RAQUEL DE SOUSA MARQUES

# Evaluation of oxidative stress and immunity in Holstein cows supplemented with macro and trace minerals during the transition period

São Paulo

2022

RAQUEL DE SOUSA MARQUES

# Evaluation of oxidative stress and immunity in Holstein cows supplemented with macro and trace minerals during the transition period

Dissertation submitted to the Postgraduate Program in Veterinary Clinic of the School of Veterinary Medicine and Animal Science of University of São Paulo to obtain the Master's degree in Sciences.

**Departament:**
Clinical Medicine

**Area:**
Veterinary Clinic

**Advisor:**

Prof. Viviani Gomes Ph.D.

**Co-Advisor:**

Prof. Camila Costa Baccili Ph.D.

São Paulo

2022

Total or partial reproduction of this work is permitted for academic purposes with the proper attribution of authorship and ownership of the rights.

**DADOS INTERNACIONAIS DE CATALOGAÇÃO NA PUBLICAÇÃO**

(Biblioteca Virginie Buff D'Ápice da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo)

4272
FMVZ

Marques, Raquel de Sousa
Evaluation of oxidative stress and immunity in Holstein cows supplemented with macro and trace minerals during the transition period / Raquel de Sousa Marques. – 2022.
97 f. : il.

Título traduzido: Avaliação do estresse oxidativo e imunidade em vacas holandesas suplementadas com macro e micro minerais durante o período de transição.

Dissertação (Mestrado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Clínica Médica, São Paulo, 2023.

Programa de Pós-Graduação: Clínica Veterinária.

Área de concentração: Clínica Veterinária.

Orientadora: Profa. Dra. Viviani Gomes.
Coorientadora: Profa. Dra. Camila Costa Baccili

1. Estresse metabólico. 2. Resposta imune. 3. Bovino de leite. 4. IgG. 5. Haptoglobina. I. Título.

Ficha catalográfica elaborada pela bibliotecária Maria Aparecida Laet, CRB 5673-8, da FMVZ/USP.

Faculdade de Medicina Veterinária e Zootecnia Universidade de São Paulo

*Comissão de Ética no Uso de Animais*

# CERTIFICADO

Certificamos que a proposta intitulada "AVALIAÇÃO DO ESTRESSE OXIDATIVO E IMUNIDADE EM VACAS HOLANDESAS SUPLEMENTADAS COM MACRO E MICRO MINERAIS DURANTE O PERÍODO DE TRANSIÇÃO", protocolada sob o CEUA nº 8207080921 (ID 009196), sob a responsabilidade de **Viviani Gomes** *e equipe; Raquel de Sousa Marques* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia Universidade de São Paulo (CEUA/FMVZ) na reunião de 03/02/2022.

We certify that the proposal "Evaluation of oxidative stress and immunity in Holstein cows supplemented with macro and trace minerals during the transition period", utilizing 60 Bovines (60 females), protocol number CEUA 8207080921 (ID 009196), under the responsibility of **Viviani Gomes** *and team; Raquel de Sousa Marques* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **APPROVED** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 02/03/2022.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 10/2021 a 01/2022 Área: Clínica Médica Veterinária

| Origem: | Animais de proprietários | | | |
|---|---|---|---|---|
| Espécie: | Bovinos | sexo: Fêmeas | idade: 1 a 5 anos | Quantidade: 60 |
| Linhagem: | Holandesa | | Peso: 340 a 700 kg | |

São Paulo, 02 de dezembro de 2022

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

Profa. Dra. Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

**EVALUATION FORM**

Autor: MARQUES, RAQUEL DE SOUSA

Title: **Evaluation of oxidative stress and immunity in Holstein cows supplemented with macro and trace minerals during the transition period**

Dissertation submitted to the Postgraduate Program in Veterinary Clinic of the School of Veterinary Medicine and Animal Science of University of São Paulo to obtain the Master's degree in Sciences.

Date: _____/_____/_____

**Committee Members**

Prof. ________________________________________________

Institution:_____________________________ Decision:_________________________

Prof. ________________________________________________

Institution:_____________________________ Decision:_________________________

Prof. ________________________________________________

Institution:_____________________________ Decision:_________________________

# DEDICATION

*To God, for life, health and determination. Making myself able to having the willpower to pursue my goals.*

*To my parents Milton and Cristina, my grandmother Alice, and my aunt Maria Alice, you are my foundation and my life. Those who make possible my dreams come true.*

*To Professor Viviani Gomes and Gecria Team for the opportunities, companionship and hard work, which makes it all so rewarding.*

*In Memoriam to Professor Fernando José Benesi, an inspiration in the Buiatrics and as a person. Missing you does not diminish, your teachings are still alive with us every day.*

# ACKNOWLEDGEMENTS

To my parents Milton and Cristina, my grandmother Alice, and my aunt Maria Alice, thank you for your affection and education of personal and professional values. For all support, for believe and invest not only in my education but also in me at all, for believe in me when neither I believed. All that I am, what I do and what I have is wholly due to you and for you.

To all my whole family, for their unconditional support, prayers and understanding throughout my studies.

To my friends at school, high school and vet school for encouraging me to always follow my goals and for celebrating with me all my joys.

Thank to my advisor, Professor Viviani Gomes. We are working together since 2011 when I was undergraduate student, since the first semester of graduation you always encouraged and inspired me as a professional, but you also made my love for dairy cattle grow up in me. In all of these years, I don't have enough words to say how grateful I am for the support and all scolding that makes me grow up, and be a better professional, thank you for dedicate your time, your knowledge, and to be an example of persistence for all team.

To my coadvisor, Camila Baccili. Since I was your intern when you were still a master's student, you taught me the first steps in clinical medicine, laboratory practices, when I still did not know anything about this research world. I am forever grateful for all your advices and all your support during my professional growth. I think I can tell, during our growth so far. Thank you so much!

To all GeCria team, Camila Martin, Fabricio Dias Torres, Karen Nascimento da Silva, Daniela Tardon, Fernanda Ramos, Nathalia Decaris, Filipe Aguera, Larissa Padilha, Janaína Santos, Bianca Santarosa, Thamires Diniz and Karina Madureira (that will always be in our hearts). To the GeCria interns Luana Camargo, Yasmin Vieira, Thalita Domingues and Natália Puzzi. Each of you help in a piece of these work, giving advices, support or just being together in this crazy moment called postgraduation.

Special thanks to Filipe that helps me in the farm period, and a lot collecting samples, wakes up early and working hard, with all the farm worksheets and data, with all my doubts in statistics, without you this wouldn’t be possible. Thanks to Nathalia, Larissa and Luana to all conversation, support and lots of laughs. And a very special

thanks to Karen that became a gift in my life during postgraduation with your friendship, your advices, lots of laughs, and for always being there when I needed it most.

To the College of Veterinary Medicine and Animal Science of the University of São Paulo, for being my home during graduation and postgraduation, with so many open doors and possibilities for learning and growth.

To Residents of Ruminants Internal Medicine and Surgery, João Marcos Coelho, Kelly Perestrelo, Bruna Feliciano, for helping with blood collections for haptoglobin analysis.

To Professor Fernando José Benesi, for all affection that you gave to us. I am so grateful our life crossing and to the opportunity of learning with the best. I imagine this moment with you present and commemorating with us, but I know wherever you are, you are proud of our job!

Thanks to all professors of Internal Medicine Department: Alice Maria Paiva Della Libera, Carla Bargi Belli, Denise Saretta Schwartz, Enrico Lippi Ortolani, Fábio Celidonio Pogliani, Lilian Gregory, Márcia de Oliveira Sampaio Gomes, Marcia Mery Kogika, Maria Cláudia Araripe Sucupira, Maria Helena Matiko Akao Larsson, Mitika Kuribayashi Hagiwara, Raquel Yvona Arantes Baccarin, Silvia Regina Ricci and Wilson Roberto Fernandes.

To all laboratory team of FMVZ-USP, Clara Mori, Cláudia Regina Stricagnolo, Maria Helena da Silva Pelissari and Dinha, you all make the lab work possible, thank you for all your help. But especially to Clara Mori, for all your patience and teaching throughout the process. For the long hours spent in the lab, the endless tubes of samples, but mostly for the conversations and laughter. It would not have been possible without you, thank you!

To all at the São Jorge farm, Bruno, Mr. Jorge and Mrs. Carla, for agreeing to carry out the study, but mainly for all assistance and reception, you welcomed me with open arms and I don't have enough words to thank you for such generosity and affection you showed me. To all farm workers: Gisele, Magal, Zelão, Paulinho and everyone who helped us directly or indirectly. I am grateful to meet dedicate employees that works with passion and helped us a lot throughout the study. I also want to thank the cows that were part of the study, the workers farm does a beautiful management job with them, so they all had a great temperament. All the work we do is hoping to improve their welfare and life quality.

To Virbac Animal Health for the financial support in the Fosfosal project, idealized by Bruno Lima in partnership with Professor Viviani Gomes, making this new study possible.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 88887.619707/2021-00.

*“One of the greatest discoveries a man makes, one of his great surprises, is to find he can do what he was afraid he couldn't do.”*

Henry Ford

**RESUMO**

MARQUES, R.S. **AVALIAÇÃO DO ESTRESSE OXIDATIVO E IMUNIDADE EM VACAS HOLANDESAS SUPLEMENTADAS COM MACRO E MICRO MINERAIS DURANTE O PERÍODO DE TRANSIÇÃO**. 2022. 97 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2023.

Mobilização excessiva de tecido adiposo durante o período de transição contribui para desregulação imunológica e estresse oxidativo e metabólico. Objetivo desta pesquisa foi avaliar a influência da suplementação mineral injetável sobre o estresse oxidativo, dinâmica da IgG e metabolização lipídica em vacas Holandesas durante o período de transição. Vacas saudáveis foram alocadas no grupo controle (C; n=32) ou suplementação mineral injetável (SM; n=34). Os animais foram tratados com três injeções (10mL) de suplemento mineral contendo fósforo, potássio, cobre, magnésio e selênio por via intramuscular, 30 dias pré-parto, no dia do parto e 15 dias pós-parto. Amostras sanguíneas foram coletadas: três semanas pré-parto (M-3); duas semanas pré-parto (M-2); uma semana pré-parto (M-1); na semana do parto (M0); uma semana pós-parto (M+1); duas semanas pós-parto (M+2) e três semanas pós-parto (M+3). O efeito do tratamento, tempo e interação tempo*tratamento foi determinada pelo procedimento MIXED (PROC-mixed, SAS), e pelo Teste t de Student para as variéveis que apresentaram interação entre tratamento e tempo. Suplementação mineral teve efeito positivo na concentração de IgG nas primíparas. Para multíparas, foram observados efeitos de IgG e haptoglobina. Resultados semelhantes foram observados para o número total de animais para IgG e haptoglobina. Ação antioxidante de GSH foi maior nas vacas tratadas para o total de animais. Não foi possível observar efeito significativo do tratamento tanto para BHB como para NEFA, porém a interação entre tratamento e tempo teve efeito significativo para BHB em vacas multíparas. Os resultados obtidos neste estudo sugerem que o protocolo de suplementação injetável melhorou a imunidade das vacas, caracterizada por um perfil anti-inflamatório e maiores concentrações séricas de IgG, além de reduzir a metabolização lipídica e estresse oxidativo.

Palavras-chave: estresse metabólico, resposta imune, bovino de leite, IgG, haptoglobina

**ABSTRACT**

MARQUES, R.S. **EVALUATION OF OXIDATIVE STRESS AND IMMUNITY IN HOLSTEIN COWS SUPPLEMENTED WITH MACRO AND TRACE MINERALS DURING THE TRANSITION PERIOD**. 2022. 97 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2023.

Excessive mobilization of adipose tissue during the transition period contributes to immune dysregulation and oxidative and metabolic stress. The aim of this research was to evaluate the influence of injectable mineral supplementation on oxidative stress, IgG dynamics and lipid metabolism in Holstein cows during the transition period. Healthy cows were allocated in the control group (C; n=32) or injectable mineral supplementation (SM; n=34). The animals were treated with three injections (10mL) of mineral supplement containing phosphorus, potassium, copper, magnesium and selenium intramuscularly, 30 days pre-partum, on the day of parturition and 15 days postpartum. Blood samples were collected: three weeks prepartum (M-3); two weeks prepartum (M-2); one week prepartum (M-1); in the week of delivery (M0); one week postpartum (M+1); two weeks postpartum (M+2) and three weeks postpartum (M+3). The effect of treatment, time and time*treatment interaction was determined by the MIXED procedure (PROC-mixed, SAS), and by Student's t test for the variables that showed interaction between treatment and time. Mineral supplementation had a positive effect on the concentration of IgG in primiparous women. For multiparous women, effects of IgG and haptoglobin were observed. Similar results were observed for the total number of animals for IgG and haptoglobin. Antioxidant action of GSH was higher in treated cows for the total number of animals. It was not possible to observe a significant treatment effect for both BHB and NEFA, but the interaction between treatment and time had a significant effect for BHB in multiparous cows. The results obtained in this study suggest that the injectable supplementation protocol improved the cows' immunity, characterized by an anti-inflammatory profile and higher serum IgG concentrations, in addition to reducing lipid metabolism and oxidative stress.

Keywords: metabolic stress, immune response, dairy cow, IgG, haptoglobin

**LIST OF FIGURES**

Figure 1 - Theoretical pattern of changes in the main physiological aspects of cow healthy during the transition period. Being ideal, the negative energy balance (NEB), inflammation, and oxidative stress are close to zero, where they do not occur in the body. And the immunocompetence and the calcemia be close to 100% of their optimal level…………………… ...........................................................................................38
Figure 2 - Cow prepartum management flowchart ...............................................46
Figure 3 - Cow postpartum management flowchart .............................................47
Figure 4 - Tubes used for blood sample collection, material obtained from each tube after laboratory processing and each laboratory analysis performed. ...................54
Figure 5 - Mean values, significant p value and differences for BHB between control and treated group for ≥ 3 lactations cows and multiparous cows ..........................62
Figure 6 - Mean values, significant p value and differences between groups in relation to control and mineral supplementation groups for TBARS, GPx and GSH in diferents parities .........................................................................................66
Figure 7 - Mean values, significant p value and differences for TBARS and TAS between control and mineral supplementation group for the cows with ≥ 3 lactations and multiparous cows.........................................................................................68
Figure 8 - Mean values, significant p value and differences between groups in relation to control and mineral supplementation groups for IgG and haptoglobin concentrations in diferents parities ..........................................................................72
Figure 9 - Mean values, significant p value and differences for haptoglobin between control and mineral supplementation for ≥ 3 lactations cows. ...............................74

## LIST OF TABLES

Table 1 - Initiation and propagation of reactive oxygen species and their interaction with organic substrates ........................................................................31
Table 2 - Initiation and propagation of reactive oxygen species and their interaction with organic substrates ........................................................................33
Table 3 - Ingredients, nutrients and minerals (% of dry matter – DM) of diets offered at prepartum and postpartum period during the experimental period. ...................48
Table 4 - Guarantee levels of prepartum and postpartum commercial premix ..49
Table 5 - Balance between energy and protein of prepartum and postpartum diet during the experimental period ........................................................................50
Table 6 - Mineral balance of prepartum diet during the experimental period.....50
Table 7 - Mineral balance of postpartum diet during the experimental period…51
Table 8 - Animal's distribution experimental groups according to parity............52
Table 9 - Samples corresponding to calving week, moment M0, divided by calving date proximity in groups ........................................................................53
Table 10 - Mean values, standard error (SE) and differences between groups in relation to control and treated groups for BHB and NEFA....................................60
Table 11 - Mean values, standard error (SE) and differences for BHB between control and treated group for for the cows with ≥ 3 lactations and multiparous cows........61
Table 12 - Mean values, standard error (SE) and differences between categories in control and treated groups for BHB and NEFA.....................................................63
Table 13 - Mean values, standard error (SE) and differences between blocks in control group and treated group for BHB and NEFA ............................................63
Table 14 - Treatment effect on incidence (%) of ketosis at the first week postpartum (M+1) by measuring serum BHB ........................................................................64
Table 15 - Mean values, standard error (SE) and differences between groups in relation to control and treated groups for biomarkers of oxidative stress ..............65
Table 16 - Mean values, standard error (SE) and differences for TBARS and TAS between control and mineral supplementation group for the cows with ≥ 3 lactations and multiparous cows........................................................................................67
Table 17 - Mean values, standard error (SE) and differences between categories in control and mineral supplementation groups for biomarkers of oxidative stress ...69

Table 18 - Mean values, standard error (SE) and differences between blocks in control and mineral supplementation groups for biomarkers of oxidative stress ...70
Table 19 - Mean values, standard error (SE) and differences between groups in relation to control and mineral supplementation groups for IgG and HAP.............71
Table 20 - Mean values, standard error (SE) and differences between control and mineral supplementation group for for HAP to cows with ≥ 3 lactations and IgG to total of animals……………...........................................................................................73
Table 21 - Mean values, standard error (SE) and differences between categories in control and mineral supplementation groups for IgG and haptoglobin ..................75
Table 22 - Mean values, standard error (SE) and differences between blocks in control group and mineral supplementation groups for IgG and haptoglobin ........75

SUMMARY

**1 INTRODUCTION ..........17**

**2 LITERATURE REVIEW ..........22**

2.1. MINERALS ..........22

2.1.1. MACRO MINERALS AND TRACE MINERALS ..........22

2.1.2. PARENTERAL MINERAL SUPLEMENTATION ..........25

2.2 OXIDATIVE METABOLISM AND ANTIOXIDANT SYSTEM ..........26

2.3 IMMUNE RESPONSE ..........30

2.4 THE TRANSITION PERIOD AND THE METABOLIC STRESS..........33

**3 OBJECTIVE ..........41**

3.1 GERAL OBJECTIVE..........41

3.2 SPECIFC OBJECTIVE ..........41

**4 MATERIAL AND METHODS..........42**

4.1 HERD SELECTION ..........42

4.2 PRE PARTUM AND IMMEDIATE POST PARTUM MANAGEMENT..........42

4.3 NUTRICIONAL MANAGEMENT..........44

4.4 ANIMAL INCLUSION CRITERIA AND EXPERIMENTAL GROUPS..........48

4.5 TREATAMENTS..........49

4.6 BLOOD SAMPLE COLLECTION..........49

4.7 SAMPLE PREPARATION FOR STORAGE..........50

4.8 ANALYSIS OF ENERGY METABOLISM BIOMARKERS ..........51

4.9 ANALYSIS OF OXIDANTS AND ANTIOXIDANTS BIOMARKERS OF OXIDATIVE STRESS ..........52

4.10 ANALYSIS OF IMMUNE BIOMARKERS ..........53

4.11 STATISTICAL ANALYSIS..........54

**6 RESULTS..........56**

6.1 ENERGY METABOLISM BIOMARKERS ..........56

6.2 OXIDANTS AND ANTIOXIDANTS BIOMARKERS OF OXIDATIVE STRESS60

6.3 IMMUNE BIOMARKERS ..........64

7 DISCUSSION .......................................................................................68

8 CONCLUSION.......................................................................................81

REFERENCES...........................................................................................82

# 1 INTRODUCTION

The constant evolution for increased productivity promotes intensification of production system, also seeking maximum financial return (WITTER, 2000). Historical gains in milk production stem in part from selection and genetic improvement, new technologies and management tools, such as estrus synchronization associated with artificial insemination (AI), advances in nutrition and use of herd health programs. As a result of these measures, dairy cows altered their metabolism and nutrients absorption, so that, although they could increase milk production, this also led to emergence of metabolic diseases or physiological overload periods, as happens during the transition period (BAUMGARD et al., 2017).

The transition period, commonly defined as three weeks pre-calving to three weeks post-calving (DRACKLEY, 1999) is the most challenging and critical period regarding the dairy cow health status during the lactation cycle. During this period, several anatomical, physiological, hormonal and metabolic changes occur. The fetal growth, with an increase in internal pressure in digestive organs associated with the great hormonal variation of this period, (CHEW et al., 1979), reduces dry matter intake (DMI) reaching 10 -30% (BELL, 1995), while energy and calcium demands for lactation increase (CHAPINAL et al., 2012).

These demands generates a greater oxygen consumption by tissues through cellular respiration in moments of greater metabolic demand. As consequence to provide the necessary energy for the onset of lactation, but results the cow enters a state of negative energy balance (NEB) (KONVICNÁ, et al., 2015). Also occurs activation of the systemic inflammatory response during calving, even in absence of microbial infections signs or other pathologies (TREVISI et al., 2012), associated with oxidative stress, due to unbalanced availability of antioxidants in presence of phenomena that increase pro-oxidants production (SORDILLO et al., 2009).

Excessive adipose tissue mobilization is a hallmark of the transition period in dairy cows that develop metabolic stress, thus disturbing physiological homeostasis (ABUELO et al., 2015) and is related to oxidative stress degree presented by animals (ZACHUT et al., 2013; CONTRERAS et al., 2017; WEBER et al., 2013). Cellular membranes are highly susceptible to oxidation, which generates lipid radicals and creates a positive feedback loop that can result in cell damage and death, consequently increasing oxidative stress (SORDILLO et al., 2013). Oxidative stress is

the result of an imbalance between oxidant and antioxidant substances, resulting either from an increase in reactive oxygen species (ROS) production or a decrease in antioxidant substances, damaging biological macromolecules, inducing metabolic disturbances and physiological disorders (TREVISAN et al., 2001; BERNABUCCI et al., 2002; BOUWSTRA et al., 2010).

During the transition period, dairy cows also experience immune dysregulation, which increases their susceptibility to infectious and metabolic diseases. Elevated concentrations of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) from excessive lipomobilization and hypoglycemia are important contributors to periparturient immune dysregulation, as glucose is essential for immune cell function. Generating excessive and unregulated responses during the transition period due to an excessive and unregulated inflammation condition, excessive lipomobilization and oxidative stress. This increase in metabolic activity results in increased ROS production and antioxidant defenses depletion during this period (SORDILLO, 2013). Oxidative stress can be monitored through antioxidant and pro-oxidant biomarkers that can be measured in serum, plasma and erythrocytes (PASSI et al., 2001).

Dairy producing cows are more susceptible to a variety of metabolic and infectious diseases during the transition period. When animals present exacerbated oxidative stress, its consequences will influence the health-related expenses of their productive life, when we add other expenses arising from production system (such as milk production loss, market value decrease, among others) alternatives for reducing oxidative stress would result in better productive performance of these animals (ABUELO et al., 2019). There is evidence for the role of other antioxidants in udder health, such as vitamin C (WEISS et al., 2007), selenium, copper, zinc, and manganese (MACHADO et al., 2013). However, information regarding the association of mineral supplementation with immune improvement and decresse inflammation and oxidative stress, it is not fully elucidated since there is still no consensus among findings.

Several strategies have been proposed and tested as a method to avoid or minimize the development of oxidative stress state during the transition period. Although the body has its own enzyme systems that remove ROS, the main antioxidants are vitamins, macro and trace minerals (ABUELO et al., 2015). In general terms, vitamins and minerals such as selenium (Se) have been shown to be effective

in counteracting oxidative stress and several diseases severity such as mastitis or metritis, both through a direct antioxidant effect and by enhancing the immune response. Most traditionally established nutritional requirements focus on deficiency situations and there is now evidence that supplementation slightly above reported requirements can improve animal health status and performance as well as milk and meat quality (CASTILLO et al., 2013).

Studies were carried out to evaluate the influence of mineral supplementation during the transition period. Soldá et al., (2017) evaluated hematological, biochemical, immunological, and antioxi-dant status in transition period dairy cows and newborn calves supplemented with a commercial mineral supplement product (magnesium, phosphorus, potassium, selenium and copper) on days 20 and 5 prepartum, and on days 2 and 7 postpartum. Was observed a decreased in concentrations of thiobarbituric acid reactive substances (TBARS), the number of total leukocyte, total protein and globulin levels, and hemoglobin levels. With an increase in activity catalase activity was increased in supplemented cows and in its calves they concluded that mineral provides beneficial effects for the categories evaluated in the study. In another study using the same commercial mineral supplement, Warken et al., (2018) evaluated metabolic parameters, immune response, milk quality and composition from dairy cows in postpartum period. Supplemented animals showed lower levels of ketone bodies and somatic cell count on experimental days 30, 45 and 60, with no changes in milk composition compared to the control group. In addition to presenting lower ROS levels and increased superoxide enzymes activity. Total protein, globulin and cytokine levels were higher in supplemented cows. Concluding that mineral supplementation in this case improved the immune response and minimized oxidative stress in dairy cows during the transition period.

The use of injectable minerals may vary in terms of administration route. In a study using injectable supplementation subcutaneously, Machado et al., 2014 used a supplement composed of 300mg of zinc, 50 mg of manganese, 25 mg of selenium, and 75 mg of copper applied in 125 multiparous cows at at 230 and 260 days of gestation, and 35 days postpartum. Was observed BHB reduction in treated group and cows diagnosed with mastitis had decreased serum SOD activity, and trace mineral supplementation increased serum SOD activity although leukocyte function was not affected by supplementation. Another study injectable supplementation

subcutaneously, evaluated the use of minerals in the face of an aflatoxin challenge. Pate and Cardoso (2018) used a 300mg of zinc, 50 mg of manganese, 25 mg of selenium, and 75 mg of copper mineral product in 29 lactating multiparous Holstein cows at the first day of experiment and day 29. They challenge the cows from day 57 to 59 with 100 μg of aflatoxin $B_1$/kg of dietary dry matter intake (DMI) administered orally via balling gun and the experiment took place until day 63. Treated cows had greater milk urea nitrogen and blood urea nitrogen. Liver concentrations of Se and Fe were greater. And tended to have greater plasma glutathione peroxidase activity. Concluding that subcutaneous injection of trace minerals maintained an adequate antioxidant response when an AF challenge was present.

Since the parenteral mineral supplementation, are usually commercial products, the way the administration should be carried out is a manufacturing companies decision whether it will be intravenously or subcutaneously. There is no comparative study in relation to these two routes of application, elucidating in which one the best mineral bioavailability occurs. In addition, the effect of using minerals looking for beneficial effects in relation to biomarkers of energy metabolism, oxidative stress and immunity in dairy cows, described in the literature, is still controversial since there is no unanimity among authors regarding the found results.

Considering that most of the studies of the transition period of cows emphasize biochemical profile and reproduction, studies relating the immune response, inflammatory response and oxidative metabolism, associating the administering injectable minerals effects are lacking. Most studies focused on immunity involving injectable minerals aimed at understanding the biomarkers of immunity for calves, or the colostrum quality that will be produced (BORDIGNON et al 2019; ALHUSSIEN et al 2021; MATTIOLI et al 2020; GLOMBOWSKY et al. 2018; TEIXEIRA et al. 2014; STOKES et al. 2019; STOKES et al. 2020; TOMASI et al., 2018). Studies seeking to understand the humoral immunity improvement in cows during the transition period moment are scarce, although it composes the first line of defense being essential to stop metabolic and infectious diseases progression or severity, that are recurrent in this period of dairy cow production. Therefore, the hypothesis of this study was was that intramuscular injectable supplementation with a commercial product containing phosphorus, potassium, magnesium, selenium, and copper and have a positive effect

on biomarkers of energy metabolism, oxidative stress and immunity in dairy cows during the transition period.

# 2 LITERATURE REVIEW

## 2.1. MINERALS

### 2.1.1. MACRO MINERALS AND TRACE MINERALS

Minerals make up about one-twentieth of body weight (b.w.) and particular mineral amount in the animal's body varies according to particular part function. Calcium (Ca) normally constitutes 1.3% b.w., phosphorus (P) 0.7%, sodium (Na) 0.16%, potassium (K) 0.19%, magnesium (Mg) 0.04% and sulfur (S) 0.15%. Mineral requirements expressed according to their type, with macro minerals being a percentage of diet dry matter, or measured in grams, while trace minerals are present in smaller amounts, in order of parts per million (ppm) or micrograms per gram (µg/g) of tissue (MULTIMIN TECHNICAL MANUAL, 2021).

About macro minerals, phosphorus is the second most abundant in body, accumulating mainly in bones and teeth (approximately 80%), where it fulfills structural functions. The remainder is found in soft tissues, where it plays a physiological role with phospholipids in cell membranes. It also composes mechanisms of signaling and regulation of enzymatic activity and is an essential component of nucleic acids (DNA and RNA) and adenosine triphosphate (ATP), contributing to acid-base balance. Inadequate levels of P generate a significant reduction in appetite and alter reproductive processes (BOWEN et al., 2020). Consequently, it is common to observe a reduction in the weaning rate, lower milk production and lower calves' survival, which also show reduced growth (HOPKINS et al., 2021). In general, the P requirement in dairy cows is met with dietary concentrations of 0.34 to 0.42%, depending on milk volume produced, lactation time, and diet composition. In breeding systems, adult cows meet their needs during the annual reproductive cycle with an amount equivalent to 12g of P/day (NRC, 2001; NASEM, 2021).

Potassium is an essential macroelement to support nerve and muscle excitability, as well as water and acid-base balance. Although most feeds used by cattle have adequate or excessive amounts of K. For lactating dairy cattle, the NRC has defined a requirement of 8-10 g K/kg DM (NRC, 2001; NASEM, 2021). Even so, there is an increase need for K at the beginning of lactation, due to this element concentration in milk, which can be a limiting factor in its production. Since potassium is a main intracellular ion in tissues, its deficiency signs is a reduction in appetite and, consequently, a low growth rate that ends up being associated with protein

deterioration metabolism. The transition to a deficiency state occurs quickly, since the body contains practically no reserves of K (SUTTLE, 1998).

Magnesium (Mg) also plays an important structural role as part of bones and teeth, simultaneously participating in processes of high biological relevance such as cell signaling being essential for nerve and muscle excitation and conduction, energy stabilization compounds, DNA and RNA molecules, and central neurotransmitters functionality. Mg participates in adenylate cyclase activation, which when stimulated forms an intracellular messenger called cAMP, necessary to modulate adrenaline cellular responses, parathyroid hormone, glucagon, adrenocorticotropin, luteinizing hormone, thyrotropin and vasopressin, among others (ROSOL et al., 1997) . NRC established that Mg requirements can be met with feeds containing 0.2% Mg/kg DM for all categories. However, the same NRC clarifies that if there is excess K in the diet, the requirement can be raised to 0.3-0.35% (11,67), for cows at the beginning of lactation the NRC proposes requirements of 0.2% Mg/kg MS (NRC, 2001; NASEM, 2016).

At least seven trace minerals can be classified essential for dairy cows, based on identification of metabolic functions. Many act as essential cofactors for enzymes involved in biochemical processes. Even marginal deficiencies can reduce growth, production, reproduction or health in cattle with few or no clinical deficiency signs. Among the essential trace minerals are cobalt (Co), copper (Cu), iodine (I), manganese (Mn), molybdenum (Mo), selenium (Se) and zinc (Zn) (ARTHINGTON & RANCHES, 2014).

Selenium (Se) is an essential trace mineral for enzymes metabolism, many that are part of the antioxidant defense system. It also plays a crucial role in insulin metabolism and is essential for formation and activation of inactive T4 thyroid hormones into active T3 (MEHDI et al., 2016), since are Se-dependent enzymes involved. Se is absorbed in the intestine, and absorption coefficient varies according to origin source (organic or inorganic). As a result, true digestibility varies between 50 and 66%, so the liver receives Se which is destined for proteins synthesis and selenoenzymes (WEISS, 2005). The most characteristic clinical presentation of Se deficiency is White Muscle Disease (incoordination, paralysis, depression and prostration, death), which affects animals in growth phase. The subclinical consequences of Se deficiency are generally by a lower antioxidant capacity, although

they overlap and are complemented by thyroid function failures. In dairy production systems, Se deficiency is associated with a higher incidence of placental retention, higher somatic cell counts, higher mastitis incidence, metritis, decreased milk production and fertility (MEHDI et al., 2016). Se plays a role in antioxidant defense by affecting neutrophil function, since it is also involved in antibodies production, vaccine response and reproduction. Se is an essential component of glutathione peroxidase (GPx) and thioredoxin reductase and appears to be the most significant trace mineral in the antioxidant system (ANDRIEU et al., 2008). Se deficiency damages cell and mitochondrial membranes, reducing neutrophils, macrophages and lymphocytes the activity and lifespan (HEFNAWY et al., 2010), beyond to affects energy metabolism by increasing insulin resistance (PICCO, 2019). In dairy cattle Se requirements are adequate in all categories with diets added to 0.3 ppm of dry matter, but whose total Se values are between 0.35 and 0.4 ppm (NRC, 2001; NASEM, 2016).

Copper (Cu) is involved in numerous physiological functions, such as hemoglobin formation, iron absorption and mobilization, and connective tissue metabolism (UNDERWOOD et al., 1999). Have an essential role in processes of high biological relevance such as protection against oxidative stress, energy metabolism, iron metabolism, angiogenesis, hypoxemia response and neuromodulation. It has action in neutrophils production, phagocytosis and pathogens elimination. Its involvement in enzymatic function has a role in antioxidants action and is necessary for antibodies production (CORAH, 1996). More specifically, it is an essential component of the antioxidant enzymes copper-zinc superoxide dismutase (Cu-Zn-SOD) and ceruloplasmin (SPEARS et al., 2008) and exerts an anti-inflammatory effect that may prevent infections and inflammation that cause oxidative tissue damage (SUTTLE, 1998). Cu uptake is low (<1.0–5.0%) in ruminants compared to values reported in non-ruminants, this malabsorption is largely due to complex interactions that occur in the rumen environment. Before the development of a functional rumen, Cu absorption is high (70-85%) but decreases dramatically after weaning (to < 10%). Is well documented that Cu requirements vary considerably with other dietary components concentrations, especially Mo (SPEARS, 2003).

### 2.1.2. PARENTERAL MINERAL SUPLEMENTATION

Usually, minerals and vitamins supply to dairy cattle occurs through mixtures (premix or unit) that are added to diet (ABUELO et al., 2014; PASCHOAL et al., 2006). Oral mineral supplementation through diet constitutes a "continuous direct supplementation" method. In contrast, parenteral supplementation constitutes a "direct discontinuous supplementation" method. Minerals contribution, regardless the administration method, must be sufficient for these elements to fulfill their functions, without causing cellular or tissue damage. For this, minerals must be strictly controlled, in order to keep within strict limits, avoiding deficiencies or toxicity (KREPLIN et al., 1994).

However, minerals and vitamins application, even being less widespread, are presented as an option, which can contribute to improve animal performance (ABUELO et al., 2014; COLLET et al., 2017). When minerals enter parenterally, they are transferred from injection site to bloodstream, preventing interactions between ingredients or antagonism between supplied minerals, as they directly reach organs and tissues, many of them being captured by liver. Parenteral route, ensures a known dose with individual application, avoiding intake fluctuations normally observed in voluntary consumption models (ARTHINGTON et al., 2014). In addition to solving the relative bioavailability problem of a mineral present in food and the absorption capacity (absorption coefficient) variability, therefore, in practical terms, it is possible to assume that its absorption is complete, which simplifies understanding of amounts with each application (PICCO, et al., 2006; FAZZIO, et al., 2017).

Injectable supplementation has a competitive advantage in relation to oral supplementation systems, when environmental conditions not guarantee an adequate or uniform mineral consumption, when the therapeutic use. On the other hand, injectable supply increases management practices with animals, requiring adequate infrastructure availability (COOKE et al., 2017).

## 2.2 OXIDATIVE METABOLISM AND ANTIOXIDANT SYSTEM

Molecular oxygen is required as an electron acceptor for efficient energy production in all living aerobic organisms. Free radicals are formed as a normal end cellular product metabolism arising from the mitochondrial electron transport chain or from stimulation of nicotinamide adenine dinucleotide phosphate (NADPH) (VALKO et al., 2007). Free radicals are defined as molecules that have at least a single unpaired electron in the outer orbit and can promote electron transfer through oxidation and reduction reactions (HALLIWEL et al., 2007).

A small amount of the oxygen consumed (2 to 5%) for the oxidation of organic compounds and energy production for cellular metabolism is reduced, producing a variety of highly reactive chemicals called reactive oxygen species (ROS). This term is used to designate free radicals related to oxygen metabolism (FERREIRA, 2007) (Table 1), as some reactive agents do not have unpaired electrons, such as hydrogen peroxide ($H_2O_2$) (CHIHUAILAF et al., 2002). The organism under normal conditions neutralizes the reactive capacity of reactive oxygenated metabolites (MOR), among which are the superoxide anion ($^{\bullet}O_2^-$), hydroxyl radicals ($OH^-$) and alkoxy radicals (AR), by the action of the antioxidant system (MILLER et al., 1993). On the other hand, $H_2O_2$ is able to cross the nuclear membrane and induce damage to the DNA molecule through enzymatic reactions (ANDERSON, 1996; NUNES, OLIVEIRA, 2006).

The ROS are a class of free radicals derived from molecular oxygen, the superoxide anion ($^{\bullet}O_2^-$) is one of the main ROS produced mainly within mitochondria and results from the electron transport chain. The $^{\bullet}O_2^-$ formation occurs when molecular oxygen receives an electron in place of a metalloprotein of the respiratory chain (Table 1). Activated neutrophils and macrophages are another important source of $^{\bullet}O_2^-$, which is enzymatically formed by NADPH oxidase during inflammatory reactions. The $^{\bullet}O_2^-$ can interact with other molecules to generate other varieties of ROS. A reaction catalyzed by superoxide dismutase (SOD) inside the mitochondrial membrane is capable of forming oxygen ($O_2$) and hydrogen peroxide ($H_2O_2$) (Table 1). (SORG, 2004).

The ROS accumulation can result in substantial tissue damage in mammals. The main targets include lipids, proteins, DNA and other macromolecules. The hydroxyl radical ($OH^{\bullet}$) is highly reactive, being formed by Fenton or Haber-Weis reactions and can lead to membrane lipid peroxidation and generation of additional ROS (SORDILO & AITKEN, 2009). The $OH^{\bullet}$ is also capable of causing oxidant-induced damage to all

components of the DNA molecule and leading to genetic mutations and abnormal protein synthesis (HALLIWEL et al., 2007; VALKO et al., 2007). Cysteine and methionine residues from proteins are particularly susceptible to oxidation by several different ROS and can lead to the reversible formation of mixed disulfides between a variety of thiol groups, such as glutathione reductase (GSH). Oxidative modification of groups of thiol proteins can regulate protein function and influence various metabolic, signaling and transcriptional processes in cells. While low levels of ROS can actually facilitate normal cell function, excessive protein oxidation can lead to cell dysfunction or premature protein degradation (EATON, 2006).

Table 1 - Initiation and propagation of reactive oxygen species and their interaction with organic substrates

| Reaction | Product |
|---|---|
| Reactive oxygen species formation | |
| $O_2$ - + e $^-$ → $•O_2^-$ | Superoxide anion |
| $2•O_2^- + 2H^{+\ SOD} \rightarrow O_2 + H_2O_2$ | Hydrogen peroxide |
| $•O_2$- + Fe+ → $O_2 + Fe^{2+}$ | Reduced iron |
| HOOH + $Fe^{2+} \rightarrow Fe^{3+} + O_2$ + OH• | Hydroxyl radical |
| Interaction with organic substrates | |
| OH• + RH ou LH → $H_2O$ + R• ou L• | Fatty acid or other oxidized organic compound |
| R• + LH → RH + L• | Oxidized fatty acid |
| L• ou R• + $O_2$ → $LO_2$• ou $RO_2$• | Lipid peroxyl radical |
| $LO_2$ + LH → L• + LOOH | Lipid peroxide |

Sorce: MULTIMIN TECHNICAL MANUAL, 2021

The production of ROS during oxygen metabolism has raised the need for the development of antioxidant defenses that can effectively capture reactive intermediates before they are able to cause oxidation of macromolecules or reduce biomolecules that have already been oxidized. . From a biological point of view, antioxidants can be defined as those that protect biological systems against deleterious effects resulting from reactions that lead to oxidation. Antioxidants can theoretically prolong the initiation phase or else inhibit the propagation phase, but cannot completely prevent oxidation (HALLIWEL et al., 2007).

Antioxidants can be didactically classified into two categories, the primary system, which are preventive inhibitors, which delay the initiation phase, preventing the

generation of reactive species or sequester these species, preventing their interaction with cellular targets. The secondary system consists of blockers of the radical chain propagation step (chain breaking), capable of effectively removing intermediate radicals, such as the peroxyl or alkoxyl radical (JORDÃO JR et al., 1998). They can also be classified according to the predominant mechanism of action (prevention, interception and repair) or by their origin, from endogenous synthesis (endogenous) or from the diet (exogenous) as is the case with carotenoids and vitamins A, C and E, which are able to supply hydrogen atoms to ROS (FERREIRA, 2007) (Table 2).

Among the most efficient antioxidants are enzymes that can directly catalyze the reduction of ROS. As is the case of the dismutation of superoxide into $H_2O_2$ and $3O_2$ catalyzed by SOD or $H_2O_2$ into $H_2O$ and $3O_2$ catalyzed by catalase. In addition, there are selenium-dependent antioxidant enzymes that have beneficial effects mediated by antioxidant selenoenzymes, due to selenocysteine residues incorporated into their active sites. Glutathione peroxidase (GPx) is the selenoenzyme most frequently associated with antioxidant functions in cattle (SMITH et al., 1997; WICHTEL, 1998). There are other bovine antioxidant selenoproteins including selenoprotein P, 5 different GPx isoforms and 3 thioredoxin reductase (TrxR) isoenzymes (GRIGNARD et al., 2005; HARA, 2001).

Glutathione peroxidase (GPx) is a metalloenzyme that forms part of the glutathione system, known as the main antioxidant system in the body. There are three types of GPx: cellular, extracellular (plasma) and phospholipid hydroperoxide glutathione peroxidase. The main known function of GPx is to inactivate MOR derivatives of aerobic metabolism, being responsible for protecting the membrane of cells that function in the presence of oxygen (MILLER et al., 1994). Also participating in the chain of reactions that catalyze the formation of prostaglandins, leukotrienes, prostacyclins and thromboxanes from arachidonic acid (STADTMAN, 1990), being related to the functioning of the immune system and the integrity of the reproductive tract (HURLEY et al., 1989).

The GPx activity can reduce a large amount of ROS resulting in reduced glutathione (GSH) it plays an important role in protecting cells against oxidative stress and toxic agents. They act as substrate or co-substrate in enzymatic reactions and react directly with ROS and lipid peroxides (BRIVIBA; SIES, 1994). Rover Junior et al., (2001) described that the activity of GSH can provide important biochemical information on

the oxidant and antioxidant relationship, allowing clinical-laboratory correlations with mutagenic processes that quantify its levels and indicate the intensity of lipid peroxidation when associated with complementary tests that trace the biochemical profile about possible diseases associated with oxidative stress. GSH are oxidized more rapidly than hemoglobin and other erythrocyte constituents, protecting them from oxidative degradation. As part of the GSH redox cycle, one $H_2O_2$ molecule is reduced to 2 $H_2O$ molecules, while 2 GSH molecules are oxidized in a GPx-catalyzed reaction (Table 2) (HALLIWELL, 2007; POPES, 1999).

Table 2 - Initiation and propagation of reactive oxygen species and their interaction with organic substrates

| Component | Location | Nutrient involved | Function |
|---|---|---|---|
| Enzymatic antioxidants | | | |
| Superoxide dismutase (SOD) | Cytosol | Cu, Zn | Enzyme that converts superoxide ion to hydrogen peroxide |
| Superoxide dismutase (SOD) | Mitocôndria | Mn, Zn | Enzyme that converts superoxide ion to hydrogen peroxide |
| Glutathione peroxidase (GPx) | Cytosol | Se | Enzyme that converts hydrogen peroxide to water |
| Catalase | Cytosol | Fe | Enzyme that converts hydrogen peroxide to water |
| Non-enzymatic antioxidants | | | |
| Ceruloplasmin | | Cu | Antioxidant protein that prevents Cu from participating in oxidation reactions |
| α- tocopherol | Membranes | Vitamin E | Stops the peroxidation of fatty acids |
| Carotene | Membranes | Carotene | Prevents fatty acid peroxidation |

Sorce: MULTIMIN TECHNICAL MANUAL, 2021

The existence of numerous antioxidants that can function both in the hydrophilic phase (cytosol and extracellular fluids) and in the lipophilic phase (membrane lipids), being essential to maintain tissue integrity during oxygen metabolism. Under normal physiological conditions, the body's own antioxidant defense systems can handle the ROS that are produced, being able to efficiently neutralize and eliminate them. Oxidative stress is a condition that occurs when ROS production exceeds the capacity of antioxidant defenses to neutralize these pro-oxidants, resulting in oxidative damage to lipids, DNA, proteins and other macromolecules (BRENNEISEN et al., 2005). The imbalance results from excessive accumulation of ROS, decreased antioxidant defenses, or a combination of these situations. Oxidative stress is often associated with numerous diseases, but it is not always known whether ROS accumulation is the cause or consequence. The general concept is that oxidative stress causes cellular damage throughout the body and can result in compromised immune and inflammatory reactions (SORDILO & AITKEN, 2009).

## 2.3 IMMUNE RESPONSE

The immune system is divided into innate and adaptive. The innate immune system is responsible for the host's first line of defense, which after an invasion, establishes an immediate response, while the adaptive immune system is activated late and induces a long-lasting specific immunity (ALBIGER et al., 2007).

Innate immunity encompasses the physical, chemical, and cellular elements of the immune system that provide immediate non-specific defense. Approximately 95% of infectious challenges are resolved by the innate system, through defense cells (neutrophils, monocytes and macrophages), humoral factors (complement, lysozyme) and cytokines (DAHA et al., 2011). The cells involved in the innate immune system are composed of cells of the monocyte/macrophage lineage, natural killer cells (NK), dendritic cells, gamma delta T cells (gdT), mucosa-associated invariant T cells (MAITs), and granulocytes (ABBAS et al., 1997). A common feature of these cell populations is their ability to recognize and destroy foreign antigens or infected cells, without the need for having previously encountered such antigens. For this purpose, such cells use phagocytosis and oxygen-dependent activities, related to the respiratory burst, and non-oxygen-dependent, fundamental functions in the destruction and elimination of the invading agent (ALBERTS et al., 2002). In addition, phagocytes

interact with the other cells that make up the immune system (lymphocytes) acting as antigen-presenting cells and, thus, participating as triggers and effectors in the generated immune response (ABBAS et al., 1997).

Among the actions of the innate system are actions such as phagocytosis performed by polymorphonuclear cells (PMN), humoral factors (such as the complement system), cytokines and PPR molecules that interact with pathogen-associated molecular patterns (PAMPs) during the initial stages of the immune response (ACKERMANN et al., 2010). The best characterized among PPRs are toll-like receptors (TLRs). Ten TLRs with diverse and sometimes overlapping PAMP affinities have been confirmed in cattle (NOVAK et al., 2013). Cytokines are considered essential during integration between animal metabolism and immune function. After an inflammatory response initiation, PMN cells migrate to the site through the process of chemotaxis. Once a pathogen is involved, the oxidative burst occurs in activated phagocytes. Changes in functions inherent to such cell populations, induced or not by exogenous factors interaction (physical injuries or intracellular infections, such as virus) leukocytes populations, can lead to a depletive state to protection provided by the immune system, characterizing by immunosuppression (SORDILLO et al., 1997).

Natural antibodies (NAbs) are an important humoral component of innate immunity. They are mostly IgM (and some IgG and IgA) produced without antigenic stimulation by B1-B cells and play an essential role in primary immune response (PLOEGAERT et al., 2011). A high proportion of NAbs binds to PAMPs with relatively low affinity, but complement activation by the classical pathway is one of the most important NAb functions (VLASOVA et al., 2021). Complement is a defense mechanism that it consists of a group of proteins (C1-C9) present in serum in an inactive form that is activated by antigen- Ab complexes (classical pathway) or by some carbohydrates (lectin pathway) or by a variety of surfaces that are not protected by natural inhibitors (alternative pathway). Apart from its direct antimicrobial effects, complement maintains Igs in soluble form by limiting the formation of harmful immune complexes and Ig precipitation (RAINARD et al., 2003).

Bovine adaptive immune responses are driven by a combination of common and unique aspects that need to be considered when developing age- and herd-specific preventative and therapeutic strategies. Adaptive immunity comprehend B cells, T

cells, antibodies, effector T cells and at least five heavy chain classes (IgM, IgG, IgA, IgD and IgE), with three IgG subclasses (IgG1, IgG2 and IgG3), two IgM subclasses (IgM1 and IgM2) and two light chain types (l and k) in bovine plasma (STANFIELD et al., 2018). In contrast to many other animals, cattle only express a limited number of variable Ig gene segments, and it is thought that Ig diversity is achieved via frequent recombinations and endogenous mutations in the CDR3 region (ZHUANG et al., 2007). Immunoglobulin functions include neutralizing antibody, complement activation, Fc receptor-mediated phagocytosis, and antibodies-dependent cellular cytotoxicity (WANG et al., 2013). IgG1 and IgG2 are highly important, with IgG1 being the most abundant in cow colostrum. IgG is important for virus and toxin neutralization and bacterial agglutination and opsonization. In cattle, IgG1 is known to be a less potent opsonin than IgG2 (MAUNSELL et al., 2019).

Acute phase proteins can be classified as positive and negative. Haptoglobin (Hp), ceruliplasmin (Cp), alpha1-acid glycoprotein (AGA), serum amyloid A (ASA) and C-reactive protein are positive acute-phase proteins, since their concentrations increase in diseases face (inflammatory and infectious). Albumin and transferrin are considered negative acute phase proteins, with a decrease in serum concentration (GONZÁLES et al., 2007; BELL, 2010; TÓTHOVÁ et al., 2011) where the concentration is directly proportional to tissue injury degree and/or inflammation (MARTYNEZ-SUBIELA, 2005).

Acute phase proteins activation is closely related to pathogen recognition mechanism by the innate immune system. This activation event occurs after breakdown of the protective physical barrier, protective mucus, mucous membranes and skin. Different microorganisms have different molecules that cause different types of immune responses, such molecules are called pathogen-associated molecular patterns (PAMPs). Pattern recognition is mainly performed by dendritic cells, monocytes and macrophages, mononuclear cells, through pattern recognition receptors (PRRs), molecules located on these cells membrane (TIZARD, 2008).

After PAMPs bind to PRRs, intracellular expression of transcription factors occurs, an important signaling pathway for immune responses. These transcription factors stimulate the synthesis of pro-inflammatory cytokines interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α). These cytokines stimulate the liver to produce acute phase proteins, especially haptoglobin in cattle. Liver is the main

producer of haptoglobin (YANG et al., 1995). However, the mammary gland, as well as other tissues that express genes, are also capable of producing it (HISS et al., 2004).

Haptoglobin is a glycoprotein that migrates in the alpha-2 globulin region in electrophoresis, in which is composed of two alpha subunits and two beta subunits, subunits combine in a β-α-α-β tetrameric chain form (MORIMATSU et al., 1991). An important haptoglobin ability is binding affinity with hemoglobin, in which when bound they form complexes described as Hp-Hb. Human haptoglobin has three subtypes due to its genetic polymorphism: Hp 1-1, Hp 1-2, Hp 2-2. In animals, the tetramer has differences, with Hp 1-1 being similar for carnivores and omnivores, while bovines have similarities with Hp 2-2 (YUEH et al., 2007). The different haptoglobin types promote different responses, Hp 1-1 promotes a reduction in reactive oxygen species (ROS) production, with antioxidant potential, binding to Hb and anti-inflammatory function, repairing and decreasing Th2 response. Hp 2-2, on the other hand, increases oxidative stress and allows inflammatory stimulus persistence, leading to a Th1 response. In modulation, haptoglobin binds to hemoglobin and decreases ROS release. Despite advances in research, there is still a need for further studies of the relationship between Hp and the modulation of immune responses (HUNTOON et al., 2008).

## 2.4 THE TRANSITION PERIOD AND THE METABOLIC STRESS

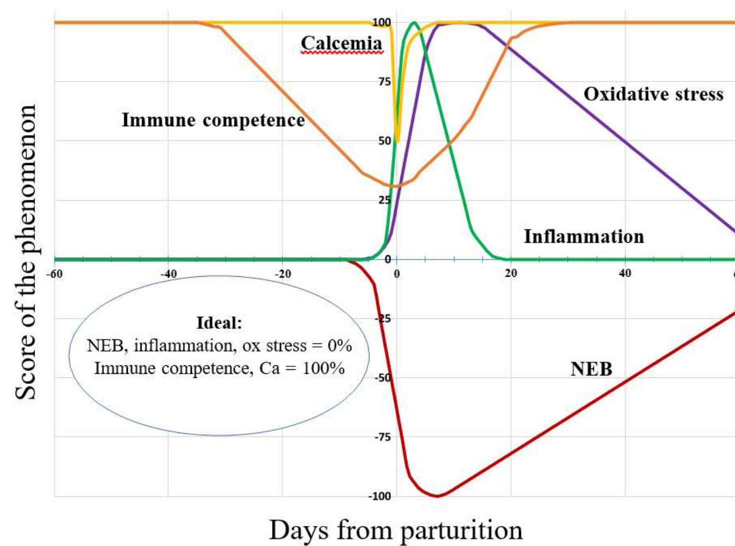
Dairy cows undergo major metabolic and physiological adaptations during the transition from pregnancy to lactation (CAMPBELL AND MILLER, 1998). The transition period, commonly defined as three weeks pre-calving to three weeks post-calving (DRACKLEY, 1999) is the most challenging and critical period regarding the dairy cow health status during the lactation cycle. During this period, several anatomical, physiological, hormonal and metabolic changes occur in the animal. Due to these changes, this is the period of greatest nutritional concern with metabolic manifestation and infectious disorders in the productive cycle (DUBUC et al., 2010).

During the peripartum period, there is a great increase in fetal growth, with an increase in internal pressure in digestive organs and a decrease in space occupied by food. This fact associated with the great hormonal variation of this period, that is, an increase in the blood concentrations of estrogen and corticoids and a decrease in the concentrations of progesterone (CHEW et al., 1979). Causing decreasing in dry matter

intake (DMI) reaching 10 -30% (BELL, 1995), while energy and calcium demands for lactation increase (CHAPINAL et al., 2012). These demands generates a greater oxygen consumption by tissues through cellular respiration in moments of greater metabolic demand. This process occurs in order to provide the necessary energy for the onset of lactation, but results in a negative energy balance (NEB) (KONVICNÁ, et al., 2015). As consequence of late calcium availability in blood, hypocalcemia occurs as a consequence of mammary gland demand for milk the synthesis (GOFF & HORST, 1997). The systemic inflammatory response during calving, even in absence of microbial infections signs or other pathologies (TREVISI et al., 2012) and oxidative stress, due to unbalanced availability of antioxidants in presence of phenomena that increase pro-oxidants production (SORDILLO et al., 2009).

These conditions are considered physiological adaptations due to state change, but when they are dramatic and prolonged (TREVISI et al., 2016) they hinder the process of homeostasis recovering and some adaptive mechanisms can be deregulated, explaining the appearance of metabolic (DRACKLEY, 1999) and infectious diseases (GOFF & HORST, 1997). Most changes occur immediately after calving, with some immune response changes occurring a few days prepartum. Thus, understanding the origin of these changes and the moment they appear seems fundamental for understanding the breaking point of homeostasis in the transition period (TREVISI & MINUTI, 2018) (Figure 1).

Figure 1. Theoretical pattern of changes in the main physiological aspects of cow healthy during the transition period. Being ideal, the negative energy balance (NEB), inflammation, and oxidative stress are close to zero, where they do not occur in the body. On the other hand the immunocompetence and the calcemia be close to 100% of their optimal level.




Sorce: TREVISI & MINUTI, 2018

Pregnancy and lactation are considered physiological stages capable of inducing metabolic stress (DRACKLEY, 1999; PICCIONE et al., 2009). During the transition period, metabolic stress describes the catabolic hypermetabolic response to this disruption in physiological homeostasis and is characterized by excessive lipomobilization, immune and inflammatory dysfunction, and oxidative stress. (ABUELO et al., 2013). These three processes are intrinsically linked and result in immunological and metabolic derangements that are associated with increased risk of metabolic and infectious diseases during this period (SORDILLO et al., 2014). Approximately 75% of the incidence of diseases such as mastitis, metritis, ketosis and displacement of the abomasum in dairy cows occurs in the first month of lactation, especially in the first 10 days after calving (LEBLANC et al., 2010).

Body fat is mobilized into bloodstream in non-esterified fatty acids (NEFA) form in early lactation, these are primarily mobilized by the liver and are either oxidized in the mitochondria to produce energy or exported in low-density lipoproteins form (TREVISI et al., 2016). NEFA are used in more than 40% of milk fat composition during the first days of lactation (BELL, 1995) and the liver metabolizes as soon as it is produced

(REYNOLDS et al., 2003), but it does not have sufficient capacity to eliminate them completely. So due to large amounts of NEFA release from adipose tissue into circulation, as a result are triglycerides accumulation in the liver (EMERY et al., 1992).

After mobilization and release of NEFA, this metabolite can be used by muscle tissue as an energy substrate, can reach the mammary gland and be transferred to milk fat, or finally can reach the liver. In the liver, NEFA will be metabolized and can follow different paths. When there is not a large amount circulating, NEFA can complete oxidation with ATP production for local use in the liver tissue. Other possibility is be re-esterification into triglycerides to be exported as an very low density lipoprotein (VLDL) to other tissues. Complete NEFA oxidation generates acetyl coenzyme A that can be used to generate energy through the Krebs cycle, however if the Krebs cycle is overloaded, acetyl Co A is diverted to produce ketones (acetoacetic acid, acetone, and β-hydroxybutyrate or BHB) due to incomplete oxidation. The last paths for NEFA is when triglycerides re-esterification and causes accumulation in liver tissue, an alteration called hepatic steatosis or fatty liver. The first two routes are desirable, but quickly saturated, and thus the third and fourth destinations, which are undesirable options, occur in a marked way in case of exacerbated mobilization (BELL, 1995; DRACKLEY, 1999; GRUMMER, 1995; ALVES, 2009; FERNANDES et al., 2012; ROSSI et al., 2004; DUFFIELD and BAGG, 2002).

Despite the importance of NEFA in early lactation, when the mobilization is very intense, ketone bodies production increases and ketosis occurs by an imbalance in body fat use in early lactation and is characterized by a state of hypoglycemia and hyperketonemia (LOOR et al., 2006). BHB levels greater than or equal to 1.2 mmol/L characterize subclinical ketosis or hyperketonemia, while values greater than or equal to 2.9 mmol/L characterize clinical ketosis or hyperketonemia (McART et al., 2012). This subclinical ketosis is a normal condition that at least 50% of all dairy cows go through a temporary period of in the first month of lactation (ZERBE et al., 2000). The high lipid reserves mobilization increases metabolic diseases occurrence (INGVARTSEN et al., 2003; CAMPOS, 2007; RODRIGUES et al., 2007). Pre and postpartum NEFA levels greater than 0.3 and 0.6 mmol/L, respectively, are associated with an increased risk of retained placenta, metritis, displaced abomasum, clinical ketosis, drop in production (683 kg in the period of 305 days) and increase in the open period (OSPINA et al., 2010; LEBLANC et al., 2010).

Some immune system functions are depressed before calving, such as neutrophil phagocytosis, lymphocytes ability to respond and produce antibodies, reduced immunoglobulins, IFNγ complement system and lysozyme (LACETERA et al., 2005). Added to the fact, that severely reduced prepartum food intake is an important factor in peripartum impairment of immunity (TREVISI et al., 2012). Immune dysfunctions before calving are due to a combination of endocrine and metabolic factors, but it is not clear whether reduced immunocompetence is a physiological condition of dairy cows or an early sign of disease induced by other events (BERTONI et al., 2009).

The metabolic challenge during the peripartum period leads to an increase in the inflammatory response, generating a greater nutrient deficit and the emergence of metabolic diseases (MANN et al., 2019). In addition, during this period, the liver also acts in the immune system regulation through immune responses, responding to pro-inflammatory cytokines by producing acute phase proteins (CECILIANI et al., 2012) in addition to their functions in metabolism. Glucose is vital for proper metabolic function and immunity because it is the main metabolic fuel for many of the immune cells (LEBLANC et al., 2010). Low glucose concentrations have been associated with less effective action of polymorphonuclear neutrophils and are often seen at the same time as reductions in reduced glutathione (GSH) concentrations (INGVARTSEN et al., 2013), both impairing host defenses. However, this pro-inflammatory condition during the transitional period cannot be considered from a negative point of view, as inflammation assists in facilitating labor (HUZZEY et al., 2009) and may play a role in homeoretic adaptations to initiation of lactation (FARNEY et al., 2013). Although endocrine and metabolic factors contribute to immune dysregulation during this period, early lactation is probably the main contributing factor (KIMURA et al., 2002).

Although the ruminant does not use glucose as a universal source for all its cells, this substrate is of fundamental importance for energy maintenance of nerve cells, mammary gland, musculature, fetal tissues and, to a lesser extent, erythrocytes (LENG; ANNISON, 1962). In dairy cows, the massive amount of energy needed to support milk production comes mostly from gluconeogenesis. This increase is probably a consequence of lactogenesis and the beginning of lactation, when there is an increase in circulating prolactin, estrogen, somatotropin and cortisol, which reduce insulin responsiveness and sensitivity. This “resistance” to insulin leads to an increase in hepatic gluconeogenesis, a reduction in glucose uptake by muscles and adipose

tissue, a reduction in lipogenesis and protein synthesis, and an increase in muscle protein degradation with the consequent release of amino acids (BELL; BAUMAN , 1997).

Hypocalcemia can also be a consequence of challenges to the immune system (ECKEL & AMETAJ, 2016) since a low concentration of calcium in the first two weeks postpartum is associated with a decrease in neutrophil functions (MARTINEZ et al., 2012). Wich indicates plasma calcium concentration maybe be a mediator in several cellular processes, including those related to immune system responses. Calcemia decreases in a dose-dependent manner with lipopolysaccharides (WALDRON et al., 2003) and severe hypocalcemia occurs in a sepsis state or with severe tissue damage such as ruminal acidosis (MINUTI et al., 2014).

Reactive oxygen species are constantly generated in cellular respiration, other products, called reactive nitrogen species (RNSs) are generated at the end of amino acid metabolism. The imbalance between ROS production and antioxidant defense systems, in former favor is called oxidative stress, a pathology in which cell membranes permeability, enzymatic functionality and muscle tone are altered. In this situation, the cellular or physiological condition of ROS high concentration or antioxidant status decreased causes molecular damage to cellular structures, with consequent alteration and impairment of vital functions (DRÖGE, 2002; RIBEIRO; GONZALEZ, 2003; HALLIWELL; GUTTERIDGE; 2007; WEIGEL, 2008). In ruminants, oxidative stress may be involved in several pathological conditions, including those most relevant to reproduction, production and general well-being. After calving, voluntary food intake is not enough to compensate for energy demand for milk synthesis, to meet the increasing energy demands, body reserves mobilization occurs predominantly in adipose tissue, leading to an increase in lipid mobilization as consequence of BEN, which may increase ROS and ERN generation (SORDILLO et al., 2013; CELI et al., 2015).

The increase in ROS production also occurs due to rapid secretory parenchyma differentiation, mammary gland intense growth and abundant onset of milk synthesis and secretion, one of the main challenges for dairy cows in the transition period is a sudden increase in needs of nutrients and oxygen (GITTO et al., 2002). These imbalances, along with decreased intake of dietary antioxidants due to a decrease in overall food intake, may lead to a pro-oxidant shift in redox balance (SORDILLO et al.,

2009; CASTILLO et al., 2005; DALLE-DONNE et al., 2005). Physiologically, the cow's body has enough antioxidant to neutralize ROS/RNS production that are continuously produced during metabolism. However, ROS/RNE production can increase as a result of pathological conditions or increased physiological processes, in addition to cows homeoretic mechanisms (increase in energy required for growth, reproduction and lactation). When an imbalance occurs between ROS/RNS generation and the body's antioxidant capacity, it leads to a shift in oxidant status that can lead to oxidative stress when there is subsequent cell or tissue damage or impairment of function (DALLE-DONNE et al., 2005).

In periparturient cows, enzymatic antioxidants represent the main antioxidant defense mechanisms in protecting cells against increased ROS. Superoxide dismutase catalyses the superoxide radical ($O_2^-$) partition into hydrogen peroxide, which is subsequently reduced to water by the GPx enzyme and is dependent on manganese (Mn), copper (Cu) and zinc (Zn) (ANDRIEU, 2008). In cows, the GPx enzyme activity depends on selenium concentration in body, in addition to serving as a body antioxidant, selenium is necessary for maintenance of other relevant biological functions, such as immune function, thyroid hormone metabolism and reproduction (SURAI, 2006; SORDILLO et al., 2013). Decreased oxidative stress through prepartum antioxidant supplementation resulted in better glucose tolerance in early lactation, suggesting that when oxidative stress is reduced, nutrient utilization may also improve (ABUELO et al., 2016).

The metabolic demands associated with late pregnancy, during calving and early lactation increase ROS production. Immune cells are particularly sensitive to oxidative stress, as their membranes contain high concentrations of polyunsaturated fatty acids (PUFAs) that are very susceptible to peroxidation and produce large amounts of ROS when stimulated and, as a consequence, the released ROS cause cellular damage to tissues (SPEARS et al., 2008). The control of oxidative metabolism in cells is extremely important for the survival of organisms in an aerobic environment. In addition to the protective effects of endogenous antioxidants, the inclusion of antioxidants is of great importance for decreasing the risk of developing diseases associated with oxidative stress. The use of antioxidant compounds found in the diet or even synthetic ones is one of the defense mechanisms against ROS that can be used in order to prevent changes or recover from them (BIANCHI; ANTUNES, 1999). The determination of the

activities of enzymatic antioxidants, such as glutathione peroxidase (GPx), reduced glutathione (GSH) and total antioxidant status (TAS) or oxidants such as thiobarbituric acid activity (TBARS) represent one of the ways to assess oxidative stress (KLECZKOWSKI et al., 2003).

# 3 OBJECTIVE

## 3.1 GERAL OBJECTIVE

The aim of this research was to evaluate the influence of injectable mineral supplementation with a commercial product containing phosphorus, magnesium, potassium, selenium and copper on oxidative stress, immune response, and biomarkers of lypolisis in Holstein cows and heifers during the transition period.

## 3.2 SPECIFC OBJECTIVE

- Evaluation of oxidative stress by enzyme activity measurements of glutathione peroxidase (GPx), glutathione reductase (GSH), total antioxidant status (TAS) and thiobarbituric acid (TBARS);
- Analysis of some imune response biomarkers: concentration of haptoglobin and total serum immunoglobulin G (IgG);
- Dynamic of lypolisis by the dynamics of beta hydroxybutyrate (BHB) and non-esterified fatty acids (NEFA) concentration.

# 4 MATERIAL AND METHODS

## 4.1 HERD SELECTION

The herd selected for this study is located in São Pedro-SP city (latitude 22º32'55" south and longitude 47º54'50" west), being characterized as a commercial herd, with Holstein cows and a total of 400 lactation cows (average production 35L/cow and total daily average production of approximately 15 thousand liters). The property has good management practices and daily monitoring by the farm's veterinarians.

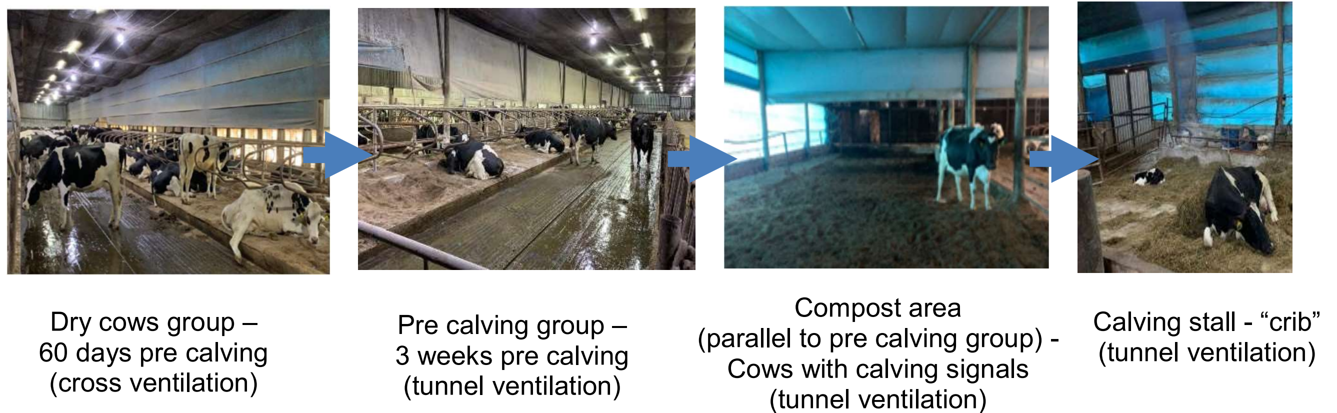
Cows monitored by veterinary diagnoses (reproductive and clinical) and have individual records of milk production and somatic cell counts (SCC) recorded in property management software (Dairy Plan®, GEA Farm Technologies - Canada). The property has two milking systems (carousel milking and robot milking), with most milking performed by the robot, while carousel milking is used for milking in the immediate postpartum period, high SCC and mastitis cows, and those that do not adapt to the robot.

## 4.2 PRE PARTUM AND IMMEDIATE POST PARTUM MANAGEMENT

Drying process takes place 70 days before calving expected date. During the dry period, approximately 60 days before calving, cows are vaccinated with different commercial vaccines against mouth and foot disease, neonatal diarrhea, keratoconjunctivitis, reproductive diseases and mastitis (Fortress 7®, Zoetis; ScourGuard 4KC®, Zoetis; Bioqueratogen®, Biogenesis Bagó ; CattleMaster Gold®, Zoetis; JVAC®, Boehringer Ingelheim).

Approximately 3 weeks pre-calving, cows transferred to a temperature-controlled closed freestall. As soon as the cow shows calving signs (vulvar mucous discharge presence, vulva swelling, udder distention, isolation from group, restlessness) or if it is close to calving expected date, she will kept separate from the group until calving (Figure 2).

Figure 2 - Cow prepartum management flowchart.

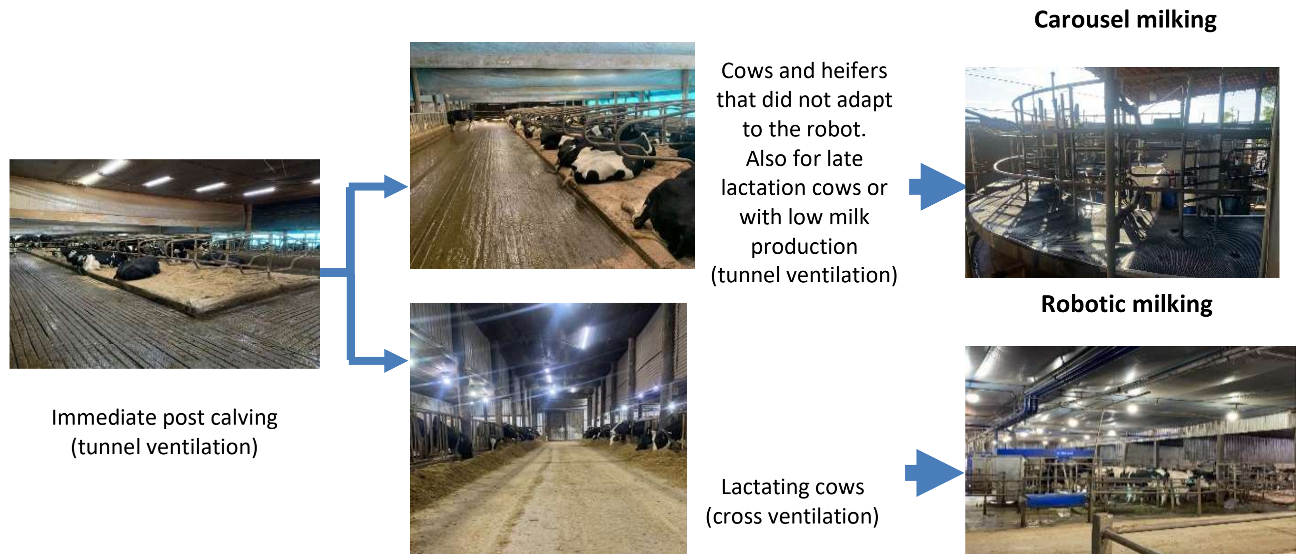



Sorce: MARQUES, 2022.

After calving, the calf is cared for colostrum administration, navel healing navel and identification by earring system. The cow has its first milking to obtain colostrum in the milking carousel, to later be transfer to the immediate postpartum batch (Figure 2), where it remains until first postpartum week. After 24 hours postpartum, cow will be separated for trimming, replacing earring identification, if necessary they will receive a pedometer system (Rescounter II®), cows intended for robotic milking receive a transponder collar (DeLaval®). Tail and ear hair trimming, udder cleaning (hair burning) which also facilitates the robot's reading during milking, and if has retained placenta (24 hours after calving) treatment is performed with ceftiofur (Ceftiomax®) associated with flunixin meglumine (Flumax®) for 3 days.

After postpartum management, the cows destined for robotic milking are divide into 4 lots. The cows are house in a free stall feedlot with cross ventilation system, divided into a resting area with two litter lines with sand, 1 feeding lane, with side access to the feedlot for a mixing cart, waiting room and milking station. (4 robots) (Figure 3). Access to the waiting room is via non-return gates in combination with two automatic gates (VMS™ entrance and separation gate that selects and redirects the cows, after identifying them by transponder collar means). Access to the waiting room is base on hour of day and/or the udder milk amount (estimated by management software).

Figure 3 - Cow postpartum management flowchart.




Sorce: MARQUES, 2022.

Cows with high SCC (> 200,000 cells/mL) and mastitis are kept in a free stall feedlot with cross ventilation system in two separate lots. These cows are milked at the carousel milking, and milk destination depends, if is from high SCC cows is return to milk tank, if is from mastitis cows, contaminated teat milk is discarded.

Cows that do not adapt to robotic milking system, cows in late lactation, or heifers that are adapting to milking, are separate into a temperature-controlled closed freestall lot for carousel milking (Figure 3).

## 4.3 NUTRICIONAL MANAGEMENT

The farm nutritionist following the recommendations of NRC (2001) requirements formulated the cows' diet. The diets formulation offered during prepartum and postpartum period is presented in table 3, while guarantee levels of the commercial premix used are presented in table 4.

Table 3 - Ingredients, nutrients and minerals (% of dry matter – DM) of diets offered at prepartum and postpartum period during the experimental period.

| Ingredients | Prepartum diet (%DM) | Postpartum diet (%DM) |
|---|---|---|
| Corn silage | 67,84 | 42,75 |
| Pre-dried | 5,78 | 2,97 |
| Cottonseed | 8,87 | 5,26 |
| AgMilk Pré parto an 330 premix | 3,18 | - |
| Pós Parto SJ premix | - | 0,43 |
| Lysine | - | - |
| Methionine | 0,09 | - |
| Soybean meal | 8,65 | 8,38 |
| DDG | 5,60 | 7,13 |
| Corn | - | 16,35 |
| Citrus pulp | - | 3,38 |
| Pre-dried silage alfalfa | - | 3,14 |
| Canola meal | - | 6,86 |
| Enerfat (Kemin) | - | 0,45 |
| Smartamine | - | 0,05 |
| Magnesium oxide | - | 0,31 |
| Sodium bicarbonate | - | 1,05 |
| Salt | - | 0,33 |
| Calcitic limestone | - | 1,17 |
| Total | 100 | 100 |
| **Nutrients** | | |
| Crude protein | 14,77 | 17,4 |
| Ethereal extract | 4,26 | 4,40 |
| Mineral residue | 5,93 | 7,26 |
| Raw fiber | - | - |
| ADF | 25,70 | 17,86 |
| Lignin | 5,65 | 3,71 |
| NDF | 44,27 | 35,49 |
| **Minerals** | | |
| Calcium | 0,41 | 1,11 |
| Phosphor | 0,38 | 0,47 |
| Potassium | 0,72 | 0,67 |

Sorce: São Jorge farm’s nutritionist, 2021.

Table 4 - Guarantee levels of prepartum and postpartum commercial premix.

| Itens | Prepartum premix[1] | Itens | Postpartum premix[2] |
|---|---|---|---|
| Calcium (min) | 55 gr/Kg | Biotin (min) | 225mg/Kg |
| Calcium (max) | 85 gr/Kg | Calcium (mín) | 150g/kg |
| Chlorine (min) | 180 gr/Kg | Calcium (max) | 220g/kg |
| Cobalt (min) | 21 mg/kg | Cobalt (min) | 94mg/kg |
| Copper (min) | 420mg/Kg | Copper (min) | 2.500mg/kg |
| Chrome (min) | 16 mg/kg | Chrome (min) | 85mg/kg |
| Sulfur (min) | 72 gr/kg | Sulfur (min) | 31g/kg |
| Fluorine (max) | 250mg/Kg | Fluorine (max) | 1.500mg/kg |
| Phosphor (min) | 25g/Kg | Phosphor (min) | 150g/kg |
| Iodine (min) | 25mg/Kg | Iodine (min) | 200mg/kg |
| Magnesium (min) | 65g/Kg | Magnesium (min) | 34g/kg |
| Manganese (min) | 700mg/Kg | Manganese (min) | 6.000mg/kg |
| Selenium (min) | 18mg/Kg | Selenium (min) | 85mg/kg |
| Sodium (min) | 24g/Kg | Vitamin A (min) | 9000.000UI/Kg |
| Zinc (min) | 2.400mg/Kg | Vitamin D (min) | 440.000UI/Kg |
| Vitamin A (min) | 250.000UI/Kg | Vitamin E (min) | 6.500UI/Kg |
| Vitamin D3 (min) | 100.000UI/Kg | Zinc (min) | 11.550mg/kg |
| Vitamin E (min) | 6.400UI/Kg | *Saccharomyces cerevisiae* | $1.25x10^{11}$CFU/Kg |
| *Saccharomyces cerevisiae* | $3,0x10^{10}$CFU/Kg | Sodium monensin | 3.400mg/kg |
| Sodium monensin | 620mg/Kg | | |

Sorce: [1]Núcleo pré-parto NA 330 ag Milk (Agroceres); [2]Núcleo Lactação AgMilk Bio

Total diet samples were sent to the Animal Nutrition Laboratory, Department of Animal Science, Federal University of Paraná for nutrients determination as described in table 5 and mineral matter as described in table 6 and 7.

Table 5 - Balance between energy and protein of prepartum and postpartum diet during the experimental period.

| | Prepartum diet | | Postpartum diet | |
|---|---|---|---|---|
| Requirements | LE (Mcal/day) | MP (g/day) | LE (Mcal/day) | MP (g/day) |
| Maintenance | 11,1 | 437 | 12,2 | 775 |
| Gestation | 3,3 | 306 | 0,0 | 0 |
| Lactation | 0,0 | 0 | 24,5 | 1.603 |
| Growth | 0,5 | 50 | 0,6 | 55 |
| Reserve | 0,0 | 0 | -1,8 | 0 |
| Total required | 14,9 | 793 | 35,5 | 2.433 |
| Total provided | 18,0 | 1.016 | 35,3 | 2.270 |
| Balance | 3,1 | 223 | -0,2 | -163 |

Recommended Liquid Energy (LE) Range (Mcal/d): (de 14,099 a 14,7497) and metabolizable protein (MP).
Sorce: Animal Nutrition Laboratory, Department of Animal Science, Federal University of Paraná, 2021.

Table 6 - Mineral balance of prepartum diet during the experimental period.

| Prepartum diet | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Macro minerals | | | | | | | Trace minerals | | | | | | | |
| Requirements (g/dL) | Ca | P | Mg | Cl | K | Na | S | Co | Cu | Fe | I | Mn | Se | Zn | Cr |
| Maintenance | 9,8 | 11,2 | 3,6 | 12,0 | 70,9 | 15,7 | 21,7 | 2,2 | 8,2 | 0,0 | 6,5 | 1,5 | 3,3 | 54,2 | 6,0 |
| Gestation | 7,8 | 4,4 | 0,3 | 0,9 | 0,9 | 1,2 | 0,0 | 0,0 | 1,4 | 15,6 | 0,0 | 0,3 | 0,0 | 10,6 | 0,0 |
| Lactation | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 |
| Growth | 1,4 | 0,8 | 0,1 | 0,1 | 0,3 | 0,2 | 0,0 | 0,0 | 0,3 | 4,5 | 0,0 | 0,3 | 0,0 | 3,2 | 0,0 |
| Total required | 18,9 | 16,4 | 4,0 | 13,0 | 72,1 | 17,1 | 21,7 | 2,2 | 9,9 | 20,1 | 6,5 | 2,0 | 3,3 | 68,0 | 6,0 |
| Total provided | 24,7 | 27,8 | 8,4 | 81,8 | 85,2 | 10,4 | 48,0 | 6,9 | 10,0 | 130,3 | 7,0 | 3,8 | 6,6 | 207,4 | 0,0 |
| Balance | 5,8 | 11,5 | 4,5 | 68,8 | 13,1 | -6,7 | 26,3 | 4,8 | 0,1 | 110,2 | 0,5 | 1,8 | 3,4 | 139,3 | 0,0 |

Macro minerals: Calcium (Ca); Phosphorus (P); Magnesium (Mg); Chlorine (Cl), Potassium (K); Sodium (Na); Sulfur (S). Trace minerals: Cobalt (Co); Copper (Cu); Iron (Fe); Iodine (I); Manganese (Mn); Selenium (Se); Zinc (Zn); Chromium (Cr).
Sorce: Animal Nutrition Laboratory, Department of Animal Science, Federal University of Paraná, 2021.

Table 7 - Mineral balance of postpartum diet during the experimental period.

| Postpartum diet | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Macro minerals | | | | | | | Trace minerals | | | | | | | |
| Requirements (g/dL) | **Ca** | **P** | **Mg** | **Cl** | **K** | **Na** | **S** | **Co** | **Cu** | **Fe** | **I** | **Mn** | **Se** | **Zn** | **Cr** |
| Maintenance | 19,6 | 22,1 | 7,0 | 24,1 | 182,3 | 31,5 | 43,5 | 43,0 | 9,3 | 0,0 | 6,5 | 1,7 | 6,5 | 108,6 | 0,0 |
| Gestation | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 | 0,0 |
| Lactation | 36,0 | 31,1 | 3,8 | 35,0 | 52,5 | 14,0 | 0,0 | 0,0 | 1,4 | 35,0 | 3,5 | 1,0 | 0,0 | 140,0 | 0,0 |
| Growth | 0,0 | 0,0 | -0,1 | 0,0 | -0,6 | -0,4 | 0,0 | 0,0 | 0,0 | -8,8 | 0,0 | -0,5 | 0,0 | -6,2 | 0,0 |
| Total required | 55,6 | 53,2 | 10,7 | 59,1 | 234,2 | 45,1 | 43,5 | 4,3 | 10,7 | 26,2 | 10,0 | 2,2 | 6,5 | 242,5 | 0,0 |
| Total provided | 121,2 | 64,7 | 34,7 | 74,5 | 174,7 | 82,7 | 59,1 | 8,9 | 16,9 | 367,4 | 15,3 | 7,3 | 10,7 | 312,7 | 0,0 |
| Balance | 65,6 | 11,5 | 24,0 | 15,4 | -59,5 | 37,5 | 15,6 | 4,6 | 6,2 | 341,2 | 5,3 | 5,1 | 4,2 | 70,3 | 0,0 |

Macro minerals: Calcium (Ca); Phosphorus (P); Magnesium (Mg); Chlorine (Cl), Potassium (K); Sodium (Na); Sulfur (S). Trace minerals: Cobalt (Co); Copper (Cu); Iron (Fe); Iodine (I); Manganese (Mn); Selenium (Se); Zinc (Zn); Chromium (Cr).
Sorce: Animal Nutrition Laboratory, Department of Animal Science, Federal University of Paraná, 2021.

## 4.4 ANIMAL INCLUSION CRITERIA AND EXPERIMENTAL GROUPS

All animals selected underwent a general physical examination and than included in the study. Heifers and cows (up to the 4th calving) pregnant on average 20-30 days before the expected calving date. Initially, the proposal would be 60 animals in two experimental groups' distribution, being group treated with mineral supplementation (n=30), and group negative control animals that received saline solution (n=30).

However, the calving date is an estimate based on the pregnancy diagnosis that was carried out by the farm's veterinarians. Due to the fact, it is not an exact date, some calvings occurred before the expected date. As the proposed protocol consists of applying the product 30 to 20 days before calving, some animals had to be included during the study. At the end we had 66 animals in total that were randomly distributed and included in Group MS – mineral supplementation (n=34), or Group C - negative control (n=32), keeping animals pairing whenever possible, treated and untreated according to parity.

Animal distribution was according to parity, being divided into 1st calving cows that are calving for the first time, multiparous cows that calved for the second time onwards and total are all cows regardless of parity, as described in table 8.

Table 8 - Animal’s distribution experimental groups according to parity.

| Parity | Treatment | | Total |
|---|---|---|---|
| | Control | Mineral supplementation | |
| Primaparous | 15 | 10 | 25 |
| Multiparous | 17 | 24 | 41 |
| Total of animals | 32 | 34 | 66 |

Primiparous: cows that are calving for the first time; Multiparous: cows that calved for the second time onwards; Total: all cows regardless of parity.
Sorce: MARQUES, 2022.

## 4.5 TREATAMENTS

Each cow of the mineral supplementation group (MS) received 10 mL of an injectable mineral supplement containing 1,400 mg of sodium glycerophosphate 5.5H2O, 2,100 mg of monosodium phosphate 2H2O, 40 mg of copper chloride 2 H2O, 60 mg of sodium chloride potassium, 250 mg of magnesium chloride and 24 mg of sodium selenite by intramuscular route. The negative control group (C) received a 0.9% saline solution at a dosage of 10 mL per animal by intramuscular route (placebo).

The treatments were performe according to following protocol: application of three doses of mineral supplement or saline solution at three moments, being at 20 to 30 prepartum, at calving day and 15 days.

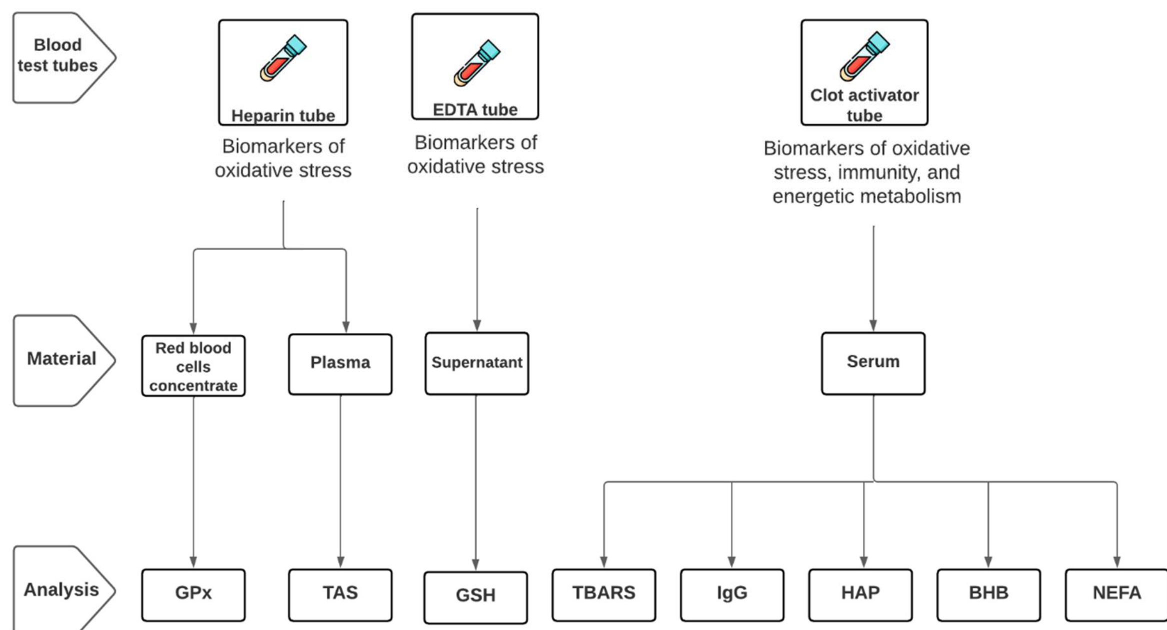
## 4.6 BLOOD SAMPLE COLLECTION

Blood samples were collected at seven moments during the period described as the transition period: three weeks prepartum (M-3); two weeks prepartum (M-2); one week prepartum (M-1); peripartum, being between calving until 6 days postpartum (M0); one week postpartum (M+1); two weeks postpartum (M+2); three weeks postpartum (M+3). Being performe on the animals' bed in the freestall itself by coccygeal vein puncture using the Vacutainer® (BD) system to obtain blood in all tubes.

For oxidants and antioxidants biomarkers of oxidative stress, heparin tubes (10mL) were use for total oxidative status (TAS) and glutathione peroxidase (GPx) dosage, EDTA tubes (4mL) for reduced glutathione (GSH) dosage and vacuum tubes with clot activator for thiobarbituric acid (TBARS) dosage. After blood sample collection, they

were processe in the laboratory, duplicate aliquoted in amber microtubes and stored at -80ºC until analysis. Vacuum tubes with clot activator (10mL) were also use to obtain blood serum for IgG, HAP, BHB and NEFA dosage. The blood serum was duplicate aliquoted into microtubes and stored at -40ºC until analysis (Figure 4).

Figure 4 – Tubes used for blood sample collection, material obtained from each tube after laboratory processing and each laboratory analysis performed.




Sorce: MARQUES, 2022.

## 4.7 SAMPLE PREPARATION FOR STORAGE

Immediately after collection, the samples were process in the laboratory. Sodium heparin tubes were centrifuged at 1900 G (or 3000 RPM) for 15 minutes in a refrigerated centrifuge at 4ºC, after centrifugation were duplicate aliquoted into amber microtubes and stored at - 80ºC until analysis to TAS dosage.

After removing and storing the plasma, buffered saline solution was added and centrifuged again at 1900 G (or 3000 RPM) for 15 minutes in a centrifuge refrigerated at 4ºC, first removing the leukocyte layer and then removing the buffered saline solution. This process was repeated until it was not possible to distinguish the leukocyte layer or it was reduced so that it did not interfere with the removal of the red blood cell mass for the determination of GPx, and then were duplicate aliquoted into amber microtubes and stored at - 80ºC until analysis.

To obtain the supernatant that was used for GSH analysis, the samples obtained with the EDTA tubes were pipetted in test tubes with distilled water and precipitant solution, after homogenization with a vortex, the sample remained at rest and later centrifuged at 1900 G (or 3000 RPM) for 5 minutes. After centrifugation, the intermediate layer was removed, duplicate aliquoted into amber microtubes and stored at - 80ºC until analysis.

The vacuum tubes with clot activator used to obtain blood serum were centrifuged at 1900 G (or 3000 RPM) for 15 minutes. After separation, the blood serum was duplicate aliquoted into microtubes and stored at -40ºC until analysis.

## 4.8 ANALYSIS OF ENERGY METABOLISM BIOMARKERS

Serum concentrations of BHB and NEFA were determined in a Labmax 240 Premium automatic biochemical analyzer (Labtest) using commercial Randox® kits (RB1007 and FA115; RANSOD and RANSEL) according to the manufacturer's recommendations.

The cut points for BHB was value < 1.2 mmol/L characterize without ketosis, values greater than or equal to 1.2 mmol/L characterize subclinical ketosis, and values greater than or equal to 2.9 mmol/L characterize clinical ketosis (McART et al., 2012).

For NEFA, the cut points were separated into prepartum and postpartum. Prepartum and postpartum serum NEFA concentrations higher than 0.3 and 0.6 mmol/L respectively, are associated with increased risk for impaired periparturient immunity and increased risk of infections (MOYES et al., 2009; OSPINA et al., 2010).

## 4.9 ANALYSIS OF OXIDANTS AND ANTIOXIDANTS BIOMARKERS OF OXIDATIVE STRESS

Determination of GSH was performed according to Beutler et al., (1963), a 200µl supernatant aliquot was added to 800µl of a Na2HPO4.12H20 (300 mmol/l) solution. After homogenization, 100µl of 0.05% DTNB (2-dinitrobenzoic acid) solution was added, the reading was performed at 412 nm within 30 seconds after DTNB addition using spectrophotometer apparatus. From standard curve values, straight-line equation was calculated and absorbance values of the analyzed samples were converted to mg/dL.

Serum GPx activity was determined in an automated biochemical analyzer, Labmax 240 Premium (Labtest) using commercial Randox® kits (RS505; RANSOD and RANSEL), as described by Paglia and Valentine (1967). Red blood cell concentraded samples were thawed and centrifuged for 3 minutes at 13000 rpm and then diluted in ice cold deionized water (300µL of red blood cell : 300µL of water) to obtain the hemolysate, with subsequent dilutions being performed according to the applicant's evaluation. To determine GPx concentration, 50µL of the hemolysate was diluted in 450µL of GPx diluent and homogenized by vortexing, a 20µL of this solution aliquot was withdrawn and diluted again in 480µL of GPx diluent. After these dilutions, the samples were analyze in an automatic analyzer. Simultaneously, the hemoglobin value of each red blood cell concentrated sample was analyze by spectrophotometry. The GPx value was obtained by dividing the resulting value from automatic analyzer by amount of hemoglobin contained in each sample, values were then converted into U/g Hb.

The determination of TAS was performed in a Labmax 240 Premium automatic biochemical analyzer (Labtest) using commercial Randox® kits (NX2332; RANSOD and RANSEL), according to manufacturer's recommendations and values obtained were expressed in mmol/L.

The determination of TBARS was performe according to BIRD et al., (1984), 3% 5-sulfosalicylic acid hydrate and 0.67% TBA were dilute in purified water at 95°C for 30 min. The initial pH of 1.8 was adjuste to 2.0 using 1M sodium hydroxide solution. For TBARS quantification, 0.25 mL of serum was adde to a test tube containing 0.25 mL of 3% 5-sulfosalicylic acid hydrate, vortexed for 10 seconds, centrifuged at 18,000 G

for 3 min and left to rest for 15 minutes at 25°C. Then, 0.25 ml of purified water (blank) or serum (samples) were dilute in 0.5 ml of 0.67% TBA solution. The solution was heated at 95°C for 30 min, and then cooled on ice for 10 min to stop reaction. After the blank and samples were equilibrated at room temperature, 300 µL was pipetted into a microplate well and the absorbance was measured at 535 nm. From standard curve values, straight line equation was calculated and absorbance values of the analyzed samples were expressed in nM of TBARS per total protein (nM/mg).

## 4.10 ANALYSIS OF IMMUNE BIOMARKERS

Serum IgG concentrations from cows and heifers were measured using an in-house sandwich ELISA assay (REBER et al., 2008). Rabbit anti-bovine IgG capture antibody (B5645; Sigma, St. Louis, MO) at 1:400 dilution in pH 9.7 sodium carbonate buffer was used to coat Immulon 4HBX plates (Thermo Corp., Milford, MA), which were incubated refrigerated (4-8°C) overnight.

After overnight incubation, the plates were washed three times with phosphate-buffered saline containing 0.5% Tween 20 (wash buffer). Serum samples were diluted 1:1,000,000 in wash buffer to determine IgG concentrations. Samples were placed in duplicate wells and incubated for one hour at room temperature. The plates were again washed three times with wash buffer for detection antibody addition, then peroxidase-conjugated rabbit anti-bovine IgG (A5295; Sigma, St. Louis, MO) was added to detect IgG that was bound to the capture antibody. Detection antibody was added to the plate diluted 1:1000 in wash buffer and incubated for 30 min.

After incubation, the plates were washed three times with a wash buffer and the substrate 2,20-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (A-9941; Sigma, St. Louis, MO) containing peroxide (30% hydrogen) was added. The plates were incubated for 20 minutes in the dark to allow color to develop. For reading, a plate reader with 405 nm filter was used. IgG concentration was determined against a nine-point serial dilution of bovine gammaglobulin standard (I5506; Sigma, St. Louis, MO), with values converted to mg/mL.

The haptoglobin concentration was determined on its ability to bind to hemoglobin (RAMOS et al., 2021), using spectrophotometry. The serum for standard curve determination was human (0.59g/L) with HAP concentration determined using a commercial kit (Beckman Coulter, Brea, CA, USA). 96-well plates were used for

spectrophotometry. Columns A1-H1 and B2-H2 were used to prepare the HAP standard curve between zero and 0.59g/L, through serial dilutions. To this, 20 µL of control serum were added to 180 µL of saline solution in wells A1 and A2, and 100 µL of saline solution in wells B1 to H1 and B2 to H2. Subsequently, 100 µL were aspirated from well A1 for serial dilution to well G1. The same procedure was performed in column 2. The last wells of columns 1 and 2 were assigned to curve blank. Subsequently, plate was filled with 90 µL of saline solution and 10 µL of tested samples, in duplicate. Each sample had its respective blank, in order to rule out influences of serum composition, as in lipidosis cases where an increase in absorbance can be observed even in the sample blank

After that, 50 µL of bovine methaemoglobin solution (40mg/dL concentration) and 50 µL of saline solution were added to the odd columns in each well of even columns. After 10 minutes of room temperature incubation, 150 µL of guaiacol substrate and 50 µL of hydrogen peroxide (0.02 mol/L) were added to all wells and incubated for 10 minutes in the dark. Optical density was determined in a spectrophotometer at a wavelength of 495nm.

## 4.11 STATISTICAL ANALYSIS

Quantitative data analysis was performed using the Statistical Analysis System for Windows (SAS® version 9.4, SAS Institute Inc., Cary, NC, USA). All quantitative variables were evaluated for the Gaussian distribution by the Guided Data Analysis function, and only the TAS variable was analyzed with the original and raw data. The concentration of IgG, TBARS and NEFA were submitted to logarithmic transformation; the GSH, haptoblobin and BHB to the inverse of the square root; and the GPx transformed into square root.

The variables were tested for fixed treatment effects (injectable mineral supplementation) and moments (M-3; M-2; M-1; M0; M+1; M+2; M+3) as well as treatment versus moment interaction effects. The fixed parity effects (primiparous and multiparous cows), moments and parity versus moment interaction effects, were analyzed separately for control and treated groups.

All effects were analyzed using the MIXED procedure (PROC-mixed, SAS), with post hoc Least Significant Difference (LSD) test. The models were tested according to

covariance structures, using the Akaike Information Criterion (AIC), statistical differences considered significant when p value ≤ 0.05.

When intereraction between treatment and moments was detected, the difference between groups in each evaluation moment was detected by the Student's t test in each prepartum and postpartum moment, considering presence of statistical difference when p value ≤ 0.05.

Quanlitative data analysis was performed using the Statistical Analysis System for Windows (SAS® version 9.4, SAS Institute Inc., Cary, NC, USA). For BHB values greater than or equal to 1.2 mmol/L, and values greater than or equal to 2.9 mmol/L were considered subclinical ketosis and clinical ketosis, respectively. Moments that presented p ≤ 0.05 were submitted to binary logistic regression analysis to estimate odd ratio and 95% confidence intervals (PROC-logistic), besides the frequency of percentage incidence (PROC-freq) and statistical differences were considered significant when p value ≤ 0.05.

# 6 RESULTS

## 6.1 LYPOLISIS BIOMARKERS

The mean±standard error results of BHB and NEFA of Holstein cows in the transition period are shown in table 10, as well as the treatment effect, time and treatment*time interaction.

Table 10 - Mean values, standard error (SE) and differences between groups in relation to control and mineral supplementation groups for BHB and NEFA.

| PARITY | VARIABLES | GROUP | | P-GROUP | P-TIME | P-GROUP*TIME |
|---|---|---|---|---|---|---|
| | | CONTROL MEAN±SE | MINERAL SUPPLEMENTATION MEAN±SE | | | |
| PRIMIPAROUS | BHB (mmol/L) | 1.35±0.03 | 1.35±0.03 | 0.9673 | 0.0079* | 0.7421 |
| | NEFA (mmol/L) | 1.01±0.06 | 0.77±0.41 | 0.2248 | 0.6718 | 0.1929 |
| MULTIPAROUS | BHB (mmol/L) | 1.26±0.03 | 1.25±0.03 | 0.4807 | <.0001* | 0.0159* |
| | NEFA (mmol/L) | 0.92±0.06 | 0.93±0.05 | 0.9673 | 0.0602 | 0.5826 |
| TOTAL | BHB (mmol/L) | 1.3±0.02 | 1.28±0.02 | 0.7753 | <.0001* | 0.2205 |
| | NEFA (mmol/L) | 0.96±0.04 | 0.88±0.03 | 0.4433 | 0.0421* | 0.7052 |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Beta hydroxybutyrate (BHB); Non esterified fatty acids (NEFA). PROC MIXED test. *Differences were considered significant when $p \leq 0.05$. Mean: mean between groups. SE: Standard error. Total animals per parity: Primiparous: 25, C=15 and MS=10; Multiparous: 41, C=17 and MS=24; Total: 66, C=32 and MS=34.
Sorce: MARQUES, 2022.

It was not possible to observe treatment effect for both BHB and NEFA for any parity. On the other hand, effect of time was detected for all categories for BHB, but only in the total number of animals for NEFA. Interaction of treatment*time was observed for BHB in multiparous dairy cows. According to BHB cut off values adopted in this research, no animal was characterized as clinical ketosis or hyperketonemia since they weren't hit values greater than or equal to 2.9 mmol/L at any of evaluated times (McART et al., 2012).

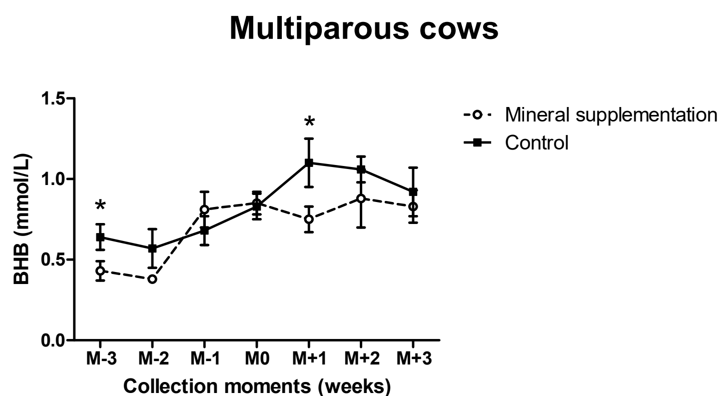
The comparison of treatments by Student t test was performed for BHB, since it was detected interaction between group*time. It was detected higher concentration of BHB for the control group compared to mineral injectable supplementation group at 3 weeks prepartum (M-3) and 1 week postpartum (M+1) collection for multiparous cows (table 11) (Figure 5).

Table 11 - Mean values, standard error (SE) and differences for BHB between control and mineral supplementation group for for the cows with ≥ 3 lactations and multiparous cows.

| VARIABLE | PARITY | COLLECTION MOMENTS | CONTROL | MINERAL SUPPLEMENTATION | P-VALUE** |
|---|---|---|---|---|---|
| | | | MEAN±SE | MEAN±SE | |
| BHB (mmol/L) | MULTIPAROUS | M-3 | 0.64±0.08 | 0.43±0.06 | 0.0587* |
| | | M-2 | 0.57±0.12 | 0.38±0.02 | 0.1621 |
| | | M-1 | 0.68±0.09 | 0.81±0.11 | 0.3770 |
| | | M0 | 0.83±0.08 | 0.85±0.07 | 0.8215 |
| | | M+1 | 1.1±0.15 | 0.75±0.08 | 0.0502* |
| | | M+2 | 1.06±0.08 | 0.88±0.18 | 0.3721 |
| | | M+3 | 0.92±0.15 | 0.83±0.1 | 0.6108 |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Beta hydroxybutyrate (BHB). Collection moments. M-3: three weeks prepartum; M-2: two weeks prepartum; M-1: one week prepartum; M0: peripartum; M+1: one week postpartum; M+2: two weeks postpartum; M+3: three weeks postpartum.**Differences between groups detected by Student's t test. Mean: mean between groups. SE: Standard error. *Differences were considered significant when p ≤ 0.05.
Total animals per group in multiparous category: Control: 17; Mineral supplementation: 24;
Sorce: MARQUES, 2022.

Figure 5 - Mean values, significant p value and differences for BHB between control and mineral supplementation group for multiparous cows.




Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Beta hydroxybutyrate (BHB). Collection moments. M-3: three weeks prepartum; M-2: two weeks prepartum; M-1: one week prepartum; M0: peripartum; M+1: one week postpartum; M+2: two weeks postpartum; M+3: three weeks postpartum. Differences between groups detected by Student's t test. Mean: mean between groups. SE: Standard error. *Differences were considered significant when p ≤ 0.05. Total animals per group in multiparous category: Control: 17; Mineral supplementation: 24;
Sorce: MARQUES, 2022.

In addition to the treatments, it was also evaluated the variation between cows' categories whithin each experimental group (Table 12 and Table 13). It was not possible to observe effects of category, time as well as category*time interaction, for

both BHB and NEFA, regardless of the group analyzed. It was only possible to observe time effect for BHB in the control group.

Table 12 - Mean values, standard error (SE) and differences between categories in control and mineral supplementation groups for BHB and NEFA.

| | | CATEGORY | | | | |
|---|---|---|---|---|---|---|
| GROUP | VARIABLES | PRIMIPAROUS | MULTIPAROUS | P-CAT | P-TIME | P-CAT*TIME |
| | | MEAN±SE | MEAN±SE | | | |
| CONTROL | BHB (mmol/L) | 0.67±0.05 | 0.75±0.04 | 0.2164 | 0.0072* | 0.7170 |
| | NEFA (mmol/L) | 1.01±0.06 | 0.92±0.06 | 0.6560 | 0.2077 | 0.8750 |
| MINERAL SUPPLEMENTATION | BHB (mmol/L) | 0.63±0.04 | 0.83±0.05 | 0.1114 | 0.0026* | 0.3451 |
| | NEFA (mmol/L) | 0.77±0.04 | 0.93±0.05 | 0.4252 | 0.5701 | 0.0650 |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Beta hydroxybutyrate (BHB); Non esterified fatty acids (NEFA). PROC MIXED test, were considered significant when p ≤ 0.05. Mean: mean between categories. SE: Standard error. *Differences were considered significant when p ≤ 0.05.
Total animals per category: Primiparous: 25, C=15 and MS=10. Multiparous: 41, C=17 and MS=24.
Sorce: MARQUES, 2022.

For the evaluation of the incidence of ketosis, values > 1.2 mmol/L or 2.9 mmol/L, which characterize subclinical and clinical ketosis, respectively, were considered. (McART et al., 2012). The incidence of ketosis did not show statistically difference for any of the evaluated parities, regardless of group. Although the control group presented higher values than the mineral supplementation group for all evaluated parities, as show in table 14.

Table 14 – Treatment effect on incidence (%) of ketosis at the first week postpartum (M+1) by measuring serum BHB.

| VARIABLE | PARITY | GROUP | INCIDENCE % (n/total) | ODDS RATIO (IC 95%) | P-VALUE |
|---|---|---|---|---|---|
| BHB (mmol/L) | PRIMIPAROUS | Control | 26.67 (4/15) | Reference | 0.2467 |
| | | Mineral supplementation | 18.33 (1/12) | 0.900 (0.383 - 1.730) | |
| | MULTIPAROUS | Control | 31.82 (4/17) | Reference | 0.5696 |
| | | Mineral supplementation | 23.53 (7/22) | 0.659 (0,157 - 2.771) | |
| | TOTAL | Control | 25.00 (8/32) | Reference | 0.8892 |
| | | Mineral supplementation | 23.53 (8/34) | 1.083 (0.351 - 3.341) | |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Beta hydroxybutyrate (BHB). Logistic regression test, were considered significant when p ≤ 0.05.
Sorce: MARQUES, 2022.

## 6.2 BIOMARKERS OF OXIDATIVE STRESS

The mean±standard error results of GPx, TBARS, GSH and TAS biomarkers of Holstein cows in the transition period are presented in table 15, as well as the treatment effect, time and treatment*time interaction.

Table 15 - Mean values, standard error (SE) and differences between groups in relation to control and mineral supplementation groups for biomarkers of oxidative stress.

| PARITY | VARIABLES | GROUP | | P-GROUP | P-TIME | P-GROUP* TIME |
|---|---|---|---|---|---|---|
| | | CONTROL MEAN±SE | MINERAL SUPPLEMENTATION MEAN±SE | | | |
| PRIMIPAROUS | GPx (U/g Hb ) | 1228.42± 39.22 | 1224.09± 44.57 | 0.7108 | 0.0733 | 0.3223 |
| | TBARS (nM/mg) | 2.65±0.17 | 1.99±0.14 | 0.3063 | <.0001* | 0.6982 |
| | GSH (mg/dL) | 3.46±0.36 | 3.59±0.32 | 0.2236 | 0.2030 | 0.6960 |
| | TAS (mmol/L) | 1.01±0.01 | 0.98±0.01 | 0.2281 | <.0001* | 0.1568 |
| MULTIPAROUS | GPx (U/g Hb ) | 801.90± 34.68 | 940.73± 29.96 | 0.0248* | 0.133 | 0.7414 |
| | TBARS (nM/mg) | 1.98±0.13 | 1.85±0.13 | 0.5898 | <.0001* | 0.0383* |
| | GSH (mg/dL) | 3.76±0.38 | 3.47±0.27 | 0.6846 | 0.4317 | 0.6287 |
| | TAS (mmol/L) | 0.99±0.01 | 0.98±0.01 | 0.3328 | <.0001 | 0.0503* |
| TOTAL | GPx (U/g Hb ) | 955.52± 28.13 | 1004.78± 25.62 | 0.2485 | 0.0074* | 0.2277 |
| | TBARS (nM/mg) | 2.29±0.11 | 1.90±0.1 | 0.2430 | <.0001* | 0.4948 |
| | GSH (mg/dL) | 3.44±0.22 | 3.7±0.18 | 0.0500* | 0.0203* | 0.6078 |
| | TAS (mmol/L) | 1.00±0.01 | 0.98±0.01 | 0.1022 | <.0001* | 0.4767 |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Glutathione peroxidase (GPx); Thiobarbituric acid (TBARS); Glutathione reductase (GSH); Total antioxidant status (TAS). PROC MIXED test. Mean: mean between groups. SE: Standard error. *Differences were considered significant when $p \leq 0.05$. Total animals per parity: Primiparous: 25, C=15 and MS=10; Multiparous: 41, C=17 and MS=24; Total: 66, C=32 and MS=34
Sorce: MARQUES, 2022.

Treatment effect was detected for the GPx variable in multiparous cows, with higher values being observed in the treated group. The GSH antioxidant enzyme was higher in cows treated with injectable mineral supplementation analyzing data from the total number of animals (Figure 6).

Figure 6 - Mean values, significant p value and differences between groups in relation to control and mineral supplementation groups for GPx and GSH for multiparous cows.

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Glutathione peroxidase (GPx); Glutathione reductase (GSH). PROC MIXED test. Mean: mean between groups. SE: Standard error. *Differences were considered significant when p ≤ 0.05. Total animals per parity: Primiparous: 25, C=15 and MS=10; Multiparous: 41, C=17 and MS=24; Total: 66, C=32 and MS=34
Sorce: MARQUES, 2022.

Table 16 - Mean values, standard error (SE) and differences for TBARS and TAS between control and mineral supplementation group for the cows with ≥ 3 lactations and multiparous cows.

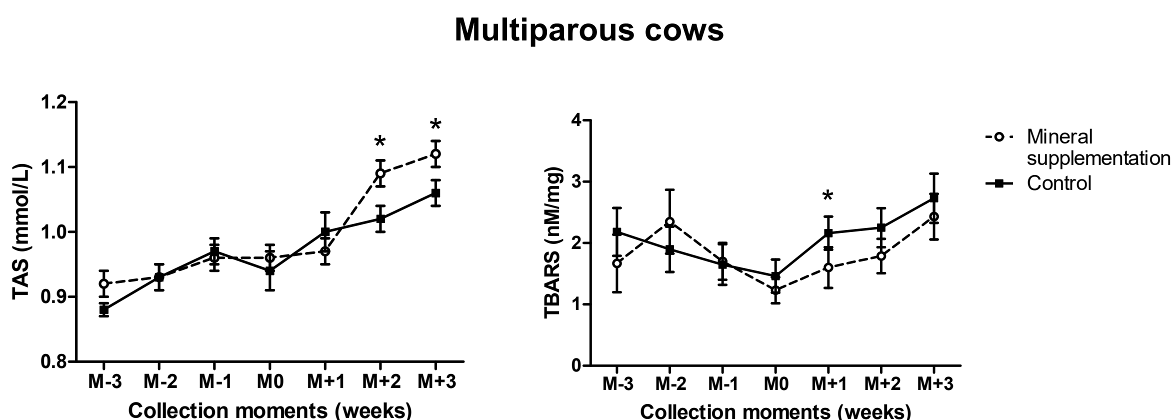
| PARITY | VARIABLE | COLLECTION MOMENTS | CONTROL | MINERAL SUPPLEMENTATION | P-VALUE** |
|---|---|---|---|---|---|
| | | | MEAN±SE | MEAN±SE | |
| MULTIPAROUS | TBARS (nM/mg) | M-3 | 2.18±0.39 | 1.67±0.47 | 0.4117 |
| | | M-2 | 1.9±0.37 | 2.35±0.52 | 0.4793 |
| | | M-1 | 1.65±0.33 | 1.7±0.3 | 0.9216 |
| | | M0 | 1.46±0.27 | 1.23±0.21 | 0.4927 |
| | | M+1 | 2.16±0.27 | 1.6±0.33 | 0.0206* |
| | | M+2 | 2.25±0.32 | 1.79±0.28 | 0.2892 |
| | | M+3 | 2.73±0.4 | 2.43±0.37 | 0.5903 |
| | TAS (mmol/L) | M-3 | 0.88±0.01 | 0.92±0.02 | 0.4341 |
| | | M-2 | 0.93±0.02 | 0.93±0.02 | 0.9860 |
| | | M-1 | 0.97±0.02 | 0.96±0.02 | 0.8839 |
| | | M0 | 0.94±0.03 | 0.96±0.02 | 0.5578 |
| | | M+1 | 1±0.03 | 0.97±0.02 | 0.4055 |
| | | M+2 | 1.02±0.02 | 1.09±0.02 | 0.0250* |
| | | M+3 | 1.06±0.02 | 1.12±0.02 | 0.0421* |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Thiobarbituric acid (TBARS); Total antioxidant status (TAS). Collection moments. M-3: three weeks prepartum; M-2: two weeks prepartum; M-1: one week prepartum; M0: peripartum; M+1: one week postpartum; M+2: two weeks

postpartum; M+3: three weeks postpartum. ** Differences between groups detected by Student's t test. Mean: mean between groups. SE: Standard error. *Differences were considered significant when p ≤ 0.05.
Total animals per group in multiparous category: Control: 17; Mineral supplementation: 24;
Sorce: MARQUES, 2022.

It was not possible to observe time effect for GSH and GPX variable regardless of parity, except for GPx variable in the total number of animals. On the other hand, there was a time effect for TBARS and TAS variables in all parity. Treatment*time interaction was observed for TBARS and TAS variables in multiparous cows. Values for the control group were higher for TBARS oxidant activity at 1 week postpartum collection for multiparous cows. The total antioxidant status (TAS) obtained higher values for the mineral supplementation group compared to control at 2 weeks (M+2) and 3 weeks postpartum (M+3) collection for multiparous cows (Table 16) (Figure 7).

Figure 7 - Mean values, significant p value and differences for TBARS and TAS between control and mineral supplementation group for multiparous cows.




Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Thiobarbituric acid (TBARS); Total antioxidant status (TAS). Collection moments. M-3: three weeks prepartum; M-2: two weeks prepartum; M-1: one week prepartum; M0: peripartum; M+1: one week postpartum; M+2: two weeks postpartum; M+3: three weeks postpartum. Differences between groups detected by Student's t test when p ≥ 0.05. *Differences were considered significant when p ≤ 0.05. Total animals per group: Control: 17; Mineral supplementation: 24.
Sorce: MARQUES, 2022.

The comparison effect between primiparous and multiparous is shown in table 17. It can only be observed for GPx variable, both in control and mineral supplementation group, not being observed for other variables independent of the group. It was possible to observe time effect for all variables in both groups, except for GSH, which had no effect in both control and mineral supplementation groups. However, the category*time interaction could only be observed for TAS variable in the control group.

Table 17 - Mean values, standard error (SE) and differences between categories in control and mineral supplementation groups for biomarkers of oxidative stress.

| GROUP | VARIABLES | CATEGORY PRIMIPAROUS MEAN±SE | CATEGORY MULTIPAROUS MEAN±SE | P-CAT | P-TIME | P-CAT*TIME |
|---|---|---|---|---|---|---|
| CONTROL | GPx (U/g Hb ) | 1128.42± 39.22 | 809.44± 34.68 | <.0001* | 0.0931 | 0.9266 |
| | TBARS (nM/mg) | 2.65±0.17 | 1.98±0.13 | 0.2171 | <.0001* | 0.8333 |
| | GSH (mg/dL) | 3.46±0.36 | 3.42±0.26 | 0.7288 | 0.7191 | 0.4190 |
| | TAS (mmol/L) | 1.01±0.01 | 0.99±0.01 | 0.4633 | <.0001* | 0.0136* |
| MINERAL SUPPLEMENTATION | GPx (U/g Hb ) | 1127.09± 44.57 | 940.73± 29.96 | 0.0117* | 0.0084* | 0.6519 |
| | TBARS (nM/mg) | 1.99±0.14 | 1.85±0.13 | 0.8541 | <.0001* | 0.0697 |
| | GSH (mg/dL) | 3.59±0.32 | 3.75±0.22 | 0.8550 | 0.1719 | 0.7595 |
| | TAS (mmol/L) | 1.99±0.14 | 1.85±0.13 | 0.5841 | <.0001* | 0.0697 |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Glutathione peroxidase (GPx); Thiobarbituric acid (TBARS); Glutathione reductase (GSH); Total antioxidant activity (TAS). PROC MIXED test, *Differences were considered significant when $p \leq 0.05$. Mean: mean between categories. SE: Standard error.
Total animals per category: Primiparous: 25, C=15 and MS=10. Multiparous: 41, C=17 and MS=24.
Sorce: MARQUES, 2022.

## 6.3 IMMUNE BIOMARKERS

The mean±standard error results of IgG and haptoglobin of Holstein cows in the transition period are presented in table 19, as well as the treatment effect, time and treatment*time interaction.

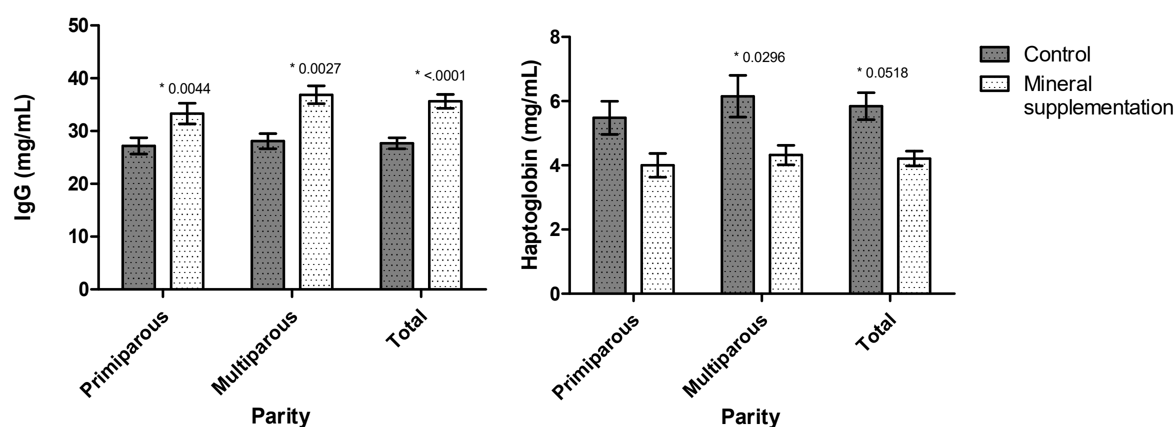
Table 19 - Mean values, standard error (SE) and differences between groups in relation to control and mineral supplementation groups for IgG and haptoglobin.

| PARITY | VARIABLES | GROUP | | P-GROUP | P-TIME | P-GROUP*TIME |
|---|---|---|---|---|---|---|
| | | CONTROL MEAN±SE | MINERAL SUPPLEMENTATION MEAN±SE | | | |
| Primiparous | IgG (mg/mL) | 27.19±1.53 | 33.32±1.96 | 0.0044* | <.0001* | 0.1413 |
| | HAP (mg/mL) | 5.48±0.52 | 4±0.37 | 0.4896 | <.0001* | 0.2329 |
| Multiparous | IgG (mg/mL) | 28.09±1.45 | 36.87±1.69 | 0.0027* | <.0001* | 0.2734 |
| | HAP (mg/mL) | 6.15±0.65 | 4.32±0.3 | 0.0296* | <.0001* | 0.1019 |
| Total | IgG (mg/mL) | 27.68±1.05 | 35.63±1.3 | <.0001* | <.0001* | 0.0303* |
| | HAP (mg/mL) | 5.84±0.42 | 4.21±0.23 | 0.0518* | <.0001* | 0.1091 |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Immunoglobulin G (IgG); Haptoglobin (HAP). PROC MIXED test, were considered significant when p ≤ 0.05. Mean: mean between groups. SE: Standard error. Total animals per parity: Primiparous: 25, C=15 and MS=10; Multiparous: 41, C=17 and MS=24; Total: 66, C=32 and MS=34
Sorce: MARQUES, 2022.

Treatment effect was detected for the IgG in primiparous, multiparous and total cows with higher values being observed in the treated group. Haptoglobin, biomarker of inflammatory process, obtained lower results for the mineral supplementation group, for multiparous and total cows (Figure 8).

Figure 8 - Mean values, significant p value and differences between groups in relation to control and mineral supplementation groups for IgG and haptoglobin concentrations in diferents parities.




Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Immunoglobulin G (IgG), Haptoglobin (HAP). PROC MIXED test, *Differences were considered significant when p ≤ 0.05. Mean: mean between groups. SE: Standard error. Total animals per parity: Primiparous: 25, C=15 and MS=10; Multiparous: 41, C=17 and MS=24; Total: 66, C=32 and MS=34
Sorce: MARQUES, 2022.

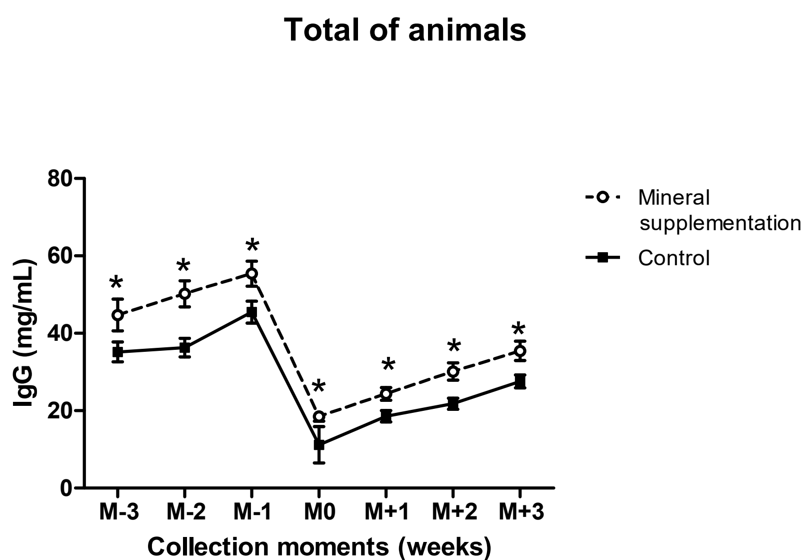
Time effect was observed for all categories on both IgG and haptoglobin variables. However, treatment*time interaction can only be observed for total animals for IgG. The IgG concentration was higher for the mineral supplementation group compared to control at all evaluated times, from 3rd prepartum week (M-3) to 3rd postpartum week (M+3), as shown in table 20 and figure 9.

Table 20 - Mean values, standard error (SE) and differences between control and mineral supplementation group for for in IgG to total of animals.

| PARITY | VARIABLE | COLLECTION MOMENTS | CONTROL | MINERAL SUPPLEMENTATION | P-VALUE** |
|---|---|---|---|---|---|
| | | | MEAN±SE | MEAN±SE | |
| TOTAL | IgG (mg/mL) | M-3 | 35.19±2.58 | 44.72±4.08 | 0.0452* |
| | | M-2 | 36.31±2.4 | 50.22±3.37 | 0.0011* |
| | | M-1 | 45.47±2.81 | 55.41±.3.22 | 0.0240* |
| | | M0 | 11.17±4.70 | 18.47±1.16 | <.0001* |
| | | M+1 | 18.56±1.48 | 24.35±1.63 | 0.0110* |
| | | M+2 | 21.82±1.44 | 30.13±2.21 | 0.0026* |
| | | M+3 | 27.56±1.68 | 35.46±2.51 | 0.0114* |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Collection moments. M-3: three weeks prepartum; M-2: two weeks prepartum; M-1: one week prepartum; M0: peripartum; M+1: one week postpartum; M+2: two weeks postpartum; M+3: three weeks postpartum. ** Differences between groups detected by Student's t test. Mean: mean between groups. SE: Standard error. *Differences were considered significant when $p \leq 0.05$. Total animals per group in ≥ 3rd calving category: Control: 12 animals; Treated: 15 animals;
Sorce: MARQUES, 2022.

Figure 9 - Mean values, significant p value and differences for IgG between control and mineral supplementation for total of animals.




Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Haptoglobin (HAP); Immunoglobulin G (IgG). Collection moments. M-3: three weeks prepartum; M-2: two weeks prepartum; M-1: one week prepartum; M0: peripartum; M+1: one week postpartum; M+2: two weeks postpartum; M+3: three weeks postpartum. * Differences between groups detected by Student's t test when $p \geq 0.05$. Total animals per group in total: 66; C=32 MS=34.
Sorce: MARQUES, 2022.

It was not possible to observe the comparison effect between primiparous and multiparous for both IgG and haptoglobin, regardless of the group analyzed, as seen in table 21. Although time effect was observed for IgG and haptoglobin in both groups, it was not possible to observe category*time interaction in any analyzed group.

Table 21 - Mean values, standard error (SE) and differences between categories in control and mineral supplementation groups for IgG and haptoglobin.

| | | CATEGORY | | | | |
|---|---|---|---|---|---|---|
| GROUP | VARIABLES | PRIMIPAROUS | MULTIPAROUS | P-CAT | P-TIME | P-CAT*TIME |
| | | MEAN±SE | MEAN±SE | | | |
| **CONTROL** | **IgG (mg/mL)** | 27.19±1.53 | 28.10±1.45 | 0.7916 | <.0001* | 0.6352 |
| | **HAP (mg/mL)** | 5.48±0.52 | 6.15±0.65 | 0.3849 | <.0001* | 0.2758 |
| **MINERAL SUPPLEMENTATION** | **IgG (mg/mL)** | 33.31±1.96 | 36.87±1.69 | 0.3369 | <.0001* | 0.7886 |
| | **HAP (mg/mL)** | 3.99±0.37 | 4.32±0.30 | 0.5469 | <.0001* | 0.6180 |

Control group did not receive injectable mineral supplementation; Mineral supplementation group received 3 doses of injectable mineral supplementation between D-30 to D-20 prepartum, on calving day and D+15 postpartum. Immunoglobulin G (IgG); Haptoglobin (HAP). PROC MIXED test, were considered significant when $p \leq 0.05$. Mean: mean between categories. SE: Standard error.
Total animals per category: Primiparous: 25, C=15 and MS=10. Multiparous: 41, C=17 and MS=24.
Sorce: MARQUES, 2022.

# 7 DISCUSSION

This research evaluated the influence of three doses of injectable mineral supplement on biomarkers of oxidants and antioxidants, energy metabolism, acute phase protein levels (haptoglobin) and dynamic of IgG in Holstein cows and heifers during the transition period. The product composition per 100 mL: sodium glycerophosphate (14g), monosodium phosphate (20.1g), copper chloride (0.4g), potassium chloride (0.6g), magnesium chloride (2.5g) and sodium selenite (0.24g).

The differential of this research can be attributed to the character of this study classified as a primary investigation in long-term including the whole transition period by weekly sampling. The most of publications regarding the injectable mineral complex on dairy cattle are represented by narrative review.

## 7.1 LYPOLISIS BIOMARKERS

During the transition period, NEB normally occurs in dairy cow as they pass from the late gestation to early lactation and thus an excessive energy demand for milk production occurs. NEFA increased levels are indicative of the increasing depletion of lipid reserves, prepartum blood NEFA level may be used for detecting cows at risk for severe NEB (LeBLANC 2006), increase the risk of clinical ketosis and metritis (OSPINA et al. 2010) as well as retained fetal membranes (CHAPINAL et al. 2011).

The effect of mineral supplementation in our study on lipolysis biomarkers could not be observed for both BHB and NEFA for mineral supplementation and control groups regardless of cow parity. It was also not possible to find differences in analysis between categories (primiparous and multiparous). This result was not expected for NEFA concentrations, since other authors who carried out studies with injectable mineral supplementation observed significant values for NEFA in at least one of the evaluated moments (OMUR et al., 2016; YAZLIK et al., 2021).

Omur et al (2016) administrate vitamins (500 000 IU vitamin A, 75 000 IU vitamin D3, 50 mg vitamin E per ml) and trace elements (2.5 mg of copper gluconate, 1.25 mg of sodium selenite, 5 mg of manganese gluconate, 5 mg of zinc gluconate per ml) in 10 multiparous brown Swiss cows. They were evaluated at the beginning of transition period, parturition and 3-weeks after the parturition, and among other findings, the results showed statistical differences for NEFA with lower values for the treated group at all evaluated times. However, Yazlık et al., (2021) found higher concentrations of

NEFA in treated animals with the same commercial products of trace mineral and vitamins (OMUR et al., 2016) but at 10 days prepartum, they analyzed 182 multiparous Holstein cows divided into 4 experimental groups (mineral supplementation, vitamin supplementation, mineral + vitamin supplementation and control group). When comparing to results of our study, their findings did not corroborate to ours. However, despite they used the same commercial product at the same concentrations (OMUR et al., 2016; YAZLIK et al., 2021) findings regarding NEFA were not similar. These different results may be associated with non-standardization of a collection date, or the technique used for sample analysis, or cow physiological factors during the transition period, or even in our study differences, due to it is a different commercial product with different concentrations of minerals. However, this divergences leads to belief that more studies should be carried out to better understand how minerals act on NEFA concentration during the transition period of dairy cows.

Although it was not possible to observe difference between the groups for NEFA and BHB, for interactions treatment*time were observed significant effect for BHB in older animals (multiparous cows) at 1 week postpartum (M+1). High serum concentration of postpartum BHB is an indicator of negative energy balance in the transition period, and it is associated with increased fat mobilization in early lactation (INGVARTSEN et al., 2000). During the 1 week postpartum period, BHB analyzes are usually performed for ketosis control (PEREIRA et al. 2013; McART et al 2012). In our study, we observed lower BHB values for mineral supplementation group compared to control for multiparous cows (0.75±0.08 vs 1.1±0.15). This event may have happened in our study given that in multiparous cows, which generally have high milk production, consequently, have a greater metabolic challenge and a more severe negative energy balance (FÜRLL et al. 2010; KREIPE et al. 2011). Therefore, injectable mineral supplementation may have helped older animals at a time of greater risk.

Our findings regarding BHB concentration for the first week postpartum corroborate with results found by Machado et al. (2014) which also carried out studies involving minerals suplemmentation during the transition period. They evaluated 250 multiparous cows that received subcutaneous mineral supplementation containing 300mg of Zn, 50 mg of Mn, 25 mg of Se e 75 mg of Cu at 230 and 260 days of gestation and 35 days postpartum. Among findings during the study, serum BHB concentrations for the group that receiving mineral supplementation was 0.27 mmol/L while control

group was 0.41 mmol/L. In our study, this difference was also observed for multiparous cows, however it has been previously reported that Cu supplementation increased metabolism of adipose tissue in steers, due to an increased response to hormones responsible for lipolysis (ENGLE et al., 2000; STAHLHUT et al., 2006). Therefore, decrease in BHB values was not expected due to lipolytic action of copper, turning still not clear how mineral supplementation decreased BHB concentration.

Macrominerals (calcium, chlorine, sulfur, phosphorus, magnesium, potassium and sodium) have significant importance in relation to dairy cow health during the transition period. Phosphorus levels are physiologically reduced during postpartum period, added to commonly lower concentrations found in high production dairy cows, phosphorus supplementation might improve the dairy cow energy condition (GIRARD & MATTE 2005; GRÜNBERG et al. 2009). Some studies showed that cows in better energy conditions have improved liver function. Grünberg et al. (2009) found that small reductions in cytosolic phosphorus levels after parturition might affect liver metabolic activity. Bertoni et al. (2008) and Trevisi et al. (2013) observed that cows with better liver function had low serum levels of NEFA and BHB. In our study, phosphorus concentration was not measured or compared between groups. Although, phosphorus dosage in the mineral supplementation composition may promotes the findings of BHB reduced levels for treated animals, due to its action in promoting liver health improvement, since this decrease in BHB levels was not expected.

The ability to predict at the cow level which animals are more likely to develop disease based on NEFA and BHBA concentrations might help producers prevent diseases proactively by focusing on management and nutritional strategies to prevent subclinical and clinical disease (OSPINA et al., 2010). For this reason, cutoff points were used for values of lipolysis biomarkers. The cut points for BHB was value < 1.2 mmol/L characterize without ketosis, values greater than or equal to 1.2 mmol/L characterize subclinical ketosis, and values greater than or equal to 2.9 mmol/L characterize clinical ketosis (McART et al., 2012). For NEFA, the cut points were separated into prepartum and postpartum. Prepartum and postpartum serum NEFA concentrations higher than 0.3 and 0.6 mmol/L respectively, are associated with increased risk for impaired periparturient immunity and increased risk of infections (MOYES et al., 2009; OSPINA et al., 2010). These values were used as a reference for the qualitative analysis of BHB through the odds ratio, but in our study, it was not

possible to observe differences between groups. This factor is mainly due to low sample number of animals per group, however, when observing the values described, mineral supplementation group presented lower BHB values for all evaluated parities (primiparous, multiparous and total), with highest differences being observed for older cows (multiparous).

## 7.2 OXIDANTS AND ANTIOXIDANTS BIOMARKERS OF OXIDATIVE STRESS

Oxidation and the production of free radicals are an integral part of aerobic metabolism. A variety of reactive oxygen species (ROS) are produced by normal metabolic processes and by certain leukocyte populations during defense against disease. Oxidative stress occurs, due to unbalanced availability of antioxidants in presence of phenomena that increase pro-oxidants production (SORDILLO et al., 2009). In periparturient cows, enzymatic antioxidants represent the main antioxidant defense mechanisms in protecting cells against increased ROS (ANDRIEU, 2008). The use of antioxidant compounds found in the diet or even synthetic ones is one of the defense mechanisms against ROS that can be used in order to prevent changes or recover from them (BIANCHI; ANTUNES, 1999). The determination of the activities of enzymatic antioxidants, such as glutathione peroxidase (GPx), reduced glutathione (GSH) and total antioxidant status (TAS) or oxidants such as thiobarbituric acid activity (TBARS) represent one of the ways to assess oxidative stress (KLECZKOWSKI et al., 2003).

This study evaluated the effect of injectable mineral suplemmentation on dynamic of TBARS (oxidant substance) and antioxidant biomarkers such as GPX, GSH and TAS in Holstein cattle primiparous and multiparous during the transition period. For primiparous cows, it was not detected the effect of mineral complex in none of analyzed variables. On the other hand, the effect of mineral supplementation was observed for GPx in multiparous cows increased values of this antioxidant substance in the supplemented group in comparison with non-treated cattle.

In addition, group*time interactions were detected for TBARS and TAS in the oldest animals from multiparous cows, observing high values for TAS and lower values of TBARS on the mineral supplementation group in the weeks +2 and +3. The antioxidant ability of GSH can only be observed for the total number of evaluated animals, with

higher values for the group supplemented with injectable minerals. The most variables had effect of time for all categories, except in the total of animals.

Previous research has revealed that increased serum Se concentration is correlated with increased GPx activity in cattle (KOLLER et al., 1984). Unfortunatelly, the measurements of serum Se concentrations were not planned in the present study. However, Bittar et al. (2017) reported increased serum Se and Mn in dairy calves treated with trace mineral injection at 1 mL/45 kg of body weight (BW). This suggests that cows that received mineral supplementation that contains Se, could also had Se increased, which was used as a co-factor for GPx production and could justify GPx increase for multiparous cows.

Enzymatic antioxidants are mainly responsible for $H_2O_2$ transformation into its less reactive forms for the organism, such as $O_2$ by catalase and $H_2O$ by GPx, being enzymes that perform similar roles despite being different from each other. Soldá et al (2016) has observed the effect of mineral supplementation on enzymatic antioxidants, they evaluated 7 Holstein cows during the transition period and their calves, after administration of two doses of injectable mineral supplementation containing magnesium, phosphorus, potassium, selenium and copper. Among the findings, animals that received the mineral supplementation were observed statistical differences in decrease values for TBARS concentrations of cows during the transition period. In addition, were observed statistical differences for catalase values in supplemented cows and their calves. In our study, we found similar results for both the oxidant and antioxidant biomarkers, although enzymatic oxidants are not the same evaluated those studies, they play the same role in the antioxidant mechanism, indicating that mineral supplementation assists the oxidative stress of dairy cows during the transition period.

Bernabucci et al. (2005) evaluated the influence of body condition score in between metabolic status and oxidative stress in periparturient dairy cows in 24 Holstein cows (10 primiparous, 7 at second calving, and 7 at third calving). Among the findings for oxidative stress biomarkers after calving, a decrease in values was observed in plasma and erythrocyte for thiol groups (such as glutathione reductase, GSH) and SOD, and an increase of ROS, TBARS, and plasma GPx. They suggested that periparturient period, particularly postpartum, as time of depleted antioxidative status which could be related to metabolic and endocrine alterations connected not only with metabolism of

foetus but also with mammary gland. The glutathione system acts as a leading cellular defense mechanism against oxidants. GSH is not only a direct ROS scavenger but also an antioxidant that has an important act in the regulation of intracellular redox status. GPx catalyzes the reduction of $H_2O_2$ to water using GSH as a cosubstrate. The capability of organisms to regenerate GSH means the cell’s success to withstand oxidative stress (JONES, 2002). Although ROS values have not been evaluated in our study, our results regarding the interaction of the glutathione system by increasing the enzymatic activity of GPx and GSH, in association with the oxidizing action of TBARS, corroborate the results found in the literature. Mineral supplementation may be associated with a reduction in the oxidant system and an increase in the antioxidant system in dairy cows during the transition period.

Silva et al., (2021) carried out a study with 142 multiparous Holstein cows in semi-arid conditions with 2 applications of subcutaneous mineral supplementation consisting of 300mg of Zn, 50 mg of Mn, 25 mg of Se and 75 mg of Cu. Cows were evaluated in the postpartum period with 3, 7, 10 and days in lactation. Among the findings, no differences were found for GPx and SOD antioxidant enzymes. Their findings differ from results of our study, the moments in which antioxidant enzymes were evaluated may have influenced the final result, since in our study we analyzed the transition period from prepartum to postpartum. Heat stress has a myriad of effects on dairy cattle throughout the life cycle, including oxidative stress (DAHL et al., 2020). Climatic conditions may have been intense to hit the point that antioxidant system was not sufficient. Abuelo et al. (2013) considered that the concentration of anti- and pro-oxidants separately is not a good indicator of oxidative stress because it is the imbalance between them that defines the oxidative stress. Although the focus of this study was not oxidative stress, but antioxidant enzymes, the lack of oxidizing substance analysis makes it not possible to observe the whole situation.

Among the findings in our study, in comparison between categories (primiparous and multiparous), only GPx showed statistical differences for the mineral supplementation group. When comparing both categories, higher GPx values were observed for younger animal. This could be an indication that mineral supplementation aimed at improving the enzymatic activity of GPx can be better utilized by this animal category. Tomasi et al (2017) evaluated the metaphylactic effect of minerals on antioxidant status of 19 newborn Holstein calves, with 2 doses of subcutaneous

mineral supplement containing copper and zinc were applied on the first day of life. The enzymatic levels of SOD and catalase were statistically significant and with higher values for the treated group in relation to the control, when the calves were 10 days old.

Warken et al (2018) also observed this same effect on enzymatic oxidants but for primiparous cows. They evaluated 12 primiparous cows that received an injectable mineral supplement containing magnesium, phosphorus, potassium, selenium and copper. In this study, greater activities of enzymatic oxidants were also observed, for catalase and SOD, in addition they observed lower levels of ROS during the evaluated periods of 3, 15, 45 and 60 days postpartum. According to our findings and those found in iterature, older animals may have a reduced ability to respond to selenoproteins, as is with GPx, as the ability of younger animals to respond to it was more evident. Thus, although further studies are needed, this may be a justification for which selenium supplementation may be related to the reduction of oxidative stress in older cows by increasing antioxidant activity.

The number of different antioxidant components in serum and tissues makes it relatively difficult to measure each antioxidant component separately. In addition, since there is a cooperation between various antioxidants, looking at one in isolation from rest may not accurately reflect their combined action. Therefore, the measurement of the total serum antioxidant status (TAS) seems a suitable biochemical parameter for evaluating the overall antioxidant status resulting from antioxidant intake or production and their consumption by the increasing glevels of oxidative stress (NEMEC et al 2000). In our study, differences were observed for TAS by group*time interaction for multiparous cows. Interaction analysis showed differences for multiparous cows during second and third postpartum weeks (M+2 and M+3). Omur et al (2016) administrate vitamins (A, D, E) and trace elements (Cu, Mn, Se, Zn) in 10 multiparous Brown Swiss cows. They were evaluated at the beginning of transition period, parturition and 3 weeks after the parturition, and among other findings they evaluated the values of total antioxidant status (TAS) to total oxidant capacity (TOC), obtaining statistically significant higher results for the group treated with injectable mineral supplementation at all times analyzed. In both studies it was possible to observe the beneficial effect of the use of injectable mineral supplementation for dairy cows during the transition period.

## 7.3 IMMUNE BIOMARKERS

The first defense mechanism of organism is the physical barriers that impede the penetration and invasion of microorganisms as the body skin, oral cavity, the mucosal surfaces of gastrointestinal and respiratory tract. Those pathogens that are eventually capable of overcoming physical barriers will be confronted by cellular mechanisms, through the innate immune response, which will grant initial resistance to infection (TIZARD, 2008). The innate, or non-specific, immunity is rapidly activated and serves as the primary immune defence in the initial stages of an infection (INGVARTSEN & MOYES, 2013), which consists in cellular and chemical mechanisms that are capable of destroying microorganisms (TIZARD, 2008). The main types of cells involved in the innate response are neutrophils, dendritic cells (DCs), natural killer cells (NK) and macrophages that recognize, phagocyte and kill pathogenic agents and, simultaneously to this attack, orchestrate responses of hosts through the synthesis of a great variety of inflammatory mediators and cytokines (ADEREM; UNDERHILL, 1999; TIZARD, 2008).

Acute phase proteins activation is closely related to pathogen recognition mechanism by the innate immune system. This activation event occurs after breakdown of the protective physical barrier, protective mucus, mucous membranes and skin. Different microorganisms have different molecules that cause different types of immune responses, such molecules are called pathogen-associated molecular patterns (PAMPs). Pattern recognition is mainly performed by dendritic cells, monocytes and macrophages, mononuclear cells, through pattern recognition receptors (PRRs), molecules located on these cells membrane (TIZARD, 2008). After PAMPs bind to PRRs, intracellular expression of transcription factors occurs, an important signaling pathway for immune responses. These transcription factors stimulate the synthesis of pro-inflammatory cytokines interleukin 1 (IL-1), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α). These cytokines stimulate the liver to produce acute phase proteins, especially haptoglobin in cattle. Liver is the main producer of haptoglobin (YANG et al., 1995). However, the mammary gland, as well as other tissues that express genes, are also capable of producing it (HISS et al., 2004).

Haptoglobin is α2-globulin synthesized by the liver during the acute-phase response (YOSHIOKA et al.,2002). In cattle, circulating concentrations of haptoglobin are negligible during healthy conditions; however, it may increase more than 100-fold during inflammatory and acute-phase reactions (MORIMATSUET al., 1992; NAKAJIMA et al., 1993). For this reason,haptoglobin has been used as a marker for infections, diseases and trauma in beef and dairy cows (Horadagoda et al., 1999). Haptoglobin can also be used as an indicator of the bovine acute-phaseresponse induced by stress or inflammation (ARAUJO et al., 2010; COOKE et al., 2011). Haptoglobin concentration was lower for the mineral supplementation group, for multiparous and total cows. The interaction between treatment*time was not observed for any of the parities analyzed.

Silva et al., (2021) carried out a study with 142 multiparous Holstein cows in semi-arid conditions with 2 applications of subcutaneous mineral supplementation consisting of 300mg of Zn, 50 mg of Mn, 25 mg of Se and 75 mg of Cu. Cows were evaluated in the postpartum period with 3, 7, 10 and days in lactation. Among his findings, were not observed differences for haptoglobin. Although our results do not agree, since in our study we observed differences with decrease values of haptoglobin for mineral supplementation group, they got higher values of haptoglobin for the control group compared to the group that received mineral supplementation. Despite this result for haptoglobin, they suggested that trace mineral supplementation resulted in a more robust innate immune system in the early postpartum period due to higher neutrophil to lymphocyte ratio, PMNL phagocytosis, and oxidative burst, as well as the intensity of the oxidative burst in addition to lower expression of the adhesion molecule L-selectin on the PMNL surface. Reinforcing that mineral supplementation improves the innate immune system.

The next line of defense is specific immunity that consists of antibodies, macrophages and T and B lymphocytes that recognize specific microorganisms. The T cells can be divided into T-helper and T-cytotoxic lymphocytes. The T-helper cells produce cytokines, such as IL-2 and interferon (IFN)-g, which are crucial for an effective cell-mediated immune response. The cytotoxic T cells recognize and eliminate cells infected with an antigen, and old or damaged immune cells that, if present, can increase the susceptibility to infections. The B lymphocytes differentiate

into plasma cells that produce antibodies or immunoglobulins (Igs), that is, IgG1, IgG2 and IgM, or memory cells (SORDILLO et al., 1997).

The effect of treatment with injectable minerals can be observed for IgG was significant in almost all parities evaluated in this study. However, the interaction between treatment*time was only observed for the total number of animals, in which all evaluated moments presented higher IgG values for the mineral supplementation group compared to the control group.

High levels of serum IgG were found at all evaluation moments (three weeks prepartum; two weeks prepartum; one week prepartum; calving week; one week postpartum; two weeks postpartum; three weeks postpartum) in the present study. Results from previous studies indicate there was a greater increase immune response in cows supplemented with trace minerals via injectable or oral ways. Yazlik et al (2021) administrate vitamins (500 000 IU vitamin A, 75 000 IU vitamin D3, 50 mg vitamin E per ml) and trace elements (2.5 mg of copper gluconate, 1.25 mg of sodium selenite, 5 mg of manganese gluconate, 5 mg of zinc gluconate per ml) in 182 multiparous Holstein cows divided into 4 experimental groups (mineral supplementation, vitamin supplementation, mineral + vitamin supplementation and control group) at 10 days prepartum. They reported that the mean percentage of phagocytosis was greater in cows supplemented with manganese, zinc, selenium and copper complex after calving.

Warken et al (2018) evaluated 12 primiparous cows that received an injectable mineral supplement containing sodium glycerophosphate (5.5H2O: 14g), monosodium phosphate (2H2 O: 20,1g), copper chloride (2H2 O: 0.4g), potassium chloride (0.6g), magnesium chloride (2.5g) and sodium selenite (0.24g). Among the finds, TNF, IL-1 and IL-6 levels were higher in cows supplemented with mineral complex (magnesium, phosphorus, potassium, selenium and copper) after 15 days post injection. Since IgG is important for virus and toxin neutralization and bacterial agglutination and opsonization, in addition to neutralizing antibody, complement activation (WANG et al 2013) among other immune functions, the increase in serum IgG dosage may be related to improvement of cow's immune status during the study period.

Alhussien et al., (2021) evaluated 42 multiparous Karan Fries cows and their calves with oral supplementation of vitamin A with 105 IU, zinc sulfate with 60 ppm and vitamin E with 2500 IU. The IgG concentration for the cows was measured in milk and blood

of the calves. Differences were observed with a higher level of IgG for both, in the group that received mineral supplementation. Despite the material evaluated in our study was blood instead of milk, there is a correlation between amounts present in the blood and milk of dairy cows. Thus, it can be stated that mineral supplementation improved the adaptive immunity response for cows in the transition period.

Few works in literature relate the concentration of serum IgG with immune capacity of the dairy cow. Bittar et al., (2020), evaluated the immune response and onset of protection from Bovine viral diarrhea virus 2 infection induced by modified-live virus vaccination 45 weaned Angus and Angus-crossbred calves (7 months old) with the influence of injectable mineral supplementation composed of 300mg of Zn, 50 mg of Mn, 25 mg of Se and 75 mg of Cu. Among the results mineral supplementation was associated with increased serum neutralizing antibodies response to BVDV1 & 2, enhanced health status, mitigation of CD4+, T-cells decrease, and reduction of T-cell activation in calves challenged with BVDV2 five days after immunization. Although the response against viruses is mediated by T cells while production of IgG is carried out by B cells, both have the same origin of APCs. It may be related to the increases in IgG generated by the administration of mineral supplementation.

### 7.4 FINAL CONSIDERATIONS

In the current study was evaluated a multimineral supplement, therefore, it was not possible to carry out individual analysis of each cow for the minerals present in product used in the treated group. Consequently, it was not possible to relate any response to a particular mineral concentration in the treatment influence. However, the study was performed in well managed farms, which did not have any history of mineral deficiencies and where the diets were also well managed. Previous research showed that a subcutaneous injection of 15 mg/mL of Cu, 5 mg/mL of Se, 60 mg/mL of Zn, and 10 mg/mL of Mn given at 1 mL/68 kg of BW resulted in greater concentrations of liver, that represents the storage pool for Se and Cu, for Cu and Se, with no difference in Zn or Mn in feedlot cattle (GENTHER et al., 2014). Thus suggesting that intramuscular application could also result in trace mineral increase in serum.

During the transition period, several disorders can occur at different intensities, such as oxidative stress, and therefore, in this study, mineral supplementation had a beneficial effect. Selenium is an important trace mineral necessary for many

physiological functions and antioxidant defense systems (SORDILLO, 2013). Selenium manifests itself in the innate immune response as selenocysteine, which is incorporated into selenoproteins by replacing protein sulfur residues Glutathione peroxidase is a primary antioxidant selenoprotein and functions to protect neutrophils during oxidative stress by reducing hydrogen peroxides to water (SUNDE et al., 1997). Teixeira et al. (2014) reported that calves supplemented with trace minerals had increased plasma GPx activity compared with calves without supplementation in the present study, similar results were found, being possible to observe significant increase of GPx in animals treated with the mineral supplement for multiparous cows category.

The injectable mineral supplementation was beneficial to health and immunity of the cows during the transition period. These phenomena can be explained duo to properties of each mineral. Selenium and copper have antioxidant power and immunology (ZANETTI et al., 1998; STOWE et al., 1988; CORTINHAS et al., 2012). Magnesium participate in enzymatic process acts as a regulator of mitochondrial function (ROSOL et al., 1997), regulates homeostasis of phosphorus (HORST 1986), and maintains the milk production and adequate fat levels due potassium levels (ISHLER 1997). Therefore, these minerals properties were able to reduce oxidation processes, which lead to the production of free radicals in the transition period of the cows, thereby minimizing oxidative stress for the animal.

The transition period increases the metabolic demand of the dairy cow due to metabolic and immunological changes from calving to the beginning of lactation. During this period, homeorhetic mechanisms are disturbed, among them lipolysis due to a negative physiological energy balance. However, oxidizing substances are generated that need to be buffered by the antioxidant system. In our study, it was possible to observe that older animals have less buffering activity for oxidizing substances. Which makes it an interesting strategy to supplement these animals in a critical period where there is a higher incidence of diseases resulting from calving such as retained placenta, metritis, mastitis, ketosis, displaced abomasum.

The interaction between treatment*time was also observed for multiparous cows, as it happened with BHB, TAS and TBARS. These findings reinforce the metabolic stress triad that involves biomarkers for energy metabolism, oxidative stress and immunity. Higher lypolisis in the control group can be associated with the oxidative

status of cows' periparturient period. The effect of injectable minerals can be observed with greater emphasis for the older cows' category, for biomarkers of energy metabolism and oxidative stress when control group values were especially higher for these categories. These findings reinforce previous findings described in the literature regarding the relationship between metabolic status and oxidative stress (BERNABUCCI et al., 2005; PEDERNERA ET AL., 2010; MACHADO et al., 2014).

# 8 CONCLUSION

The application of injectable mineral supplementation with a commercial product containing phosphorus, magnesium, potassium, selenium and copper in Holstein cows and heifers during the transition period, suggests that:

- Reduced lipid metabolization, although it was not possible to observe significant differences between groups, the interaction between group*time for the BHB variable, showed a decrease in its concentration for the group treated at a critical moment in the postpartum period.
- Reduced oxidative stress in treated animals by increasing enzymatic activity of GPx (multiparous cows) and GSH (total animals).
- Improved immunity of cows, characterized by an anti-inflammatory profile by the haptoglobin biomarker that had its levels reduced compared to the control group and higher serum IgG concentrations for the group that received the treatment at all times analyzed.

**REFERENCES**

ABBAS, A. K.; LICHTMAN, A. H.; POBER, J. S. **Cellular and molecular immunology**. Philadelphia: W. B. Saunders, 3. ed., 494 p., 1997.

ABUELO, A.; ALVES-NORES, V.; HERNANDEZ, J.; MUINO, R.; BENEDITO, J.L.; CASTILLO, C. Effect of parenteral antioxidant supplementation during the dry period on postpartum glucose tolerance in dairy cows. **J. Vet. Intern. Med**., v.30, p.892–898, 2016.

ABUELO, A.; HERNANDEZ, J.; BENEDITO, J.L.; CASTILLO, C. The importance of the oxidative status of dairy cattle in the periparturient period: Revisiting antioxidant supplementation. **J. Anim. Physiol. Anim. Nutr**., v.99, p.1003–1016, 2015.

ABUELO, A.; HERNANDEZ,J.; BENEDITO, J. L.; CASTILLO, C. Oxidative stress index (OSi) as a new tool to assess redox status in dairy cattle during the transition period. **Animal**, v.7, n.8, p.1374–1378, 2013.

ABUELO, A.; HERNANDEZ,J.; BENEDITO, J. L.; CASTILLO, C. Redox Biology in Transition Periods of Dairy Cattle: Role in the Health of Periparturient and Neonatal Animals. **Antioxidants**, v.8, n.20, 2019

ABUELO, A.; HERNANDEZ,J.; BENEDITO, J. L.; CASTILLO, C. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. **Journal of Animal Physiology and Animal Nutrition**. v.99 n.1003–1016, 2014.

ACKERMANN,MR.; DERSCHEID, R.; ROTH, JA. Innate Immunology of Bovine Respiratory Disease. **Vet Clin North Am Food Anim Pract**. v.26, n.2, p.215–228, 2010.

ALBERTS, B.; JOHNSON, A.; LEWIS, J.; RAFF, M.; ROBERTS, K.; WALTER, P. **Molecular biology of the cell**. New York: Garland, 4. Ed, 1616 p., 2002.

ALBIGER, B.; DAHLBERG, S.; HENRIQUES-NORMARK, B.; NORMARK, S. Role of the innate immune system in host defence against bacterial infections: focus on the toll-like receptors. **Journal of Internal Medicine**, v.261, n.6, p.511-528, 2007.

ALHUSSIEN, MN.; TIWARI, S.; PANDA, B.S.K.; PANDEY, Y.; LATHWAL, S.S.; DANG, A.K. Supplementation of antioxidant micronutrients reduces stress and improves immune function/response in periparturient dairy cows and their calves. **Journal of Trace Elements in Medicine and Biology** v.65, p.126718, 2021.

ALVES, L. Free radicals, lipid peroxidation and the antioxidant system in the blood of cows and newborn calves around calving. Part B **Comparative Biochemistry and Physiology** v. 143, p. 391–396, 2009.

ANDERSON, D. Antioxidant Defences Against Reactive Oxygen Species Causing Genetic And Other Damage. **Mutation Research**, v. 350, n. 1, p.103-108, 1996.

ANDRIEU, S. Is there a role for organic trace element supplements in transition cow health? **The Veterinary Journal**. v.176, p. 77-83, 2008.

ARTHINGTON, JD.; MORIEL, P.; MARTINS, P. G. M. A.; LAMB, G. C.; HAVENGA, L. J. Effects of trace mineral injections on measures of performance and trace mineral status of pre- and postweaned beef calves. **J. Anim. Sci**. v.92, p. 2630–2640, 2014.

BALLET, N.; ROBERT, J. C.; WILLIAMS, P. E. V. Vitamins in forages. **Forage evaluation in ruminant nutrition.** p. 399-431, 2000.

BARGO, F.; MULLER, L. D.; DELAHOY, J. E. Milk response to concentrate supplementation of high producing dairy cows grazing at two pasture allowances. **Journal of Dairy Science**, v. 85, p. 1777-1792, 2002.

BAUMGARD, L. H.; COLLIER, R. J.; BAUMAN, D. E. A 100-Year Review: Regulation of nutrient partitioning to support lactation, **Journal of Dairy Science**, v. 100, n. 12, 2017

BELL, A. W., Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. **Journal of Animal Science**, v. 73, p. 2804–2819, 1995.

BELL, A. W.; BAUMAN D. E. Adaptations of glucose metabolism during pregnancy and lactation. **Journal of Mammary Gland Biology and Neoplasia**. v. 2, n. 3, p. 265-278, 1997.

BELL, MJ. The effect of improving cow productivity, fertility, and longevity on the global warming potential of dairy systems. Journal of Dairy Science. v.94, n.7, p.3662-3678, 2010.

BERNABUCCI, U.; RONCHI, B.; LACETERA, N.; NARDONE, A. Influence of body condition score on the relationship between metabolic status and oxidative stress in periparturient dairy cows. **Journal of Dairy Science**, v. 88, p. 2017–2026, 2005.

BERNABUCCI, U.; RONCHI, B.; LACETERA, N.; NARDONE, A. Markers of oxidative status in plasma and erythrocytes of transition dairy cows during hot season. **Journal of Dairy Science**, v. 85, n. 9, p. 2173–2179, 2002.

BERTONI G., MINUTI A. & TREVISI E. Immune system, inflammation and nutrition in dairy cattle. Animal Production Science. v.55, p.943-948, 2009.

BIANCHI, M. L. P.; ANTUNES, L. M. G. Radicais livres e os principais antioxidantes da dieta. **Revista de Nutrição**, v. 12, n. 2, p. 123-130, 1999.

BORDIGNON, R.; VOLPATO, A.; GLOMBOWSKY, P.; SOUZA, CF.; BALDISSERA, MD.; SECCO, R.; PEREIRA, WAB.; LEAL, MLR.; VEDOVATTO, M.; SILVA, AS. Nutraceutical effect of vitamins and minerals on performance and immune and antioxidant systems in dairy calves during the nutritional transition period in summer. **Journal of Thermal Biology**. v.84 p.451–459, 2019.

BOUWSTRA, R. J.; NIELEN, M.; NEWBOLD, J. R.; JANSEN, E. H. J. M.; JELINEK, H. F.; VAN WERVEN, T. Vitamin e supplementation during the dry period in dairy cattle. Part ii: oxidative stress following vitamin e supplementation may increase clinical mastitis incidence postpartum. **Journal of Dairy Science**, v. 93, n. 12, p. 5696–5706, 2010.

BOWEN M. K.; CHUDLEIGH, F.; DIXON, R. M.; SULLIVAN, M. T.; SCHATZ, T.; OXLEY, T. The economics of phosphorus supplementation of beef cattle grazing northern Australian rangelands. **Animal Production Science**, n.60, v.5, p.683–69. 2020.

BRENNEISEN, P., STEINBRENNER, H., SIES, H. Selenium, oxidative stress, and health aspects. **Molecular Aspectsof Medicine**, v. 26, p. 256–267, 2005.

BRIVIBA, K.; SIES, H. Non-enzymatic antioxidants defence system. **Natural antioxidants in human health and disease.** p. 107-128, 1994.

CAMPBELL, M. H.; MILLER, J. K. Effect of supplemental dietary vitamin E and zinc on reproductive performance of dairy cows and heifers fed excess iron. **Journal of Dairy Science**, v. 81, p. 2693–2699, 1998.

CAMPOS, R.; GONZÁLEZ, F.; COLDEBELLA, A.; LACERDA, L. Indicadores do metabolismo energético no pós-parto de vacas leiteiras de alta produção e sua relação com a composição do leite. **Ciência Animal Brasileira**, v. 8, n. 2, p. 241-249, 2007.

CASTILLO, C.; HERNANDEZ, J.; BRAVO, A.; LOPEZ-ALONSO, M.; PEREIRA, V.; BENEDITO, J.L. Oxidative status during late pregnancy and early lactation in dairy cows. **Vet. J.,** v.169, p.286–292, 2005.

CASTILLO, C.; PEREIRA, V.; ABUELO, A.; HERNANDEZ, J. Effect of supplementation with antioxidants on the quality of bovine milk and meat production. **Sci. World J**., v.2013, p.616098, 2013.

CECILIANI, F.; CERON, J.J.; ECKERSALL, P.D.; SAUERWEIN, H. Acute phase proteins in ruminants. **Journal of Proteomics**, v.75, n.14, p.4207-4231, 2012.

CELI, P.; GABAI, G. Oxidant/Antioxidant Balance in Animal Nutrition and Health: The Role of Protein Oxidation. **Front. Vet. Sci.** v.2, p.48., 2015.

CHAPINAL, N.; CARSON, M.; DUFFIELD, T.F.; CAPEL, M.; GODDEN, S.; OVERTON, M.; SANTOS, J.E.; LEBLANC, S.J. The association of serum metabolites with clinical disease during the transition period. **J Dairy Sci**. n.94, p.4897-4903. 2011.

CHEW, B. P.; ERB, R. E.; FESSLER, J. F. Effects of ovariectomy during pregnancy and of prematuraly induce parturation on progesterone, estrogens, and calving traits. **Journal of Dairy Science**, v. 62, p. 557-566, 1979.

CHIHUAULAF, R.H.; CONTRERAS P.A.; WITTWER F.G. Pathogenesis of oxidative stress: consequences and evaluation in animal health. **Vetereniry of Méxican**.,v. 33, n. 3, p. 265-283, 2002.

CONTRERAS, G.A.; STRIEDER-BARBOZA, C.; RAPHAEL, W. Adipose tissue lipolysis and remodeling during the transition period of dairy cows. **J. Anim. Sci. Biotechnol.**, v.8, p.41, 2017.

COOKE, R. F. SCHUBACH, K. M.; MARQUES, R. S.; PERES, R. F. G.; SILVA, L. G. T.; CARVALHO, R. S.; CIPRIANO, R. S.; BOHNERT, D. W.; PIRES, A. V.; VASCONCELOS, J. L. M. Effects of temperament on physiological, productive, and reproductive responses in Bos indicus beef cows. **Journal of Animal Science**., v. 95, n. 1, p. 1-8, 2017

CORAH, L. Trace mineral requirements of grazing cattle. **Animal Feed Science and Technology**. v.59, p.61-70 1996.

DAHA, M.R. Grand challenges in molecular innate immunity. **Front. Immunol**. v.2, p.16–17. 2011

DAHL, G.E.; TAO, S.; LAPORTA, J. Heat Stress Impacts Immune Status in Cows Across the Life Cycle. **Front. Vet. Sci**. v.7, p.116, 2020.

DALLE-DONNE, I.; SCALONI, A.; GIUSTARINI, D.; CAVARRA, E.; TELL, G.; LUNGARELLA, G.; COLOMBO, R.; ROSSI, R.; MILZANI, A. Proteins as biomarkers of oxidative/nitrosative stress in diseases: The contribution of redox proteomics. **Mass Spectrom. Rev.**, v.24, p.55–99, 2005.

DRACKLEY, J. K. Biology of Dairy Cows During the Transition Period: the Final Frontier? **Journal of Dairy Science,** v. 82 p.2259–2273, 1999.

DROGE, W. Free Radicals In The physiological control of cell function. **Physiology Reviews**, v. 82, p. 47–95, 2002.

DUBUC, J. DUFFIELD, T F.; LESLIE, K E.; WALTON, J S.; LEBLANC, S J. Risk factors for postpartum uterine diseases in dairy cows. **Journal of Dairy Science**, v.93, n.12, p.5764-5771, 2010.

DUFFIELD, T. F.; LISSEMORE, K. D.; MCBRIDE, B. W.; LESLIE, K. E. Impact of Hyperketonemia in Early Lactation Dairy Cows on health and Production. **Journal of Dairy Science**, 2002.

DUREL, L.; O'NEILL, P.; VAN WYL, L.; ROSE, C.; BATARD, A. **MULTIMIN TECHNICAL MANUAL**, Virbac, p.100, 2021.

EATON, P., Protein thiol oxidation in health and disease: Techniques for measuring disulfides and related modifications in complex protein mixtures. **Free Rad. Biol**. Med. v.40, p.1889–1899, 2006.

ECKEL, E.F., AMETAJ, B.N. Invited review: role of bacterial endotoxins in the etiopathogenesis of periparturient diseases of transition dairy cows. **J. Dairy Sci**. v.99 n.8, p.5967–5990. 2016

EMERY, R. S.; LIESMAN, J. S.; HERDT, T. H. Metabolism of long-chain fatty acids by ruminant liver. **Journal Nutrition**, v. 122, p. 832-837, 1992.

FARNEY, J.K.; MAMEDOVA, L.K.; COETZEE, J.F.; KUKANICH, B.; SORDILLO, L.M.; STOAKES, S.K.; MINTON, J.E.; HOLLIS, L.C.; BRADFORD, B.J. Anti-inflammatory salicylate treatment alters the metabolic adaptations to lactation in dairy cattle. **Am. J. Physiol. Regul. Integr. Comp. Physiol**., v.305, p.R110–R117, 2013.

FAZZIO, L.E.; ROSA, D.E.; PICCO, S.J.; MATTIOLI, G.A. Assessment of Cu-Zn EDTA Parenteral Toxicity in Calves. **Biol Trace Elem Res**. v.179, n.2, p.213-217, 2017.

FERNANDES, V.; MACHADO, L. P.; KOHAYAGAWA, A.; SAITO, M. E.; YONEZAWA, L. A. Lesão oxidativa eritrocitária e mecanismos antioxidantes de interesse na medicina veterinária, **Revista de Ciências Agroveterinárias**. v. 8, n. 1, p. 84-94, 2012.

FERREIRA, F. Stress oxidativo e dano oxidativo muscular esquelético: influência do exercício agudo inabitual e do treino físico. **Revista Portuguesa de Ciências do Desporto**, v. 7, n. 2, p. 257–275, 2007.

GITTO, E., REITER, R.J., KARBOWNIK, M., TAN, D.X., GITTO, P., BARBERI, S., BARBERI, I. Causes of oxidative stress in the pre- and perinatal period. **Biol. Neonate** v.81, p.146–157, 2002.

GLOMBOWSKY, P.; SILVA, AS.; SOLDÁ, NM.; GALLI, GM.; BIAZUS, AH.; CAMPIGOTTO, G.; BOTTARI, NB.; SOUSA, RS.; BRISOLA, MC.; STEFANI, LM.;

BALDISSERA, MD.; LEAL, MLR.; MORSCH, VM.; SCHETINGER, MRC.; MACHADO, G. Mineralization in newborn calves contributes to health, improve the antioxidant system and reduces bacterial infections, **Microbial Pathogenesis** v.114 p.344–349, 2018.

GOFF, J. P.; HORST R. L. Physiological changes at parturition and their relationship to metabolic disorders. **Journal of Dairy Science**, v. 80, p. 1260–1268, 1997.

GOFF, J.P.; HORST, R.L. Physiological Changes at Parturition and Their Relationship to Metabolic Disorders. **Journal of Dairy Science**. v.80, n.7, p.1260-1268. 1997.

GONZÁLEZ, F. H. D. BARCELLOS, J. O.; OSPINA, H.; RIBEIRO, L. A. O Uso do perfil metabólico para determinar o status nutricional em gado de corte. **Perfil metabólico em ruminantes e seu uso em nutrição e doenças nutricionais.** Porto Alegre: UFRGS, 2007.

GRIGNARD, E., MORIN, J., VERNET, P., DREVET, J.R.,. GPX5 orthologs of the mouse epididymis-restricted and sperm-bound selenium-independent glutathione peroxidase are not expressed with the same quantitative and spatial characteristics in large domestic animals. **Theriogenology,** v.64, p.1016–1033, 2005.

GRUMMER, R. R. Etiology of lipid-related metabolic disorders in periparturient dairy cows. **Journal of Dairy Science**, v. 76, p. 3882–3896, 1995.

HALLIWELL, B.; GUTTERIDGE, J. M. Free radicals in biology and medicine. **New York: Oxford University Press**, 2007.

HARA, S. Effects of selenium deficiency on expression of selenoproteins in bovine arterial endothelial cells. **Biol. Pharmaceut**. **Bull**. v.24, p.754–759, 2001

HEFNAWY, A.E.G; TÓRTORA-PÉREZ, J.L. The importance of selenium and the effects of its deficiency in animal health. **Small Ruminant Research**. v.89, n.2–3, p.185-192, 2010.

HISS, S.; MIELENZ, M.; BRUCKMAIER, R.M.; SAUERWEIN, H. Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. **J. Dairy Sci**., n.87, p. 3778-3784, 2004.

HUNTOON, K. M.; RUSSELL, L.; TRACY, E.; BARBOUR, K. W.; LI, Q.; SHRIKANT, P. A.; BERGER, F.G.; GARRETT-SINHA, L.A.; BAUMANN, H. A unique form of haptoglobin produced by murine hematopoietic cells supports B-cell survival, differentiation and immune response. **Molecular Immunology**. v.55, n.3–4, p.345-354, 2013.

HURLEY, W.L., R.M. DOANE. Recent developments in the roles of vitamins and minerals in reproduction. **J. Dairy Sci**. v.72 p.784-804, 1989.

HUZZEY, J.M.; DUFFIELD, T.F.; LEBLANC, S.J.; VEIRA, D.M.; WEARY, D.M.; VON KEYSERLINGK, M.A. Short communication: Haptoglobin as an early indicator of metritis. **J. Dairy Sci**. v.92, p.621–625, 2009.

INGVARTSEN, K.L.; DEWHURST, R.J.; FRIGGENS, N.C. On the relationship between lactational performance and health: Is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. **Livest. Prod. Sci**., v.83, p.277–308, 2003.

INGVARTSEN, K.L.; MOYES, K. Nutrition, immune function and health of dairy cattle. **Animal**, v.7, n.1, p.112-122, 2013.

JONES DP. Redox potential of GSH/GSSG couple: Assay and biological significance. **Methods Enzymol**., p. 93-112, 2002.

JORDÃO Jr A. A.; CHIARELLO P. G.; BERNARDES, M. S. M.; HELIO VANNUCCHI, H. Peroxidação lipídica e etanol: papel da glutationa reduzida e da vitamna E. MEDICINA, RIBEIRÃO PRETO. n. 31, p.434-449, 1998.

KIMURA, K.; GOFF, J.P.; KEHRLI, M.E., Jr.; HARP, J.A.; NONNECKE, B.J. Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. **J. Dairy Sci**., v.85, p.1437–1444., 2002.

KLECZHOWSKI, M.; KLUCINSKI, W.; SIKORA, J.; JDANOCVIEZ, M.; DZIEKAN, P. Role of antioxidants in the protection against oxidative stress in cattle: non-enzymatic mechanisms (part 2). **Polish Journal of Veterinary Sciences**, n. 6, p. 301–308. 2003.

KONVICNÁ, J.; VARGOVÁ, M.; PAULÍKOVÁ, I.; KOVÁC, G.; KOSTECKÁ, Z. Oxidative stress and antioxidant status in dairy cows during prepartal and postpartal periods. **Acta Vet. Brno**, v.84, p.133–140., 2015.

KREPLIN, C.; YAREMCIO B. Effects of Nutrition on Beef Cow Reproduction. **Practical information for Alberta's agriculture industry**. 1994.

LACETERA, N., SCALIA, D., BERNABUCCI, U., RONCHI, B., PIRAZZI, D., NARDONE, A., Lymphocyte functions in overconditioned cows around parturition. **J. Dairy Sci**. v.88, p.2010–2016, 2005.

LEBLANC, S. J. Monitoring metabolic health of dairy cattle in the transition period. **The Journal of Reproduction and Development,** v. 56, p. 29–35, 2010.

LENG, R. A.; ANNISON, E. F. Metabolic activities of sheep erythrocytes. I. Glycolytic activities. **Australian Journal of Agricultural Research**, v. 13, p. 31-43, 1962.

LOOR J. J. ; DANN H. M. ; EVERTS R. E.; OLIVEIRA R.; GREEN C. A.; JANOVICKGURETZKY N. A.; LITHERLAND N.; RODRIGUEZ-ZAS S. L.; LEWIN H. A.; DRACKLEY J. K. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. **Physiological Genomics**, v. 27, n. 1, p. 29–41, 2006.

MANN, S. SIPKA, AS.; GRENIER, JK. The degree of postpartum metabolic challenge in dairy cows is associated with peripheral blood mononuclear cell transcriptome changes of the innate immune system. **Developmental and Comparative Immunology**, v.93, p.28-36, 2019.

MARTINEZ, N., RISCO, C.A., LIMA, F.S., BISINOTTO, R.S., GRECO, L.F., RIBEIRO, E.S., MAUNSELL, F., GALVÃO, K., SANTOS, J.E., Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. **J. Dairy Sci**. v.95, n.12, p.7158–7172, 2012.

MARTYNEZ-SUBIELA, S.; CERON, J.J.; ECKERSALL, P.D. Acute phase proteins in dogs and cats: current knowledge and future perspectives. **Vet. Clin. Pathol**. v.34, n.2, p.85-99. 2005.

MATTIOLI, G.A.; ROSA, D.E.; TURIC, E.; PICCO, S.J.; RAGGIO, S.J.; MINERVINO, A.H.H.; FAZZIO, L.E. Effects of Parenteral Supplementation with Minerals and Vitamins on Oxidative Stress and Humoral Immune Response of Weaning Calves. **Animals**. v.10, n.8, p.1298, 2020.

MAUNSELL, F.P.; CHASE, C. Mycoplasma Bovis: Interactions With the Immune System and Failure to Generate an Effective Immune Response. **Vet Clin North Am Food Anim Pract**. v.35, n.3, p.471–83, 2019.

McART, J.A.A.; NYDAM, D.V.; OETZEL, G.R. Epidemiology of subclinical ketosis in early lactation dairy cattle. **Journal of Dairy Science**. v.95, n.9, p.5056-5066, 2012.

MEHDI, Y.; DUFRASNE, I. Selenium in Cattle: A Review. **Molecules**. v.21, n.4, p.545, 2016.

MILLER, J. K.;. MADSEN F. C; LYONS T. P.; JACQUES K. A.. Transition metals, oxidative status, and animal health: Do alterations in plasma fast-acting antioxidant lead to disease in livestock? Biotechnology in the feed industry. **Proc. Alltech's Tenth Annu. Symp. ed. Nottingham Univ. Press, Nottingham, UK.** p. 283–301, 1994.

MILLER, J.K., E. BRZEZINSKA-SLEBODZINSKA, F.C. MADSEN. Oxidative stress, antioxidants and animal function. **J. Dairy Sci**. v.76, p.2812-2823. 1993.

MINUTI, A., AHMED, S., TREVISI, E., PICCIOLI-CAPPELLI, F., BERTONI, G., JAHAN, N., BANI, P., Experimental acute rumen acidosis in sheep: consequences on clinical, rumen, gastrointestinal permeability conditions, and blood chemistry. J**. Anim. Sci.** v.92, n.9, p.3966–3977, 2014.

MORIMATSU, M.; SYUTO, B.; SHIMADA, N.; FUJINAGA, T.; YAMAMOTO, S.; SAITO, M.; NAIKI, M. Isolation and characterization of bovine haptoglobin from acute phase sera. **Journal of Biological Chemistry**, v.266, n.18, p.11833-11837, 1991.

MOYES, K.M.; LARSEN, T.; FRIGGENS, N.C.; DRACKLEY, J.K.; INGVARTSEN, K.L. Identification of potential markers in blood for the development of subclinical and clinical mastitis in dairy cattle at parturition and during early lactation. **Journal of Dairy Science**. v.92, n.11, p.5419-5428, 2009.

MURATA, H., SHIMADA, N., YOSHIOKA, M. Current research on acute phase proteins in veterinary diagnosis: An overview. **The Veterinary Journal**, v.168, p.28–40, 2004.

NASEM: National Academies of Sciences, Engineering and Medicine. **Nutrient Requirements of Beef Cattle**: Eighth Revised Edition. National Academies Press, 2016.

NASEM: National Academies of Sciences, Engineering and Medicine. **Nutrient Requirements of Dairy Cattle**: Eighth Revised Edition. National Academies Press, 2021.

NEMEC, M. DROBNIČ-KOŠOROK, M. SKITEK, Z. PAVLICA, S. GALAC, J. BUTINAR. Total Antioxidant Capacity (TAC) Values and Their Correlation with Individual Antioxidants in Healthy Beagles. **Acta Vet. Brno**, v.69, n.4: 297-303, 2000

NOVAK, K. Functional Polymorphisms in Toll-Like Receptor Genes for Innate Immunity in Farm Animals. **Vet Immunol Immunopathol,** v.157, n.1-2, p.1–11, 2014.

NRC: NATIONAL RESEARCH COUNCIL. Committe on Animal Nutrition. Nutrient requeriments of beef cattle. 6. ed. Washingyon: **National Academy of Science**, 2011.

NUNES, E.; OLIVEIRA, S. Radicais livres: conceito, doenças, estresse oxidativo e antioxidantes. **Open Journal Systems**. 2006

OSPINA, P.A., NYDAM, D.V., STOKOL, T., OVERTON, T.R., Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. **J. Dairy Sci**. v.93, p.1596–1603, 2010.

PASSI, S.; STANCATO A.; COCCHI M. A monitoring of oxidative stress of ageing and ageing-related diseases. **Prog. Nutr**. v.3, p. 35–58, 2001.

PATE, R. T.; CARDOSO F. C.. Injectable trace minerals (selenium, copper, zinc, and manganese) alleviate inflammation and oxidative stress during an aflatoxin challenge in lactating multiparous Holstein cows. **J. Dairy Sci.** v.101, p.8532–8543, 2018.

PICCIONE, G.; CAOLA, G.; GIANNETTO, C. Selected biochemical serum parameters in ewes during pregnancy, post-parturition, lactation and dry period. **Animal Science Papers and Reports**, v. 27, n. 4, p. 321–330, 2009.

PICCO, S.J. **Nutrición Mineral y Vitamínica de Bovinos**. 2ª edición. Capítulo 1. 2019.

PICCO, S.J.; MATTIOLI, G.A.; FAZZIO, L.E.; ROSA, D.; FURNUS, C.C.; DULOUT, F.N. Genotoxic and Clastogenic effect of Parenteral Cu-Ca EDTA supplementation in Cattle. **Basic and Applied Genetics**, vol. 17 p. 33 – 39, 2006.

PLOEGAERT, T.C.; TIJHAAR, E.; LAM, T.J.; TAVERNE-THIELE, A.; VAN DER POEL, J.J.; VAN ARENDONK, J.A.; SAVELKOUL, H.F.J.; PARMENTIER, H.K. Natural Antibodies in Bovine Milk and Blood Plasma: Variability Among Cows, Repeatability Within Cows, and Relation Between Milk and Plasma Titers. **Vet Immunol Immunopathol**, v.144, n.1-2, p.88–94, 2011.

RAINARD P. The Complement in Milk and Defense of the Bovine Mammary Gland Against Infections. **Vet Res**. v.34, n.5, p.647–70, 2003.

RAMOS, J.S.; MADUREIRA, K.M.; SILVA, K.N.; BOSCO, K.A.; MORITA, L.M.; GUIMARÃES, J.E.; GOMES, V. Haptoglobin and its association with naturally occurring diseases in Holstein heifer calves. **Arq. Bras. Med. Vet. Zootec**. v.73, n.03, 2021.

REYNOLDS, C. K., AIKMAN, P. C., LUPOLI, B., HUMPHRIES, D. J., BEEVER, D. E. Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. **Journal of Dairy Science**,. v.86, p.1201-1217, 2003.

RIBEIRO, L., GONZÁLEZ, F. Perfil metabólico de borregas corriedale em pastagem nativa do Rio Grande do Sul. **Acta Scientiae**, v. 31, p. 167–170, 2003.

ROSOL, C.; CAPEN, C. Calcium-regulating hormones and diseases of abnormal mineral calcium, phosphorus, magnesium metabolism. **Clinical biochemistry of domestic animals**, v.5, p. 619–702, 1997

ROSSI, E.; FAURE, P.; LAFOND, J. L.; RICHARD, M. J.; FAVIER, A.; HALIMI, S. Vitamin E improves the free radical defense system potential and insulin sensitivity of rats fed high fructose diets. **The Journal of Nutrition**, v. 127, p. 103–107, 2004.

ROVER JÚNIOR, L.; HÖEHR, N.F.; VELLASCO, A. P. Sistema antioxidante envolvendo o ciclo metabólico da glutationa associado a métodos eletroanalíticos na avaliação do estresse oxidativo. **Química Nova**, v. 24, n. 1, p. 112-119, 2001.

SMITH, K. L.; HARRISON, J. H.; HANCOCK, D. D. Effect of vitamin E and selenium supplementation on incidence of clinical mastitis and duration of clinical symptoms. **Journal of Dairy Science**, v.67, p.1293-1300, 1997.

SOLDÁ, N.M; GLOMBOWSKY, P.; CAMPIGOTTO, G.; BOTTARI, N.B.; SCHETINGER, M.R.C.; MORSCH, V.M.; FAVERO, J.F.; BALDISSERA, M.D.; SCHOGOR, A.N.L.B.; BARRETA, D.; MACHADO, G.; SILVA, A.S. Injectable mineral supplementation to transition period dairy cows and its effects on animal health. **Comp Clin Pathol** v.26, p.335–342, 2017.

SORDILLO, L.M. et al., Immunobiology of the mammary gland. **Journal of Dairy Science**, v.80, n.8, p.1851-1865, 1997.

SORDILLO, L.M. Selenium-dependent regulation of oxidative stress and immunity in periparturient dairy cattle. **Vet. Med**. p.154045, 2013.

SORDILLO, L.M.; AITKEN, S.L. Impact of oxidative stress on the health and immune function of dairy cattle. **Vet. Immunol. Immunopathol**. v.128, p.104–109, 2009.

SORDILLO, L.M.; MAVANGIRA, V. The nexus between nutrient metabolism, oxidative stress and inflammation in transition cows. **Anim. Prod. Sci**. v.54, p.1204–1214, 2014.

SORG, O. Oxidative stress: a theoretical model or a biological reality? **C. R. Biol**. v.327, p.649–662, 2004.

SPEARS, J. W.; WEISS W. P. Role of antioxidants and trace elements in health and immunity of transition dairy cows. **Veterinary Journal**, v. 176, n. 1, p. 70-76, 2008.

STANFIELD, R.L.; HAAKENSON, J.; DEISS, T.C.; CRISCITIELLO, M.F.; WILSON, I.A.; SMIDER, V.V. The Unusual Genetics and Biochemistry of Bovine Immunoglobulins. **Adv Immunol**. v.137, p.135–64, 2018

STOKES, R.; RALPH, A.; MICKNA, A.; CHAPPLE, W.; SCHROEDER, A.; IRELAND, F.A.; SHIKE, D.W. Effect of an injectable trace mineral at the initiation of a 14 day CIDR protocol on heifer performance and reproduction. **Transl Anim Sci**. v.1, p.458–66. 2019.

STOKES, R.S.; VOLK, M.J.; IRELAND, F.; SHIKE, D.W. Effects of maternal supplementation with an injectable trace mineral on subsequent calf performance and inflammatory response. **Journal of Animal Science**, v.97, n.11, p. 4475–4481, 2019.

SURAI, P.F. **Selenium in Ruminant Nutrition**. Selenium in Nutrition and Health, Nottingham University Press: Nottingham, UK,; p. 487–587, 2006.

SUTTLE, N.F., JONES, D.G. Recent developments in trace element metabolism and function: trace elements. Disease resistance and immune responsiveness in ruminants. **J. Nutr**. v.119, p.1055–1061, 1998.

TEIXEIRA, A.G.V.; LIMA, F.S.; BICALHO, M.L.S.; KUSSLER, A.; LIMA, S.F.; FELIPPE, M.J.; BICALHO, R.C. Effect of an injectable trace mineral supplement containing selenium, copper, zinc, and manganese on immunity, health, and growth of dairy calves. **J Dairy Sci**. v.97, n.7, p1–11, 2014.

TIZARD, I. R**. Imunologia veterinária**: uma introdução. 6. ed. São Paulo: Roca, 2008.

TOMASI, T. ; VOLPATO, A.; PEREIRA, W. A. B.; DEBASTIANI, L. H.; BOTTARI, N. B.; MORSCH, V. M.; SCHETINGER, M. R. C.; LEAL, M. L. R.; MACHADO, G.; DA SILVA, A. S. Metaphylactic effect of minerals on the immune response, biochemical variables and antioxidant status of newborn calves. **J Anim Physiol Anim Nutr**. v.102, n.4, p.819-824, 2018.

TOTHOVA, C.S.; NAGY, O.; SEIDEL, H.; KONVICNA, J.; FARKASOVA, Z.; KOVAC, G. Acute phase proteins and variables of protein metabolism in dairy cows during the pre-and postpartal period. **Acta Vet Brno.** n.77. p.51-57. 2011.

TREVISAN, M.; BROWNE, R.; RAM, M.; MUTI, P., FREUDENHEIM, J., CAROSELLA, A. M.; ARMSTRONG, D. Correlates of markers of oxidative status in the general population. **American Journal of Epidemiology**, v. 154, p. 348–356, 2001.

TREVISI, E., AMADORI, M., COGROSSI, S., RAZZUOLI, E., BERTONI, G. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. **Res. Vet. Sci**. v.93, p.695–704, 2012.

TREVISI, E., MOSCATI, L., AMADORI, M. Chapter 9- Disease-Predicting and prognostic potential of innate immune responses to noninfectious stressors: human and animal models. In: Amadori, M. (Ed.), **The Innate Immune Response to Noninfectious Stressors. Elsevier Inc., The Netherland**, p. 209–235, 2016.

TREVISI, E.; MINUTI, A. Assessment of the innate immune response in the periparturient cow. **Research in Veterinary Science**. Sci. v.14, p.285–292, 2018.

UNDERWOOD, J.P.; DRACKLEY, J. K.; DANN, H. M.; DOUGLAS, G. N.; GURETZKY, N. A. J.; LITHERLAND, N. B.; LOOR, J. J. Physiological and pathological adaptations in dairy cows that may increase susceptibility to periparturient diseases and disorders. **Journal Animal Science**. v. 4, p. 323– 344, 1999.

VALKO, M., LEIBFRITZ, D., MONCOL, J., CRONIN, M.T., MAZUR, M., TELSER, J., Free radicals and antioxidants in normal physiological functions and human disease. **Int. J. Biochem. Cell. Biol**. v.39, p.44–84, 2007.

VLASOVA, A.N.; SAIF, L.J. Bovine Immunology: Implications for Dairy Cattle. **Frontiers in Immunology**. v.12, p.6 43206, 2021.

WALDRON, M. R.; OVERTON T. R. Nutritional management of transition dairy cows: strategies to optimize metabolic health. **Journal of Dairy Science**, v. 87, p. 105– 119, 2003.

WANG, K., WU, L., DOU, C., GUAN, X., WU, H., LIU, H., Research advance in intestinal mucosal barrier and pathogenesis of Crohn's disease. **Gastroenterol. Res. Pract**. p.9686238, 2013.

WEBER, C.; HAMETNER, C.; TUCHSCHERER, A.; LOSAND, B.; KANITZ, E.; OTTEN, W.; SINGH, S.P.; BRUCKMAIER, R.M.; BECKER, F.; KANITZ, W.; Variation in fat mobilization during early lactation differently affects feed intake, body condition, and lipid and glucose metabolism in high-yielding dairy cows. **J. Dairy Sci**. v.96, p.165–180, 2013.

WEIGEL, R. Avaliação do metabolismo oxidativo e da histopatologia renal e hepática de ovinos intoxicados por cobre e tratados com tetratiomolibdato e vitaminas antioxidantes. 2008. 150 f. **Dissertação (Mestrado em Clínica Médica)** – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2008.

WEISS, W. P.; SMITH, K. L.; HOGAN, J. S. Effect of forage to concentrate ratio on disappearance of vitamins a and e during in vitro ruminal fermentation. **Journal of Dairy Science**, v. 78, p. 1837-1842, 1995.

WEISS, W.P., HOGAN, J.S. Effects of dietary vitamin C on neutrophil function and responses to intramammary infusion of lipopolysaccharide in peri- parturient dairy cows. **Journal of Dairy Science** v.90, p.731–739, 2007.

WICHTEL, J.J. A review of selenium deficiency in grazing ruminants part 2: Towards a more rational approach to diagnosis and prevention. **New Zealand Vet. J**. v.46, p.54–58, 1998.

WITTWER, F. **Marcadores bioquímicos no controle de problemas metabólicos nutricionais em gado de leite**. In: PERFIL METABÓLICO EM RUMINANTES: SEU USO EM NUTRIÇÃO E DOENÇAS NUTRICIONAIS. p.53-62, 2000.

ZACHUT, M.; HONIG, H.; STRIEM, S.; ZICK, Y.; BOURA-HALFON, S.; MOALLEM, U. Periparturient dairy cows do not exhibit hepatic insulin resistance, yet adipose-specific insulin resistance occurs in cows prone to high weight loss. **Journal of Dairy Science**. v.96, n.9, p.5656-5669, 2013.

ZERBE, H.; SCHNEIDER, N.; LEIBOLD, W.; WENSING, T.; KRUIP, T.A.; SCHUBERTH, H.J. Altered functional and immunophenotypical properties of neutrophilic granulocytes in postpartum cows associated with fatty liver. **Theriogenology**. v.54, p.771–786, 2000.