E 20,000	0.18	0.07
Rumensin 90 <sup>5</sup>	0.02	0.01
Energy Booster 100 <sup>6</sup>	-	1.91
MetaSmart Dry <sup>7</sup>	-	0.18

<sup>1</sup>Values represent averages of weekly samples.

<sup>2</sup>Ag Processing Inc., Emmetsburg, IA.

<sup>3</sup>The Mosaic Co., Plymouth, MN.

<sup>4</sup>The trace mineral premix contained 99.85% of ground corn, 0.04% of cobalt sulfate, 0.05% of EDDI (ethylenediamine dihydriodide), and 0.06% of sodium selenite.

<sup>5</sup>Elanco Animal Health, Indianapolis, IN.

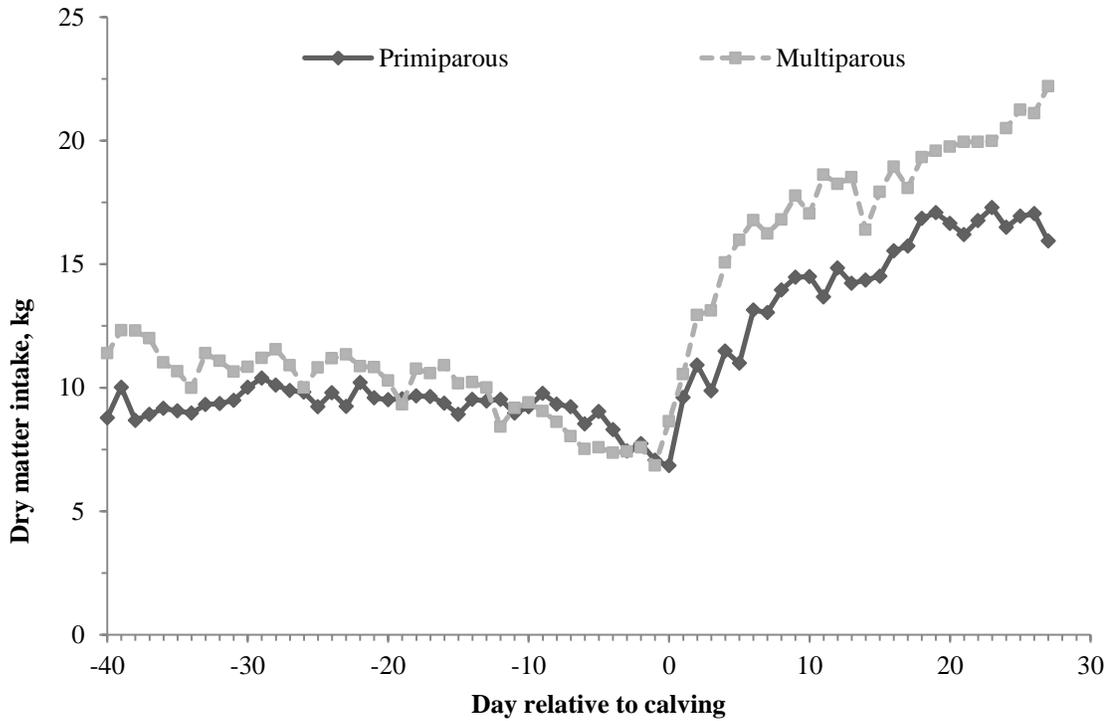
<sup>6</sup>Milk Specialties Global, Eden Prairie, MN.

<sup>7</sup>Adisseo Inc., Antony, France.

**Table 2.3.** Chemical composition of the basal diets

Nutrient (DM basis) <sup>1</sup>	Prepartum period	Postpartum period
CP, %	11.59	17.47
Available protein, %	10.57	16.47
Soluble protein, % of CP	34.57	30.67
ADF, %	37.26	21.72
NDF, %	56.89	34.55
Lignin, %	5.54	3.75
NFC, %	23.46	38.45
Starch, %	8.47	20.35
ESC (simple sugars), %	4.34	3.07
Crude fat, %	2.04	4.45
Ash, %	8.22	7.71
NE <sub>l</sub> , Mcal/kg	1.30	1.66
NE <sub>m</sub> , Mcal/kg	1.14	1.66
NE <sub>g</sub> , Mcal/kg	0.58	1.06
Ca, %	0.96	0.99
P, %	0.21	0.33
Mg, %	0.37	0.35
K, %	1.58	1.61
Na, %	0.11	0.37
Fe, ppm	353.57	397.67
Zn, ppm	49.57	47.83
Cu, ppm	14.71	10.67
Mn, ppm	80.14	43.17
Mo, ppm	0.73	1.22
S, %	0.24	0.30
Chloride ion, %	1.06	0.51
DCAD, mEq/100g	0.00	23.83

<sup>1</sup>Values, provided by DairyOne Laboratories Inc. (Ithaca, NY), represent averages of samples composited monthly.



**Figure 2.1.** Dry matter intake of periparturient dairy cows. Primiparous (n = 30) and multiparous (n = 29) cows were fed from d  $-39 \pm 4.6$  to 27 from parturition a basal diet to meet or exceed (NRC, 2001) requirements. Treatments were top-dressed at the morning feeding at a rate of 200 g/d for the prepartum and 250 g/d for the postpartum period. Parity, time, and parity  $\times$  time effects,  $P < 0.001$ . Values represent mean; pooled SEM = 0.69.

**Table 2.4.** Colostrum variables of periparturient dairy cows

Variable	Treatment <sup>1</sup>					SEM <sup>2</sup>	P-value		
	CTL	ITM	ITMAOX	OTM	OTMAOX		Parity	Treatment	Parity × treatment
Yield, kg	5.05	6.82	7.87	6.61	6.54	1.18	0.629	0.592	0.844
Fat, %	6.09 <sup>bc</sup>	8.77 <sup>a</sup>	4.81 <sup>c</sup>	7.55 <sup>ab</sup>	6.85 <sup>b</sup>	0.98	0.019	0.080	0.693
Protein, %	10.76	11.16	10.94	11.19	11.32	0.54	0.828	0.949	0.244
SNF, %	13.64	14.60	14.70	14.44	14.70	0.68	0.544	0.789	0.189
SCS <sup>3</sup>	6.03	6.14	6.67	5.66	6.20	0.53	0.360	0.571	0.131
IgA, mg/mL	0.68	0.74	0.83	0.54	0.65	0.15	0.029	0.944	0.382
IgG, mg/mL	56.52	46.87	46.97	46.83	51.22	7.10	0.145	0.771	0.870
IgM, mg/mL	6.06 <sup>a</sup>	4.12 <sup>b</sup>	6.53 <sup>a</sup>	6.01 <sup>a</sup>	5.05 <sup>ab</sup>	0.97	0.535	0.096	0.543

<sup>a,b</sup>Means with different superscript letters differ ( $P < 0.10$ ) within row.

<sup>1</sup>Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants.

<sup>2</sup>Pooled SEM.

<sup>3</sup>SCS =  $[\log_2(\text{SCC}/100,000) + 3]$ .

**Table 2.5.** Milk variables among different parities up to 27 days postpartum

Variable <sup>1</sup>	Parity			P-value						
	Primiparous	Multiparous	SEM <sup>2</sup>	Parity	Time	Treatment	Parity × Time	Parity × treatment	Time × treatment	Parity × time × treatment
Milk yield, kg/d	25.68	37.14	1.07	< 0.001	< 0.001	0.779	< 0.001	0.392	0.426	0.088
3.5% FCM, <sup>3</sup> kg/d	32.08	46.76	1.62	< 0.001	0.011	0.988	0.912	0.729	0.497	0.220
ECM, <sup>4</sup> kg/d	31.34	45.26	1.56	< 0.001	0.033	0.966	0.755	0.893	0.481	0.150
Fat, %	4.90	5.09	0.18	0.416	< 0.001	0.963	0.556	0.051	0.814	0.004
Fat yield, kg/d	1.27	1.86	0.07	< 0.001	0.109	0.945	0.591	0.727	0.323	0.088
Protein, %	3.15	3.23	0.04	0.317	< 0.001	0.926	0.221	0.798	0.499	0.031
Protein yield, kg/d	0.81	1.19	0.03	< 0.001	0.082	0.984	0.273	0.611	0.716	0.999
SNF, %	8.71	8.61	0.06	0.234	< 0.001	0.651	0.396	0.067	0.376	0.677
SNF yield, kg/d	2.27	3.26	0.10	< 0.001	< 0.001	0.961	0.366	0.435	0.568	0.953
MUN, mg/dL	9.47	9.64	0.32	0.707	< 0.001	0.292	0.767	0.425	0.895	0.454
SCS <sup>5</sup>	3.46	2.51	0.29	0.029	< 0.001	0.429	0.535	0.243	0.835	0.571

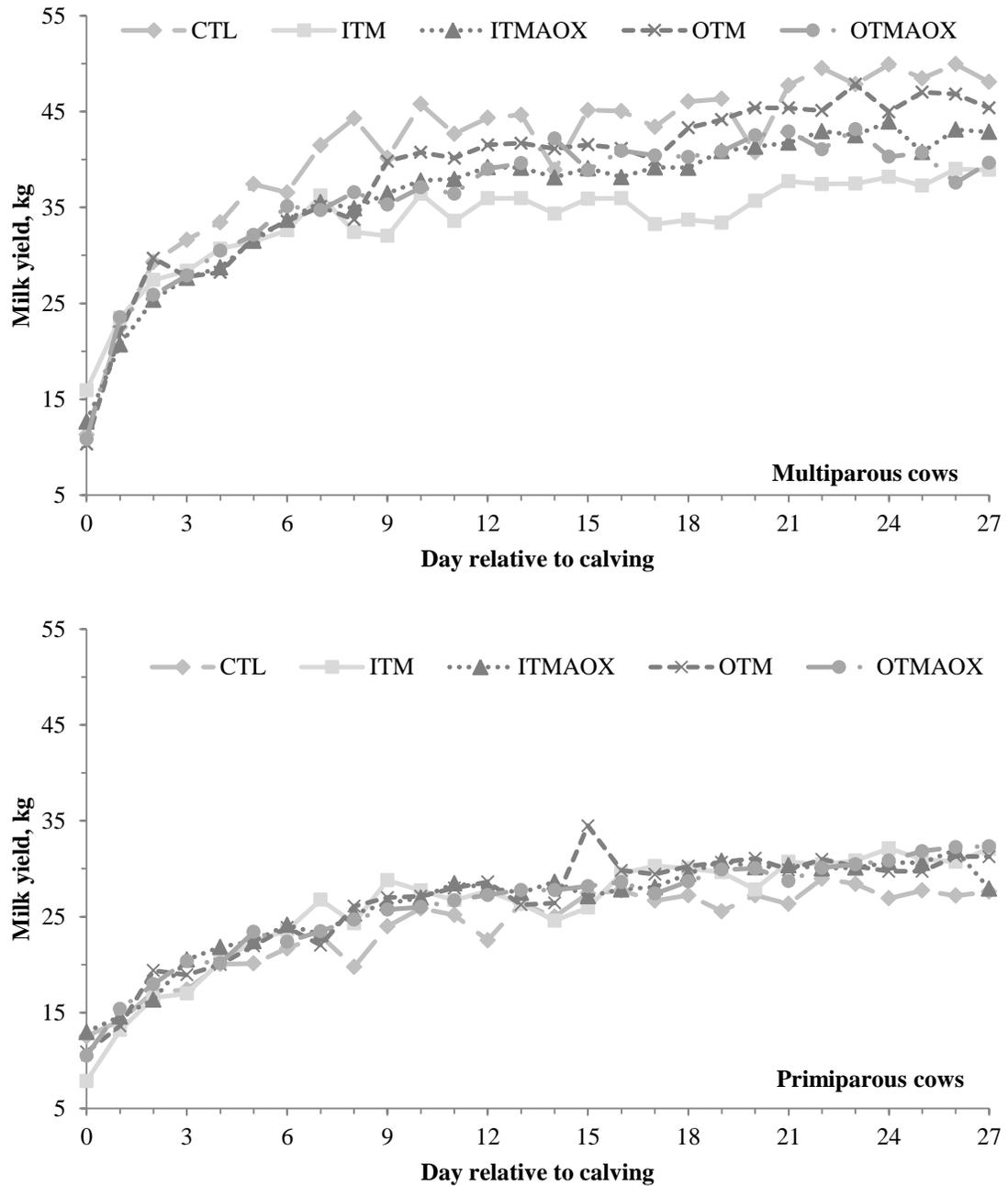
<sup>1</sup>Except for milk yield, samples were collected once weekly from consecutive p.m. and a.m. milkings (Wednesday and Thursday), and composition was a calculated composite of each milking corrected for milk yield.

<sup>2</sup>Pooled SEM.

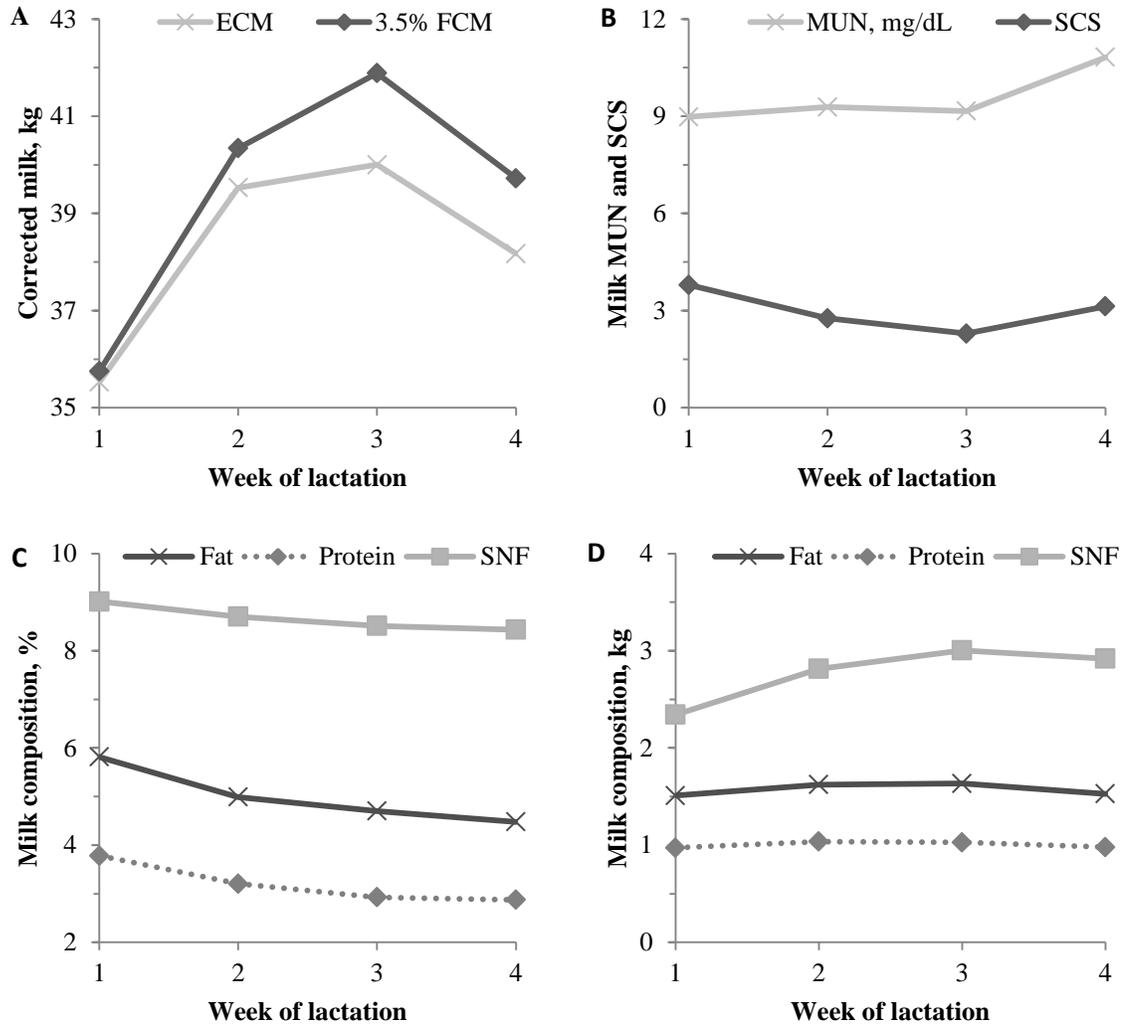
<sup>3</sup>3.5% FCM, kg/d = {[milk yield, kg/d × 0.4324] + [milk fat, kg/d × 16.2162]}.

<sup>4</sup>ECM, kg/d = {[milk yield, kg/d × 0.327] + [milk fat, kg/d × 12.86] + [milk protein, kg/d × 7.65]}.

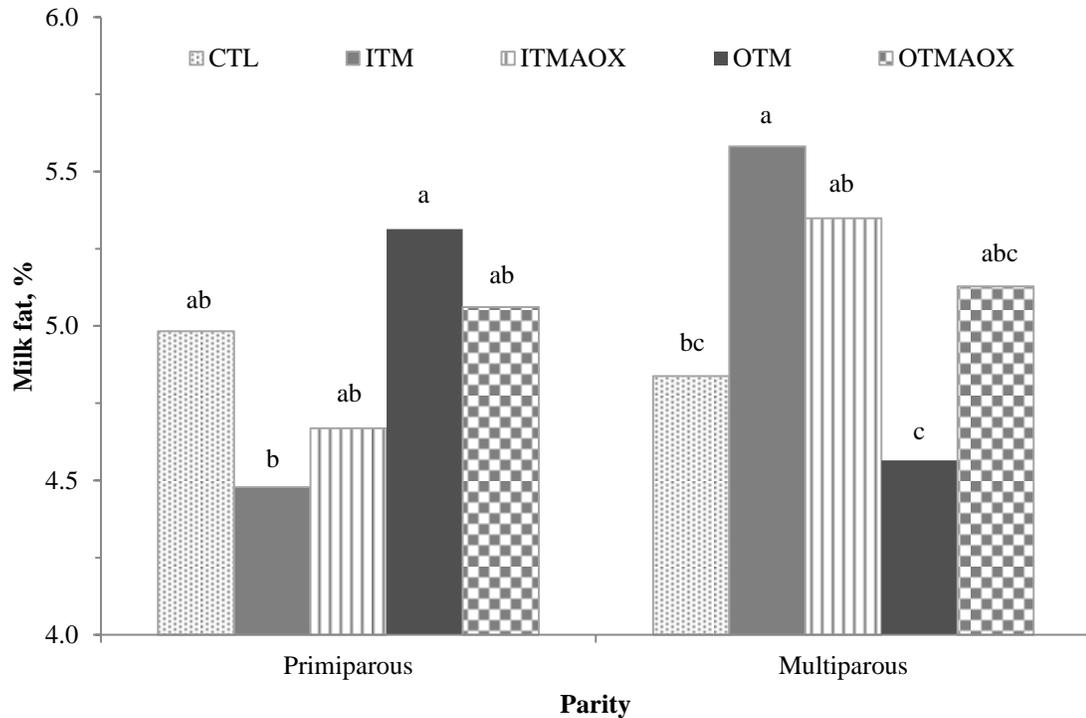
<sup>5</sup>SCS = [log<sub>2</sub>(SCC/100,000) + 3].



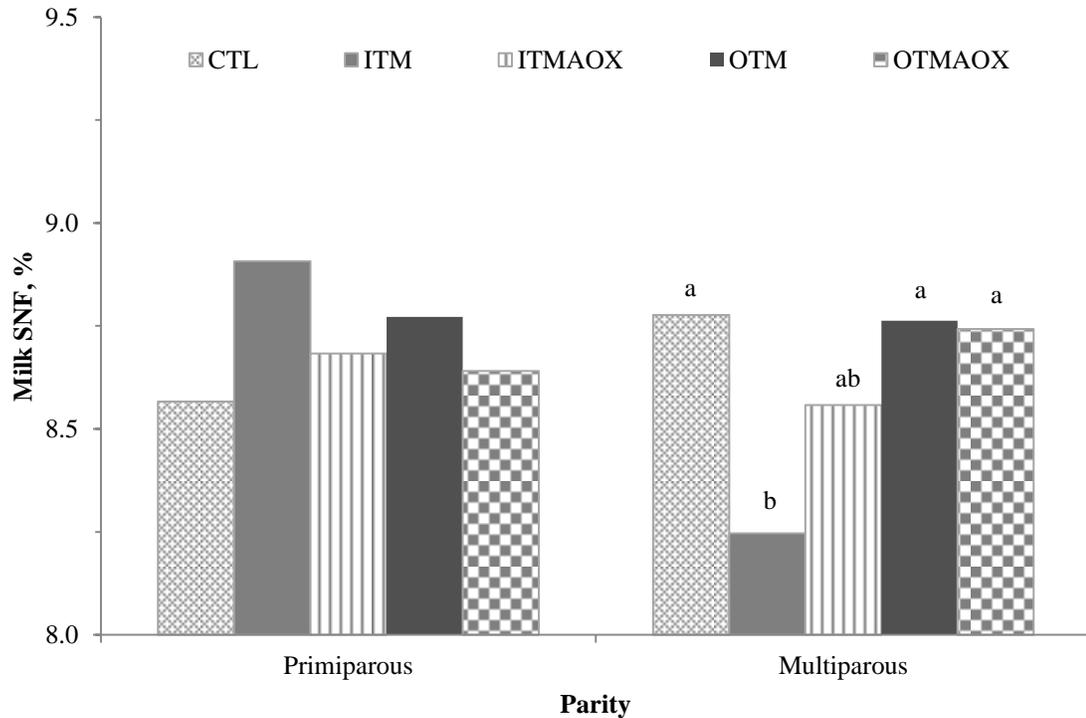
**Figure 2.2.** Effects of treatment within parity on milk production of early lactation cows. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Parity, time, and parity  $\times$  time,  $P < 0.001$ ; parity  $\times$  time  $\times$  treatment,  $P = 0.08$ . Values represent mean; primiparous cows pooled SEM = 2.93, and multiparous cows pooled SEM = 2.99.



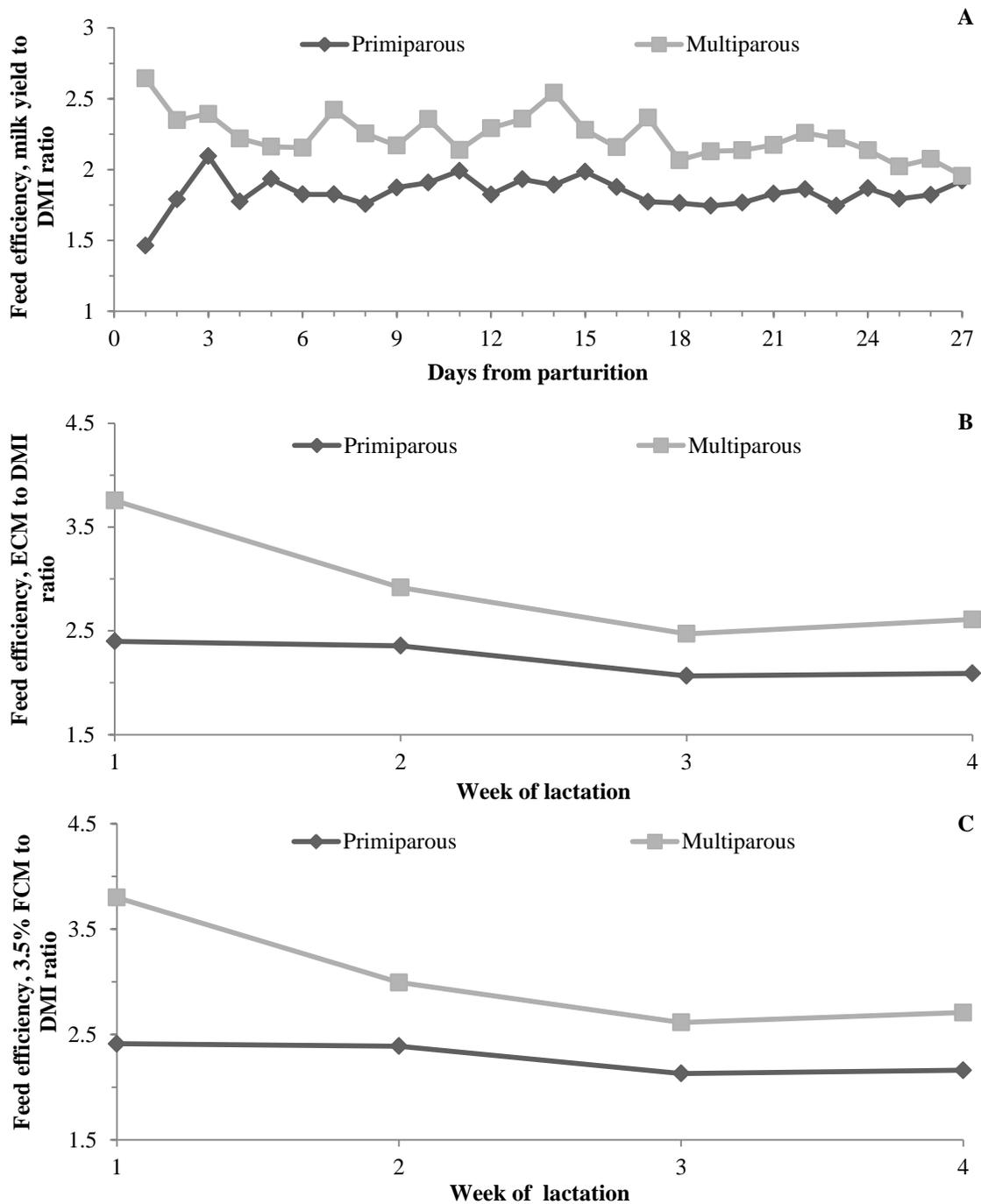
**Figure 2.3.** Effect of time on energy- and 3.5% fat-corrected milk production, and milk composition of early lactation cows. P-values for time effect are presented on Table 2.5. Values represent mean. A) For ECM, 3.5% FCM, B) MUN, SCS, C) fat content, protein content, SNF content, D) fat yield, protein yield, and SNF yield, pooled SEM = 1.55, 1.65, 0.01, 0.29, 0.20, 0.04, 0.07, 0.08, 0.03, and 0.10, respectively.



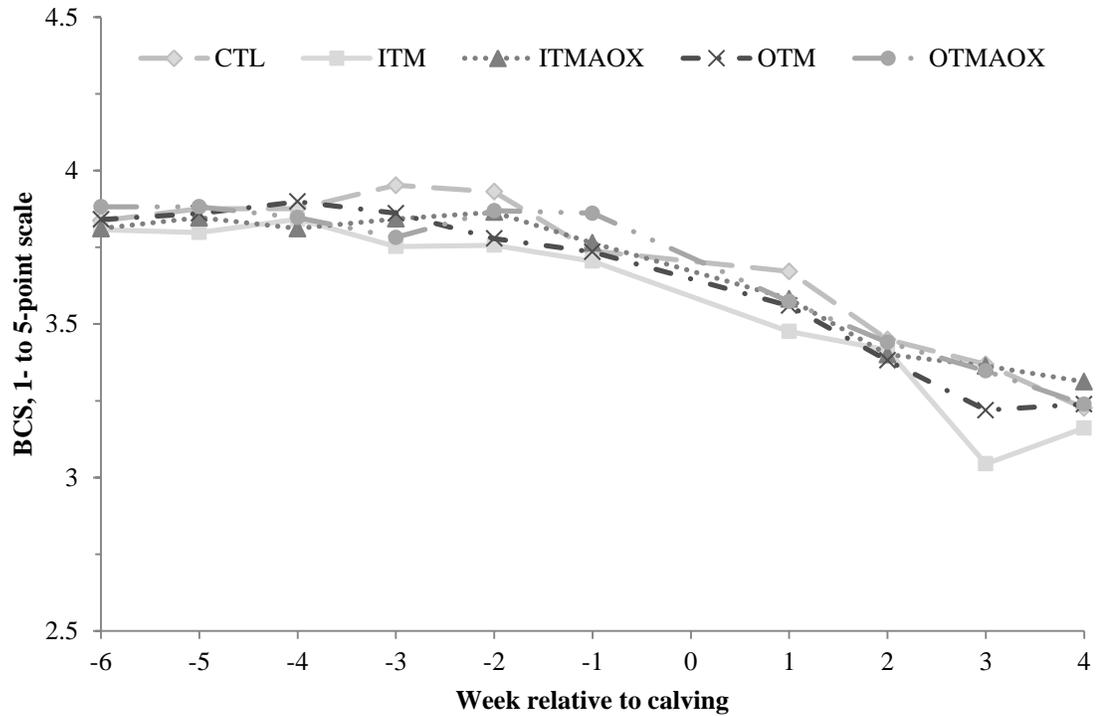
**Figure 2.4.** Effect of treatment within parity on milk fat percent of early lactation cows. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Different superscript letters represent difference of treatments within parity ( $P < 0.10$ ). Parity  $\times$  treatment,  $P = 0.05$ ; parity  $\times$  time  $\times$  treatment,  $P = 0.004$ . Values represent mean; primiparous cows pooled SEM = 0.38, and multiparous cows pooled SEM = 0.36.



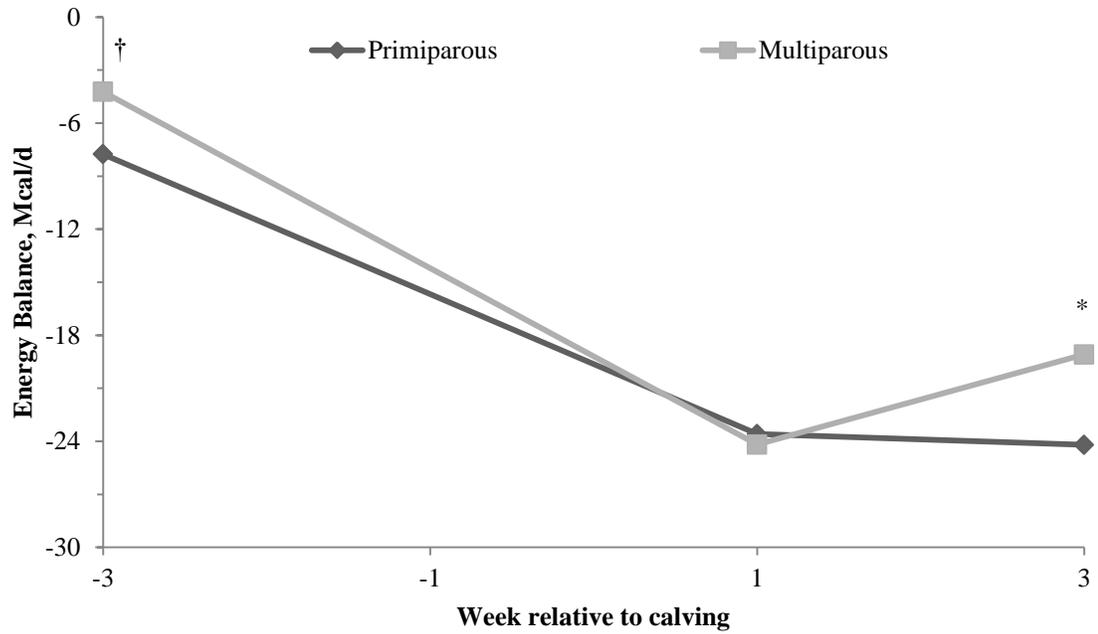
**Figure 2.5.** Effect of treatment within parity on solids non-fat percentage of early lactation cows. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Different superscript letters represent difference of treatments within parity ( $P \leq 0.03$ ). Parity  $\times$  treatment,  $P < 0.07$ . Values represent mean; primiparous cows pooled SEM = 0.13, and multiparous cows pooled SEM = 0.14.



**Figure 2.6.** Feed efficiency of early lactation cows. A) Milk yield to DMI ratio (parity,  $P < 0.001$ ; parity  $\times$  time,  $P = 0.004$ ); B) ECM to DMI ratio (parity,  $P < 0.001$ ; time,  $P < 0.001$ ; parity  $\times$  time,  $P < 0.03$ ); and C) 3.5% FCM to DMI ratio (parity,  $P < 0.001$ ; time,  $P = 0.001$ ; parity  $\times$  time,  $P < 0.05$ ). Values represent mean; for milk yield to DMI, ECM to DMI, and 3.5% to DMI ratios, pooled SEM = 0.13, 0.19, and 0.20, respectively.



**Figure 2.7.** Effect of treatment body condition score of periparturient dairy cows. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Time,  $P < 0.001$ ; treatment  $\times$  time,  $P = 0.09$ . Values represent mean, pooled SEM = 0.07.



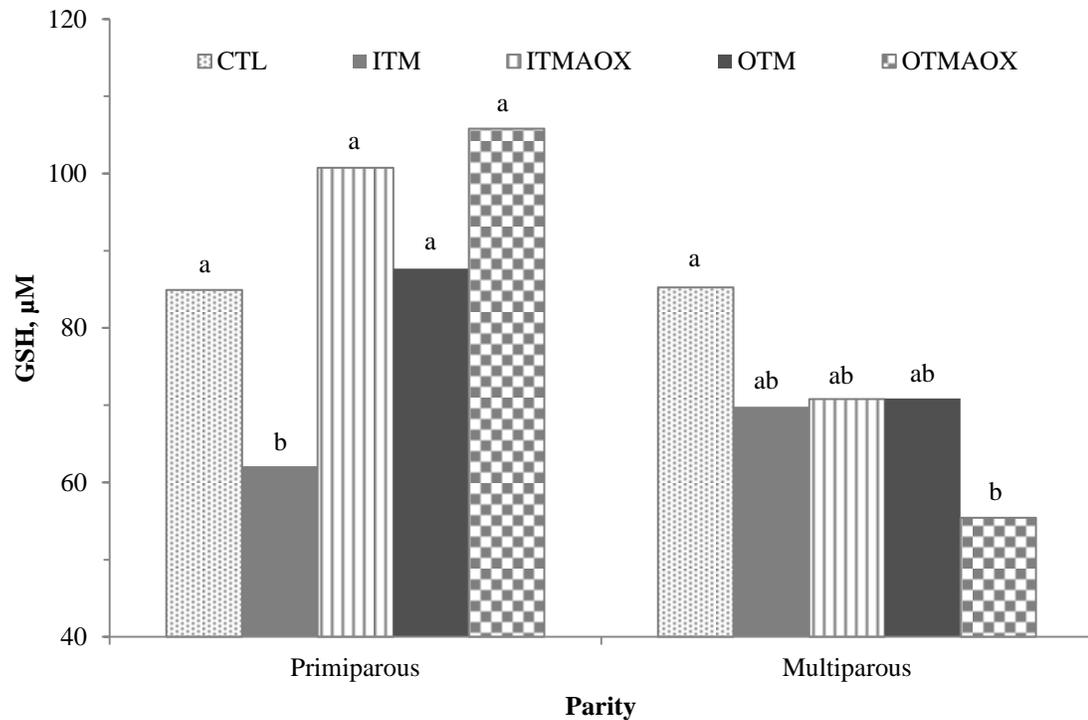
**Figure 2.8.** Calculated energy balance of periparturient dairy cows. Animals were fed from d -39  $\pm$  4.6 to 27 from parturition a basal diet to meet or exceed (NRC, 2001) requirements. † $P < 0.10$ , and \* $P < 0.01$  within time. Time,  $P < 0.001$ ; parity,  $P = 0.08$ ; and parity  $\times$  time,  $P = 0.06$ . Values represent mean, pooled SEM = 1.47.

**Table 2.6.** Health issues of early postpartum cows until 14 days after parturition

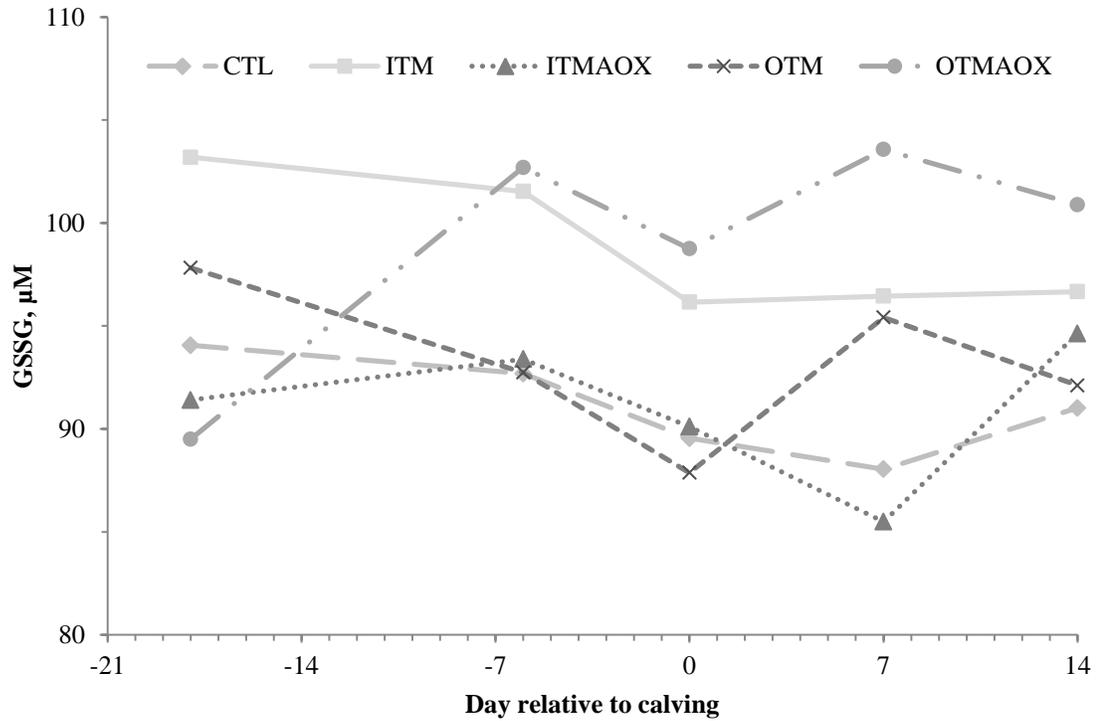
Variables <sup>1</sup>		Displaced abomasum	Clinical ketosis	Twins	Dystocia	Retained Placenta	Metritis
Treatment <sup>2</sup>	CTL	0	0	1	1	1	2
	ITM	2	3	1	2	1	2
	ITMAOX	1	3	0	1	3	3
	OTM	0	1	1	0	3	0
	OTMAOX	0	3	0	1	1	0
Parity	Primiparous	0	3	0	4	4	6
	Multiparous	3	7	3	1	5	1

<sup>1</sup>Number of cases occurred from d 0 to 14 from parturition.

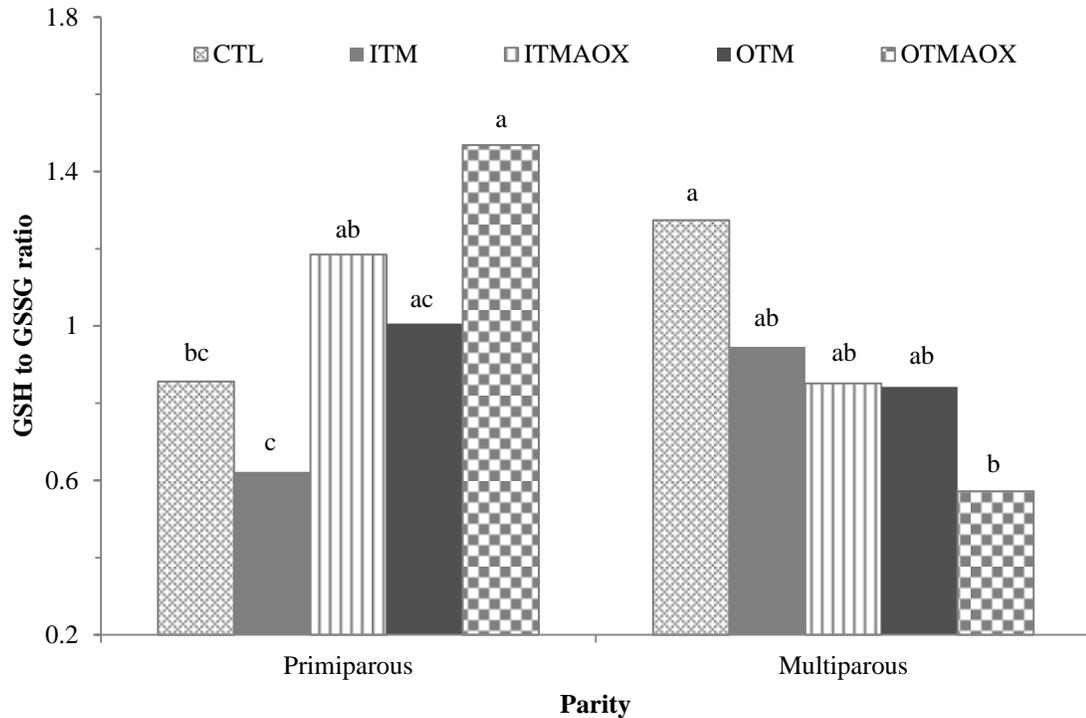
<sup>2</sup>Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants.



**Figure 2.9.** Effect of treatment within parity on erythrocyte reduced glutathione concentrations in periparturient cows. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Different superscript letters represent difference of treatments within parity ( $P \leq 0.10$ ). Parity,  $P < 0.01$ ; time,  $P < 0.07$ ; parity  $\times$  treatment,  $P = 0.03$ . Values represent mean; primiparous cows pooled SEM = 10.04, and multiparous cows pooled SEM = 10.13.



**Figure 2.10.** Effect of treatment on erythrocyte oxidized glutathione concentrations in periparturient cows. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Time  $\times$  treatment,  $P = 0.09$ . Values represent mean; pooled SEM = 7.09.



**Figure 2.11.** Effect of treatment within parity on erythrocyte reduced over oxidized glutathione ratio in periparturient cows. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Parity  $\times$  treatment,  $P = 0.03$ . Different superscript letters represent difference of treatments within parity ( $P \leq 0.10$ ). Values represent mean; primiparous cows pooled SEM = 0.22, and multiparous cows pooled SEM = 0.22.

**Table 2.7.** Effect of treatments on comet variables in periparturient dairy cows

Variables	Tail length, px	DNA in tail, <sup>1</sup> %	Tail moment	
Treatment <sup>2</sup>	CTL	3.10 <sup>a</sup>	5.94 <sup>a</sup>	22.89 <sup>a</sup>
	ITM	2.26 <sup>ab</sup>	5.00 <sup>ab</sup>	19.35 <sup>ab</sup>
	ITMAOX	1.57 <sup>b</sup>	3.85 <sup>b</sup>	11.15 <sup>b</sup>
	OTM	2.57 <sup>a</sup>	5.26 <sup>a</sup>	19.71 <sup>a</sup>
	OTMAOX	2.83 <sup>a</sup>	6.63 <sup>a</sup>	29.78 <sup>a</sup>
SEM <sup>3</sup>	0.40	0.86	6.79	
P-value	Parity	0.355	0.656	0.679
	Time	0.376	0.419	0.347
	Treatment	0.029	0.061	0.009
	Parity × time	0.530	0.500	0.460
	Parity × treatment	0.781	0.610	0.880
	Time × treatment	0.492	0.829	0.403
	Parity × time × treatment	0.170	0.224	0.149
	CAO <sup>4</sup>	0.551	0.676	0.358
	CTM <sup>5</sup>	0.014	0.017	0.004
CAS <sup>6</sup>	0.090	0.306	0.078	

<sup>a,b</sup>Means with different superscript letters differ ( $P < 0.05$ ) within column.

<sup>1</sup>Power > 0.999 for treatment effect.

<sup>2</sup>Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants.

<sup>3</sup>Pooled SEM.

<sup>4</sup>CAO = effect of treatments containing dietary antioxidants (ITMAOX and OTMAOX) versus treatments not containing dietary antioxidants (ITM and OTM), excluding CTL.

<sup>5</sup>CTM = effect of treatments containing organic trace minerals (OTM and OTMAOX) versus treatments not containing organic trace minerals (ITM and ITMAOX), excluding CTL.

<sup>6</sup>CAS = effect of treatments containing any type of supplementation (ITM, ITMAOX, OTM and OTMAOX) versus control (CTL).

**CHAPTER THREE**

**RESPONSE OF DAIRY COWS SUPPLEMENTED WITH DIETARY  
ANTIOXIDANTS AND CHELATED TRACE MINERALS TO  
INTRAMAMMARY BACTERIAL CHALLENGE**

**ABSTRACT**

The aim of this study was to evaluate the response of early lactation dairy cows supplemented with dietary antioxidants and organic trace minerals to intramammary bacterial challenge. Sixty pregnant Holstein cows were blocked based on date of expected parturition, parity (primiparous vs. multiparous), and initial body weight to a randomized complete block design study. Cows were individually fed a basal diet appropriate for physiological state formulated to meet or exceed National Research Council recommendations from d  $-39.3 \pm 4.6$  to 38 from parturition. In addition, cows were top-dressed treatments at a rate of 200 g/d for prepartum and 250 g/d for postpartum animals, as: 1) no supplemental antioxidant nor trace minerals (Cu, Mn, and Zn; **CTL**), 2) trace minerals provided as 100% sulfates without antioxidant (**ITM**), 3) trace minerals provided as 100% sulfates with antioxidant (**ITMAOX**), 4) trace minerals provided as 50% of sulfates and 50% of chelates without antioxidant (**OTM**), and 5) trace minerals provided as 50% of sulfates and 50% of chelates with antioxidant (**OTMAOX**).

Treatments were iso-mineral except CTL; all were iso-methionine. Intake and milk yield were recorded daily. Animals were vaccinated against *Escherichia coli* at dry-off,  $-27.3 \pm 4.6$ , and 14 d from calving. On d 28 postpartum, the right front quarter of each animal was infused with  $17,369.81 \pm 1,488.00$  CFU of *E. coli* P4:O32 diluted in 5 mL of sterile PBS and the contralateral quarter with the same volume of sterile PBS. Sterile milk samples were collected at 0 (prior to infusion), 6, 12, 18, 24, 36, 48, 96, and 192 h, and blood samples at 0, 24, 48, 96, 192 h from challenge. Quarters infused with bacteria had greater concentrations of *E. coli* and somatic cell score (SCS) compared to the control quarters. The supplementation with either dietary antioxidants or trace minerals or both increased milk yield and feed efficiency and decreased milk urea nitrogen. Use of dietary antioxidants increased SCS. Animals supplemented with chelated trace minerals had lower dry matter intake, milk yield and solids non-fat content, and higher SCS and milk fat content than animals fed the inorganic sources. Reduced over oxidized glutathione ratio increased and comet measurements decreased up to 48 h after challenge. Results suggest that supplementation greater than NRC (2001) recommendations may not be beneficial to primiparous, although production variables were improved in multiparous cows supplemented with inorganic trace minerals.

## INTRODUCTION

Mastitis costs approximately 2 billion dollars every year to the American dairy industry (Sordillo, 2011). These enormous economic losses are due to a decrease in milk production and quality, treatment of cases, extra labor, and replacement of some animals. Coliform pathogens are an important class of microorganisms that cause intramammary infections. These organisms populate the gastrointestinal tract of warm blooded animals and are excreted with the feces into the environment. Environmental exposure is the main cause of coliform IMI compared to the transfer of pathogens from infected to uninfected animal, and 85% coliform mastitis cases show clinical signs of infection (Hogan and Smith, 2003). Nemeth et al. (1994) found similar *Escherichia coli* isolates in feces and in mastitic milk. Lipopolysaccharide (**LPS**) is an endotoxin present in the bacterial wall of *E. coli* and other coliform pathogens (Burvenich et al., 2003; Hogan and Smith, 2003) that is able to induce a defense response by the host in low doses, while elevated doses may induce lethal shock (Lohuis et al., 1988a,b). At time of cell replication or death, the endotoxin is released from the bacteria (Burvenich et al., 2003), causing disruption of blood flow and then affecting secretory cells (Shuster et al., 1991). Clinical signs of the disease may include abnormal appearance of the milk, swelling of the udder, a drop in feed intake, decrease in milk production, dehydration, and diarrhea (Wilson and González, 2003). However in severe cases diarrhea, rumen stasis, paralysis, hypothermia, hypersalivation, very high pulse rate, and lethal shock may also occur (Burvenich et al.,

2003; Wenz et al., 2006). Severity of *E. coli* mastitis is host dependent (Burvenich et al., 2003; Rainard and Riollet, 2006) and it can be influenced by parity, stage of lactation, SCC, genetic resistance, nutritional status, pre-existing diseases, and other cow factors (Paape et al., 2002).

Inflammation processes such as mastitis can worsen oxidative stress in the cow by stimulating reactive oxygen species (**ROS**) production (Burvenich et al., 2003; Sordillo et al., 2008) or decreasing the antioxidant capacity (O'Boyle et al., 2006). An elevated oxidative load may lead to dysfunction of immunity and increased metabolic stress (Sordillo et al., 2009). Reactive oxygen species cause peroxidation of lipids (Miller et al., 1993) that, under oxidative stress, negatively affects immune cells which contain high concentrations of polyunsaturated fatty acids in their membranes (Spears and Weiss, 2008). Decreased PMN count, function (Cai et al., 1994; Diez-Fraile et al., 2003), and viability (Ditcham et al., 1996) during the periparturient period is one of the causes of increased susceptibility to intramammary infection (**IMI**; Vangroenweghe et al., 2004b, 2005), when most of the severe cases happen (Vandeputte-Van Messom et al., 1993). Likewise, the velocity in which PMN influx into the mammary gland can alleviate the severity of mastitis (Vandeputte-Van Messom et al., 1993). The *E. coli* infection usually shows rapid progression due to the fast multiplication of the microorganism (Kornalijnslijper et al., 2004) and bacteria count in milk is related to severity (Shuster et al., 1996; Vandeputte-Van Messom et al., 1993). However, elevated pre-infection SCC might be inhibitory to the initial growth of the pathogen (Shuster et al., 1996), thus controlling the severity of cases.

Dietary antioxidants, such as ethoxyquin and tertiary butyl hydroquinone, scavenge free radicals by binding to their unpaired electrons in the fatty acids or in the media and blocking oxidation (Bowman et al., 2008), thereby alleviating the effects of oxidative stress. Vázquez-Añón et al. (2008), when feeding dietary antioxidants with different sources of fat, reported the dietary antioxidants could increase glutathione peroxidase activity, but an increase in antioxidant status and superoxide dismutase activity was only seen when treatments were fed in combination with oxidized fat. Wang et al. (2010) observed a decrease in H<sub>2</sub>O<sub>2</sub> concentration and an increase in total antioxidant capacity for cows supplemented with dietary antioxidants. In addition, Wang et al. (1997) reported a higher ratio of reduced over oxidized forms of glutathione (**GSH:GSSG**) in the duodenal and ileal tissues of poultry supplemented with ethoxyquin.

Trace minerals also play a role in reducing the load of ROS. After intramammary *E. coli* challenge, heifers fed organic Cu showed lower *E. coli* count and milk yield tended to be higher from 2 days until 5 days after initial infection compared to heifers fed an inorganic source of Cu (Scaletti and Harmon, 2012). Supplementation of organic Zn was observed to reduce incidence of IMI (Spain et al., 1993) but no difference in somatic cells count (**SCC**) or milk production was reported. However, results of the influence of Zn on SCC are contradictory (Kellogg, 1990). In an experiment on a commercial dairy farm, dairy cows fed a mix of organic trace minerals from 21 days prepartum to 250 days postpartum displayed no difference in milk yield, energy-corrected milk (ECM), and 3.5% fat-corrected milk (FCM), but these animals tended to have lower SCC in milk

(DeFrain et al., 2009). Rabiee et al. (2010) found no difference in SCC for cows fed organic trace mineral in a meta-analysis that included twenty experiments.

The objective of this experiment was to evaluate the effects of feeding dietary antioxidants and organic trace minerals on the response of early lactation dairy cows to intramammary bacterial challenge (**IMC**). We hypothesized that animals supplemented with both dietary antioxidants and organic trace minerals would show an improved recovery against the intramammary challenge due to a possible synergistic effect of the treatments.

## **MATERIAL AND METHODS**

### ***Animals, Diets, Experimental Design, and Treatments***

The use and care of all animals were approved by the University of Missouri Animal Care and Use Committee. This experiment was conducted as a randomized complete block design with cow as experimental unit. Sixty pregnant Holstein cows housed at University of Missouri Foremost Dairy Center were blocked based on date of expected parturition, parity [primiparous (**PP**) and multiparous cows (**MP**;  $3.17 \pm 1.25$  parities)], and initial BW. All cows enrolled on this study had a current locomotion score lower than two (Sprecher et al., 1997). All multiparous cows had SCC lower than 150,000 cells/mL of milk for the last two months of the previous lactation, were dried-off

at least 15 days prior to the beginning of the trial, and blocks were balanced for milk yield based on the previous lactation milk production. Cows within block were randomly assigned to receive to one of five treatments: 1) no supplemental dietary antioxidants or trace minerals (**CTL**); 2) trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants (**ITM**); 3) trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants (**ITMAOX**); 4) trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants (**OTM**); and 5) trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants (**OTMAOX**). The dietary antioxidant supplemented was Agrado Plus (Novus International, St. Charles, MO), and chelated Cu, Mn, and Zn were supplied as metal methionine hydroxy analog chelates (Mintrex, Novus International). Treatments were iso-mineral and provided at least 100% of the (NRC, 2001) requirements for Cu, Mn, and Zn, except CTL; all were made iso-methionine by supplemental Ca-salt of 2-hydroxy-4-methylthio-butanoic acid (MHA feed supplement, Novus International). Treatments were top-dressed and mixed into the top portion of the diet immediately prior to morning feeding to prepartum and postpartum cows at a rate of 200 and 250 g/d, respectively, using ground corn as carrier (Table 3.1). Dry and lactating cows were fed a basal diet, formulated to meet or exceed predicted requirements for all nutrients (NRC, 2001). Basal diets were provided as TMR for ad libitum (intake to achieve about 10% refusals). Cows were fed once (0700 h) and twice (1400 and 0700 h) daily during the prepartum and postpartum periods respectively, from  $-39 \pm 4.6$  to 38 d relative to

parturition via individual feeding system (American Calan Inc, Northwood, NH). Cows were trained how to use the feeding system at least one week prior the beginning of the trial. Intake was recorded daily throughout the experiment. Feeds were sampled weekly and dry matter (**DM**) content was determined by forced-air oven drying at 55°C for 72 h. Diets were adjusted each week for ingredient DM content. Weekly feed samples were ground through a 0.2-mm sieve and combined to monthly composites. Table 3.2 and Table 3.3, respectively, show the ingredient and chemical composition of the diets. Water was available for ad libitum consumption throughout the experimental period. Lactating cows were milked twice daily (1800 and 0600 h) and production was recorded for each milking. Feed efficiency was evaluated by calculation of ratio of milk production over DMI.

All cows were intravenously administered 500 mL of 23% calcium gluconate at the day of parturition to minimize any problems associated with calcium metabolism or hypocalcaemia. Animals were vaccinated against *Escherichia coli* (Enviracor J-5, Pfizer Animal Health, Kalamazoo, MI) at dry-off,  $-27.3 \pm 4.6$ , and 14 d from parturition.

### ***Intramammary bacterial challenge***

Intramammary bacterial challenge was induced by the infusion of *E. coli* strain P4:O32, a strain isolated from a clinical case of mastitis (Bramley et al., 1976). Prior to the challenge, 10 mL of sterile brain heart infusion broth (Sigma-Aldrich, St Louis, MO) was inoculated with one colony-forming unit (**CFU**) of the bacteria and incubated for 12 h at 37°C. After the incubation period, stock solution was kept at 4°C while the concentration of bacteria in the solution was determined. One ml of the stock solution

was serially diluted in sterile PBS to the ratio of 1:1,000,000 after incubation, then 1 mL of the diluted solution was plated onto the surface of tryptic soy agar plate with 5% of sheep blood (Northeast Laboratory, Waterville, ME) and incubated 24 h at 37°C. The concentration of *E. coli* P4:O32 was then determined based on plate count, the stock solution was diluted with sterile PBS to yield a final concentration of 3,500 CFU/mL and kept at 4°C until challenge.

On d 28 from postpartum, immediately after the morning milking, the right front mammary quarter was cleaned with a cotton swab soaked in 70% ethanol, and 5 mL of the final solution containing *E. coli* were infused using a sterile 6-mL syringe fitted with a sterile teat cannula (Jorgensen Laboratories, Inc., Loveland, CO). Following infusion, the teat and then mammary gland were massaged from the bottom up to distribute the solution. The contralateral quarter was also aseptically cleaned and the same volume of sterile PBS was infused as well as teat and gland were massaged as describe previously. Following challenge, 1-ml sample of the infused solution was serially diluted up to a ratio of 1:1,000 then plated using the same techniques described above to determine the actual infusion dose of *E. coli*.

The dose chosen in this study ( $1.75 \times 10^4$  CFU of *E. coli*/infusion) was based on previous studies done in multiparous and primiparous cows (Vandeputte-Van Messom et al., 1993; Vangroenweghe et al., 2004b), where they used  $10^4$  CFU of the same strain of bacteria and were able to elicit a moderate immune response in the animals.

### ***Milk Sampling and analysis***

**Sampling.** Samples were collected at 0 (prior to infusion), 6, 12, 18, 24, 36, 48, 96, and 192 h after infusion, immediately before animals were milked, except for hours 6 and 18 that were off the milking time. For sampling, each quarter was cleaned with a cotton swab soaked in 70% ethanol, then manually stripped at least 3 times. Following stripping, samples were aseptically collected from each quarter for determination of *E. coli* CFU in milk, and milk composition (fat, protein, solids non-fat – **SNF**, milk urea nitrogen – **MUN**, and **SCC**). For milk composition, samples were collected and stored using a preservative (Broad Spectrum Microtab, Advanced Instruments Inc.), After sampling, samples were immediately inserted in ice then transferred and stored within 15 min from collection at either -20°C for further analysis of bacteria in milk or at 4°C until analyzed for composition within 48 h by a certified DHIA laboratory (Mid-South Dairy Records, Springfield, MO).

If *E. coli* was not found after plating the samples, 0.1 CFU was assigned as the value. Somatic cells score (**SCS**) was calculated using the equation reported by Jamrozik and Schaeffer (2012):  $SCS = [\log_2(SCC/100,000) + 3]$ .

**Analysis.** Concentration of *E. Coli* CFU in milk was determined by plating 10 µL of milk onto the surface of blood agar plates and incubating for 24 h at 37°C.

#### ***Blood Samples and Rectal Temperature***

Blood was collected immediately after morning milking from the coccygeal vessels into evacuated tubes with K<sub>3</sub>-EDTA (Monoject, Tyco Healthcare Group LP, Mansfield, MA), and Na-heparin (BD Vacutainer, Franklin Lakes, NJ) at 0 (immediately

before challenge), 24, 48, 96, and 192 h from IMC, processed and stored for further analysis of glutathiones, and comet assay.

**Glutathiones.** Red blood cell total and oxidized (GSSG) glutathione were determined by enzymatic colorimetric methods using a commercial kit (Cayman Chemical, Ann Arbor, MI) Samples were analyzed in triplicate and all methods were validated in our laboratory. Reduced glutathione was mathematically calculated by subtracting GSSG from total glutathione, and the ratio of glutathiones was calculated by dividing the reduced by the oxidized glutathione. Inter- and intra-assay coefficients of variation were 0.94 and 2.72% for total glutathione, and 1.70 and 1.69% for GSSG, respectively.

**Comet assay.** Comet assay is able to detect DNA damage (Ersson and Moller, 2011) using electrophoresis for migration of the damaged DNA. The extent of migration correlated with the amount of DNA damage to a cell (Oliveira and Johansson, 2012). The most useful measurements are tail length, percent DNA in tail, and tail moment (product of tail length and percent DNA in tail) because it bears a linear relationship to the break frequency (Collins, 2004). A commercial kit (CometAssay, Trevigen Inc., Gaithersburg, MD) was used to run this assay. Processes were performed under dimmed light. First, lysis solution A was prepared by mixing 100 mL of lysis solution (part of the kit) with 1 mL of DMSO. Lysis solution A was chilled at 4°C for at least 20 min prior to use. The samples were removed from the -80°C freezer and 1 mL of PBS was added to the frozen media and mixed together. The samples were centrifuged for at 200 x g for 10 min at 4°C. One milliliter was pipette out while leaving the pellet at the bottom of the tube.

Next, 200  $\mu\text{L}$  of PBS was added to the sample in the tube. Low-melting agarose (LMAgarose, Trevigen Inc.) was melted in the microwave for no more than 10 s with the cap loosened. Then, the bottle with melted agarose was placed in a 37°C water bath for 20 min to cool. Tubes of 0.6 mL were placed in a tube heater at 37°C and then 300  $\mu\text{L}$  of the molten agarose gel was added to the 0.6-mL tubes. Agarose gel remained at 37°C until applied to sample slide. After adding the agarose gel to the 0.6-mL tubes, the pellet was re-suspended from the sample tubes. Thirty  $\mu\text{L}$  of cells were added from the sample tube to the 0.6-mL tubes containing agarose gel, and mixed very well. Immediately following, 10  $\mu\text{L}$  was added on to the 96-well comet slide (Trevigen Inc.). The pipette tip was used to spread the agarose gel containing cells over the sample area to ensure complete coverage. After all samples are applied to the 96-well comet slide, the slide was placed in the dark at 4°C for 30 min. Following this step, the slide was immersed in pre-chilled lysis solution A and left at 4°C for 14 h. After incubation, alkaline unwinding solution was prepared by combining 0.8 g of NaOH pellets, 500  $\mu\text{L}$  of 200 mM EDTA (Trevigen Inc.), and 99.5 mL of nanopure water for every 100 mL made. It was ensured that the pH was greater than 13 and allowed chilling time prior to use. Next, the slide immersed in lysis solution A was removed and excess was drained off. The slide was then immersed in the freshly prepared and chilled alkaline unwinding solution and left the slide immersed for 20 min at room temperature in the dark. 500 mM EDTA stock solution was prepared by mixing 40 mL of 200 mM EDTA (pH = 10) with 60 mL of nanopure water for every 100 mL made. The pH was adjusted to 8 following the combination of listed ingredients. Next, 1 L of alkaline electrophoresis solution was

created by mixing 8 g of NaOH pellets with 2 mL of 500 mM EDTA. After the pellets were dissolved, nanopure water was added to solution until final volume of solution reached 1 L. The pH was checked to be greater than 13. Next, the solution was chilled to 4°C prior to use. Nine hundred mL of pre-chilled alkaline electrophoresis solution were added to the Comet Assay ES TANK (Trevigen Inc.). The slide was placed in the slide tray and covered with the slide tray overlay. The power supply was set to 21 volts and applied to slide for 40 min. After the electrophoresis had ended, the slide was removed from the tank and drained any excess solution from the slide. Then, the slide was immersed twice in nanopure water for 5 min each time and then immersed in 70% ethanol for 5 min as well. The slide was dried at 37°C for 15 min and then stored in the dark at room temperature with controlled humidity. When all slides were processed and ready to be stained, TE buffer was prepared by adding 5 mL of 2 M Tris (Fisher Scientific, Fair Lawn, NJ), 2 mL of EDTA 500 mM pH = 8, and nanopure water completed up to 1 L. The pH was adjusted to 7.5. Next, SYBR Gold solution was prepared by diluting 1 µL of SYBR Gold Nucleic Acid Gel Stain (Life Technologies, Eugene, OR) in 30 mL of TE buffer, and stored in the dark at 4°C. Twenty µL of SYBR Gold Solution was added to each well of the 96-well comet slide and then the slide was stained for 30 min at room temperature in the dark. Following this step, the slide was gently tapped to remove excess SYBR Gold Solution and then immersed briefly in nanopure water. The slide was allowed to dry completely for approximately 15 min in the dark at 37°C. Next, the samples were viewed by epifluorescence microscopy at 494 nm excitation, 521 nm emission, and 100 ms of exposure time at 20 x magnification, and a

picture was taken. Using CometScore software (TriTek Corp., Sumerduck, VA), no more than 10 cells per photo were selected and then the average of the measured values was assigned one for each sample.

Rectal temperature was measured at 0 (immediately before challenge), 6, 12, 18, 24, 36, 48, 96, and 192 h from challenge.

### ***Statistical Analysis***

A multiparous cow assigned to the CTL treatment was found unable to use the feeding system at the beginning of the experiment and by consequence displaced abomasum occurred. Her data was excluded from the statistical analysis. Averages of DMI, milk production, and feed efficiency from 3 days prior to infusion were used as the pre-challenge values for the respective variables. The data was analyzed using SAS 9.3 (SAS Institute Inc., Cary, NC). The influence option in the model statement for MIXED procedure was used to detect extreme observations. Extreme observations were removed from the data set if they presented at least two of the following characteristics: standardized residual ( $r_i$ ) > 2.5 standard deviations, high leverage ( $h_{ii} \geq 2pn^{-1}$ , where  $n$  = number of observations and  $P$  = number of parameters), and cook's distance ( $D_i$ ) > 1 (Kaps and Lamberson, 2009). Results indicated that none of the observations were outliers. All the variables were evaluated for normality using the UNIVARIATE procedure and normalized if necessary. For normalization, infused bacteria, bacteria count, feed efficiency, milk fat and MUN, reduced over oxidized glutathione ratio, and comet tail length and moment data were  $\log_{10}$ -transformed before analysis. For DMI and comet percentage of DNA in tail, milk protein, and milk SNF, square-root, -2

exponential, and 2 exponential transformations were used, respectively. *P*-values are shown based on normalized data, except for bacteria count and ratio of reduced over oxidized glutathiones, however, means and SEM are presented as non-normalized values.

Data were analyzed as a randomized complete block design using the MIXED procedure of SAS with the repeated statement for variables measured over time. For DMI, milk production, glutathiones, comet assay, and feed efficiency, the statistical model included fixed effects as treatment, parity, and time as well as the resultant interactions. Block and block within treatment and lactation were the specified terms for the random statement. Time was the specific term for the repeated statement with block within treatment and parity as subject, whereas either autoregressive order one or compound symmetry were the covariance structures selected based on the lowest Akaike's information criterion (Littell et al., 1998). For milk *E. coli* CFU and components, the procedure used was the same as describe above; however, it also included the effect of quarter in the model and all the resultant interactions. The random statement included block within treatments, parity and quarter as a term, and was also used as subject for the repeated statement. The infused bacteria data did not use the repeated statement in the MIXED procedure similar to the other models. Additionally the time and quarter effect and associated interactions were excluded from the model, and block was the term for the random statement. Three orthogonal contrasts were used to interpret effect of treatments: 1) effect of treatments containing dietary antioxidants (ITMAOX and OTMAOX) versus treatments not containing dietary antioxidants (ITM and OTM), excluding CTL (CAO); 2) effect of treatments containing organic trace

minerals (OTM and OTMAOX) versus treatments not containing organic trace minerals (ITM and ITMAOX), excluding CTL (CTM); and 3) effect of treatments containing any type of supplementation (ITM, ITMAOX, OTM and OTMAOX) versus control (CTL; CAS). The PDIFF option was used to separate least square means. Significance was declared at  $P \leq 0.05$ , and tendency was considered when  $0.05 < P \leq 0.10$ . Data are reported as least squares means  $\pm$  SEM.

## RESULTS

### *Bacteria*

The number of *E. coli* CFU infused ( $17,369.81 \pm 1,488.00$  CFU of *E. coli*/infusion) did not differ between parities ( $P = 0.20$ ), treatments ( $P = 0.35$ ), or parity  $\times$  treatment interaction ( $P = 0.48$ ). Quarters infused with bacteria ( $0.67 \pm 0.16$   $\log_{10}$ CFU/mL) had greater ( $P < 0.001$ ) concentrations of *E. coli* compared to the control quarters ( $-0.67 \pm 0.16$   $\log_{10}$ CFU/mL). In addition, concentrations of *E. coli* in milk of control quarters of PP and MP did not differ, but the infected quarters did differ ( $P < 0.001$ ) between each other ( $0.20 \pm 0.20$  vs.  $1.13 \pm 0.20$   $\log_{10}$ CFU/mL, respectively). Concentrations of *E. coli* before infusion were not different between quarters and parities (Figure 3.1). In the infected quarters, *E. coli* concentrations in milk peaked at 12 h ( $2.61 \pm 0.31$   $\log_{10}$ CFU/mL) in MP while the peak was at 24 h ( $1.35 \pm 0.30$   $\log_{10}$ CFU/mL) in

PP; concentrations at peak differed ( $P < 0.001$ ) among parities. Curiously, in PP, *E. coli* concentrations decreased ( $P < 0.001$ ) between 12 and 24 h. Bacteria count in the infused quarters of PP reached pre-infusion levels at 96 h. For MP, concentrations of *E. coli* in milk started declining after 18 h, and at 192 h were not significantly different from 0 h. Interestingly, increased concentrations of the bacteria in the control quarter were found at 24 h in MP compared to the pre-infusion measurement ( $P < 0.01$ ).

### ***Dry Matter Intake***

From d -1 to 0 (day of infusion) a drop ( $P < 0.001$ ) of 42% in DMI was observed for PP and 61% for MP. For the first 3 days after infusion, DMI was higher ( $P \leq 0.028$ ) in PP than in MP, and after d 4 intake was not different between parities.

Dry matter intake in PP was not different between treatments, no difference in DMI was observed between treatments within a single time-point for PP, however, CTL had slightly higher DMI than the other treatments from d 7 postpartum, through the end of the experiment. Also, DMI was restituted to pre-infusion levels on d 2 from challenge. In contrast, DMI for MP on the ITM treatment declined less ( $12.01 \pm 2.18$  kg/d) compared to ITM, OTM, and OTMXAOX ( $P < 0.05$ ), but not different from ITMAOX. Multiparous cows fed ITMAOX restored their pre-IMC levels of DMI on d 3, ITM on d 4, and OTM on d 7; the CTL and OTMAOX did not reach ( $P \leq 0.06$ ) the pre-IMC levels after challenge for the period analyzed. Figure 3.2Figure 3.3 show the effect of treatments on DMI in PP and MP animals. Animals fed chelated trace minerals had lower ( $P < 0.01$ ) DMI compared to animals fed the inorganic forms of the minerals.

### ***Milk***

Milk yield was affected ( $P < 0.001$ ) only by time and for the parity  $\times$  time interaction. The actual nadirs of milk yield were not different among parities and occurred on d 1 from infusion. However, comparing to d -1, milk yield dropped 41% in PP and by 67% in MP following the IMC. In addition, PP restored milk yield to pre-IMC levels on d 7 from infusion, but MP never did ( $P < 0.001$ ). Multiparous cows had greater milk yield on d -1 ( $P < 0.001$ ) compared to PP ( $42.98 \pm 1.91$  vs.  $30.36 \pm 1.88$  kg/d, respectively), but on d 2 and 3 PP was higher ( $P < 0.04$ ). From d 4 on, milk yield did not differ anymore among parities (Figure 3.4). The supplementation with either dietary antioxidants or trace minerals or both tended ( $P = 0.09$ ) to increase milk yield, also, animals supplemented with chelated trace minerals had lower milk yield ( $P < 0.04$ ) compared to animals fed the inorganic sources.

Fat percent in milk of MP ( $3.57 \pm 0.16\%$ ) tended to be greater (treatment,  $P = 0.06$ ) than in milk of PP ( $3.14 \pm 0.15\%$ ). At 6 h from IMC, fat concentration peaked in milk ( $4.97 \pm 0.22\%$ ) and the nadir occurred at 12 h ( $1.34 \pm 0.22\%$ ). After 18 h from IMC ( $3.99 \pm 0.28\%$ ), fat percent was restored preinfusion levels (Figure 3.5A). A tendency on parity  $\times$  time  $\times$  treatment interaction was seen ( $P = 0.07$ ), but fat content in milk after *E. coli* intramammary challenge was highly variable (pooled SEM = 0.80). Animals supplemented with chelated trace minerals had higher percentage of fat ( $P < 0.06$ ) in milk compared to animals fed the inorganic sources.

Protein percent in milk tended to be higher (parity,  $P = 0.09$ ) for MP ( $3.27 \pm 0.06\%$ ) than PP ( $3.10 \pm 0.06\%$ ) cows. Infected quarters did not change protein concentration in milk for the first 12 h from IMC, but then protein increased ( $P < 0.001$ )

compared to 0 h, peaking at 24 h ( $3.87 \pm 0.11\%$ ). After peak concentration, milk protein decreased through 48 h ( $3.52 \pm 0.13\%$ ; compared to 0 h and 24 h,  $P < 0.01$ ) maintaining these low levels until the end of the experiment. Interestingly, protein content of the control quarter had similar behavior to the infected quarter. Protein concentration reached its nadir at 12 h ( $2.70 \pm 0.09\%$ ;  $P = 0.01$  compared to 0 h) and plateau at 36 h ( $3.18 \pm 0.09\%$ ) from IMC (Figure 3.5B).

The *Escherichia coli* infused quarter had lower ( $P < 0.001$ ) SNF percent than the PBS infused quarter ( $7.29 \pm 0.10$  vs  $8.00 \pm 0.10\%$ , respectively). In addition, percentage of SNF in MP ( $7.43 \pm 0.11\%$ ) was lower ( $P = 0.001$ ) than in PP ( $7.87 \pm 0.11\%$ ). Solids non-fat declined ( $P < 0.001$ ) after infusion for both of the parities. The first nadir was at 18 h ( $6.74 \pm 0.19\%$  for PP and  $6.50 \pm 0.19\%$  for MP) post-infusion, then concentrations of SNF rose at 24 h ( $7.89 \pm 0.16\%$  for PP and  $7.22 \pm 0.19\%$  for MP), and a second nadir was seen at 36 h ( $7.24 \pm 0.18\%$  for PP and  $6.23 \pm 0.20\%$  for MP). After 36 h, concentrations of SNF rose in PP, reaching pre-IMC levels at 96 h ( $8.57 \pm 0.16\%$ ) and tended ( $P = 0.07$ ) to surpass the pre-IMC concentration at 192 h ( $8.77 \pm 0.16\%$ ). A similar type of curve occurred for MP, although pre-IMC levels were reached only at 192 h ( $8.35 \pm 0.19\%$ ) post-challenge. The nadir of the infected quarter was much lower ( $P < 0.001$ ) than the control quarter. Comparing to 0 h, both quarters had similar SNF percent only at 192 h post-IMC (Figure 3.5C). Within time, treatments were not different at 0, 6, and 12 h from challenge. At 18 h, OTM was the lowest and statistically differed from ITMAOX and OTMAOX; at 24 and 36 h, ITM was the greatest and statistically differed ( $P \leq 0.07$ ) from CTL and OTM, and also tended to differ ( $P < 0.10$ ) from OTMAOX at

36 h; at 48 h, OTM tended to be the lowest ( $P \leq 0.06$ ) and ITM the greatest ( $P \leq 0.04$ ). At 96 h this difference decreased, and ITM kept the greatest ( $P < 0.06$ ). Finally, at 192 h, OTMAOX was the highest but only tended to differ ( $P < 0.07$ ) from ITMAOX (Figure 3.6). Animals supplemented with chelated trace minerals had lower SNF content ( $P < 0.10$ ) in milk compared to animals fed the inorganic sources.

Parity affected ( $P = 0.03$ ) concentration of MUN in milk ( $11.15 \pm 0.63$  vs.  $12.68 \pm 0.64\%$  in PP and MP, respectively) during the IMC period. Milk urea nitrogen peaked at 24 h after IMC ( $13.85 \pm 0.72$  in PP vs.  $16.84 \pm 0.76\%$  in MP;  $P < 0.01$ ). In PP, MUN at 48 h was not different from pre-IMC levels, but at 96 and 192 h it was lower than 0 h ( $P < 0.04$ ). In MP, MUN was higher until 48 h then lower at 96 and 192 h compared to 0 h ( $P < 0.01$ ). Peak in both quarters was at 24 h, and the control quarter ( $16.09 \pm 0.67\%$ ) peaked higher ( $P = 0.040$ ) than the *E. coli*-challenged quarter ( $14.59 \pm 0.72\%$ ). Hour 0 had similar MUN as hour 48, but levels declined ( $P \leq 0.001$ ) after that (Figure 3.5D). Figure 3.7 illustrates MUN percent in milk within parity. The supplementation with either dietary antioxidants or trace minerals or both decreased ( $P < 0.03$ ) milk concentrations of MUN.

Somatic cell score was highly different ( $P < 0.001$ ) from 0 h for all the collection points regardless of parity. After 6 h for PP and 12 h for MP, SCS never was lower than 5.42. Peak of SCS was at 18 h for PP ( $7.76 \pm 0.36$ ) and at 36 h for MP ( $7.40 \pm 0.39$ ). There was no difference of parity for magnitude of SCS at the peak. After peak, SCS declined over time for both of the parities but never reached pre-IMC SCS ( $2.77 \pm 0.27$  for PP vs.  $2.53 \pm 0.28$  for MP) levels (Figure 3.8). Infected quarter SCS ( $7.05 \pm 0.14$ ) was

greater ( $P < 0.001$ ) than the control quarter ( $4.67 \pm 0.12$ ). Effects of time and quarter ( $P < 0.001$ ) on SCS are depicted in Figure 3.5E. Treatment effects within parity on SCS in milk and are shown in Figure 3.9. Use of dietary antioxidants increased ( $P < 0.01$ ) SCS compared to treatments that did not use (CAO). Animals supplemented with chelated trace minerals had higher SCS ( $P = 0.03$ ) compared to animals fed the inorganic sources.

### ***Feed Efficiency***

Feed efficiency increased ( $P < 0.001$ ) on d 0 ( $2.90 \pm 0.19$  of feed efficiency) compared to d -1 ( $1.90 \pm 0.17$ ). Days 1 and 2 were not different from d -1, but from d 3 on feed efficiency significantly declined ( $P \leq 0.03$ ) by approximately 20% compared to day before IMC, reaching nadir on d 5 ( $1.48 \pm 0.18$ ) from infusion. No difference on feed efficiency was observed between d 3 and 10. Supplementation of any form improved ( $P < 0.01$ ) feed efficiency compared to the control group in cows submitted to IMC.

### ***Metabolic Variables***

Rectal temperature was only affected by time ( $P = 0.002$ ). Comparing to 0 h ( $39.16 \pm 0.13^\circ\text{C}$ ), temperature dropped ( $P = 0.04$ ) at 6 h ( $38.78 \pm 0.13^\circ\text{C}$ ), and remained lower ( $P \leq 0.06$ ) until 18 h ( $38.78 \pm 0.15^\circ\text{C}$ ). At 24 h ( $39.08 \pm 0.12^\circ\text{C}$ ) rectal temperature rose back to pre-infusion temperature, but by 48 h ( $38.75 \pm 0.13^\circ\text{C}$ ) declined to reach the nadir ( $P < 0.03$ ). After the nadir, temperature increased and was not different from preinfusion levels through the end of the experiment

Total glutathione was higher ( $P < 0.001$ ) in PP ( $181.53 \pm 2.72 \mu\text{M}$ ) than MP ( $161.25 \pm 2.78 \mu\text{M}$ ). Animals supplemented with chelated trace minerals tended to increase ( $P = 0.07$ ) total glutathione compared to animals being supplemented inorganic

forms of trace minerals. Reduced glutathione was affected by parity ( $P = 0.03$ ); PP had  $86.33 \pm 4.32 \mu\text{M}$  against  $74.42 \pm 4.40 \mu\text{M}$  of GSH in MP. Additionally, in PP, ITM had the lowest concentrations of GSH; in MP, CTL had the highest, and OTMAOX the lowest concentrations of GSH in red blood cells (treatment,  $P = 0.03$ , and parity  $\times$  treatment effect,  $P = 0.01$ ; Figure 3.10). Concentrations of GSH decreased from 0 h ( $94.77 \pm 2.89 \mu\text{M}$ ) until the nadir at 48 h ( $88.01 \pm 2.91 \mu\text{M}$ ) post-IMC ( $P = 0.001$ ). Then, at 96 h ( $93.22 \pm 2.91 \mu\text{M}$ ), it returned to similar concentrations as pre-challenge, although it tended to decline ( $P < 0.06$ ) until 192 h ( $90.69 \pm 2.90 \mu\text{M}$ ). A tendency was observed for a parity effect ( $P = 0.09$ ) on GSSG concentrations ( $95.15 \pm 3.41$  in PP vs.  $87.33 \pm 3.47 \mu\text{M}$  in MP). Time tended to affect ( $P < 0.08$ ) GSH to GSSG ratio; it increased ( $P \leq 0.02$ ) at 24 and peaked at 48 h compared to 0 h. At 96 h, it was not different from 0 h anymore, although it increased ( $P < 0.03$ ) again at 192 h, having comparable levels to 24, 48, and 96 h. Also, there was a significant by parity  $\times$  treatment interaction ( $P < 0.04$ ; Figure 3.11).

Primiparous cows tended to have ( $P = 0.06$ ) a greater percentage of DNA in the tail of the comet, and a significantly greater ( $P < 0.02$ ) tail length and moment compared to MP ( $2.49 \pm 0.22$  vs.  $1.88 \pm 0.23$  sqrt%,  $0.35 \pm 0.09$  vs.  $0.02 \pm 0.09$  log<sub>10</sub>px, and  $0.83 \pm 0.14$  vs.  $0.27 \pm 0.15$  log<sub>10</sub>, respectively). In all the comet measurements, a time effect was observed ( $P \leq 0.04$ ; Figure 3.12). Compared to the moment immediately before infusion, a decline ( $P < 0.05$ ) was observed for all variables at 24 h and extended until 48 h. An increase then occurred at 96 h such that pre-IMC levels were achieved.

Animals supplemented with dietary antioxidants had a greater (contrast CAO;  $P = 0.04$ ) tail moment compared to animals not supplemented with antioxidants.

## DISCUSSION

The immune response triggered after IMC was responsible for initiating several symptoms such as decline in DMI and milk production, change in body temperature, and a decrease in milk quality. Some authors (Burvenich et al., 2003; Mehrzad et al., 2002) have studied the influence of parity on animal immune response. They observed that younger cows have an increased cellular function of immune cells that allows them to mount a more effective immune response. This enhance immune function gives them the ability to clear the infection sooner than older cows even with high infusion doses of the pathogen; because of this, more severe cases of mastitis were observed in multiparous than primiparous animals. The immune response activated against pathogenic or inflammatory insult quickly disrupts homeostasis on the day of the insult, and DMI and milk yield declined following challenge (Shuster et al., 1996; Waldron et al., 2003, 2006). Inflammatory processes produce cytokines responsible for anorexia (Le Floc'h et al., 2004). Independent of treatment, after an initial decrease, DMI in PP from d 2 on was observed to not differ from pre-infusion levels. However, in MP, this DMI recovery response was delayed, and in some cases (CTL and OTMAOX) never reached pre-

infusion levels. Interestingly, an antagonistic effect on DMI was observed when organic minerals and dietary antioxidants were combined, yielding similar DMI results to the control group. Moreover, we observed animals supplemented with inorganic sources of trace minerals presented higher DMI than animals supplemented with the organic sources. Other authors (Scaletti and Harmon, 2012; Scaletti et al., 2003) did not observe any treatment differences in DMI of heifers submitted to intramammary *E. coli* challenge. We observed similar results in primiparous cows. As discussed in greater detail below, somatic cells may have been the cause of decreased DMI in chelated trace minerals-fed animals.

The nadir of milk yield was at the day after infection which was probably when cellular function in the mammary gland was most affected, as discussed below. Any type of treatment supplementation was beneficial in increasing milk production during *E. coli* mastitis. Additionally, perhaps related to DMI, animals fed inorganic trace minerals showed higher milk yield than animals fed the chelated forms. This finding is inconsistent with reports from other researchers (Scaletti and Harmon, 2012; Scaletti et al., 2003); however, these researchers only tested the effect of different sources of Cu in heifers.

Attenuated milk synthesis caused 20% reduction in feed efficiency in this experiment. Decrease in milk synthesis allows nutrients to be directed towards clearance of the pathogen, restitution of cell function, and the tentative of reestablishing homeostasis. Feed efficiency was improved in animals using any type of supplements because the inclusion of any supplements increased milk yield but did not affect DMI.

Studies to better evaluate how mastitis could affect feed efficiency in a long period of time and possible association with recurrent cases would be useful, as to the current study only examined a short period of time, up to 10 days from the onset of the disease.

Our findings on *E. coli* count are consistent with the literature (Bannerman et al., 2008; Vangroenweghe et al., 2004b). The infected quarter showed rapid proliferation of the pathogen while the control quarter was not affected (Bannerman et al., 2004), with exception of hour 24 in multiparous cows. The numerical increase in *E. coli* concentrations in some of the control quarters after challenge may have been caused by an accidental contamination and minor infection of the control quarter at the time of challenge even after the cleaning process. Several authors associated the velocity of PMN influx into the mammary gland to the clearance of the pathogen. In primiparous cows, faster influx of PMN led to faster elimination of the insult (Vangroenweghe et al., 2004b), but in multiparous cows this influx was delayed as well the clearance of *E. coli* (Vandeputte-Van Messom et al., 1993). We used a similar infusion dose of *E. coli* P4:O32 as these authors and our data are in agreement with their findings. Curiously, we did not detect differences of parity on SCS on animals submitted to intramammary bacterial challenge, which may agree with the fact of MP have decreased PMN function compared with PP as discussed above. Interestingly, in this case, the increase in SCS that occurred in animals fed organic trace minerals could be the cause of decreased DMI and milk yield. Mastitis causes apoptosis of the mammary epithelial cells (Burvenich et al., 2007), this situation may have caused accentuated damage of the secretory tissue, consequently declined milk production and DMI. In this experiment, we observed a

considerable loss of quarters after challenge, which led to a sustained loss of milk yield, however loss of quarters were not recorded. Possible an accidental contamination caused the elevation in SCS within the control quarter, however in lesser extent compared to infected quarters.

A decrease in milk SNF content was observed immediately after IMC at a time coincident with the decrease in milk yield post-challenge. This decrease in milk SNF is in accordance with milk production results for PP and MP, the decline in production of the *E. coli* infected quarters as indicated by (Vangroenweghe et al., 2004a; b), and declined milk yield on animals fed organic trace minerals. Burvenich and co-workers (2007), reviewed the events that influence the inflammatory response against *E. coli* infections. When phagocytes reach the mammary gland, they ingest milk fat and casein, decreasing phagocytic function and inducing apoptosis of these cells. During the far-off dry period, the absence of milk fat or proteins helps with enhancing PMN efficiency for clearance of pathogens. The appearance of these products in early lactation diminishes the phagocytic activity of immune cells. Milk fat may also participate on lipid peroxidation processes following ROS release in the challenged mammary gland. Increased lipid peroxidation and free radical production could further interfere on the functionality of immune cells (Spears and Weiss, 2008). The low fat content observed at 0 h could be due to the sampling protocol, where milk was collected from a full udder after few manual strips of milk from the teat, and so not homogenized as a whole. Also, elevation of protein content in milk might have diminished the ability of the phagocytes to combat the infection (Burvenich et al., 2007). Increase in protein content occurred 6 h after the increase in

SCS as observed during intramammary LPS challenge by (Hinz et al., 2012) who also associated protein content during an IMC to the migration of PMN to the mammary gland and release of proteolytic enzymes into the gland. Boehmer et al. (2008) reported serum proteins in milk, probably due to the disruption of the blood-milk barrier by inflammatory processes during infection such as cytokine production and altered vascular permeability of the mammary epithelium. Milk nitrogen urea, product of the metabolism of amino acids, is higher in MP than PP, suggesting damage of this membrane, although this membrane is highly permeable to urea. However, any supplementation used in this experiment was able to keep the MUN concentrations lower than the control group.

Contradicting several trials (Bannerman et al., 2008, 2004; Vangroenweghe et al., 2004b), temperature dropped after infusion, and in combination with other factors above, suggesting a possibility that a septic shock could occur, then raised back to 0 h levels but curiously dropped again. Although all these changes were observed, temperature only varied between  $38.75 \pm 0.13$  and  $39.38 \pm 0.13^{\circ}\text{C}$  what may not express important biological value during an IMC. The other experiments cited above showed variation of rectal temperature higher than  $1^{\circ}\text{C}$ .

Glutathiones are very important in scavenging ROS from the body; they metabolize  $\text{H}_2\text{O}_2$  to less harmful compounds such as  $\text{H}_2\text{O}$  by oxidizing GSH to GSSG in a reversible reaction (Nelson et al., 2008). Reduced glutathione is predominant during normal cellular conditions and GSSG under stress (O'Boyle et al., 2006). As a result, during periods of increased oxidative stress, the GSH over GSSG ratio decreases (Lykkesfeldt and Svendsen, 2007). When GSH was unable to saturate glutathione

peroxidase, elimination of  $H_2O_2$  was linearly related with GSH concentrations (Michiels et al., 1994). Thus elevation in GSH concentrations may strengthen the antioxidant defense of the cells (Lykkesfeldt and Svendsen, 2007). In situations of stress such as IMC, concentrations of total glutathione seems to decline as observed by (Settivari et al., 2006), when endophyte-infected fescue was fed to rats and downregulated the hepatic production of total glutathione. Jhambh et al. (2013) observed a decline in SOD and GSH concentrations and an increase in lipid peroxidation in cows with clinical mastitis. Multiparous cows showed declined concentrations of total glutathione, GSH, and GSSG compared to PP. Independent of the parity, GSH:GSSG ratio was not different, thus GSH is not being spared. Interestingly, GSH and GSSG concentrations decreased up to 48 h, but the GSH:GSSG ratio increased up to the same time. Reeds and Jahoor (2001) concluded that in well-nourished individuals, an increase in GSH reactions can increase GSH synthesis, but it may be accompanied by a fall of its concentrations. This suggestion might indicate that the synthesis of GSH could be stimulated in order to protect the gland against the detrimental effects of ROS. In PP under ITM and MP under OTMAOX, the decline in GSH concentration was responsible for a lower GSH:GSSG ratio.

Glutathione protects cells against harmful endogenous and exogenous agents, including protection against effects on DNA and protein synthesis, and amino acid transport (Lafleur et al., 1994). It is very interesting to analyze the behavior of GSH and Comet variables together. The reaction of oxidizing GSH was increased and its concentration decreased until 48 h, and, at the same time, DNA damage was lowered as indicated by decreased comet measurements. In periods of apparent accentuated stress,

such as the time period when SCS peaked, and when milk production and intake were decreased, the organism seems to prioritize the maintenance and integrity of the DNA.

To the best of our knowledge, this is the first experiment that evaluated the effect supplementation of blended trace minerals and synthetic dietary antioxidants on the response of cows from different parities submitted to intramammary bacterial challenge. The study of more mechanistic effects on use of organic trace minerals, either blended with other minerals or blended with different forms of the compounds, and dietary antioxidants would be very helpful to fully understand the physiology of animals under severe stress, here demonstrated as mastitis.

## **CONCLUSION**

Under intramammary bacterial challenge, results of this study suggest that supplementation over (NRC, 2001) requirements may not be beneficial to primiparous cows. However, production variables were improved in multiparous cows supplemented with inorganic trace minerals. Dry matter intake and milk production were affected by SCS, and it is suggestive that PMN function was more affected in multiparous than in primiparous cows. Milk quality was altered in infected and control quarters. Feed efficiency declined after infusion. In cases of accentuated stress, the organism seems to

prioritize the maintenance and integrity of the DNA. Our findings may suggest that multiparous had gone through more severe clinical signs of the infection than primiparous cows. The drop in dry matter intake and milk yield as well as alteration of milk quality could be used as an indicator of coliform mastitis.

**Table 3.1.** Composition of the treatments

Item (%)	Treatments <sup>1,2</sup>				
	CTL	ITM	ITMAOX	OTM	OTMAOX
Cu, sulfate <sup>3</sup>	-	0.30	0.30	0.15	0.15
Mn, sulfate <sup>4</sup>	-	0.86	0.86	0.43	0.43
Zn, sulfate <sup>5</sup>	-	0.84	0.84	0.42	0.42
Cu, chelate <sup>6</sup>	-	-	-	0.21	0.21
Mn, chelate <sup>7</sup>	-	-	-	0.60	0.60
Zn, chelate <sup>8</sup>	-	-	-	0.83	0.83
Antioxidant <sup>9</sup>	-	-	2.01	-	2.01
HMTBa <sup>10</sup>	1.55	1.55	1.55	-	-
Corn, ground	98.45	96.45	94.44	97.36	95.35

<sup>1</sup>Prepartum and postpartum animals were supplemented 200 and 250 g/d of the mix, respectively.

<sup>2</sup>Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants.

<sup>3</sup>Copper sulfate contained 25.2% of Cu.

<sup>4</sup>Manganese sulfate contained 22.2% of Mn.

<sup>5</sup>Zinc sulfate contained 36% of Zn.

<sup>6</sup>Copper chelate contained 15% of Cu (Mintrex, Novus International, St. Charles, MO).

<sup>7</sup>Manganese chelate contained 13% of Mn (Mintrex, Novus International).

<sup>8</sup>Zinc chelated contained 16% of Zn (Mintrex, Novus International).

<sup>9</sup>Agrado Plus (Novus International).

<sup>10</sup>Ca-alt of 2-hydroxy-4-methylthio-butanoic acid (MHA feed supplement, Novus International).

**Table 3.2.** Ingredient composition of the basal diets

Item	Prepartum period	Postpartum period
DM, <sup>1</sup> %	61.49	47.61
Ingredients, % of DM		
Corn silage	21.21	35.40
Alfalfa haylage	-	7.08
Alfalfa hay	-	7.87
Brome hay	54.79	-
Corn, ground	9.90	24.55
Soybeans hulls, ground	10.61	4.13
Brewer's grain, wet	-	4.92
Soybean meal, 47.5% CP	-	6.29
Amino Plus <sup>2</sup>	-	3.44
Blood, meal	0.53	0.98
Sodium bicarbonate	-	0.79
Salt white	0.22	0.30
Limestone, ground	-	1.08
Calcium chloride dihydrate	1.24	-
Potassium carbonate	-	0.33
Magnesium oxide	0.21	0.18
Magnesium sulfate heptahydrate	0.88	-
Dynamate <sup>3</sup>	-	0.33
Trace mineral premix <sup>4</sup>	0.10	0.10
ADE premix	0.11	0.06
Vitamin E 20,000	0.18	0.07
Rumensin 90 <sup>5</sup>	0.02	0.01
Energy Booster 100 <sup>6</sup>	-	1.91
MetaSmart Dry <sup>7</sup>	-	0.18

<sup>1</sup>Values represent averages of weekly samples.

<sup>2</sup>Ag Processing Inc., Emmetsburg, IA.

<sup>3</sup>The Mosaic Co., Plymouth, MN.

<sup>4</sup>The trace mineral premix contained 99.85% of ground corn, 0.04% of cobalt sulfate, 0.05% of EDDI (ethylenediamine dihydriodide), and 0.06% of sodium selenite.

<sup>5</sup>Elanco Animal Health, Indianapolis, IN.

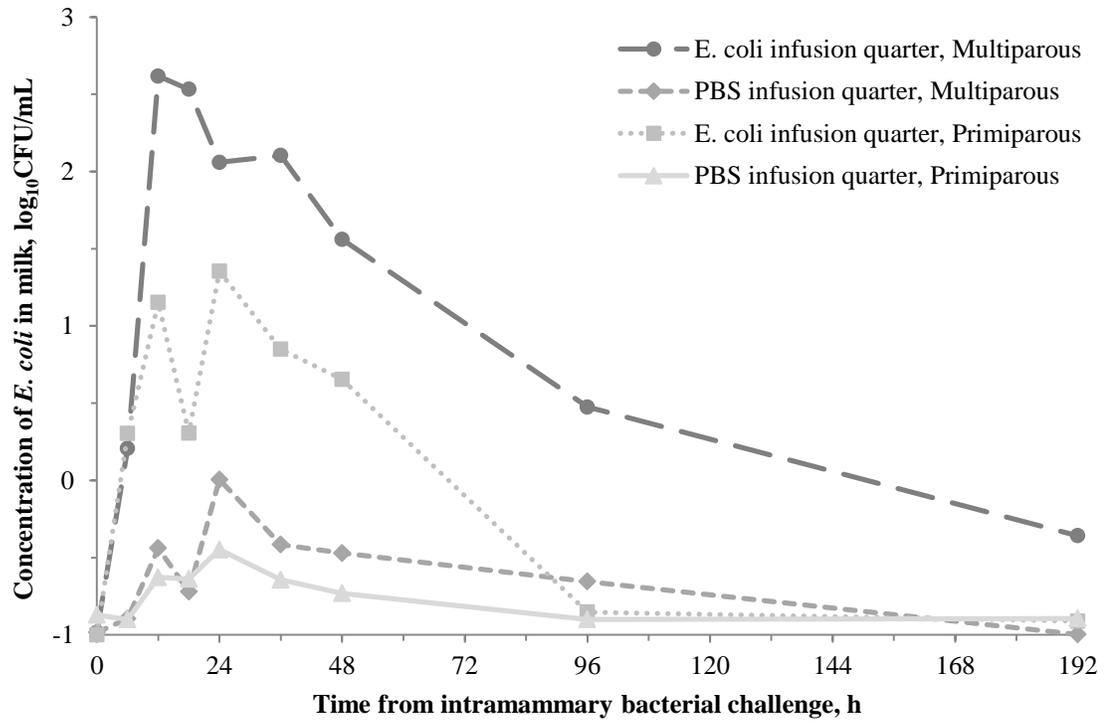
<sup>6</sup>Milk Specialties Global, Eden Prairie, MN.

<sup>7</sup>Adisseo Inc., Antony, France.

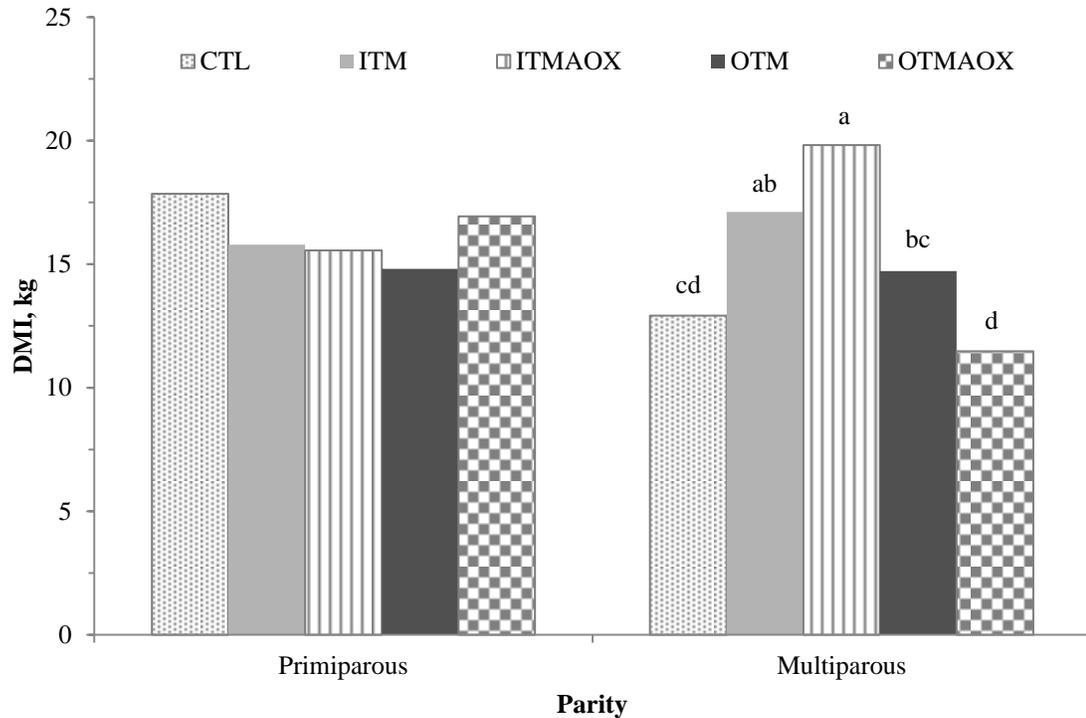
**Table 3.3.** Chemical composition of the basal diets

Nutrient (DM basis) <sup>1</sup>	Prepartum period	Postpartum period
CP, %	11.59	17.47
Available protein, %	10.57	16.47
Soluble protein, % of CP	34.57	30.67
ADF, %	37.26	21.72
NDF, %	56.89	34.55
Lignin, %	5.54	3.75
NFC, %	23.46	38.45
Starch, %	8.47	20.35
ESC (simple sugars), %	4.34	3.07
Crude fat, %	2.04	4.45
Ash, %	8.22	7.71
NE <sub>i</sub> , Mcal/kg	1.30	1.66
NE <sub>m</sub> , Mcal/kg	1.14	1.66
NE <sub>g</sub> , Mcal/kg	0.58	1.06
Ca, %	0.96	0.99
P, %	0.21	0.33
Mg, %	0.37	0.35
K, %	1.58	1.61
Na, %	0.11	0.37
Fe, ppm	353.57	397.67
Zn, ppm	49.57	47.83
Cu, ppm	14.71	10.67
Mn, ppm	80.14	43.17
Mo, ppm	0.73	1.22
S, %	0.24	0.30
Chloride ion, %	1.06	0.51
DCAD, mEq/100g	0.00	23.83

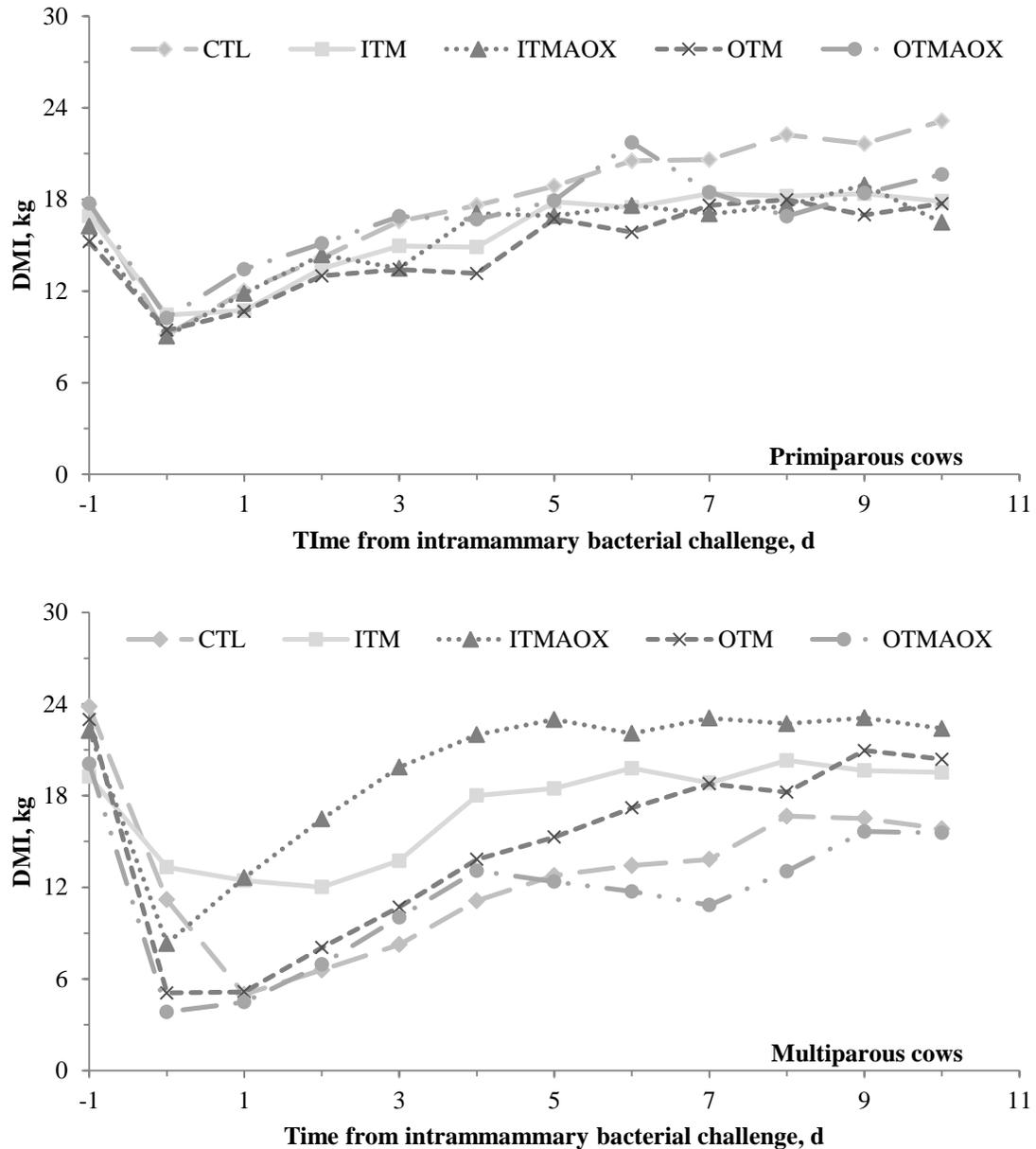
<sup>1</sup>Values, provided by DairyOne Laboratories Inc. (Ithaca, NY), represent averages of samples composited monthly.



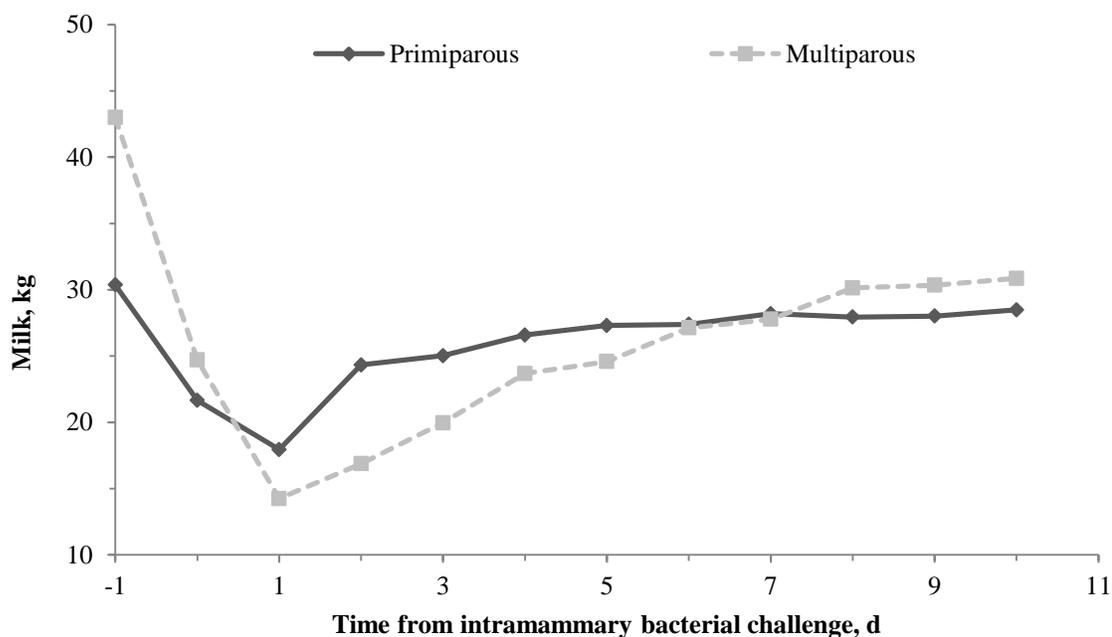
**Figure 3.1.** Concentrations of *Escherichia coli* in milk of cows submitted to intramammary bacterial challenge. Right front quarter of animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*, and PBS was infused in the left front quarter as a control. Parity,  $P < 0.01$ ; quarter,  $P < 0.001$ ; time,  $P < 0.001$ ; parity  $\times$  time,  $P < 0.03$ ; parity  $\times$  quarter,  $P < 0.01$ ; quarter  $\times$  time,  $P < 0.001$ ; and parity  $\times$  quarter  $\times$  time,  $P < 0.03$ . Values represent mean, pooled SEM = 0.31.



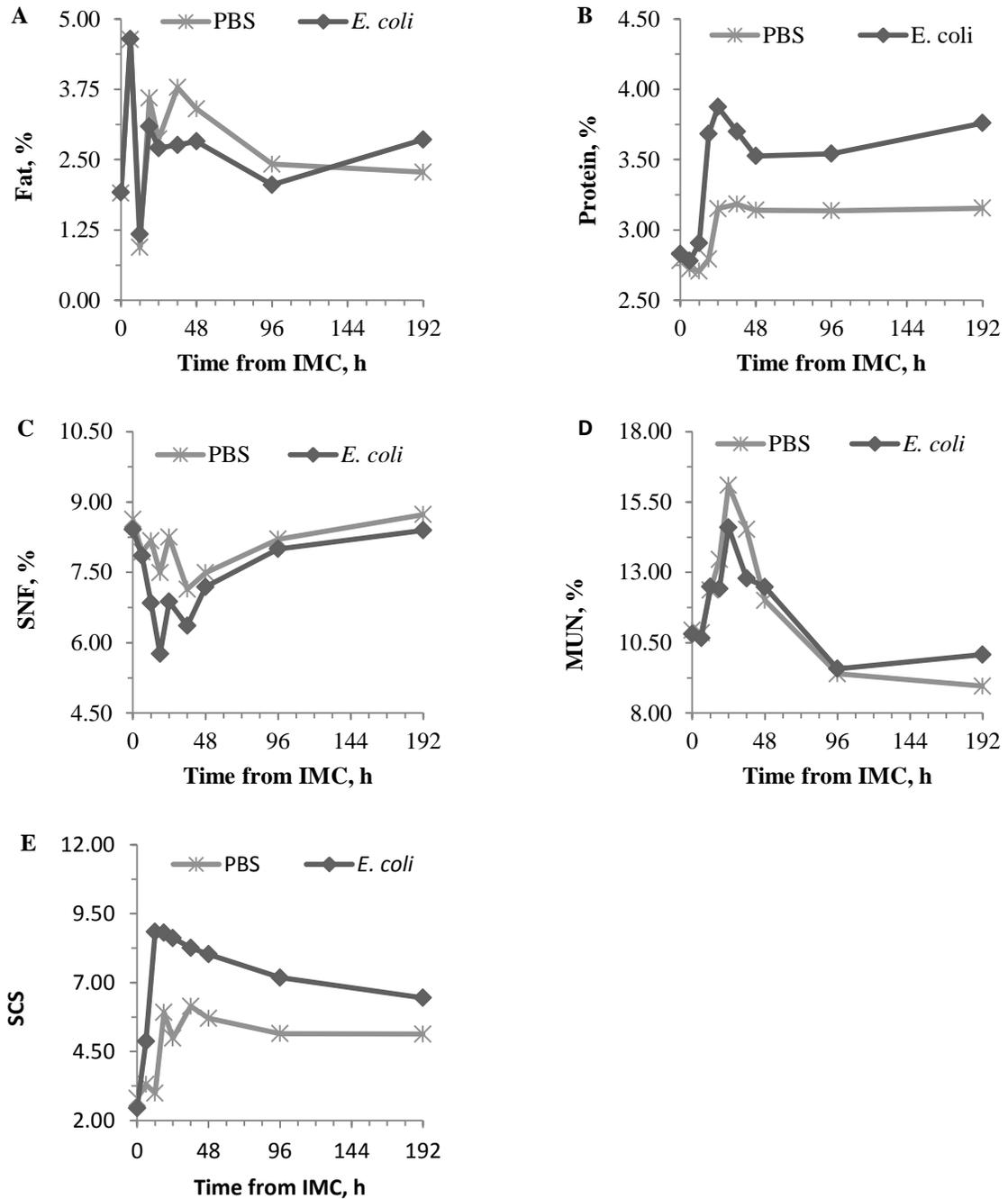
**Figure 3.2.** Effect of treatment within parity on dry matter intake in early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Time,  $P < 0.001$ ; parity  $\times$  time,  $P < 0.001$ ; parity  $\times$  treatment,  $P = 0.02$ ; and parity  $\times$  time  $\times$  treatment,  $P = 0.04$ . Different superscript letters represent difference of treatments within parity ( $P \leq 0.10$ ). Values represent mean; primiparous cows pooled SEM = 1.59, and multiparous cows pooled SEM = 1.62.



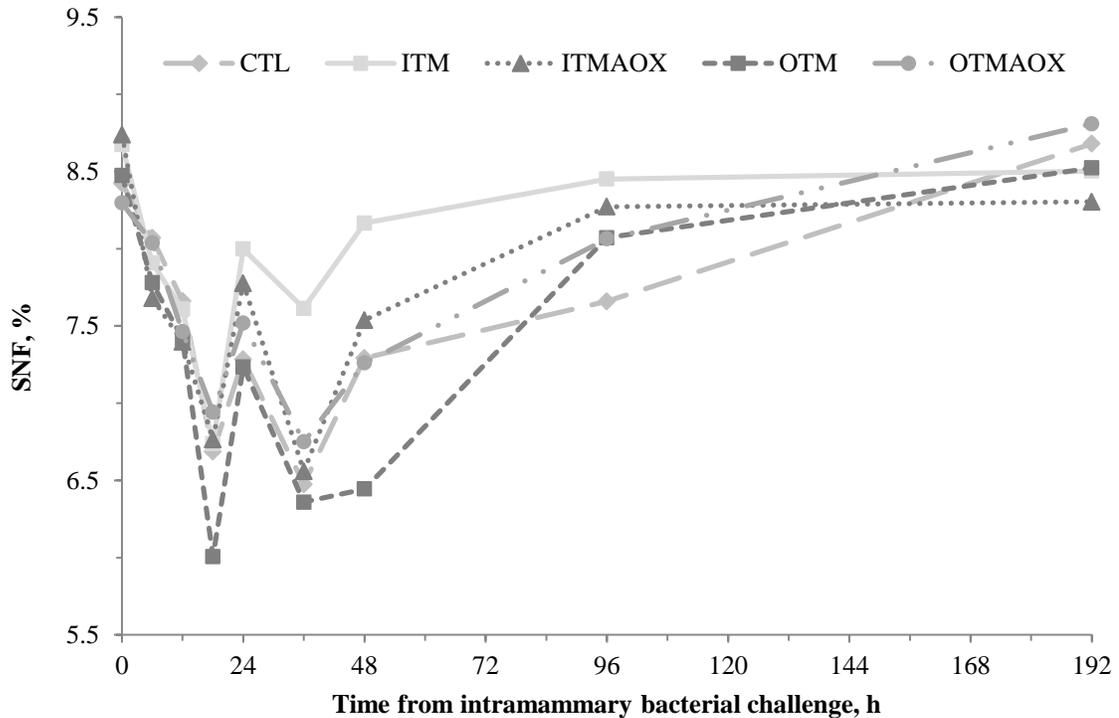
**Figure 3.3.** Effects of treatment within parity on dry matter intake in early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Time,  $P < 0.001$ ; parity  $\times$  time,  $P < 0.001$ ; parity  $\times$  treatment,  $P = 0.02$ ; and parity  $\times$  time  $\times$  treatment,  $P = 0.04$ . Values represent mean; primiparous cows pooled SEM = 2.22, and multiparous cows pooled SEM = 2.28.



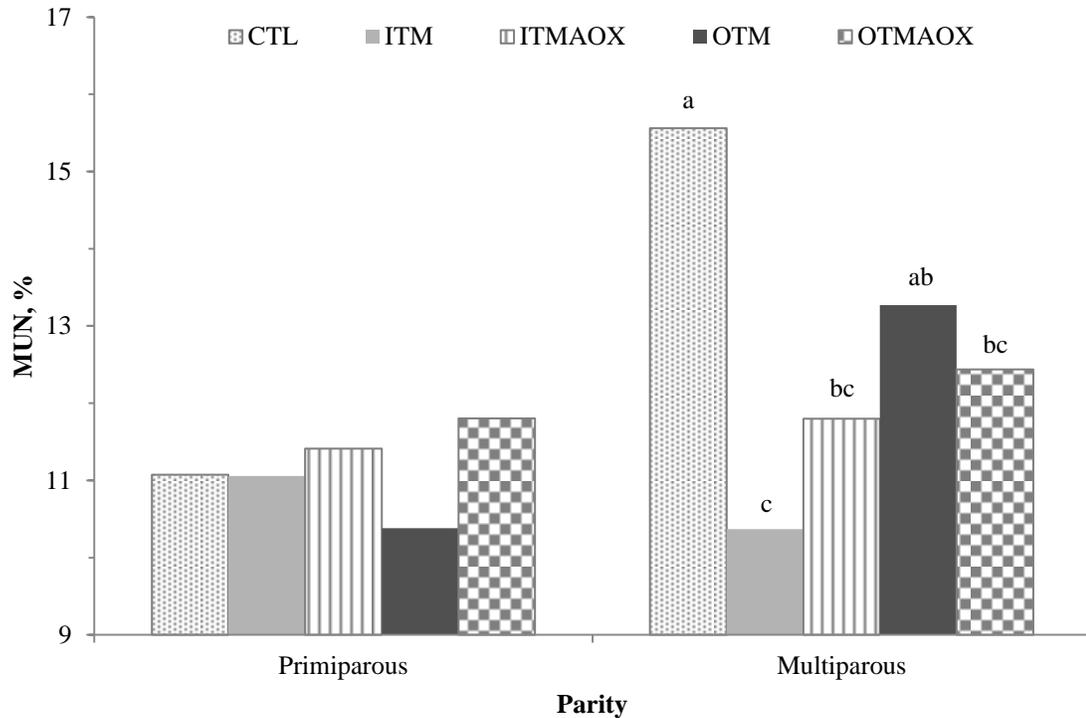
**Figure 3.4.** Milk production of early lactation cows submitted to intramammary bacterial challenge. Primiparous (n = 30) and multiparous (n = 29) were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Cows and fed from d  $-39 \pm 4.6$  to 38 from parturition a basal diet to meet or exceed (NRC, 2001) requirements. Treatments were top-dressed at the morning feeding at a rate of 200 g/d for the prepartum and 250 g/d for the postpartum period. Time and parity  $\times$  time,  $P < 0.001$ . Values represent mean, pooled SEM = 1.90.



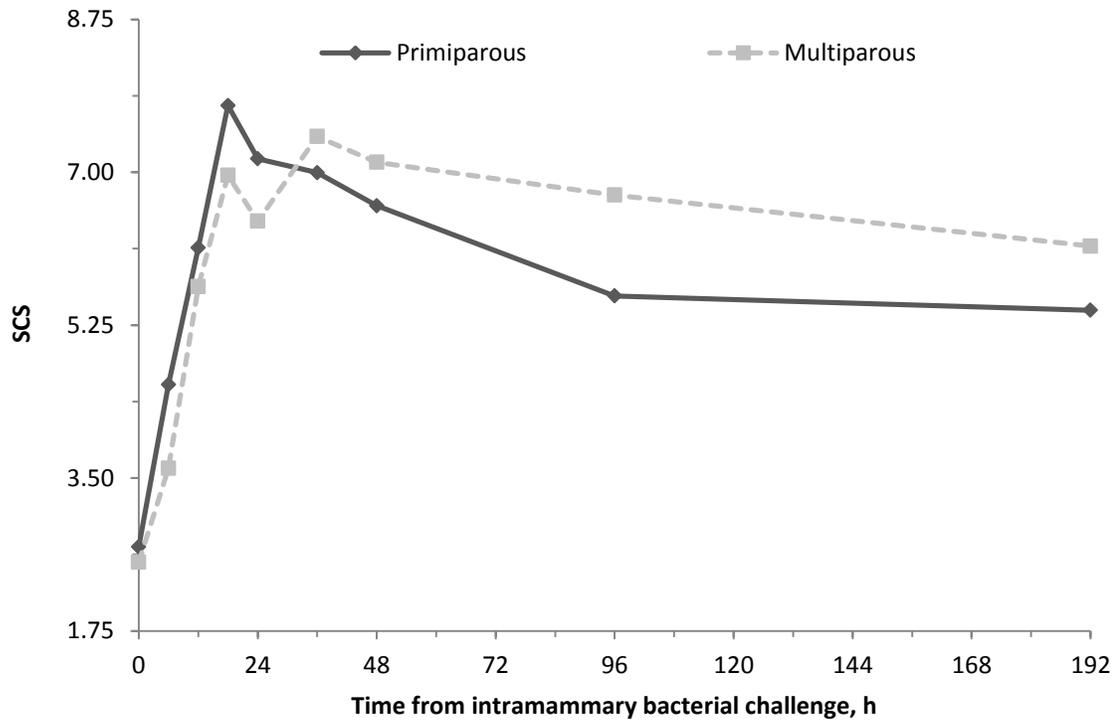
**Figure 3.5.** Effect of quarter on milk composition of early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with 5 mL of solution containing  $17,369.81 \pm 1,488.00$  CFU of *E. coli* in the right front quarter, and the same volume of PBS in the left quarter. *P*-values for quarter  $\times$  time effect are, respectively, 0.07,  $< 0.001$ ,  $< 0.001$ ,  $< 0.04$ , and  $< 0.001$ . Values represent mean. For percentage of fat, protein, SNF, MUN, and SCS in milk, pooled SEM = 0.04, 0.10, 0.17, 0.70, and 0.32, respectively.



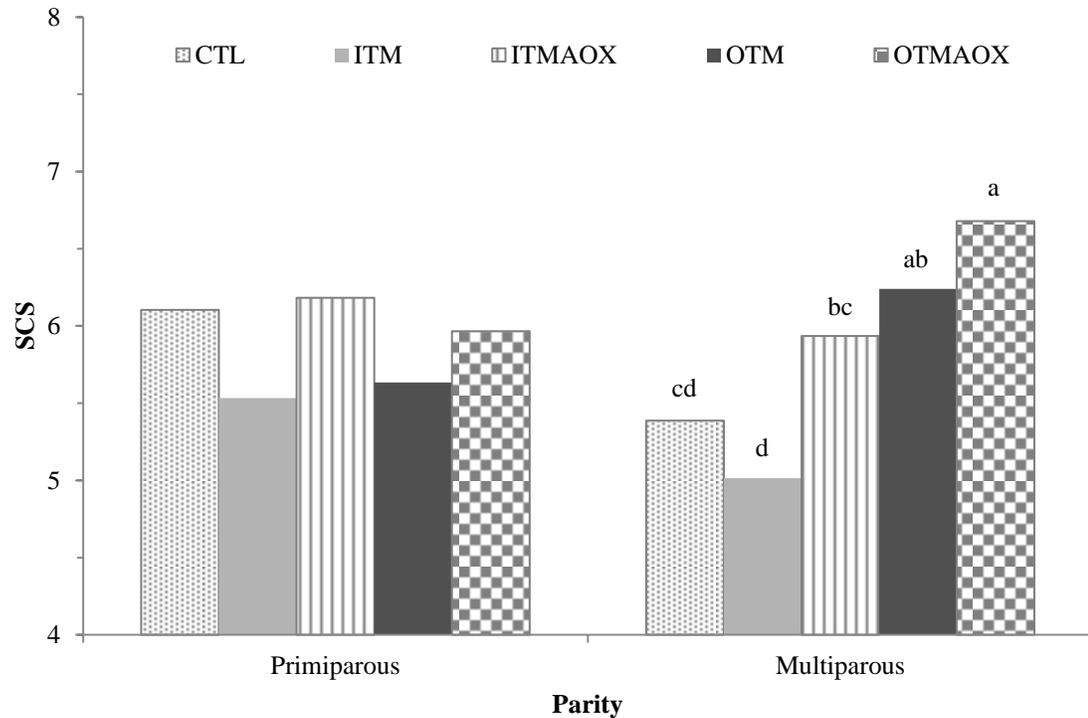
**Figure 3.6.** Effects of treatment on percentage of solids non-fat in early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Parity,  $P = 0.001$ ; quarter,  $P < 0.001$ ; time,  $P < 0.001$ ; parity  $\times$  time,  $P < 0.001$ ; time  $\times$  treatment,  $P < 0.05$ ; quarter  $\times$  time,  $P < 0.001$ ; and parity  $\times$  time  $\times$  treatment,  $P = 0.08$ . Values represent mean, pooled SEM = 0.27.



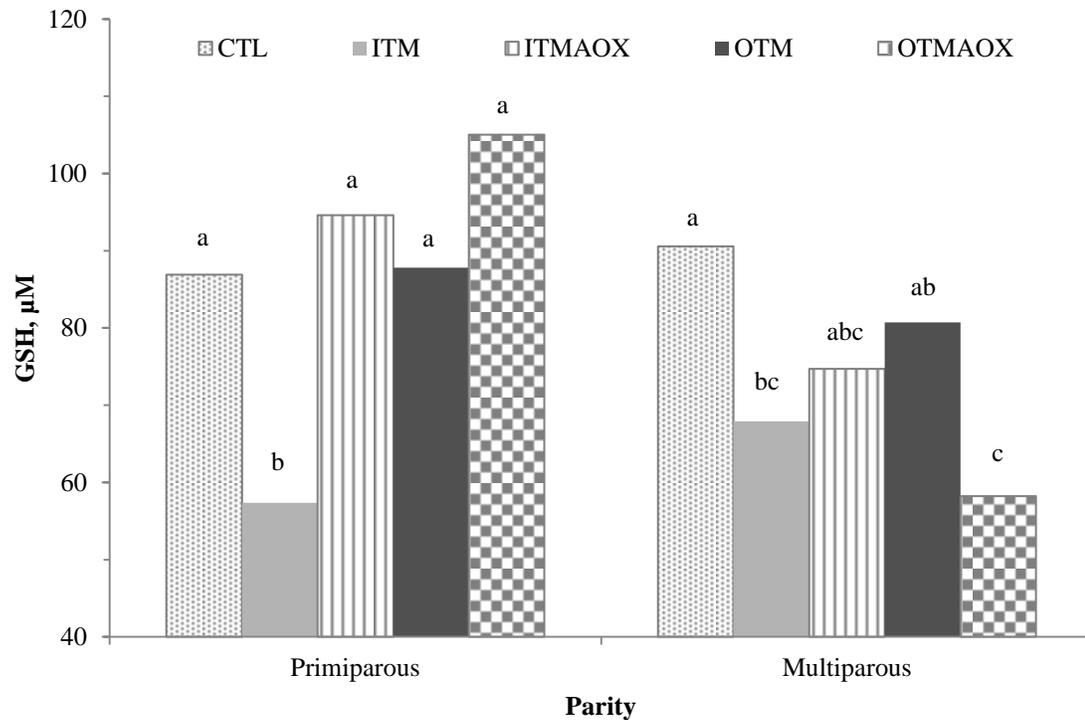
**Figure 3.7.** Effect of treatment within parity on percentage of milk urea nitrogen in early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Parity,  $P = 0.03$ ; time,  $P < 0.001$ ; parity  $\times$  time,  $P < 0.001$ ; parity  $\times$  treatment,  $P < 0.06$ ; quart  $\times$  time,  $P < 0.04$ ; time  $\times$  treatment,  $P = 0.02$ ; and parity  $\times$  time  $\times$  treatment,  $P = 0.06$ . Different superscript letters represent difference of treatments within parity ( $P \leq 0.10$ ). Values represent mean; primiparous cows pooled SEM = 0.97, and multiparous cows pooled SEM = 0.99.



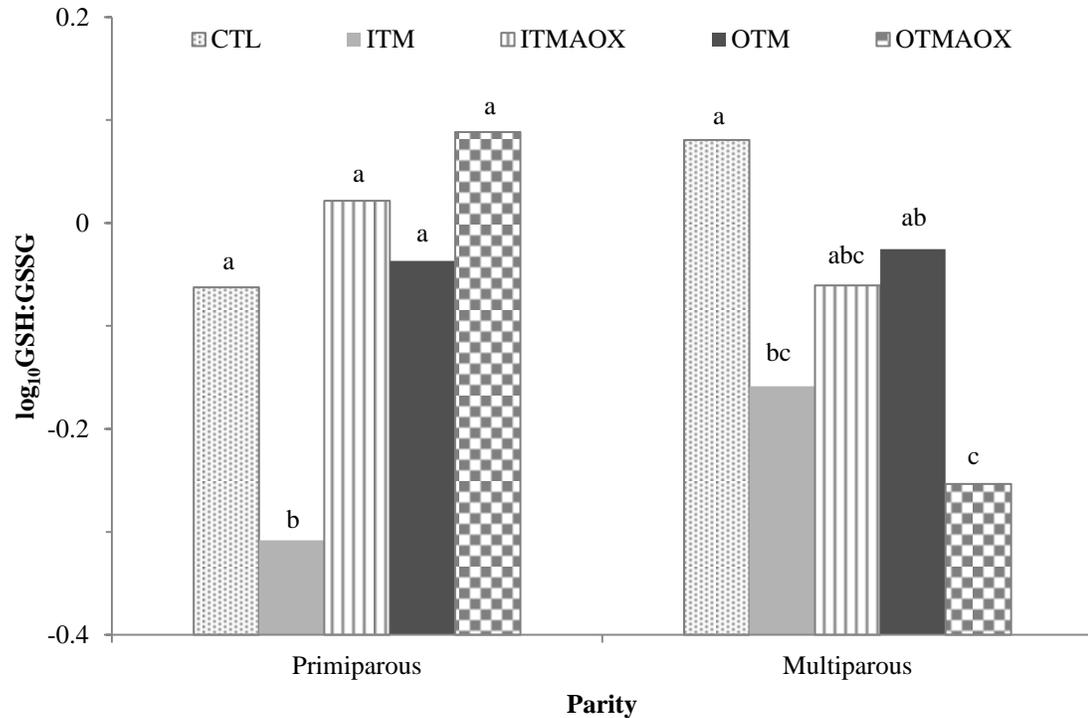
**Figure 3.8.** Effect of parity within time on somatic cell score in early lactation cows submitted to intramammary bacterial challenge. Primiparous (n = 30) and multiparous (n = 29) were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Quarter,  $P < 0.001$ ; time,  $P < 0.001$ ; treatment,  $P < 0.02$ ; parity  $\times$  time,  $P < 0.01$ ; parity  $\times$  treatment,  $P = 0.07$ ; quart  $\times$  time,  $P < 0.001$ ; and parity  $\times$  quarter  $\times$  time,  $P < 0.07$ . Different superscript letters represent difference of treatments within parity ( $P \leq 0.10$ ). Values represent mean; primiparous cows pooled SEM = 0.30, and multiparous cows pooled SEM = 0.34



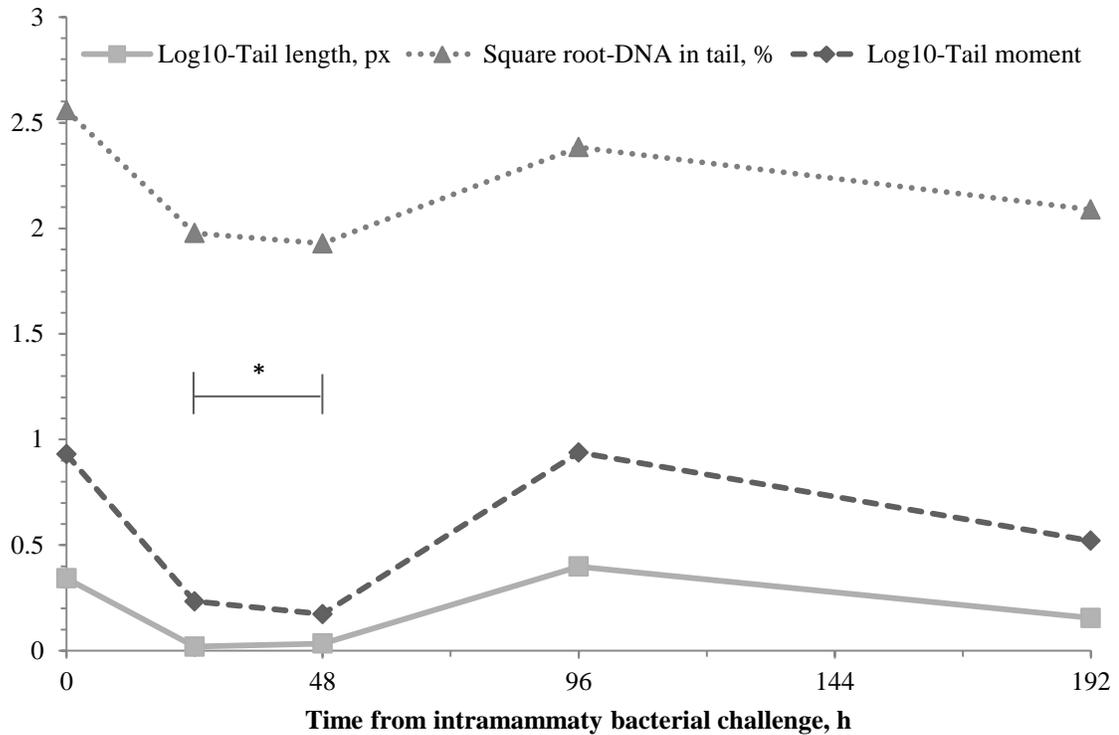
**Figure 3.9.** Effect of treatment within parity on somatic cell score in early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Quarter,  $P < 0.001$ ; time,  $P < 0.001$ ; treatment,  $P < 0.02$ ; parity  $\times$  time,  $P < 0.01$ ; parity  $\times$  treatment,  $P = 0.07$ ; quart  $\times$  time,  $P < 0.001$ ; and parity  $\times$  quarter  $\times$  time,  $P < 0.07$ . Different superscript letters represent difference of treatments within parity ( $P \leq 0.10$ ). Values represent mean; primiparous cows pooled SEM = 0.28, and multiparous cows pooled SEM = 0.31.



**Figure 3.10.** Effect of treatment within parity on erythrocyte reduced glutathione in early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Parity,  $P = 0.03$ ; treatment,  $P = 0.03$ ; parity  $\times$  treatment,  $P = 0.01$ . Different superscript letters represent difference of treatments within parity ( $P \leq 0.10$ ). Values represent mean; primiparous cows pooled SEM = 8.69, and multiparous cows pooled SEM = 8.87.



**Figure 3.11.** Effect of treatment within parity on the ratio of erythrocyte reduced over oxidized glutathione in early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. Treatments: CTL = no supplemental dietary antioxidants or chelated trace minerals; ITM = trace minerals provided as 100% sulfates of Cu, Mn, and Zn without supplemental dietary antioxidants; ITMAOX = trace minerals provided as 100% sulfates of Cu, Mn, and Zn with supplemental dietary antioxidants; OTM = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn without supplemental dietary antioxidants; and OTMAOX = trace minerals provided as 50% sulfates of Cu, Mn, and Zn plus 50% chelated Cu, Mn, and Zn with supplemental dietary antioxidants. Treatment,  $P = 0.05$ ; time,  $P < 0.08$ ; and parity  $\times$  treatment,  $P < 0.04$ . Different superscript letters represent difference of treatments within parity ( $P \leq 0.10$ ). Values represent mean; primiparous cows pooled SEM = 0.08, and multiparous cows pooled SEM = 0.08.



**Figure 3.12.** Effect of time on comet variables in early lactation cows submitted to intramammary bacterial challenge. Animals were challenged with  $17,369.81 \pm 1,488.00$  CFU of *E. coli*. For all variables, parity,  $P \leq 0.06$ ; time,  $P \leq 0.04$ ; and for tail moment, parity  $\times$  time  $\times$  treatment,  $P < 0.02$ . \* $P < 0.05$  from 0 h for the respective variable. Values represent mean;  $\log_{10}$ tail length pooled SEM = 0.11, sqrtDNA in tail (%) pooled SEM = 0.22, and  $\log_{10}$ tail moment pooled SEM = 0.20.

**CHAPTER FOUR**

**INTEGRATED OVERVIEW FROM THE STUDY OF PERIODS OF DECLINED**

**IMMUNE COMPETENCE IN DAIRY COWS SUPPLEMENTED**

**WITH DIETARY ANTIOXIDANT AND CHELATED**

**TRACE MINERALS**

The experiments presented in Chapters 2 and 3 have contributed to our understanding on effects of parity, dietary supplements, and the interaction of both in dairy cows during periods of known immune dysfunction. In Chapter 2, the supplements were tested in periparturient dairy cows. This period is when cows undergo a variety of metabolic and immunologic changes which increase the susceptibility to metabolic disorders and infectious diseases (Goff, 2006; Sordillo and Aitken, 2009). Treatments did not affect dry matter intake. Milk yield was not different over time between treatments in primiparous cows. However CTL showed slightly increased milk production in multiparous cows. Primiparous cows under ITM have decreased protein and SNF contents. Animals fed ITM had slightly lower BCS. Animals fed organic trace minerals had higher DNA damage. Glucose, non-esterified fatty acids, and  $\beta$ -hydroxybutyrate were not affected by treatment. Multiparous cows subjected to the combination of dietary antioxidants and organic trace minerals had increased rabies antibody titers, suggesting enhanced adaptive immune response. In Chapter 3, we evaluate the response of supplemented early lactation dairy cows submitted to experimental *Escherichia coli*

mastitis. Supplementation over NRC (2001) requirements may not be beneficial to primiparous cows, although production variables were improved when multiparous cows were supplemented with inorganic trace minerals. Dry matter intake and milk production were affected by SCS. Milk quality was altered in infected and control quarters. Feed efficiency declined after infusion. In cases of accentuated stress, the organism seems to prioritize the maintenance and integrity of the DNA.

Experiment presented in Chapter 3 is a continuation of the one presented in Chapter 2. In the first instance, comparison among parities agrees with several published research articles that primiparous are healthier than multiparous animals. Burvenich et al. (2007) suggested that older the cow is more debilitate animals will be. These animals showed greater metabolic status demonstrated by metabolites analyzed in serum, and immune status by erythrocyte glutathione and increased rabies titers even under more severe negative energy balance. Interestingly, DNA damage was not affected by time, probably because parturition and this transition between periods is a programmed event. Therefore, the organism may prepare itself to protect its genetic code. Now, during intramammary bacterial challenge, the improved response of primiparous cows was due to the faster recruitment of immune cells and its ability to neutralize the pathogen as described by Burvenich et al. (2003), which may have been influenced by increased health and metabolic status during the transition period. The efficiency of the vaccine against *E. coli* also may have been greater than in multiparous cows based on extrapolation of rabies antibody titers data, thus playing a role in boosting the immune system. Consequently, the severity of the infection in primiparous animals decreased

compared to multiparous cows. The ability of the adaptive immune system to control the severity of the disease could be affected by the suggested increased oxidative status in these animals.

During the transition period, treatments most affected milk variables in multiparous cows and health in all the animals. Little is known about mechanisms of how trace minerals affect milk component synthesis, but it should be a broad and interesting area of research. Health was improved in animals receiving organic trace minerals, but effects on health are dependent of many other events besides treatments which probably have a secondary effect. Several authors did not show effect of treatment on clinical health incidence (Nocek et al., 2006; Hackbart et al., 2010). Effects on immune-related metabolites suggest more mechanistic action of trace minerals which may lead to improved health events. However, other studies from our laboratory (Revelo, 2012) did not find differences on PMN function between cows receiving placebo, inorganic, or organic trace minerals. Additionally, the increased rabies antibody titers observed in multiparous cows receiving OTMAOX could be due to decreased GSH:GSSG ratio observed suggesting increase oxidative status, thus stimulating the adaptive immune system (Seifried et al., 2007). After bacterial challenge, production variables were negatively affected by organic compared to inorganic forms of trace minerals. Revelo (2012) suggested that organic trace minerals stimulate pathways of PMN involved in antigen recognition and immune response. Following, we observed that feeding chelated trace minerals increased SCS. In our case, greater concentrations of PMN inside the challenged mammary gland have been negatively associated with production variables.

Dry matter intake and milk production were affected by SCS, however function of PMN has been negatively associated with fat and protein content inside the infected quarter. In cases of accentuated stress, the organism seems to prioritize the maintenance and integrity of the DNA, through protective compounds as glutathione independently of treatment and parity.

It would be valuable to better profile the antioxidative and oxidative statuses of the animals as well as gene expression. Identifying and elucidating mechanisms affected by different parities and treatments would improve our knowledge and help to better determine, at the farm level, management strategies for periods of increased stress and decreased immune competence. In addition, to better evaluate the effects of dietary antioxidants themselves, a treatment should have been included, and it would be composed of basal diet plus supplementation of only the synthetic antioxidant. This way the experiment would be designed as a  $3 \times 2$  factorial.

In conclusion, primiparous are healthier and fight intramammary infection more efficiently than multiparous cows. Synthetic dietary antioxidants had no effect on dairy cows. Transition period was not affected by supplementation of trace minerals over requirements, even though organic sources decreased DNA damage and improved health during early postpartum period. However, inorganic trace minerals improved production variables during intramammary bacterial challenge compared to organic sources, most helping multiparous cows. In my opinion, NRC (2001) requirements for multiparous cows should be increased in order to boost immunity and alleviate negative effects in a possible case of mastitis.

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## VITA

Ricardo de Oliveira Rodrigues was born in Mauá, SP, Brazil, on September 18, 1988. While attending school in the city, he spent his weekends and school vacations at his family farm, working with horses and dairy cows. He then went on to study Animal Science at São Paulo State University in Botucatu, SP, Brazil, where he worked with Dr. José Vasconcelos (Zequinha) and was first exposed to research. In his last semester in college, Ricardo first came to USA as an intern under advisory of Dr. Matthew Waldron at University of Missouri. After the completion of the internship, he obtained his Bachelor of Science degree in Animal Sciences from São Paulo State University in December of 2010. In April of 2011, Ricardo come back to USA and continued working under the same supervisor, but now as a research specialist. Then, in January of 2012, he started his Master's degree under mentorship of Dr. Waldron at the University of Missouri in the Division of Animal Sciences. Following the completion of his Master's degree in May of 2014, Ricardo will remain at the University of Missouri to pursue a doctoral degree.