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COLLEGE OF ANIMAL SCIENCE AND FOOD ENGINEERING**

THIAGO HENRIQUE DA SILVA

**Alternative strategies and new insights into immunology, health, and performance of dairy
cattle**

Pirassununga

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Concentration Area:
Animal Quality and Production

Advisor:
Prof. Dr. Arlindo Saran Netto

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BIOGRAPHICAL SKETCH

Thiago Henrique da Silva, son of Antonio Carlos da Silva and Eli Aparecida Pavan da Silva, brother of Antonio Carlos da Silva Junior, was born on July 24, 1987 in Piracicaba, São Paulo, Brazil. Throughout his childhood period, Thiago spent his school vacations at his grandmother's beef and dairy farm, which was fundamental to his interests in livestock production. Moreover, during his childhood, he spent 10 years studying music, which was essential for his knowledge expansion in arts. He is married to Iuli Caetano da Silva Brandão Guimarães since March 2019, and father of Luca Guimarães da Silva, his life inspiration.

He got his degree in veterinary from Federal University of Uberlândia, Brazil, and as a veterinary student, Thiago got involved in extra-curricular activities under the supervision of Dr. Ricarda Maria dos Santos, Dr. Marcelo Emílio Beletti, and Dr. Edmundo Benedetti, people who inspired his academic and veterinary practice career. During his undergraduate studies in veterinary medicine, Thiago always was involved in scientific research focusing on the biology of reproduction. In addition, he was involved in dairy cattle extension programs, helping producers make informed decisions and implement solutions to improve milk quality, dairy profitability, and operational efficiency.

In the middle of 2013, Thiago joined the FMVZ/USP Master program in Nutrition and Animal Production under Dr. Francisco Palma Rennó supervision, where he was granted a scholarship by the FAPESP. During this period, Thiago focused his efforts on the evaluation of dietary additives in dairy cows' metabolism and performance.

In 2015, Thiago joined Suporte Consultoria Pecuária where he provided veterinary support to Nestlé partners farms.

In 2017, Thiago was approved to join FZEA/USP Doctoral Program in Animal Science. Also, he was awarded a FAPESP scholarship. During his doctoral studies, Thiago spent 12 months as a visiting researcher in Dr. Vinicius Silva Machado and Dr. Michael Ballou's laboratory at Texas Tech University. There in the USA he got involved with several research projects focused on management, nutrition, immunology, and health of dairy cattle.

Finally, Thiago, during his academic life, has published papers in several prestigious journals of veterinary and animal science.

DEDICATION

This thesis is dedicated to the one who made me a dad: my first son Luca. Also, I dedicate it to my wife Iuli, for her endless love, support, and encouragement.

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To God, first and always.

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“Be the change that you wish to see in the world.”

- Mahatma Gandhi -

ABSTRACT

SILVA, T.H. **Alternative strategies and new insights into immunology, health, and performance of dairy cattle.** 2021. 148p Thesis (Doctorate) – College of Animal Science and Food Engineering, University of São Paulo, Pirassununga, 2021.

There are two critical phases in dairy animals' life. Still in the young phase, calves usually pass through a turbulent moment when they are moved from the individual housing phase to a group housing system after weaning. Another critical moment in dairy cattle's life is the transition from a non-lactating pregnant state to a non-pregnant lactating state in mature cows. Numerous studies have identified the negative impact of these phases on the homeorretic and homeostatic mechanisms. Therefore, the objective of the first study was to evaluate the effect of supplementing an ultra-diluted product in the first 112 days after grouping of Holstein heifers on the incidence of diseases and performance. Also, its carryover impacts up to first lactation was evaluated. Although there was no improvement on performance during the supplementation period, a lower incidence of diarrhea was detected for animals consuming the ultra-diluted product. In addition, a lower culling rate was detected at the raising phase after supplementation. A study was also conducted herein to test the potential effect of an ultra-diluted product on polymorphonucleate leukocytes (PMNL) function and respiratory disease of Holstein calves after grouping. No changes were detected in PMNL function or scores for respiratory disease. Regarding the impact of the transition period on mature dairy cows, a trial was performed to evaluate the effect of injectable trace mineral supplementation (Se, Cu, Zn and Mn) at dry-off, close-up, and 35 days after calving on immunity, incidence of diseases, reproductive outcomes, milk production, and survivability of dairy cows undergoing the transition period in semi-arid environment. No effects of injectable trace minerals were detected in productive and reproductive performance; however, a lower incidence of metritis and stillbirths was detected for cows supplemented. Also, greater PMNL function was detected for supplement treated group. Finally, in view of the need to evaluate a new method with greater feasibility that could replace or reduce the redundancy of techniques currently used in immune system evaluation of early postpartum dairy cows, a study was conducted to evaluate the association of natural antibodies (NAb; IgM and IgG), total immunoglobulins (Ig; M and G), and PMNL function of dairy cows on day 3 after calving. A positive correlation was detected between NAb_{IgM} and IgM. In addition, IgM was positively associated with PMNL function of dairy cows.

RESUMO

SILVA, T.H. **Estratégias alternativas e novidades em imunidade, saúde e desempenho de bovinos leiteiros.** 2021. 148f Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2021.

Na pecuária leiteira existem duas fases críticas na vida dos animais. Ainda na fase jovem, as bezerras passam por um turbulento momento em que são movidas da fase de criação individual para uma criação em grupos após o desaleitamento. Outro momento crítico da vida dos bovinos leiteiros é a transição das vacas adultas de um estado gestante não-lactante para um estado não-gestante lactante. Inúmeros estudos têm identificado o impacto negativo destas fases na vida dos animais em relação à súbita mudança dos mecanismos homeorréticos e homeostáticos. Diante disso, foi avaliado o efeito da suplementação de um produto ultra-diluído nos primeiros 112 dias após o agrupamento de bezerras da raça Holandês sobre a incidência de doenças e produtividade de bezerras até a primeira lactação. Embora não se tenha observado melhora no desempenho dos animais no período de suplementação, menor incidência de diarreia foi detectada para animais consumindo o produto ultra-diluído. Além disso, menor taxa de descarte foi detectada na fase de criação após a suplementação. Aqui também foi conduzido um estudo para testar o potencial efeito de um produto ultra-diluído sobre atividade de leucócitos polimorfonucleados (PMN's) e índices de doenças respiratórias de bezerras da raça Holandês após o agrupamento. Não foi detectada nenhuma alteração na função dos PMN's nem nos escores de doença respiratória. Em relação ao impacto do período de transição de vacas leiteiras, foi avaliado o efeito da suplementação injetável de micro-minerais (Se, Cu, Zn e Mn) na secagem, pré-parto a 35 dias após o parto sobre a imunidade, incidência de doenças, produtividade e sobrevivência de vacas leiteiras passando o período de transição em clima semi-árido. Não foi detectada melhora no desempenho produtivo e reprodutivo dos animais, entretanto menor incidência de metrite e natimortos foi detectada para as vacas tratadas com o suplemento. Também, maior funcionalidade de PMN's foi detectada para o grupo tratado. Finalmente, diante da necessidade de se avaliar uma técnica com maior exequibilidade que poderia substituir ou reduzir a redundância de técnicas atualmente empregadas na avaliação do sistema imune de vacas leiteiras no início da lactação, foi conduzido um estudo para avaliar a associação de anticorpos naturais (AN; IgM e IgG), imunoglobulinas totais (Ig; M e G) e a função de PMN's no terceiro dia após o parto de vacas Holandesas. Foi detectada correlação positiva entre AN_{IgM} e IgM. Além disso, IgM foi positivamente associada à função dos PMN's.

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CHAPTER 1 – GENERAL INTRODUCTION

There are two most critical moment of the dairy cattle's life. The first one is the transition period of post-weaned growing heifers, where the animals are moved from an individual to a group housing system. This usual on-farm strategy may generate a social stress on the animals, increasing health disorders, negatively impacting their productivity (BOLT et al., 2017). The second critical moment is the transition of a dairy cow between a pregnant non-lactating state to a non-pregnant lactating state, which has been negatively associated to cow's life since it is not carefully managed (DRACKLEY, 1999). Usually, the transition period brings up a physiological stress through an imbalance between the oxidant production and availability of antioxidant defense system. This phenomenon generates the so-called oxidative stress, which is recognized to impair the cow's immune system leading to several disorders during this period (ABUELO et al., 2019).

Additionally, several methodologies have been performed to measure the immune status of dairy cows (SORDILLO, 2016). However, through the laboratory difficulties and redundancy of analysis performed in studies with dairy cows, a new feasible method to assess the immune status needs to be evaluated to replace all other variables used for now. Few studies have identified the role of natural antibodies (Nabs) in health, immunity, and performance of post-partum dairy cows (VAN KNEGSEL et al., 2007; MACHADO et al., 2014b; VAN ALTENA et al., 2016). These natural antibodies are produced spontaneously by the long-lived, self-renewing B1 B-cells, in the absence of an apparent antigenic stimulation (HAMILTON; LEHUEN; KEARNEY, 1994). Thus, the association of natural antibodies, total immunoglobulins, and polymorphonuclear leukocytes function of post-partum dairy cows is needed since these molecules may be an important marker of immune status of dairy cows.

The objective of this literature review is to discuss the published data related to 1) the social stress present in the transition from an individual to a group housing system of weaned growing heifers and the effect of ultra-diluted medicines during this phase; 2) the oxidative stress consequences during the transition period of dairy cows and the effect of injectable trace minerals on immunity, health, and performance during this period; and finally 3) the association of serum natural antibodies with immune variables of post-partum dairy cows.

Calves undergoing the transition period from individual to group housing system

The biggest challenge facing modern livestock is to increase the efficiency of animal protein production in order to sustain the onward population growth. It, however, must be made causing minimal negative impact on the environment. There is a concern not only scientific but also political about the need to adopt livestock practices that guarantee the conservation of natural resources combined with the economic viability of the on-farm business. The replacement heifers raising system has a strong impact on profit of a dairy farm. The growth rate of a heifer is strongly related to its age at first calving (AFC), which is essential for expense reductions due to onset of lactation. In Brazilian dairy farms, milk production is the main source of income, reaching 85% or more of all income generated by on-farm dairy business. However, this value depends on the farm technological level, whereas more technology and knowledge applied to dairies are associated to greater income from milk sold due to reduced culling rates in this type of dairies (personal communication).

To be efficient in raising heifers and make them an income source in dairy business, the growth rate of this category must be maximized from birth. The key points that define raising heifer efficiency are age at puberty (AP) and AFC. Heifers calving between 23 to 24.5 months of age are more efficient in first lactation than heifers calving later or earlier (ETTEMA; SANTOS, 2004).

The return on investment made in heifer raising phase depends on the milk that will be produced by these animals, in other words, the speed with which heifers calving and start their first lactation is the critical point to increase the profitability of on-farm dairy business. It is known that reproductive efficiency has a fundamental role in achieving the goals of raising phase, however, other factors are described since birth in order to achieve the desired AFC (KRPÁLKOVÁ et al., 2014).

The growth performance of young animals requires a multifactorial approach that involves fundamental management details, such as: dry-cow vaccination to improve colostrum quality; satisfactory colostrum feeding management; effective navel healing; hygiene procedures; prevention, detection and treatment of diseases such as diarrhea, pneumonia, and tick-borne diseases, consistent nutritional procedures, gentle weaning process (AKINS, 2016), and, finally, strategies to mitigate stress during transition from individual to group housing system (HULBERT; BALLOU, 2012). As characterized by Lynch (2010), the stress faced by an animal can bring long-

lasting physiological disorders that limit genetic expression throughout the period from raising to when they become matures. It causes a strong negative impact on profitability of on-farm dairy business (HULBERT; MOISÁ, 2016).

Impact of stress on young dairy animals

From birth to raising phase, there are several managements, which are potential stressors faced by dairy calves. These stressors can be divided into 2 main events: inflammatory (parturition, dehorning, and castration); or physiological (transport, weaning period, grouping and regrouping, change of location, climatic factors, and exposure to different pathogens; CALLAN; GARRY, 2002; DUFF; GALYEAN, 2007; HULBERT; MOISÁ, 2016). All these factors may negatively interfere on animal performance.

Stress is a broad and complex concept that is difficult to define concisely. Before and during the 20th century, the scientific community concern for a precise definition of stress increased, which culminated in the increase of new concepts about this topic. Hippocrates may have been the first author to describe the term “stress”, in which he applies the concept of balance and disharmony. In other words, Hippocrates conceives stress as a health essential state that the disturbance of this balance would manifest itself as a disease. In 1878, Claude Bernard recognized stress as an internal environment that is more stable than external changes and that this stability is essential for life.

Later, Walter B. Cannon (1914, 1929) describes this stable state via physiological regulation and modulation which he called homeostasis. Cannon's studies served as basis for the evolution of stress studies by Selye who created a biological theory for stress called “The General Adaptation Syndrome” (SELYE, 1946). This theory consists of three universal phases of dealing with a threat or stimulus, which are: 1) initial alarm reaction similar to Cannon's concept of fight or flight; 2) adaptation stage that is associated with resistance to an external stimulus; and finally, 3) the stage of exhaustion, which culminates in organism death, if the adverse effect is not removed. Sterling and Eyer (1981) conceptualize the active process by which animals respond daily as homeostasis, which is nothing more than achieving stability through changes.

Once the animal identifies the stressor, the system organizes a biological response in which, according to the level of stress, it can be autonomic, neuroendocrine, immune, and behavioral, or

a combination of all of them. Stress (chronic or acute) is not easily defined (SEGERSTROM; MILLER, 2004), but it is well known that chronic or severe stress can alter the biological system by interrupting homeostasis, leading the animal to pathological conditions (MOBERG; MENCH, 2000).

Animals may suffer from two main types of stress: the physical stress which are those caused by extreme temperatures, limited food or water, fatigue or injuries; or psychological stress that comprises the novelties of the animal's life, the fear, isolation, insertion in a new group, changes in routine and/or environment (GRANDIN, 1997; LYNCH, 2010). The neuroendocrine response, stimulated by a stressor, can activate the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenomedullary (SAM) axes, which promote the release of glucocorticoids (cortisol) and/or catecholamines (adrenaline and norepinephrine). This close relationship between the central nervous system (autonomous system) and the hypothalamic-pituitary axis plays an important role in regulating systems in response to stress (ADER; COHEN; FELTE, 1995).

The classic stressful stimulus acts on the HPA and SAM axes, in which chronic stress is mainly linked to the high reactivity of HPA axis that raise cortisol levels (passive response). On the other hand, acute responses are associated with reactivity of SAM axis that releases catecholamines - adrenaline (fight / flight; TORRES; NOWSON, 2007). In review, Grandin (1997) reports the plasmatic level of cortisol as an efficient way to measure the amount of stress in animals, and demonstrates the effect of weaning on basal levels of this hormone. Still in this study, it shows the effect of animal restraint, which increase plasma cortisol levels.

The nutrients ingested in periods when the animals undergoing stress are directed to other systems responsible by animal's survival, instead of depositing muscle and growth, for example (ELSASSER; KAHL, 2002). In addition, nutrient intake can be impaired when the animal is under stress (DUFF; GALYEAN, 2007). The deficiency of nutrient intake by calves can lead to dysfunction of the immune system, consequently increasing the susceptibility of these animals to diseases (DRACKLEY, 2008). This dysfunction is mainly caused by inadequate energy and protein intake, which influence adaptive immunity, cytokine production, complement system, phagocytic functions, and IgA antibody concentrations (DUFF; GALYEAN, 2007). In addition to the nutritional impact on immune system, it is also recognized that the functional capacity of neutrophils is responsive to stress, including suppression of phagocytosis and oxidative burst as

well as expression of leukocyte adhesion molecules, decreasing the efficiency of action of the immune system to fight infections (BURTON, 1995).

Other management factors such as change of housing system, change of routine, formation of groups, climate, ambient temperature, humidity, ventilation, dust particles, and exposure to different pathogens lead animals to new challenges (CALLAN; GARRY, 2002; DUFF; GALYEAN, 2007). These challenges are not always isolated from the effect of nutritional changes in weaning studies.

Since 1878, Pasteur has demonstrated that cold stress increases mortality rate of chickens infected with anthrax compared to chickens infected without stress. As already mentioned, the anti-inflammatory and immunosuppressive power of glucocorticoids leads to an exacerbation of diseases. This fact shows that environmental factors are stressful to animals and that they can increase the incidence of diseases. It is a fact that confinement increases the performance of animals and consequently profitability, however confinement itself is a stressor to the animals (GRANDIN, 1997). Additionally, it provides greater contact of animals with pathogens due to higher concentration of microorganisms by area. It also allows greater animal-animal contact with a high possibility of transmitting pathogens, which requires good management practices to prevent diseases and poor performance (SMITH; AL-MAMUN; GRÖHN, 2017).

The switch from an individual milk-feeding system to a post-weaned heifers' group has been a common management strategy adopted for many dairy farms (NAHMS, 2007). This practice aims to decrease the high labor and facilities cost involved in individual milk-feeding system. However, decreased performance during the grouping phase has been observed, which may be due to stressors related to social changes or due to the higher exposition to novel microbes (HULBERT; BALLOU, 2012). Bøe and Færevik (2003) observed that when calves are regrouped or a strange animal is added to the group, it may take up to 15 d for social interactions and locomotor activities to return to basal levels. Supporting this information, Hulbert and Ballou (2012) detected reduced performance among grouped-calves, in which the authors explained to be due to an increased locomotor activity and decreased inactivity. In fact, other researchers found that grouped-housed or paired calves spent less time feeding and more time performing other locomotor behaviors, while individually and isolated calves spent more time in recumbency (WARNICK; ARAVE; MICKELSEN, 1977; CHUA et al., 2002). Thus, this increased activity probably contributed to decreased efficiency and utilization of metabolizable energy for growth.

Furthermore, it is well-reported the negative association between grouping stress and immune system, which may support high risk of disease (HULBERT; MOISÁ, 2016). For example, abrupt weaning and disruption of the social group affected immune responsiveness, specifically suppressing IFN γ response to keyhole limpet hemocyanin of calves (HICKEY; DRENNAN; EARLEY, 2005). From a different perspective of the negative impact of immune function on performance, acute stress may have limited suppressive effects on immune cells. Notwithstanding, Lochmiller and Deerenberg (2000) declared that despite the low amount of leukocyte total cells compared to the total body weight of the animals, an activated acute phase immune response can decrease performance and feed efficiency.

In this way, it can be said that there are numerous other sources of stress which can affect animal performance bringing a strong negative economic impact to the system (WEARY; JASPER; HÖTZEL, 2008; HULBERT; MOISÁ, 2016). Several management resources can be used to improve animal performance during the raising phase. In terms of stress, and its consequences, alternative strategies such as ultra-diluted products or as usually called homeopathic medicines, can be used to replace synthetic substances to increase animal performance. It could help to mitigate growing heifers stress reducing the incidence of diseases and improving animal performance.

Ultra-diluted remedies as an alternative strategy to reduce diseases in dairy cattle

In the treatment and prevention of infectious diseases caused by bacteria in the livestock production system, antimicrobial drugs have been used as the first choice for decades. However, these drugs are no longer well accepted by modern consumers (DOEHRING; SUNDRUM, 2016) due to their high negative impact on bacterial resistance worldwide (LAXMINARAYAN et al., 2013).

The agencies that regulate research have paid special attention to the risks that antibiotics residues present in animal origin foods may bring to the population's health. Few countries, not including Brazil (REGITANO; LEAL, 2010), have efficient data collection in order to estimate the amount of antimicrobial drugs marketed annually for animal husbandry. However, it is estimated that 70% of veterinary drugs marketed in Brazil are antimicrobial substances (THIELE-BRUHN, 2003; DÍAZ-CRUZ; BARCELÓ, 2007). The European Parliament issued a resolution on 27 October 2011 that imposes severe measures on antimicrobial use. These measures were taken in an

attempt to reduce risks to human and animal health, which may have a strong influence on export market requirements.

Therefore, in view of the need for economic and material development without harming the environment research on alternative therapies that improve health and provide better performance in livestock is necessary. In this context, we can mention the ultra-diluted products use, an alternative therapy that has been little studied, as a strategy to be used both in preventing and curing diseases in production systems (ARLT et al., 2009). This product can reduce the indiscriminate use of antimicrobials and increase the livestock profit in addition to decreasing bacterial resistance.

Ultra-diluted medicine was created by the German Samuel Hahnemann (1755-1843), at the end of the 18th century, and consists of the supply of extremely diluted doses of a certain substance, which in high doses would cause the disease in healthy individuals. It is believed that the energy contained in the harmful agent is transferred to the water molecule and thus the animals have their nerve endings sensitized to fight it, without the disease itself. A controversial study published in the journal *Nature* in 1988 (DAVENAS et al., 1988) by the group of French immunologist Beveniste, reported that high dilutions of anti-IgE were able to promote the degranulation of basophils as well as the anti-IgE itself in high concentrations (Figure 1), confirming the hypothesis that water had a memory capable of stimulating such cell. John Maddox, the editor of the journal *Nature* at the time, was impressed by Beveniste's findings, but under pressure from the scientific community he formed a team to test these effects, however no results similar to Beveniste's were detected. Therefore, any data was published about it (BALL, 2004).

Figure 1. Scheme of the Bevenites' study reporting basophils being degranulated (metachromasia) with high dilutions of anti-IgE. Source: own authorship adapted from Bellavite et al. (2006) and created by BioRender software.

On the other hand, Belon et al. (1999) gathered data from 5 European laboratories that tested the inhibition of basophil degranulation with highly diluted histamine (15th-19th dilution) when stimulating it with anti-IgE. In 4 of the laboratories the effect was significantly different, showing that the highly diluted histamine was able to inhibit the degranulation of basophils by anti-IgE. This fact and others lead to a tireless discussion among researchers in an attempt to explain and elucidate whether homeopathy has an effect on the organism or not, thus demonstrating the need to carry out consistent studies to evaluate such products.

Studies in the literature that demonstrate the effectiveness of using ultra-diluted compounds on health and performance of heifers are still scarce. On the other hand, some studies regarding mastitis, metritis, and parasites infestations treatment in adult dairy cows have been found (MATHIE; CLAUSEN, 2015; DOEHRING; SUNDRUM, 2016).

In a data compilation from studies published between 1981 and 2014, in which they tested the effectiveness of ultra-diluted treatment in adult cows, only 51.8% of trials showed a positive effect in prevention and cure of diseases related to reproductive system and mammary gland inflammation of adult cows (DOEHRING; SUNDRUM, 2016). However, the wide variation among the results can be explained due to different contexts, such as: diseases, pathogens, type of infection (naturally occurring or artificial), types of animals, management conditions and treatment procedures. There is also a difference in the ultra-diluted products and form of supply. In addition, there was different form of disease diagnostics performed by different researchers. Finally, the category of animal also influences the result of the studies. In the literature review performed by Doebling and Sundrum (2016) the data found were either adult cows or suckling calves, demonstrating the needs to well-designed studies regarding the impact of ultra-diluted products on heifer health and performance.

Impact of oxidative stress in dairy cows undergoing the transition period

The transition period is when a dairy cow moves from late pregnancy into lactation. This period has been reported as the most challenged period of cow's life (DRACKLEY, 1999). The exact length of time of transition period has been differently classified among authors, but the most used definition is as the last 3 weeks before calving up to 3 weeks after calving (GRUMMER, 1995). High incidence of infectious and metabolic disorders has been reported during this time (LEBLANC; LESLIE; DUFFIELD, 2010). De Vries, Olson, and Pinedo (2010) identified high risk of culling during the periparturient period. The authors detected that the daily hazards of culling first peaked approximately 30 d after calving. Additionally, the authors observed high risk of culling again around 280 DIM, for older cows. This last finding can be attributed at least in part due to the difficulties during the transition period (OVERTON; WALDRON, 2004). All peripartum dairy cow experience, to a greater or lesser degree insulin resistance (DE KOSTER; OPSOMER, 2013), reduced feed intake coupled with lipolysis, negative nutrient balance, weight loss (GRUMMER, 1995; DRACKLEY, 1999), hypocalcemia (GOFF, 2008), immune modulation (SORDILLO, 2016), and uterine bacterial contamination (SHELDON et al., 2006).

The transition period is characterized by a series of adaptations to the demands of colostrogenesis and lactogenesis, which, when dysregulated, can lead the transition dairy cows to

develop diseases due to its negative impact on immune system. Kimura et al. (2002) studying the effect of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows, detected that, not only the parturition alters the immune function of a transition dairy cow, but also the metabolic demands associated with the onset of lactation negatively impacted the composition of immune cells. The underlying mechanism of the metabolic regulation is named homeorhesis, a process that drives milk production during this period. This mechanism allows the animal to adapt to the challenges imposed by the change from a pregnancy non-lactating state to a non-pregnancy lactating state (BAUMAN; CURRIE, 1980). Thus, an imbalanced homeorhetic process may redirect nutrients that are essentials for dairy cow's survivability. Therefore, a disordered homeorhetic control, which redirect essential nutrients from cow's health, results homeostasis disorders reflecting in diseases during this period. Eventually, a disorder of one metabolic process will impact on the efficiency of others (LEAN; SAUN; DEGARIS, 2013). All these information allow us to affirm that all the metabolic processes are intricately linked.

During the periparturient period, the high productive dairy cows face an overwhelmed metabolism that has led to the emergence of new metabolic diseases. In this regard, in face of reduced nutrient intake linked to an aggressive nutrient requirement of dairy cows undergoing the transition period, a hypermetabolic catabolic system response is developed, whereas a high oxygen consumption is needed through normal cellular respiration in order to provide the energy requirement for the onset of lactation (ABUELO et al., 2019). Most reactive oxygen species (ROS) found in healthy tissues likely result from increased cellular metabolism and energy generation by the mitochondria. It came from the normal mitochondrial electron transport chain or from peroxisomal β -oxidation during fatty acid metabolism. The latest is an especially important source of ROS in transition dairy cows. In addition, in phagocytic immune cells, the respiratory burst to kill microbial pathogens generates a high amount of ROS (BABIOR, 1999). As a result of this hypermetabolic catabolic process in transition dairy cows, an imbalance of oxidants molecules from oxygen metabolism and availability of dietary antioxidants may be detected. This phenomenon may produce the so-called oxidative stress (OS). In other words, OS is the accumulation of oxidizing agents such as ROS, which may induce deleterious effects on immune system since an antioxidant defense system is not able to counteract their effects (ABUELO et al., 2019).

Although ROS is essential normal cellular metabolism such as the control of immune and inflammatory responses, a ROS accumulation may be dangerous to tissue macromolecules such as DNA, protein, and lipids. The first target of ROS damage are lipids, also the products of the lipid peroxidation during the transition period of dairy cows can damage cellular membranes and organelles, which altogether alter cellular functions and signal transduction. Sordillo and Aitken (2009) showed that oxidative stress contributes to a dysfunctional inflammatory response in dairy cows undergoing the transition period. It is due to imbalances in ROS expression and the availability of antioxidant defenses. Thus, it is important to host tissue regulates the accumulation of ROS to physiologic conditions through antioxidant defenses to optimize immune cell function (SORDILLO, 2016).

Trace mineral supplementation as an alternative to mitigate oxidative stress

Certain trace minerals are proven to be effective against ROS reducing the severity of dairy cattle diseases (SPEARS; WEISS, 2008). This effect may be through a direct antioxidant effect and/or improving the immune response (ABUELO et al., 2013). The trace minerals with antioxidant effects may quench oxidants directly or forming part of enzymatic redox molecules that convert ROS to less reactive metabolites (SORDILLO, 2016). Among the ROS detoxifying enzymes systems associated with minerals include catalase, superoxide dismutase, and selenium-dependent enzymes (SORDILLO; AITKEN, 2009). The latest, is the most studied antioxidant defense enzyme in dairy cattle in which cytosolic glutathione peroxidase (GPX1) and thioredoxin reductase (TRX1) are important enzymes in this group. Both enzymes have selenocysteine residues incorporated into their active sites and work by reducing H_2O_2 and fatty acid hydroperoxides to less reactive water and alcohols, respectively (SORDILLO, 2016). Other enzyme systems that play a critical role in preventing the formation of oxygen radicals is the cytosolic superoxide dismutase and mitochondrial superoxide dismutase, which are copper plus zinc-dependent, and manganese plus zinc-dependent, respectively (SORDILLO, 2016). Additionally, vitamin A, C, and E are proven to be effective against ROS (ABUELO et al., 2019).

Although micronutrients have an important role against oxidant components, it is significant that blood concentration of vitamins and minerals decreasing in dairy cows around the time of calving due to decreased feed intake and high requirements associated with metabolic stress

at this moment (SPEARS; WEISS, 2008). Sciorsci et al. (2020) showed that blood ROS levels undergo an expressive increase from 180-210 days of pregnancy onwards. Furthermore, Sordillo et al. (2007) and Abuelo et al. (2013) reported high levels of plasma lipid hydroperoxides and oxidative stress index, respectively, during the first month postpartum. Further, Bernabucci et al. (2002) reported that summer conditions increase oxidative stress in transition dairy cows. The most studied role of trace minerals on immunocompetence is their involvement in antioxidant enzyme activity, which fight against the dangerous ROS. As aforementioned, high levels of pro-oxidants metabolites, found during the transition from late gestation to peak lactation of dairy cows, are associated with dysfunctional immune cell responses (SORDILLO, 2013). Thus, a trace mineral deficiency may impair immunity and health of transition dairy cows (SPEARS; WEISS, 2008; SORDILLO, 2013).

In a retrospective study performed by Enjalbert et al. (2006) involving 2080 herds, higher odds of diseases, such as metritis, mastitis, and lameness were detected in adult cattle when marginal and deficient trace minerals blood levels were present. Corroborating with these findings, a consistent association between trace minerals supplementation and decreased incidence of mastitis has been demonstrated in the literature (SORDILLO; AITKEN, 2009). Even feeding rations that meet the trace minerals requirements the treatment with injectable trace minerals may be a good strategy to ensure a known dose of these elements without voluntary intake oscillation, as related to transition dairy cows (GRUMMER, 1995), particularly those undergoing heat stress (ADIN et al., 2009). Through injectable trace minerals supplementation, the nutrients are absorbed directly into the bloodstream from subcutaneous tissues, do not suffering antagonist interferences in the gastrointestinal environment (SPEARS, 2003).

Injectable trace mineral supplements have been used in dairy cattle in order to decrease the ROS impact on health, reproduction, and performance. Bicalho et al. (2014) found that ITMS treatment increase serum levels of Se, Zn, and Cu, which are important trace minerals in the antioxidant defense system of dairy cows. Pate and Cardoso (2018) detected that aflatoxin challenged cows treated with two subcutaneous injection of trace minerals had greater liver selenium (Se) concentration and higher plasma glutathione peroxidase activity compared to non-treated cows. These findings indicate a positive antioxidant response in aflatoxin challenged cows treated with injectable trace minerals. In addition, Machado et al. (2014a) detected increased superoxide dismutase activity for ITMS treated cows compared to control cows through 100 DIM.

Some positive effects of ITMS treatment on udder health traits have been shown. Machado et al. (2013) observed that 3 subcutaneous injection of trace minerals were effective to improve udder health; since a decreased linear somatic cell count (SCC) and lower incidence of subclinical and clinical mastitis were detected to trace minerals treated cows. Corroborating these findings, in the study of Ganda et al. (2016), injectable trace mineral supplementation reduced the incidence of chronic clinical mastitis in primiparous cows and tended to increase subclinical mastitis cure in cows with 3 or more lactations.

Regarding reproductive efficiency, in the study of Michael, Storch, and Smith (2004), who treated cows 4 weeks before calving and 4 weeks before breeding with micro mineral injections, the authors related higher reproductive performance when treated dairy cows with ITMS. Still, a tendency of about 9 percentage points more cows pregnant for ITMS group compared to controls group at 80 DIM was detected. This better reproductive performance may be through the decreased incidence of stillbirth parturitions and endometritis events reported for injectable trace mineral supplemented cows (MACHADO et al., 2013). Although studies have reported positive effects of ITMS on health and reproductive performance, there are no studies reporting better milk production. Also, a lack of studies has been observed in the literature regarding the effects of ITMS on dairy cows undergoing the transition period in hot environment.

New insights into the early lactation dairy cow immunology: The natural antibodies

Diseases in dairy cows, through direct and indirect effects, continue to be an important cause of economic losses to the dairy industry (LIANG et al., 2017). About 30-50% of cows suffering of some disease are affected by some disease around the time of calving (LEBLANC; LESLIE; DUFFIELD, 2010). The production diseases of the dairy cows are caused by an inability of them to cope with the metabolic demands imposed by the improved genetic selection. Among the challenges which provide high incidence of diseases in dairy cows we can relate the inconsistency of feed intake, the poor feeding and nutritional management, the unsuitable environment, inefficient reproductive programs, or combinations among these factors (MULLIGAN; DOHERTY, 2008).

The dysregulated immune system around the time of calving due to altered metabolism imposed by the progressive milk yield is the most important factor related to high incidence of

diseases at this phase. It is because an exacerbated or inadequate inflammatory response can favor poor health outcomes (LEBLANC, 2020). Due to the substantial nutrient demand increase to support the lactation, dairy cows have to adapt to several metabolic changes such as uncoupling of the somatotrophic axis with peripheral insulin resistance and high degree of lipolysis to fuel the milk production (LEBLANC, 2020). Additionally, the dairy cows facing a hard time during the transition period; since they must cope with a transient reduction of nutrient intake, which may result in negative balance of energy, protein, minerals, and vitamins (LEAN; SAUN; DEGARIS, 2013).

The non-esterified fatty acids (NEFA) generate through negative energy balance of dairy cows undergoing the transition period are associated with the risk of metabolic and infectious diseases (LEBLANC, 2020) due to their negative impact on immune system (INGVARTSEN; MOYES, 2013). McArt et al. (2013) reported that about 35% of peripartum cows have NEFA and 45% have BHB above thresholds related with metabolic disease. Fatty acids coming from body fat mobilization during the negative energy balance has been negative associated to many immune variables such as IgM secretion, cytokine production, cell viability, phagocytosis, diapedesis, and antigen presentation. In addition, the ketone bodies such as β -hydroxybutyrate (BHB) formed during the body fat mobilization has been related to be inhibitory effect on immunity due to its negative impact at lymphocyte blastogenesis, and all the immune variables mentioned to fatty acids. In this way, the immune system function appears to be a great player that decides the limit between the metabolic health and disease in dairy cows (INGVARTSEN; MOYES, 2013).

To evaluate the impact of any intervention strategy on immune status of dairy cattle a reliable and accurate method must be performed since immune system has a complex network which can be easily misunderstood. Sordillo (2016) in her excellent literature review, reports the main methods usually performed to assess the immune function in dairy cattle. The number of leukocytes has been used at clinical practice to clarify the immune status of an individual. The blood concentrations of circulating acute phase proteins has been assessed to verify any not specific infection as well. Additionally, the activity of isolated immune cells in vitro has been performed to assess immune function. Currently, the application of genomic, proteomic, and metabolomic methodology has brought important on immune response during several diseases. Others have used humoral components of the immune system, including natural antibodies (NAbs) and total

immunoglobulins (Igs) as markers of immunocompetence of dairy cows (HERR; BOSTEDT; FAILING, 2011; BANOS et al., 2013).

Natural antibodies and total Igs concentration in blood were reported to be positively correlated in goats (CECCHINI; RUFRANO; CAPUTO, 2019). Additionally, circulating NAbs concentration and PMN function have similar relationship with disease risk and metabolic imbalance during the transition period of dairy cows (VAN KNEGSEL et al., 2007; THOMPSON-CRISPI; MIGLIOR; MALLARD, 2013; MACHADO et al., 2014b). In addition, circulating leukocyte counts have been correlated with circulating total Igs concentrations around parturition (HERR; BOSTEDT; FAILING, 2011).

These Nabs are found in the serum of healthy, non-immunized individuals (HERZENBERG et al., 1986; MADI et al., 2009). They are produced spontaneously by the long-lived, self-renewing B1 B-lymphocytes, in the absence of an apparent antigenic stimulation (HAMILTON; LEHUEN; KEARNEY, 1994). Keyhole limpet hemocyanin (KLH) has been used to measure Nabs because it is a metalloprotein derived from the *Megathura crenulata*, a giant keyhole limpet that lives up to 33 m deep into the Pacific Ocean. Therefore, it is assumed that dairy cattle are naïve to KLH and only non-specific Igs, such as NAbs could recognize it (STAR et al., 2007). Natural antibodies are essentially formed from IgM isotype (BOES, 2000). However, protective roles of other Nabs isotypes such as IgG and IgA have been suggested (MACHADO et al., 2014b; ROTHSTEIN, 2016; PALMA et al., 2018; CECCHINI; RUFRANO; CAPUTO, 2019). Thus, besides the difficulty to handle with fresh blood to study the leukocytes functions of dairy cows such as phagocytosis and oxidative burst, the analysis of several immune methods may be redundant, having an important economic impact on research costs and on-farm veterinary practices.

REFERENCES

- ABUELO, A. et al. 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. et al. Redox biology in transition periods of dairy cattle: Role in the health of periparturient and neonatal animals. **Antioxidants**, v. 8, n. 1, 2019.
- ADER, R.; COHEN, N.; FELTE, D. Psychoneuroimmunology: interactions system and the immune system. **The Lancet**, v. 345, n. 8942, p. 99–103, 1995.

ADIN, G. et al. Effects of cooling dry cows under heat load conditions on mammary gland enzymatic activity, intake of food and water, and performance during the dry period and after parturition. **Livestock Science**, v. 124, n. 1–3, p. 189–195, set. 2009.

AKINS, M. S. Dairy Heifer Development and Nutrition Management. **Veterinary Clinics of North America - Food Animal Practice**, v. 32, n. 2, p. 303–317, 2016. Available on: <<http://dx.doi.org/10.1016/j.cvfa.2016.01.004>>.

ARLT, S. et al. Efficacy of homeopathic remedies as prophylaxis of bovine endometritis. **Journal of Dairy Science**, v. 92, n. 10, p. 4945–4953, 2009. Available on: <<http://dx.doi.org/10.3168/jds.2009-2142>>.

BABIOR, B. M. NADPH Oxidase: An Update. **Blood**, v. 93, n. 5, p. 1464–1476, 1999.

BALL, P. **The memory of water. The life and work of Jacques Benveniste taught us valuable lessons about how to deal with fringe science.** Disponível em: <<http://www.nature.com/news/2004/041004/full/news041004-19.html>>. Accessed on: 1 January 2021.

BANOS, G. et al. Identification of Immune Traits Correlated with Dairy Cow Health, Reproduction and Productivity. **PLoS ONE**, v. 8, n. 6, 12 jun. 2013.

BAUMAN, D. E.; CURRIE, W. B. Partitioning of Nutrients During Pregnancy and Lactation: A Review of Mechanisms Involving Homeostasis and Homeorhesis. **Journal of Dairy Science**, v. 63, n. 9, p. 1514–1529, 1980.

BELLAVITE, P. et al. Immunology and homeopathy. 2. Cells of the immune system and inflammation. **Evidence-based Complementary and Alternative Medicine**, v. 3, n. 1, p. 13–24, 2006.

BELON, P. et al. Inhibition of human basophil degranulation by successive histamine dilutions: Results of a European multi-centre trial. **Inflammation Research**, v. 48, n. SUPPL. 1, p. 2–4, 1999.

BERNABUCCI, U. et al. 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.

BICALHO, M. L. S. et al. Effect of trace mineral supplementation on selected minerals, energy metabolites, oxidative stress, and immune parameters and its association with uterine diseases in dairy cattle. **Journal of Dairy Science**, v. 97, n. 7, p. 4281–4295, 2014.

BØE, K. E.; FÆREVIK, G. Grouping and social preferences in calves, heifers and cows. **Applied Animal Behaviour Science**, v. 80, p. 175–190, 2003.

BOES, M. Role of natural and immune IgM antibodies in immune responses. **Molecular Immunology**, v. 37, 2000. Available on: <www.elsevier.com/locate/molimm>.

BOLT, S. L. et al. Pair housing of dairy calves and age at pairing: Effects on weaning stress, health, production and social networks. **PLoS ONE**, v. 12, n. 1, p. 1–18, 2017.

BURTON, J. L. Supplemental chromium: its benefits to the bovine immune system. **Animal Feed Science and Technology**, v. 53, n. 2, p. 117–133, 1995.

CALLAN, R. J.; GARRY, F. B. Biosecurity and bovine respiratory disease. **The Veterinary Clinics Food Animal Practice**, v. 18, p. 57–77, 2002.

CECCHINI, S.; RUFRANO, D.; CAPUTO, A. R. Natural antibodies and their relationship with total immunoglobulins and acquired antibody response in goat kid (*Capra hircus*, L. 1758) serum. **Veterinary Immunology and Immunopathology**, v. 211, p. 38–43, 2019.

CHUA, B. et al. Effects of pair versus individual housing on the behavior and performance of dairy calves. **Journal of Dairy Science**, v. 85, n. 2, p. 360–364, 2002. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(02\)74082-4](http://dx.doi.org/10.3168/jds.S0022-0302(02)74082-4)>.

DAVENAS, E. et al. Human basophil degranulation triggered by very dilute antiserum against IgE. **Nature**, v. 333, n. 30, p. 816–818, 1988.

DE KOSTER, J. D.; OPSOMER, G. Insulin resistance in dairy cows. **Veterinary Clinics of North America - Food Animal Practice**, v. 29, n. 2, p. 299–322, 2013. Disponível em: <<http://dx.doi.org/10.1016/j.cvfa.2013.04.002>>.

DE VRIES, A.; OLSON, J. D.; PINEDO, P. J. Reproductive risk factors for culling and productive life in large dairy herds in the eastern United States between 2001 and 2006. **Journal of Dairy Science**, v. 93, n. 2, p. 613–623, 2010. Available on: <<http://dx.doi.org/10.3168/jds.2009-2573>>.

DÍAZ-CRUZ, M. S.; BARCELÓ, D. Recent advances in LC-MS residue analysis of veterinary medicines in the terrestrial environment. **TrAC - Trends in Analytical Chemistry**, v. 26, n. 6, p. 637–646, 2007.

DOEHRING, C.; SUNDRUM, A. Efficacy of homeopathy in livestock according to peer-reviewed publications from 1981 to 2014. **Veterinary Record**, v. 179, n. 24, p. 1–13, 2016.

DRACKLEY, J. K. ADSA foundation scholar award: Biology of dairy cows during the transition period: The final frontier? **Journal of Dairy Science**, v. 82, n. 11, p. 2259–2273, 1999.

DRACKLEY, J. K. Calf Nutrition from Birth to Breeding. **Veterinary Clinics of North America - Food Animal Practice**, v. 24, n. 1, p. 55–86, 2008.

DUFF, G. C.; GALYEAN, M. L. Board-invited review: Recent advances in management of highly stressed, newly received feedlot cattle. **Journal of Animal Science**, v. 85, n. 3, p. 823–840, 2007.

ELSASSER, T. H.; KAHL, S. Adrenomedullin has multiple roles in disease stress: Development and remission of the inflammatory response. **Microscopy Research and Technique**, v. 57, n. 2, p. 120–129, 2002.

ENJALBERT, F.; LEBRETON, P.; SALAT, O. Effects of copper, zinc and selenium status on performance and health in commercial dairy and beef herds: Retrospective study. **Journal of Animal Physiology and Animal Nutrition**, v. 90, n. 11–12, p. 459–466, 2006.

ETTEMA, J. F.; SANTOS, J. E. P. Impact of age at calving on lactation, reproduction, health, and income in first-parity Holsteins on commercial farms. **Journal of Dairy Science**, v. 87, n. 8, p. 2730–2742, 2004. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(04\)73400-1](http://dx.doi.org/10.3168/jds.S0022-0302(04)73400-1)>.

GANDA, E. K. et al. Effects of injectable trace mineral supplementation in lactating dairy cows with elevated somatic cell counts. **Journal of Dairy Science**, v. 99, n. 9, p. 7319–7329, 1 set. 2016.

GOFF, J. P. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. **Veterinary Journal**, v. 176, n. 1, p. 50–57, 2008.

GRANDIN, T. Assessment of Stress during Handling and Transport. **Journal of Animal Science**, v. 75, n. 1, p. 249–257, 1997.

GRUMMER, R. R. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. **Journal of Animal Science**, v. 73, n. 9, p. 2820–33, set. 1995. Available on: <<http://www.ncbi.nlm.nih.gov/pubmed/8582873>>.

HAMILTON, A. M.; LEHUEN, A.; KEARNEY, J. F. Immunofluorescence analysis of B-1 cell ontogeny in the mouse. **International Immunology**, v. 6, n. 3, p. 355–361, mar. 1994.

HERR, M.; BOSTEDT, H.; FAILING, K. IgG and IgM levels in dairy cows during the periparturient period. **Theriogenology**, v. 75, n. 2, p. 377–385, 2011. Available on: <<http://dx.doi.org/10.1016/j.theriogenology.2010.09.009>>.

HERZENBERG, L. A. et al. The LY-1B Cell Lineage. **Immunological Reviews**, v. 93, n. 1, p. 81–102, 1986.

HICKEY, M.-C.; DRENNAN, M.; EARLEY, B. The effect of abrupt weaning of suckler calves on the plasma concentrations of cortisol, catecholamines, leukocyte, acute-phase proteins **End of Project Report**, July, p. 2847–2855, 2005.

HULBERT, L. E.; BALLOU, M. A. Innate immune responses and health of individually reared Holstein calves after placement into transition-pens 23 d after weaning. **Journal of Dairy Research**, v. 79, n. 3, p. 333–340, 2012.

HULBERT, L. E.; MOISÁ, S. J. Stress, immunity, and the management of calves1. **Journal of Dairy Science**, v. 99, n. 4, p. 3199–3216, 2016.

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

KIMURA, K. et al. Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. **Journal of Dairy Science**, v. 85, n. 6, p. 1437–1444, 2002. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(02\)74211-2](http://dx.doi.org/10.3168/jds.S0022-0302(02)74211-2)>.

KRPÁLKOVÁ, L. et al. Effect of prepubertal and postpubertal growth and age at first calving on production and reproduction traits during the first 3 lactations in Holstein dairy cattle. **Journal of Dairy Science**, v. 97, n. 5, p. 3017–3027, 2014. Available on: <<http://dx.doi.org/10.3168/jds.2013-7419>>.

LAXMINARAYAN, R. et al. Antibiotic resistance-the need for global solutions. **The Lancet Infectious Diseases**, v. 13, n. 12, p. 1057–1098, 2013.

LEAN, I. J.; SAUN, R. Van; DEGARIS, P. J. Mineral and antioxidant management of transition dairy cows. **Veterinary Clinics of North America - Food Animal Practice**, v. 29, n. 2, p. 367–386, 2013.

LEBLANC, S. J. Review: Relationships between metabolism and neutrophil function in dairy cows in the peripartum period. **Animal**, v. 14, n. S1, p. s44–s55, 2020.

LEBLANC, S. J.; LESLIE, K. E.; DUFFIELD, T. F. Metabolic Predictors of Displaced Abomasum in Dairy Cattle. **Journal of Dairy Science**, v. 88, n. 1, p. 159–170, 2010.

LIANG, D. et al. Estimating US dairy clinical disease costs with a stochastic simulation model. **Journal of Dairy Science**, v. 100, n. 2, p. 1472–1486, 2017. Available on: <<http://dx.doi.org/10.3168/jds.2016-11565>>.

LOCHMILLER, R. L.; DEERENBERG, C. Trade-offs in evolutionary immunology: Just what is the cost of immunity? **Oikos Journal**, v. 88, n. 1, p. 87–98, 2000.

LYNCH, E. M. **Thesis. Characterization of physiological and immune-related biomarkers of weaning stress in beef cattle**. 2010. National University of Ireland, 2010.

MACHADO, V. S. et al. Effect of an injectable trace mineral supplement containing selenium, copper, zinc, and manganese on the health and production of lactating Holstein cows. **Veterinary Journal**, v. 197, p. 451–456, 2013.

MACHADO, V. S. et al. The effect of injectable trace minerals (selenium, copper, zinc, and manganese) on peripheral blood leukocyte activity and serum superoxide dismutase activity of lactating Holstein cows. **Veterinary Journal**, v. 200, n. 2, p. 299–304, 2014a.

MACHADO, V. S. et al. Short communication: Relationship between natural antibodies and postpartum uterine health in dairy cows. **Journal of Dairy Science**, v. 97, n. 12, p. 7674–7678, 2014b.

MADI, A. et al. Organization of the autoantibody repertoire in healthy newborns and adults revealed by system level informatics of antigen microarray data. **Proceedings...** National Academy of Sciences of the United States of America, v. 106, n. 34, p. 14484–14489, 2009.

MATHIE, R. T.; CLAUSEN, J. Veterinary homeopathy: Meta-analysis of randomised placebo-controlled trials. **Homeopathy**, v. 104, n. 1, p. 3–8, 2015.

MCART, J. A. A. et al. Elevated non-esterified fatty acids and β -hydroxybutyrate and their association with transition dairy cow performance. **Veterinary Journal**, v. 198, n. 3, p. 560–570, 2013.

MICHAEL, N.; STORCH, A.; SMITH, W. **Injectable micro-minerals (Multimin) prove to be an effective and essential route of micro-mineral supplementation for lactating dairy cows.** Available on: <https://synbios.com.mx/wp-content/uploads/2018/02/SumariodeInvestigacion_Multimin90_MicorMineralSupLactatingDairyCows_ingles.pdf>. Accessed on 4 January 2020.

MOBERG, G. P.; MENCH, J. A. **The biology of animal stress: basic principles and implications for animal welfare.** First edition. CABI, 2000.

MULLIGAN, F. J.; DOHERTY, M. L. Production diseases of the transition cow. **Veterinary Journal**, v. 176, n. 1, p. 3–9, 2008. Available on: <<http://dx.doi.org/10.1016/j.tvjl.2007.12.018>>.

NAHMS (NATIONAL ANIMAL HEALTH MONITORING SYSTEM). **Dairy 2007 Heifer Calf Health and Management Practices on U.S. Dairy Operations, 2007.** USDA:APHIS:VS. USDA. Available on: <https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_ir_CalfHealth_1.pdf>.

OVERTON, T. R.; WALDRON, M. R. Nutritional management of transition dairy cows: Strategies to optimize metabolic health. **Journal of Dairy Science**, v. 87, n. SUPPL. 1, p. E105–E119, 2004. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(04\)70066-1](http://dx.doi.org/10.3168/jds.S0022-0302(04)70066-1)>.

PALMA, J. et al. Natural antibodies – Facts known and unknown. **Central European Journal of Immunology**, v. 43, n. 4, p. 466–475, 2018.

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. **Journal of Dairy Science**, v. 101, n. 9, p. 8532–8543, 2018.

REGITANO, J. B.; LEAL, R. M. P. Comportamento e impacto ambiental de antibióticos usados na produção animal brasileira. **Revista Brasileira de Ciência do Solo**, v. 34, n. 3, p. 601–616, 2010 (in portuguese).

ROTHSTEIN, T. L. Natural antibodies as rheostats for susceptibility to chronic diseases in the aged. **Frontiers in Immunology**, v. 7, n. 4, 2016.

SCIORSCI, R. L. et al. Oxidative status along different stages of pregnancy in dairy cows. **Large Animal Review**, v. 26, p. 223–228, 2020.

SEGERSTROM, S. C.; MILLER, G. E. Psychological stress and the human immune system: A meta-analytic study of 30 years of inquiry. **Psychological Bulletin**, v. 130, n. 4, p. 601–630, 2004.

SELYE, H. The general adaptation syndrome and the diseases of adaptation. **The Journal of Clinical Endocrinology**, v. 6, n. 2, p. 118–131, 1946.

SHELDON, I. M. et al. Defining postpartum uterine disease in cattle. **Theriogenology**, v. 65, n. 8, p. 1516–1530, 2006.

SMITH, R. L.; AL-MAMUN, M. A.; GRÖHN, Y. T. Economic consequences of paratuberculosis control in dairy cattle: A stochastic modeling study. **Preventive Veterinary Medicine**, v. 138, p. 17–27, 2017. Available on: <<http://dx.doi.org/10.1016/j.prevetmed.2017.01.007>>.

SORDILLO, L. M. et al. Shifts in thioredoxin reductase activity and oxidant status in mononuclear cells obtained from transition dairy cattle. **Journal of Dairy Science**, v. 90, n. 3, p. 1186–1192, 2007. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(07\)71605-3](http://dx.doi.org/10.3168/jds.S0022-0302(07)71605-3)>.

SORDILLO, L. M. Selenium-Dependent Regulation of Oxidative Stress and Immunity in Periparturient Dairy Cattle. **Veterinary Medicine International**, p. 15045, 2013.

SORDILLO, L. M. Nutritional strategies to optimize dairy cattle immunity. **Journal of Dairy Science**, v. 99, n. 6, p. 4967–4982, 2016.

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

SPEARS, J. W. Comparative Trace Element Nutrition: Trace Mineral Bioavailability in Ruminants. **Journal of Nutrition**, v. 133, n. 11, p. 1506–1509, 2003. Available on: <<https://academic.oup.com/jn/article-abstract/133/5/1506S/4558538>>.

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. Available on: <<http://dx.doi.org/10.1016/j.tvjl.2007.12.015>>.

STAR, L. et al. Natural humoral immune competence and survival in layers. **Poultry Science**, v. 86, n. 6, p. 1090–1099, 2007. Available on: <<http://dx.doi.org/10.1093/ps/86.6.1090>>.

STERLING, P.; EYER, J. Biological basis of stress-related mortality. **Social Science and Medicine**, v. 15, n. 1, p. 3–42, 1981.

THIELE-BRUHN, S. Pharmaceutical antibiotic compounds in soils - A review. **Journal of Plant Nutrition and Soil Science**, v. 166, n. 2, p. 145–167, 2003.

THOMPSON-CRISPI, K. A.; MIGLIOR, F.; MALLARD, B. A. Incidence Rates of Clinical Mastitis among Canadian Holsteins Classified as High, Average, or Low Immune Responders. **Clinical and Vaccine Immunology**, v. 20, n. 1, p. 106–112, 2013.

TORRES, S. J.; NOWSON, C. A. Relationship between stress, eating behavior, and obesity. **Nutrition**, v. 23, n. 11–12, p. 887–894, 2007.

VAN ALTENA, S. E. C. et al. Bovine natural antibodies in antibody-dependent bactericidal activity against *Escherichia coli* and *Salmonella Typhimurium* and risk of mastitis. **Veterinary Immunology and Immunopathology**, v. 171, p. 21–27, 2016.

VAN KNEGSEL, A. T. M. et al. Natural Antibodies Related to Energy Balance in Early Lactation Dairy Cows. **Journal of Dairy Science**, v. 90, n. 12, p. 5490–5498, 2007.

WARNICK, V. D.; ARAVE, C. W.; MICKELSEN, C. H. Effects of Group, Individual, and Isolated Rearing of Calves on Weight Gain and Behavior. **Journal of Dairy Science**, v. 60, n. 6, p. 947–953, 1977. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(77\)83968-4](http://dx.doi.org/10.3168/jds.S0022-0302(77)83968-4)>.

WEARY, D. M.; JASPER, J.; HÖTZEL, M. J. Understanding weaning distress. **Applied Animal Behaviour Science**, v. 110, n. 1–2, p. 24–41, 2008.

HYPOTHESES

- 1- Ultra-diluted complex would improve health and performance of growing heifers immediately after grouping up to their first lactation;
- 2- Ultra-diluted complex supplementation would improve the polymorphonuclear leukocyte function of weaned Holstein calves immediately after grouping management reducing respiratory scores;
- 3- Injectable trace mineral supplementation would improve incidence of health disorders, reproductive and productive performance, culling rate, innate immune activity, and antioxidant status of dairy cows undergoing the transition period in hot environment;
- 4- Natural antibodies, total immunoglobulins, and polymorphonuclear leukocyte function variables measured in the early postpartum period of dairy cows will be associated to each other.

OBJECTIVES

- 1- The chapter 2 study was undertaken to test the effectiveness of an ultra-diluted combination as a prophylactic strategy on health, metabolism, and performance of weaned Holstein calves immediately after grouping;
- 2- The aim of chapter 3 study was to evaluate the immune function, performance, and respiratory scores of weaned Holstein calves immediately after grouping;
- 3- The objective of chapter 4 study was to evaluate the effect of an injectable trace mineral supplementation containing copper, selenium, zinc, and manganese at dry-off, 20 days prior to expected day of calving, and 35 days postpartum on incidence of postpartum diseases, reproductive outcomes, culling rate, milk production, hematological parameters, peripheral blood polymorphonuclear leukocytes activity, concentrations of antioxidant enzymes, levels of oxidative stress markers, and circulating haptoglobin in dairy cows undergoing the transition period in hot-months;
- 4- Finally, the objective of chapter 5 study was to investigate the associations between natural antibodies (Nabs) and total serum immunoglobulin (Igs) concentrations, both from IgG and IgM isotypes, with several variables of peripheral polymorphonuclear leukocyte (PMN) function in early postpartum dairy cows.

CHAPTER 2 – Prophylactic use of an ultra-diluted complex on health, metabolism, and performance of weaned Holstein calves and its carryover impacts up to first-lactation

PAPER SUBMITTED TO JOURNAL OF DAIRY SCIENCE (attachment A).

ULTRA-DILUTED COMPLEX IN WEANED CALVES

Prophylactic use of an ultra-diluted complex on health, metabolism, and performance of weaned Holstein calves and its carryover impacts up to first-lactation

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ABSTRACT

The study aimed to evaluate the effect of an ultra-diluted complex supplementation as a prophylactic strategy on the incidence of diseases, metabolism, and performance of weaned Holstein calves immediately after grouping. Additionally, the objective was to investigate the carryover effects on heifer-raising and first-lactation period, in a double-blind, placebo-controlled trial. A total of 184 weaned Holstein calves were allocated to 8 paddocks in a completely randomized design experiment with blocks. During a 112 days period, the animals received a total mixed ration and were randomly allocated into two groups: 1) control (basal diet + calcium carbonate, top-dressed at 30 g/animal/d - placebo), or 2) ultra-diluted (basal diet + TopVita™-Real H, top-dressed at 30 g/animal/d – *Sulphur*: 10^{-60} + *Viola tricolor*: 10^{-14} + *Caladium seguinum*: 10^{-30} + *Zincum oxydatum*: 10^{-30} + *Phosphorus*: 10^{-60} + *Cardus marianus*: 10^{-60} + *Colibacillinum*: 10^{-30} + *Podophyllum*: 10^{-30} + Vehicle: calcium carbonate; q.s. 1kg). Incidence of diseases, days in treatment, and mortality were recorded daily by the research team. Performance and blood sampling were performed at enrollment and every 28 days until the end of the study period (112 days after enrollment). The digestive problems incidence was higher for the control group. Additionally, the control calves had more cumulative days per calf affected by tick-borne disease. Blood analysis revealed that control calves had increased mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration levels than ultra-diluted calves on d 28 after enrollment and had increased serum albumin and total protein concentration on d 84, as well. Furthermore, a higher albumin to globulin ratio was found to control calves than ultra-diluted group at enrollment day; however, on d 56, the inverse was observed. On d 28 after enrollment, control calves had increased serum cortisol concentration. Regarding body measurements, higher wither height and body depth was detected in ultra-diluted calves, on d 28 and d 84, respectively. Finally, a higher culling rate was observed in the control group at the heifer-raising period. In conclusion, a lower incidence of digestive problems and days under treatment for tick-borne disease were detected in the ultra-diluted group. However, ultra-diluted product did not improve performance at the post-weaning, heifer-raising, and first-lactation periods.

Key Words: culling, digestive problems, grouping, stress

INTRODUCTION

Despite the lower nutrient demand of weaned growing calves compared to milk-feeding calves, satisfactory growth rates should be maintained in this category, otherwise, these animals will not achieve the proper weight and height required for appropriate reproductive performance and healthy first-calving (ETTEMA; SANTOS, 2004). The cost involved in raising dairy replacement heifers strongly contributes to the total dairy business expense (HEINRICHS, 1993). Therefore, a lower impact of diseases on weaned growing calves' performance would play a great role in dairies' profitability. In Brazilian farms, the incidence of tick-borne disease caused by *Anaplasma spp.* and *Babesia spp.*, from birth until 180 days of age, may affect about 58% of the animals, being among the most common causes of death in post-weaned growing calves (MENDONCA, 2015). Regarding digestive disorders, despite of the high infection degree, allopathic treatments, and stress during the pre-weaning phase, weaning procedure may be one of the most tragic gastrointestinal transformations in calves biology and is close associated with impaired GIT health (KHAN; WEARY; VON KEYSERLINGK, 2011; KHAN et al., 2016). Furthermore, stress provided by animal movements can precipitate respiratory problems (LUNDBORG; SVENSSON; OLTENACU, 2005; SVENSSON; LIBERG, 2006). Besides, the commingling management increases the risk of pathogen exposure compared to individual calves raised in hutches (CALLAN; GARRY, 2002).

The selective pressure exerted by antimicrobial misuse outside of human medicine has produced antimicrobial resistance worldwide (HOLMES et al., 2016). The scientific and commercial community has encouraged the development of alternative strategies to combat antimicrobial resistance in livestock (BALLOU; DAVIS; KASL, 2019). From this perspective, ultra-diluted products - so-called homeopathic remedies - have attracted producers' interest during the past few years in order to reduce the abusive antibiotic use in farm animals. However, ultra-diluted product effectiveness has still to be demonstrated in well-designed studies. In a recent review, Doehring and Sundrum (2016) related the precariousness of the methodologies used in livestock studies to prove ultra-diluted effectiveness. These authors identified high heterogeneity of the results when clinical studies were analyzed. According to Kaptchuk (2001), the 'gold standard' design to prove the efficacy of a product is the randomized-controlled trials. Thus, unless evidence of efficacy of ultra-diluted products was not performed by a double-blind randomized-

controlled trial, any recommendation of replacing or reducing antibiotics by these remedies should be given.

The homeopathy, or as herein mentioned ultra-diluted remedies, was conceived in 1796 by Samuel Hahnemann, who believed that a diluted form of a substance that causes symptoms of a disease in healthy people, would heal similar symptoms in sick people (HAHNEMANN, 1810). Also, the potency of these remedies can be increased through a specific dilution and shaking of the base substance (plant, mineral, or animal in origin) in a solvent substance (e.g., ethanol; Ebert et al., 2017). From that, Hahnemann creates the simile principle, in other words, “like cures like” (*similia similibus curentur*). Furthermore, not only the patient's symptoms are important for this concept, besides that, patient's behavior, constitution, and conditions are taken into account to find the right remedy for each person (DOEHRING; SUNDRUM, 2016). Through the difficulty in treating a single individual prophylactically in a dairy herd scenario, the concept of homotoxicological remedies has been used. The homotoxicological concept is a single combination of ultra-diluted medicines for all herd (ARLT et al., 2009). Therefore, herein we used a homotoxocological complex to treat all the animals enrolled in the ultra-diluted group.

Although there is a lot of well-designed studies on milk-fed calves, a lack of research on the alternatives to improve post-weaned dairy calves' health and performance has been noticed. Regarding ultra-diluted products, the information is even more scarce in the current literature. To date, no study has been conducted to demonstrate the effect of standardized ultra-diluted combination on weaned dairy calves up to their first-lactation. This study was undertaken to test the effectiveness of an ultra-diluted combination as a prophylactic strategy on health, metabolism, and performance of weaned Holstein calves immediately after grouping. We hypothesized that the ultra-diluted complex supplemented as a homotoxicological remedy would reduce the incidence of diseases, which would be translated into higher performance of weaned Holstein calves immediately after grouping up to first-lactation.

MATERIALS AND METHODS

All activities performed in this study were reviewed and approved by the University of Sao Paulo Institutional Animal Care and Use Committee (#3547081117).

Animals and Facilities

The study was conducted in a commercial dairy farm located in southeast of Sao Paulo state, Brazil, from April 30, 2017, to November 15, 2017; the follow-up period continued until 25 November 2020. The farm milked 1700 Holstein cows 3 times per day in a double 30-stall parallel milking parlor. Before weaning, milk-fed calves were raised individually in suspended cages. After weaning, the calves were housed in 8 dry-lot pens (approximately 23 animals each pen), consisting of vegetated open lots (i.e., corrals) with shaded areas. The animals were fed a total mixed ration (TMR) *ad libitum* with free access to water. The diet was formulated to meet or exceed the NRC (2001) nutrient requirements for weaned growing calves. The ingredients and chemical composition included in the basal diet are presented in Table 1.

Experimental design, Treatment Allocation, and Inclusion criteria

This study was a double-blind, randomized, placebo-controlled trial. The experiment was designed as completely randomized with blocks. The weaned Holstein calves were blocked according to the weaning date and, within each block, randomly assigned to one of the two treatments immediately after grouping, according to the Microsoft Excel™ RAND command. A total of 184 weaned Holstein calves (83.01 ± 7.9 days old; 112.5 ± 11.7 kg) were allocated in 8 paddocks (experimental unit). At the day of grouping after milk-feeding phase, the animals were randomly allocated into two different groups: (1) Control: untreated controls ($n=93$; basal diet + calcium carbonate, top-dressed at 30g/animal/d – placebo); and (2) Ultra-diluted: treated with a ultra-diluted complex ($n = 91$; basal diet + TopVita™ - Real H, top-dressed at 30 g/animal/d - Sulphur: 10^{-60} + Viola tricolor: 10^{-14} + Caladium seguinum: 10^{-30} + Zincum oxydatum: 10^{-30} + Phosphorus: 10^{-60} + Cardus marianus: 10^{-60} + Colibacillinum: 10^{-30} + Podophyllum: 10^{-30} + Vehicle: q.s. 1kg – calcium carbonate; TopVita™ – Real H Company, Campo Grande, MS, Brazil). Calves were included in the study if they did not present clinical signs associated with tick-borne disease, digestive disorder, and pneumonia. No calf exclusion was required.

The ultra-diluted product as well as the placebo treatment were incorporated into the corn ground portion (300 g/kg of corn ground as-fed). This mix was fed as a top-dressing on the total mixed ration (TMR) daily in the morning for each pen. The exact amount of the mix was achieved by multiplying the number of animals per pen by 100 g of the mix. It was expected that calves would consume approximately 30 g/d of ultra-diluted or placebo product.

Data Collection and Case Definition

Both groups placebo and ultra-diluted treatment were blinded to the attending personnel, research team, and veterinarians who assessed clinical signs of the animals. To achieve blinding of research and farm personnel, treatment allocation and administration were performed by a veterinarian from the research team in the mornings. Calves were visually inspected by the research team members daily from enrollment until 112 days of the enrollment. In addition to the research group monitoring, all the animals were visually monitored daily by trained employees and veterinarians from the farm. The tick-borne disease was tracked daily by the presence of clinical signs of paleness of mucous membranes, increased heart and respiratory rates, pyrexia, weakness, lethargy, anorexia (KOCAN et al., 2010), and behavior changes (OLIVEIRA JÚNIOR et al., 2018). The clinical examination included measurement of rectal temperature, evaluation of the color of mucous membranes (mouth, eyes, and vulva; 1 = normal, 2 = pale, 3 = icteric), and hydration degree (1 = hydrated, 2 = moderate dehydration, 3 = severe dehydration). In addition, a blood sample was taken at the moment of diagnostic from a small ear vessel by puncture with a 27-gauge needle and collected into a heparin-treated capillary tube. Capillary tubes were spun at $14,490 \times g$ for 5 min using a microhematocrit centrifuge (FANEM®, Mod. 211, São Paulo, Brazil). A microhematocrit reading card was used to determine packed cell volume (PCV). A calf with a hematocrit $\leq 20\%$ was positive for tick-borne disease since blood smear examination has also been positive by any of the disease-causing pathogens. The blood smears were prepared by the Romanowsky stains (Kit Panótico Rápido®, Laborclin) and microscopically observed for the detection of *A. marginale* and *Babesia spp.* in blood. Digestive problems were characterized by diarrhea and bloat. Respiratory problems were characterized by increased respiratory frequency associated with fever and presence of increased lung sounds at auscultation. Data regarding the incidence of diseases, days in treatment, and mortality were recorded daily by the research team.

All diets were mixed as TMR and offered once daily at 07:00 h. Diets were fed for 50 g refusals for each kg offered. The TMR and ort samples of each pen were taken daily during the week of the 28th day of each period to provide a composite sample (4 samples, one per period). Immediately after collections, samples were stored at -20°C until chemistry analysis (ESALQLab, Piracicaba, SP, Brazil). Dry matter, mineral matter, ether extract, starch, crude protein, and neutral detergent fiber (NDF) were analyzed on Foss NIR 5000 equipment, according to the user manual. The calibration to estimate the nutritional quality variables was developed by the Dairy One Forage Lab (Ithaca, NY, USA) and validated with independent and local samples from Brazil by ESALQLab - Department of Animal Sciences - ESALQ / USP, SP, Brazil. It is worth mentioning that NIR is already a methodology recognized by AOAC [AOAC 991.01 - Moisture in Forage. AOAC 989.03 - Fiber (Acid Detergent) and Protein (Crude) in Forages]. Total digestible nutrients (TDN) were calculated according to the NRC (2001).

Body weight and body measurements were assessed at enrollment and every 28 days until the end of the study period (112 days after enrollment). Through the practical and reliable technique, body weight was estimated using calibrated weight tapes measuring heart girth circumference (HEINRICHS; ROGERS; COOPER, 1992). These measurements were used to calculate the average daily gain (ADG) during the study period (final weight–initial weight/period in days). Additionally, measures of withers height (cm), body depth (cm), and hearth girth (cm) were taken using a hipometer. The average daily gain of withers height (cm/d), body depth (cm/d), and hearth girth (cm/d) were calculate performing the difference between the final and initial measures divided per the period in days. The pen-level body weight gain to feed ratio was calculated using the following equation:

$$\text{BW G: F} = \frac{\text{pen level ADG (BW)}}{\text{pen level DMI}}$$

Blood sampling and analysis

Blood was collected for a random subset of 40 calves per group to determine evidence of inflammation and stress. Blood samples were collected at enrollment, 28, 56, 84, and 112 days later by jugular venipuncture using a Vacutainer tube without anticoagulant and a Vacutainer tube with EDTA, and a 20-gauge $\times$ 2.54-cm Vacutainer needle (Becton, Dickinson and Company, Franklin Lakes, NJ). After collection, tubes were immediately placed in a cooler containing iced water and

transported to a commercial laboratory (DAC, Clinical Analysis, Pirassununga, SP, Brazil) within 2h after collection. Blood samples collected without anticoagulant were centrifuged for serum separation. After, serum samples were used to chemistry profile (total protein, albumin, albumin to globulin ratio, globulin, glucose, blood urea nitrogen, aspartate aminotransferase, gamma-glutamyl transferase, creatinine) and cortisol analysis. The inter and intra-assay coefficients of variation for serum cortisol were 12.4 and 4.6 %, respectively. Samples collected with EDTA were evaluated for complete blood cell counts, using a hematology analyzer (IDEXX Procyte DX, Westbrook, ME).

Retrospective data collection

The carryover effects of ultra-diluted treatment of weaned calves on the heifer-raising period and first lactation outcomes were evaluated using data collected retrospectively for all the animals. Data regarding culling and first-calving age during the heifer-raising period; first-lactation 305-d milk yield, retention of fetal membranes, days to first artificial insemination (AI), and culling up to 60 days in milk (DIM) during the first-lactation period were extracted from the farms' database software (Ideagri softwareTM, Belo Horizonte, MG, Brazil).

Statistical analysis

Based on previously reported data, we expected that ultra-diluted treatment would decrease digestive problems in 15 percentage units (e.g. 50% to 35%). Considering a significance level of $\alpha = 0.05$ and a power of 80%, a total of 90 calves would be enough for each treatment group.

Descriptive statistics were undertaken using the chi-square and ANOVA functions of SAS 9.4 (SAS Institute Inc., Cary, NC). The relative risk of clinical tick-borne diseases, digestive problems, pneumonia, mortality (during the experimental period), and culling rate (during the heifer-raising period) were assessed by fitting the data using Poisson regression in GENMOD procedure of SAS, with a log link and Poisson distribution. Variables offered to the models included treatment (which was forced into all the models), dam parity (nulliparous or primiparous + multiparous), navel health in milk-fed period (normal or inflamed), dystocia (yes or no). Variables associated with the outcome at $P \leq 0.2$ were offered to the multivariable model. A manual backward stepwise selection procedure was used to build the final models, with variables at $P \leq 0.05$ being retained as main effects. Pen served as the experimental unit for all outcomes. Because

of the low number of dead animals during supplementation period and through the low number of animals affected by fetal membrane retention and culled up to 60 DIM, during first-lactation, the proportion difference between treatments were tested by Fisher's exact test using PROC FREQ procedure of SAS 9.4.

To evaluate the effect of the ultra-diluted complex on performance measures, cell blood count, chemical profile, and cortisol throughout the five sampling points (enrollment day, 28, 56, 84, and 112 days later), repeat measures models, ordered by time, were fitted by multiple mixed linear models using the MIXED procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). The experimental unit was the pen. To account appropriately for within-calf residues correlation, in repeated measures models, a first-order autoregressive covariance structure [AR(1)] was applied for all models. This variance-covariance structure is indicated for equally spaced data collection and assumes correlations decline as a function of time. The variable treatment, time, and their interaction were forced into all statistical models even in the absence of statistical significance. Parity (Nulliparous or Primiparous + Multiparous), navel health (normal or inflamed), dystocia (yes or no), and the own variable value from enrollment collection, were offered to all models as a covariate to control confounding effects. For multivariate models above, independent variables and their respective interactions were kept when $P \leq 0.05$. Block effect and pen were included as random errors. Repeated observations within a pen were modeled using a compound symmetry residual variance-covariance structure. Cumulative days in allopathic treatment during the 112 experimental days, the interval from birth to first-calving, whole lactation milk yield (305 d), and interval from calving to the first postpartum AI were analyzed by the MIXED procedure of SAS with a model that included the fixed effects of treatment. Block and pen were included as random errors. Repeated observations within a pen were modeled as previously described. Block effect was considered the enrollment date of the groups.

Dry matter and nutrients intake were analyzed with data from 4 pens/treatment. Daily values were averaged to week mean (-3 and +3 days relative to the 28th day of each period) and analyzed as repeated measures. The fixed effects included treatment, time, the interaction between treatment, and time. Block and pen were included as a random experimental error in the model. Visual assessment of the distribution plots of the studentized residuals were used to confirm that the residuals were normally distributed. Least square means and proportions are reported for all

variables evaluated. For all the analyses, differences detected at $P \leq 0.05$ were considered significant.

RESULTS

Descriptive statistics

Parity of the dam or embryo transfer, calving problem, navel infection during the milk-feeding period, average body weight (kg) at enrollment, and average age (days) at enrollment were not different for calves in ultra-diluted and treatments (Table 2).

Disease incidence and cumulative days in treatment

The effect of treatment on adjusted incidences of tick-borne disease, digestive disorder, pneumonia, and mortality are presented in Table 3. Ultra-diluted treatment did not decrease the incidence of tick-borne disease during 112 days after grouping ($P = 0.13$). The tick-borne incidence was 93.5% and 85.7% for calves enrolled in the control and ultra-diluted treatment, respectively. Similarly, ultra-diluted treatment did not decrease the proportion of pneumonia incidence during the period (Table 3; $P = 0.89$). Otherwise, incidence of digestive disorder was reduced by ultra-diluted treatment (53.3 vs. 30.8% for control and ultra-diluted calves, respectively; $P < 0.001$). Although treatment difference was observed in digestive disorder outcome, ultra-diluted treatment did not improve the mortality rate at the post weaning period (Table 3). Regarding days in treatment, we detected fewer days in allopathic tick-borne disease treatment for the ultra-diluted treatment group compared to control calves (Table 4; $P = 0.007$). On the other hand, we did not observe days in treatment difference for digestive disorder and pneumonia incidences (Table 4; $P = 0.62$ and $P = 0.81$, respectively).

Blood variables

The effect of ultra-diluted treatment on white blood cell, neutrophil, lymphocyte counts, neutrophil to lymphocyte ratio, and red blood cell variables are presented in Table 5. Interactions between treatment and time are depicted in Figure 1. Neither white blood cells count non neutrophil to lymphocyte ratio were different between the treatments ($P > 0.05$). However, MCH and MCHC

were higher in control group on d 28 after the enrollment compared to ultra-diluted group (Table 5 depicted in Figure 1; $P = 0.05$ and $P = 0.01$, respectively).

The effect of ultra-diluted product supplementation on blood chemical variables is presented in Table 6. Ultra-diluted product did not influence serum AST, GGT, creatinine, urea, globulin, and glucose concentration ($P > 0.05$). However, albumin, total protein, albumin to globulin ratio, and cortisol blood concentration were affected by the treatment depends on the study day. The dynamic of these serum metabolites are demonstrated in Figure 2. Calves enrolled in the control group had increased serum concentration of albumin ($P = 0.01$) and total protein ($P = 0.04$) in comparison to ultra-diluted calves on d 84 (Figure 2A and 2B, respectively). Additionally, serum albumin to globulin ratio was increased for control calves at enrollment day in comparison to ultra-diluted treated calves ($P = 0.004$). Contrariwise, a higher albumin to globulin ratio was detected in ultra-diluted treated calves on d 56 after enrollment (Figure 2C; $P = 0.05$). Serum cortisol level was greater for control calves on d 28 after enrollment (Figure 2D; $P = 0.03$).

Nutrients intake and performance

The effect of ultra-diluted treatment on dry matter intake and nutrients and performance are presented in Table 7 and Table 8, respectively. Dry matter and nutrient intakes did not differ between the treatments ($P > 0.05$). Also, no differences were detected in body weight from enrollment day up to 112 days ($P > 0.05$). Besides, no differences were detected in ADG (Table 8). Although no differences were detected in ADG during the experimental period, the dynamic of this variable throughout the study is depicted in Figure 3A. Also, gain to feed ratio is presented in Figure 3B. Regarding body measurements, we detected a higher wither height and wither height gain for ultra-diluted calves on d 28 and between 0-28 days after enrollment, respectively (Figure 3A and 3B; $P < 0.05$ and $P = 0.004$). Also, the ultra-diluted calves had higher body depth on d 84 (Figure 4A; $P = 0.05$), although a higher body depth gain was detected to control calves between 29-56 and 85-112 days after enrollment (Figure 4B; $P < 0.05$). We did not detect difference in hearth girth circumference between the treatments throughout the study ($P > 0.05$).

Heifer-raising period and subsequent first-lactation outcomes

Retrospective outcomes from the heifer-raising period and subsequent first-lactation are presented in Table 9. We observed a higher culling rate to control calves in comparison to ultra-

diluted group (Ultra-diluted relative risk = 0.86; $P = 0.006$). However, no differences were detected in days to first calving, first-lactation 305-d milk yield, days to first artificial insemination, retained fetal membranes incidence, and culling rate up to 60 days in milk ($P > 0.05$).

DISCUSSION

The ultra-diluted complex utilized in this study has been recommended to improve disorders of hematologic, gastrointestinal, and respiratory systems. Ultra-diluted complex did not decrease the incidence of tick-borne disease nor it affected the incidence of pneumonia during the post-grouping period. However, reduced incidence of digestive problems was detected for calves. Furthermore, fewer days in tick-borne disease allopathic treatment was observed for calves that received ultra-diluted supplementation. Neither white blood cells count non neutrophil to lymphocyte ratio were different between the groups. However, RBC variables were slightly altered between the groups throughout the study. The amounts of MCH and MCHC were higher in control group on day 28 after the enrollment compared to ultra-diluted group. Regarding serum metabolites, albumin, total protein, albumin to globulin ratio, and cortisol concentration were affected by the treatment depends on the day of study. Since AST, GGT, creatinine, and urea levels did not differ between the treatments, we concluded that ultra-diluted complex had no impact on liver and kidney function. No difference was detected in performance between the groups during the early post-grouping calves' period, heifer-raising period, and first lactation. Nevertheless, a lower culling rate was detected in ultra-diluted group during the heifer-raising period.

In this study, all the animals when in milk-feeding system were reared individually in a covered area. Individual housing allows controlled management of the neonatal calves to adapt to the ex-utero environment and prevents the horizontal transmission of disease through calf-to-calf contact (HULBERT; BALLOU, 2012). The switch from an individual milk-fed system to a post-weaned calves' group has been a common management strategy adopted for many dairy farms. This practice aims to decrease the high labor cost involved in an individual milk-feeding system. However, decreased performance during the grouping phase has been observed, which may be due to stressors related to social changes or due to the higher exposition to novel microbes (HULBERT; BALLOU, 2012). Notwithstanding, Lochmiller and Deerenberg (2000) declared that despite the

low amount of leucocyte total cells compared to the animal total body weight, an activated acute phase immune response can decrease performance and feed efficiency.

Herein in this study, ultra-diluted treatment decreased the incidence of digestive problems during the post-weaning period. Through the several compounds included in the ultra-diluted product, we are not able to indicate which single compound is acting on digestive physiology to decrease the digestive problems. However, *Viola tricolor*, *Zincum oxidatum*, *phosphorus*, *Cardus mariannus*, *colibacillum*, and *podophyllum*, which were included in the ultra-diluted formula have been used as a medicine to treat digestive problems, in the homeopathic medicine practice. The homeopathy theory explains the effect due to electromagnetic energy of these substances on autonomic nervous system (MISHRA et al., 2011). Still, regarding the ultra-diluted effects on health, cumulative days of allopathic tick-borne disease treatment was reduced for ultra-diluted treated calves. Fortuoso et al. (2018) was the only similar study that used a combination of substances in an ultra-diluted manner as a prophylactic alternative to minimize bacterial infection and prevent neonatal diarrhea in milking calves. These authors detected 50% of efficacy on control of diarrhea using *Colibacillinum*, *Mercurius dulcis*, *Chinchona officinalis*, *Enterococcinum*, *Podophyllum peltatum*, and *Colocynthis* substances, diluted on the centesimal scale. However, the low number of experimental units in this study reduces the statistical reliability to affirm such finding.

A higher MCH and MCHC levels were detected in control calves at day 28 after enrollment, although both groups were in normal bovine reference range (11-17pg and 30-36% for MCH and MCHC, respectively). Significantly higher MCH and MCHC may be the result of immature red cells in circulation and simultaneously incorporation of an increased amount of hemoglobin at the time of their release (KUMAR; PACHAURI, 2000). Even though any statistical difference was detected on tick-borne disease incidence between the treatments (93.5 vs. 85.7% in control and ultra-diluted treated calves, respectively), the cumulative days in allopathic treatment for control calves affected by tick-borne disease was greater in comparison to ultra-diluted treated calves. Herein, we may suggest a slight level of anemia in control animals, reflected by MCH and MCHC alteration levels on day 28. Moreira (2017) detected first tick-borne disease event about 125 d of age in heifers. It may be consistent with disease's pathogenesis and common postweaning practices that expose calves to outdoor group housing for the first time at approximately 90 d of age (OLIVEIRA JÚNIOR et al., 2018).

The albumin metabolite reflects liver functionality, and its lack of production may indicate liver dysfunction or even dehydration in animals. The albumin level was higher to control calves at day 84 after enrollment. This effect was reflected in higher total protein at the same day of study. As the incidence of digestive problems was greater to control calves, we may suggest that dehydration in this group was the main cause for these results, corroborating Guzelbektes et al. (2007) study. At day 56 after enrollment, the control group had reduced proportion of albumin to globulin ratio. The albumin to globulin ratio is an important blood variable to evaluate the presence of infection in the organism. In this study, the level of serum cortisol was greater on day 28 to control group. Further, serum cortisol is tightly associated to control of pro- and anti-inflammatory signals to immune system tissues (HULBERT; MOISÁ, 2016). Thus, we speculate that the higher proportion and duration of diseases detected in control groups - such as incidence of diarrhea and days in treatment for tick-borne disease, respectively – provided these alterations in blood biochemical and hormonal profile. Collectively, blood analysis results suggest that the systemic inflammatory state of ultra-diluted calves was improved.

Although no effects of detected on dry matter and nutrients intake, a higher wither height, and body depth was detected to ultra-diluted treated calves at 28 and 84 days after enrollment, respectively. Diseases and activated immune system may uptake nutrients from bloodstream which would be used for growth (LOCHMILLER; DEERENBERG, 2000). Therefore, we hypothesized that due to the lower proportion of animals affected by digestive problems and fewer cumulative treatment days for tick-borne diseases in ultra-diluted group, the nutrients such as protein and energy were used more efficiently in this group. However, at the end of the study (112 d after enrollment), no difference was detected for body measurements between calves enrolled in ultra-diluted or control group.

Regarding the heifer-raising period after the ultra-diluted supplementation period, a lower culling rate was observed in ultra-diluted group compared to control calves. Carryover consequences of diseases in the heifer-raising period have been reported for lifetime in dairy animals (WATHES et al., 2014). Nevertheless, no differences were detected in 305-d milk yield, days to first AI, retained placenta incidence, and culling up to 60 DIM between the groups during the first-lactation. It may be explained through the increased culling rate observed to control calves during the heifer-raising period. Those removed animals would probably impair health,

reproductive outcomes, and milk yield in first-lactation control calves compared to ultra-diluted treated calves, but it was not confirmed.

CONCLUSIONS

In conclusion, prophylactic use of ultra-diluted complex did not improve performance of weaned Holstein calves, nor it had an impact on the heifers-raising period and subsequent first-lactation. However, reduced proportion of digestive problems, fewer cumulative treatment days for tick-borne disease, and a lower culling rate in the heifer-raising period were detected in ultra-diluted treated group. In addition, in specific days after enrollment, systemic inflammation status of calves was improved by ultra-diluted complex based on circulating biomarkers of inflammation and stress. More research is needed to prove the effectiveness of ultra-diluted products on farm animals since there is a lack of well-designed studies in the literature.

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CONFLICT OF INTEREST

This research was funded by a grant from Real-H company. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Table 1. Ingredients and chemical composition of basal diet (% of DM, otherwise stated)

Ingredients	
Corn silage	36.7
Grass hay (Tifton-85)	11.9
Citric pulp	12.8
Ground corn	17.9
Soybean meal	18.6
Mineral and vitamins	1.6
Sodium Bicarbonate	0.4
Mycotoxin adsorbent	0.1
Salt	0.1
Yeast complex	0.1
Biotin	0.01
Chemical composition	
Dry matter (% OM)	50.0
Organic matter	42.8
Ash	7.2
NDF ¹	38.0
Starch	24.0
Crude protein	14.8
Ether extract	2.9

¹Neutral detergent fiber

Table 2. Descriptive statistics of treatment groups.

Item	Treatment		P-value
	Control ¹	Ultra-diluted ¹	
Total number of calves included in the study (%)	93 (50.5)	91 (49.5)	-
Parity of the dam			
Nulliparous (%)	13.2	5.68	0.22
Multiparous (%)	42.9	44.3	-
Embryo transfer (%)	43.9	50	-
Navel infection			
Yes (%)	12.1	19.3	0.18
No (%)	87.9	80.7	-
Calving problem			
Dystocic (%)	28.6	28.4	0.98
No dystocic (%)	71.4	71.6	-
Average body weight (kg) at enrollment ($\pm$ SE)	113.6 (10.8)	111.9 (12.7)	0.94
Average age (days) at enrollment ($\pm$ SE)	82.4 (7.6)	83.6 (8.3)	0.92

Table 3. Incidence of disease and mortality of weaned Holstein calves fed control or ultra-diluted complex during 112d.

Item	Treatment	
	Control ¹	Ultra-diluted ¹
Tick-Borne Disease		
Incidence, %	93.5	85.7
Relative Risk, 95% CI	<i>Baseline</i>	0.93 (0.84 - 1.02)
P-value		0.13
Digestive disorders		
Incidence, %	53.3	30.8
Relative Risk, 95% CI	<i>Baseline</i>	0.60 (0.58 - 0.61)
P-value		<0.0001
Pneumonia		
Incidence, %	52.2	50.6
Relative Risk, 95% CI	<i>Baseline</i>	0.99 (0.86 - 1.14)
P-value		0.89
Mortality		
Dead calves, %	1.09	0
Relative Risk, 95% CI	<i>Baseline</i>	0.98 (0.96 - 1.01)
P-value		0.50

¹Control = calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping;

Table 4. Cumulative days per weaned Holstein calves affected by health conditions fed control or ultra-diluted complex during 112d.

Condition	Treatment		SEM	P-value
	Control	Ultra-diluted		
Tick-Borne disease, d	6.3	4.8	0.48	0.007
Diarrhea, d	2.4	2.5	0.34	0.62
Pneumonia, d	6.5	6.8	1.11	0.81

¹Control = calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping;

Table 5. Blood cell count and hemoglobin of weaned Holstein calves fed control or ultra-diluted complex during 112d.

Item	Treatment			P-value		
	Control ¹	Ultra-diluted ¹	SEM	Treatment	Time	Treatment x Time
White blood cell, x 10 ³ /μL	11.4	11.9	0.41	0.37	<0.0001	0.68
Neutrophils	2.9	2.8	0.30	0.90	<0.0001	0.55
Lymphocytes	7.4	7.9	0.31	0.27	<0.0001	0.17
Monocytes	1.06	1.04	0.11	0.83	<0.0001	0.36
As % of total						
Neutrophils	25.5	24.1	2.50	0.70	<0.0001	0.58
Lymphocytes	64.7	65.6	2.50	0.81	<0.0001	0.60
Monocytes	9.4	9.0	1.10	0.76	<0.0001	0.08
Neutrophil:Lymphocyte	0.62	0.66	0.19	0.90	<0.0001	0.43
Red blood Cells, x 10 ⁶ /μL	5.1	5.0	0.13	0.75	<0.0001	0.74
Hemoglobin, g/dL	7.65	7.5	0.19	0.60	<0.0001	0.84
Hematocrit, %	21.6	22.4	0.81	0.48	0.0001	0.68
MCV, fL ²	43.5	46.1	2.32	0.50	0.08	0.60
MCH, pg ³	15.3	15.2	0.24	0.76	<0.0001	0.05
MCHC, % ⁴	35.8	35.1	0.78	0.54	<0.0001	0.04
Platelets, x 10 ³ μL	339.7	296.8	22.9	0.24	<0.0001	0.67

¹Control = calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping;

²Mean corpuscular volume

³Mean corpuscular hemoglobin

⁴Mean corpuscular hemoglobin concentration

Table 6. Blood metabolites of weaned Holstein calves fed control or ultra-diluted complex during 112d.

Item	Treatment		SEM	P-value		
	Control ¹	Ultra-diluted ¹		Treatment	Time	Treatment x Time
AST ² , U/L	72.8	71.3	2.73	0.62	<0.0001	0.86
GGT ³ , U/L	22.6	20.2	1.91	0.46	<0.0001	0.83
Creatinine, mg/dL	0.82	0.81	0.07	0.78	0.0003	0.46
Urea, mg/dL	31.9	30.6	1.97	0.64	<0.0001	0.41
Albumin, g/dL	3.10	3.10	0.04	0.77	<0.0001	0.0005
Globulin, g/dL	3.14	3.15	0.06	0.91	<0.0001	0.08
Total protein, g/dL	6.22	6.27	0.08	0.70	<0.0001	0.01
Albumin:Globulin	1.02	1.01	0.02	0.86	<0.0001	0.002
Glucose, mg/dL	70.5	69.4	3.05	0.77	<0.0001	0.06
Cortisol, µg/dL	0.94	0.98	0.08	0.75	0.16	0.008

¹Control = calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping;

²Aspartate-aminotransferase;

³Gamma-glutamyltransferase;

Table 7. Dry matter and nutrient intake of weaned Holstein calves fed control or ultra-diluted complex during 112d.

Item	Treatment		SEM	P-value		
	Control ¹	Ultra-diluted ¹		Treatment	Time	Treatment x Time
Group intake, kg/d						
Dry matter	3.09	3.16	0.14	0.73	<0.0001	0.53
Crude protein	0.47	0.47	0.02	0.81	<0.0001	0.47
Starch	0.77	0.78	0.04	0.84	<0.0001	0.37
NDF	1.12	1.16	0.05	0.61	<0.0001	0.66
Ether extract	0.09	0.09	0.004	0.58	<0.0001	0.31
Dry matter, % BW	2.06	2.14	0.09	0.54	<0.0001	0.47
NDF, % BW	0.80	0.80	0.03	0.52	0.0004	0.59

¹Control = calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping;

Table 8. Body weight (BW) and growth measurements of weaned Holstein calves fed control or ultra-diluted complex during 112d.

Item	Treatment		SEM	P-value		
	Control ¹	Ultra-diluted ¹		Treatment	Time	Treatment x Time
Mean BW, kg	140.2	137.7	2.09	0.47	<0.0001	0.29
BW, kg						
0d	112.3	112.0	2.22	0.94	-	-
28d	125.0	122.5	2.22	0.49	-	-
56d	135.4	133.1	2.23	0.52	-	-
84d	153.5	149.5	2.23	0.27	-	-
112d	174.6	171.2	2.23	0.34	-	-
BW ADG, kg/d	0.55	0.52	0.03	0.57	<0.0001	0.43
Mean Gain:Feed, kg/kg	0.18	0.17	0.01	0.51	0.01	0.30
Mean wither height, cm	98.9	99.4	0.25	0.13	<0.0001	0.009
Initial	91.8	92.1	0.32	0.44	-	-
Final	106.1	106.2	0.32	0.68	-	-
Wither height ADG, cm/d	0.13	0.13	0.004	0.61	0.01	0.004
Mean body depth, cm	50.2	50.5	0.17	0.22	<0.0001	<0.0001
Initial	45.1	45.1	0.26	0.9	-	-
Final	55.3	55.4	0.26	0.84	-	-
Body depth ADG, cm/d	0.09	0.09	0.003	0.59	<0.0001	0.0004
Mean heart girth, cm	115.9	115.4	0.78	0.67	<0.0001	0.14
Initial	106.1	107.5	0.94	0.31	-	-
Final	126.1	124.8	0.94	0.34	-	-
Heart girth ADG, cm/d	0.18	0.14	0.02	0.28	0.0003	0.47

¹Control = calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d);
 Ultra-diluted = calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping;

Table 9. Retrospective outcomes from the heifer-raising period and subsequent lactation of weaned Holstein calves fed control or ultra-diluted complex during 112d.

Item	Treatment	
	Control ¹	Ultra-diluted ¹
Heifer-raising period		
Culling		
Rate, %	18.7	5.5
Relative risk (95% CI)	<i>Baseline</i>	0.86 (0.77-0.96)
P-value		0.0064
Days to first calving		
95% CI	784 (758-810)	797 (770-823)
P-value		0.36
Subsequent first-lactation period		
First-lactation, 305-d milk yield, kg		
95% CI	10,501 (9667.7 - 11335)	10,508 (9667.2 - 11349)
P-value		0.99
Days to first AI		
95% CI	92 (76.9 - 107.2)	93 (78.2 - 108)
P-value		0.9
Retained Placenta		
Incidence, %	5.8	7.2
Relative risk (95% CI)	<i>Baseline</i>	1.01 (0.93-1.1)
P-value ²		0.72
Cull up to 60 days in milk		
Dead cows, %	2.2	2.2
Relative risk (95% CI)	<i>Baseline</i>	1.0 (0.96-1.04)
P-value ²		0.69

¹Control = calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping;

²Fisher's exact test

Figure 1. Effect of ultra-diluted treatment on A) Mean Corpuscular Hemoglobin, and B) Mean corpuscular hemoglobin concentration. Control = weaned Holstein calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = weaned Holstein calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping. An asterisk (*) indicates a $P < 0.05$.

Figure 2. Effect of ultra-diluted treatment on concentration of A) Albumin, B) Total protein, C) Albumin:Globulin ratio, and D) Cortisol. Control = weaned Holstein calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = weaned Holstein calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping. An asterisk (*) indicates a $P < 0.05$.

Figure 3. Effect of ultra-diluted treatment on A) Average daily gain, B) Gain:Feed ratio, C) Withers height, and D) Withers height gain. Control = weaned Holstein calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = weaned Holstein calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping. An asterisk (*) indicates a $P < 0.05$.

Figure 4. A) Body depth, and B) Body depth gain. Control = weaned Holstein calves received placebo treatment (calcium carbonate, top-dressed at 30 g/animal/d); Ultra-diluted = weaned Holstein calves received ultra-diluted treatment (TopVita™ - Real H, top-dressed at 30 g/animal/d), both during 112 d after grouping. An asterisk (*) indicates a $P < 0.05$.

REFERENCES

- ARLT, S. et al. Efficacy of homeopathic remedies as prophylaxis of bovine endometritis. **Journal of Dairy Science**, v. 92, n. 10, p. 4945–4953, 2009. Available on: <<http://dx.doi.org/10.3168/jds.2009-2142>>.
- BALLOU, M. A.; DAVIS, E. M.; KASL, B. A. Nutraceuticals: An Alternative Strategy for the Use of Antimicrobials. **Veterinary Clinics of North America - Food Animal Practice**, v. 35, n. 3, p. 507–534, 2019.
- BØE, K. E.; FÆREVIK, G. Grouping and social preferences in calves, heifers and cows. **Applied Animal Behaviour Science**, v. 80, p. 175–190, 2003.
- CALLAN, R. J.; GARRY, F. B. Biosecurity and bovine respiratory disease. **The Veterinary Clinics Food Animal Practice**, v. 18, p. 57–77, 2002.
- CHUA, B. et al. Effects of pair versus individual housing on the behavior and performance of dairy calves. **Journal of Dairy Science**, v. 85, n. 2, p. 360–364, 2002. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(02\)74082-4](http://dx.doi.org/10.3168/jds.S0022-0302(02)74082-4)>.
- DOEHRING, C.; SUNDRUM, A. Efficacy of homeopathy in livestock according to peer-reviewed publications from 1981 to 2014. **Veterinary Record**, v. 179, n. 24, p. 1–13, 2016.
- EBERT, F. et al. Randomized, blinded, controlled clinical trial shows no benefit of homeopathic mastitis treatment in dairy cows. **Journal of Dairy Science**, v. 100, n. 6, p. 4857–4867, 2017.
- ETTEMA, J. F.; SANTOS, J. E. P. Impact of age at calving on lactation, reproduction, health, and income in first-parity Holsteins on commercial farms. **Journal of Dairy Science**, v. 87, n. 8, p. 2730–2742, 2004. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(04\)73400-1](http://dx.doi.org/10.3168/jds.S0022-0302(04)73400-1)>.
- FORTUOSO, B. F. et al. Homeopathic treatment as an alternative prophylactic to minimize bacterial infection and prevent neonatal diarrhea in calves. **Microbial Pathogenesis**, v. 114, n. November 2017, p. 95–98, 2018. Available on: <<https://doi.org/10.1016/j.micpath.2017.11.041>>.
- GUZELBEKTES, H.; COSKUN, A.; SEN, I. Relationship between the degree of dehydration and the balance of acid-based changes in dehydrated calves with diarrhoea. **Bulletin of the Veterinary Institute in Pulawy**, v. 51, n. 1, p. 83–87, 2007.
- HAHNEMANN, S. **Organon der rationellen Heilkunde**. Dresden, Germany: Arnold, 1810. v. 1
- HEINRICHS, A. J. Raising Dairy Replacements to Meet the Needs of the 21st Century. **Journal of Dairy Science**, v. 76, n. 10, p. 3179–3187, 1993. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(93\)77656-0](http://dx.doi.org/10.3168/jds.S0022-0302(93)77656-0)>.
- HEINRICHS, A. J.; ROGERS, G. W.; COOPER, J. B. Predicting Body Weight and Withers Height in Holstein Heifers Using Body Measurements. **Journal of Dairy Science**, v. 75, n. 12, p. 3576–3581, 1992. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(92\)78134-X](http://dx.doi.org/10.3168/jds.S0022-0302(92)78134-X)>.

HICKEY, M.-C.; DRENNAN, M.; EARLEY, B. **END OF PROJECT REPORT The effect of abrupt weaning of suckler calves on the plasma concentrations of cortisol, catecholamines, leukocyte, and acute-phase proteins.** July, p. 2847–2855, 2005.

HOLMES, A. H. et al. Understanding the mechanisms and drivers of antimicrobial resistance. **The Lancet**, v. 387, n. 10014, p. 176–187, 2016.

HULBERT, L. E.; BALLOU, M. A. Innate immune responses and health of individually reared Holstein calves after placement into transition-pens 23 d after weaning. **Journal of Dairy Research**, v. 79, n. 3, p. 333–340, 2012.

HULBERT, L. E.; MOISÁ, S. J. Stress, immunity, and the management of calves1. **Journal of Dairy Science**, v. 99, n. 4, p. 3199–3216, 2016.

KAPTCHUK, T. J. The double-blind, randomized, placebo-controlled trial. **Journal of Clinical Epidemiology**, v. 54, n. 6, p. 541–549, 2001.

KHAN, M. A. et al. Invited review: Transitioning from milk to solid feed in dairy heifers. **Journal of Dairy Science**, v. 99, n. 2, p. 885–902, 2016. Available on: <<http://dx.doi.org/10.3168/jds.2015-9975>>.

KHAN, M. A.; WEARY, D. M.; VON KEYSERLINGK, M. A. G. Invited review: Effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. **Journal of Dairy Science**, v. 94, n. 3, p. 1071–1081, 2011. Available on: <<http://dx.doi.org/10.3168/jds.2010-3733>>.

KOCAN, K. M. et al. The natural history of *Anaplasma marginale*. **Veterinary Parasitology**, v. 167, n. 2–4, p. 95–107, 2010.

KUMAR, B.; PACHAURI, S. P. Haematological profile of crossbred dairy cattle to monitor herd health status at medium elevation in Central Himalayas. **Research in Veterinary Science**, v. 69, n. 2, p. 141–145, 2000.

LOCHMILLER, R. L.; DEERENBERG, C. Trade-offs in evolutionary immunology: Just what is the cost of immunity? **Oikos Journal**, v. 88, n. 1, p. 87–98, 2000.

LUNDBORG, G. K.; SVENSSON, E. C.; OLTENACU, P. A. Herd-level risk factors for infectious diseases in Swedish dairy calves aged 0–90 days. **Preventive Veterinary Medicine**, v. 68, n. 2–4, p. 123–143, 2005.

MENDONCA, F. L. de M. **Thesis. Utilização de isolados de *Anaplasma marginale* de baixa virulência em protocolos de imunização de bezerros: Análise dos efeitos pós-vacinais e proteção contra o desafio natural.** 2015. UFMG (in portuguese), 2015. Available on: <<https://repositorio.ufmg.br/handle/1843/SMOC-9VVNMW>>.

MISHRA, N. et al. An exploratory study on scientific investigations in homeopathy using medical analyzer. **Journal of Alternative and Complementary Medicine**, v. 17, n. 8, p. 705–710, 2011.

MOREIRA, G. H. F. A. **Fatores de risco e impacto da tristeza parasitária bovina**. PhD dissertation. 2017. Universidade Federal de Minas Gerais, Belo Horizonte, Brazil (in portuguese), 2017.

NRC. **Requirements of Dairy Cattle**. Seventh Revised Edition, 2001. Seventh ed. [s.l: s.n.]

OLIVEIRA JÚNIOR, B. R. et al. Short communication: Tick-borne disease is associated with changes in feeding behavior in automatically fed weaned dairy calves. **Journal of Dairy Science**, v. 101, n. 12, p. 11256–11261, 2018. Available on: <<http://dx.doi.org/10.3168/jds.2018-14637>>.

SVENSSON, C.; LIBERG, P. The effect of group size on health and growth rate of Swedish dairy calves housed in pens with automatic milk-feeders. **Preventive Veterinary Medicine**, v. 73, n. 1, p. 43–53, 2006.

WARNICK, V. D.; ARAVE, C. W.; MICKELSEN, C. H. Effects of Group, Individual, and Isolated Rearing of Calves on Weight Gain and Behavior. **Journal of Dairy Science**, v. 60, n. 6, p. 947–953, 1977. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(77\)83968-4](http://dx.doi.org/10.3168/jds.S0022-0302(77)83968-4)>.

WATHES, D. C. et al. Heifer fertility and carry over consequences for life-time production in dairy and beef cattle. **Animal**, v. 8, n. SUPPL. 1, p. 91–104, 2014.

CHAPTER 3 – *Short Communication:* Prophylactic use of an ultra-diluted complex on polymorphonuclear leukocyte function, and respiratory scores of weaned Holstein calves immediately after grouping

PAPER SUBMITTED TO REVISTA BRASILEIRA DE ZOOTECNIA (Attachment B)

ULTRA-DILUTED COMPLEX IN WEANED CALVES

***Short communication:* Prophylactic use of an ultra-diluted complex on polymorphonuclear leukocyte function, and respiratory scores of weaned Holstein calves immediately after grouping**

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ABSTRACT

The aim of the study was to evaluate the effect of an ultra-diluted complex supplementation as a prophylactic strategy on immunity, respiratory scores, and performance of weaned Holstein calves immediately after grouping. Thirty-six weaned Holstein female calves (80.4 ± 1.3 days old; 105.6 ± 10.4 kg) were allocated to 6 pens ($n=6$ per pen) in a completely randomized design experiment in a double-blind, placebo-controlled trial. During a 28 days period, animals received a total mixed ration and were enrolled into two different groups ($n=18$ per group): 1) Control (basal diet + calcium carbonate, top-dressed at 30 g/animal/d – ultra-diluted placebo vehicle), or 2) ultra-diluted complex (basal diet + TopVita™-Real H, top-dressed at 30 g/animal/d – *Sulphur*: 10^{-60} + *Viola tricolor*: 10^{-14} + *Caladium seguinum*: 10^{-30} + *Zincum oxydatum*: 10^{-30} + *Phosphorus*: 10^{-60} + *Cardus marianus*: 10^{-60} + *Colibacillinum*: 10^{-30} + *Podophyllum*: 10^{-30} + Vehicle: calcium carbonate; q.s. 1kg). Blood samples were collected from each calf at enrollment and 28 days later to assess polymorphonuclear leukocyte (PMNL) function and blood cell counts. Body weight was assessed at enrollment and 28 days later at the end of study. To respiratory-screening process, a calf scoring system modified for calves in group pens was used. There was no effect of prophylactic ultra-diluted treatment on PMNL functions, nor it had effect on neutrophils and lymphocytes count and their ratio. In addition, the ultra-diluted product did not affect body weight and ADG. Further, no effect was observed in respiratory scores throughout the study period. In conclusion, ultra-diluted complex did not improve the PMNL function and blood cell counts, nor did it had impact on performance of weaned Holstein calves immediately after grouping.

INTRODUCTION

Despite of diet changes, individual milk-feeding calves usually experience a new environment and social interaction after weaning process. It has been made in order to increase labor efficiency, since fewer workers are needed to monitor the animals. These new housing systems follow weaning have several novel items such as feeding and drinking equipment. This moment may be a stressful period, since this is the first time, they experience real social contact with other mates. Studies have identified the benefit of group pen systems before weaning, either similar or older mate, since it may decrease the stress caused by this regrouping strategy (DE PAULA VIEIRA; VON KEYSERLINGK; WEARY, 2012; BOLT et al., 2017). Finally, milk-feeding rearing method, which has been wide used in dairy farms, may impact stress levels during the process of regrouping after weaning management (BOLT et al., 2017).

Stress has been associated with immune system failure, since several hormones such as cortisol and catecholamine have been linked to an inefficient immune system functionality (ALHUSSIEN; DANG, 2020). Hulbert and Ballou (2012) detected negative impact of grouping strategy on immune functionality of weaned dairy calves, even though they grouped 3 animals each pen in comparison with individual housing weaned animals. Indeed, respiratory disease was the single largest cause of weaned heifer deaths in the U.S. (NAHMS, 2007). Thus, alternatives need to be studied in order to decrease respiratory disease incidence during this phase.

Ultra-diluted remedy was conceived in 1796 by Samuel Hahnemann. It is a diluted form of a substance that causes symptoms of a disease in healthy people when in high doses. However, in the diluted form, it would heal similar symptoms in sick people (HAHNEMANN, 1810). The early concepts consist in treat the specific symptoms of a single individual, however, in a herd perspective, the concept of homotoxicological remedies has been suggest. Homotoxicological remedies, herein used, use a single ultra-diluted medicine for a set of individuals, favoring its use by veterinarians around the world (ARLT et al., 2009). Although, the ultra-diluted technology is widespread among the dairy producers, more well-designed studies must be assessed to confirm its reliability (DOEHRING; SUNDRUM, 2016). The aim of this study was to evaluate the immune function, performance, and respiratory scores of weaned dairy calves immediately after grouping.

MATERIALS AND METHODS

All procedures performed in this study were approved by the Animal Care and Use Committee (IACUC, protocol #20018-02). Based on previous blood samples analysis from the studied herd, baseline neutrophils count in weaned calves housed in superhutches was anticipated to average 4×10^6 cells/mL and standard deviation = 1×10^6 cells/mL, with an assumption that ultra-diluted treatment would reduce neutrophil count by at least 25% (1×10^6 cells/mL unit). To detect this reduction, 17 calves per treatment for each group was required to ensure a power (1- β error probability) of 0.80. To ensure this number of animals per treatment, herein we enrolled 18 animals each group.

Between February 2020 and March 2020, 36 clinically healthy weaned Holstein calves were randomly selected at a commercial dairy located in west Texas, USA. The dairy milked 3700 Holstein cows three times daily in a 70-stall rotary milking parlor. After being weaned about 60 ± 1 d of age, the calves remained at hutches until 80.4 ± 1 d of age, when the calves were moved to the group pen. The calves were housed in outdoor, plastic superhutches with an attached pen. Superhutches, is a large calf hutch (6×3 m) that provides transitional housing for a small group of 6 weaned calves per group. A total of 6 superhutches (6 animals each) were enrolled for this study. The superhutches were bedded with cottonseed hulls and equipped with a feed bunk that provided space for 6 calves and 1 water bowl. When moved into groups, the calves continued to receive the same starter fed in the milk-feeding period. The animals had *ad libitum* access to water and starter. The experimental period lasted 28 days after enrollment date.

The weaned growing calves were allocated into 2 treatments at the day of grouping (3 superhutches/group and $n = 18$ animals/group): (1) Control: untreated controls (basal diet + calcium carbonate, top-dressed at 30g/animal/d – placebo); or 2) Ultra-diluted complex : treated with a ultra-diluted complex (basal diet + TopVita™ - Real H, top-dressed at 30 g/animal/d - *Sulphur*: 10^{-60} + *Viola tricolor*: 10^{-14} + *Caladium seguinum*: 10^{-30} + *Zincum oxydatum*: 10^{-30} + *Phosphorus*: 10^{-60} + *Cardus marianus*: 10^{-60} + *Colibacillinum*: 10^{-30} + *Podophyllum*: 10^{-30} + Vehicle: q.s. 1kg – calcium carbonate; TopVita™ – Real H Company, Campo Grande, MS, Brazil).

Blood samples were collected from each heifer at enrollment and 28 days later, from the jugular vein using a Vacutainer tube with lithium heparin, a Vacutainer tube with potassium EDTA, and a 20-gauge x 2.54-cm Vacutainer needle (Becton, Dickinson and Company, Franklin Lakes,

NJ). After collection, the heparinized blood was stored in an ice chest without any ice to preserve their phagocytic and oxidative burst capacity (SELLERS; HULBERT; BALLOU, 2013). The EDTA and coagulated blood samples were transported to the laboratory on ice. The heparinized and EDTA blood samples were processed in the same day of collection for measures of hematology and *ex vivo* PMNL responses.

Leukocyte count and differentials were performed using a hematology analyzer (IDEXX Procyte DX, Westbrook, ME), and the variables of interest were neutrophil count and neutrophils to lymphocytes ratio. Flow cytometry was used to determine the phagocytosis and oxidative burst capacity of peripheral PMNL and the quantification of the adhesion molecule L-selectin (CD62L), with minor modifications (HULBERT et al., 2011). To measure the phagocytic and oxidative burst capacity, 100 μ L of heparinized whole blood were incubated for 15 min in an ice bath. After this initial incubation, 20 μ L of a 100 μ M solution of dihydrorhodamine (Invitrogen, Carlsbad, CA), and 20 μ L of a 10^9 cfu/mL propidium iodine labeled *E. coli* suspension were added to the blood, and then incubated in a 38.5°C water bath for 10 min (negative controls were incubated in an ice bath for 10 min). Then, samples were immediately placed on an ice bath for 5 min, and erythrocytes were hypotonically lysed, and washed with PBS. Dual-color flow cytometry was performed using an Attune flow cytometer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA). The PMN population was gated using forward and side scatter plots. The mean fluorescence intensity (MFI) and proportion of PMNs that performed phagocytosis and oxidative burst were acquired using the optical filters BL3 (excited by a 488 nm laser on a 695/40 filter) and BL1 (excited by a 488 nm laser on a 530/30 filter), respectively. Negative controls were used to determine negative and positive signals on the BL1 by BL3 scatterplot used to assess PMNs that performed phagocytosis and oxidative burst. Phagocytosis and oxidative burst indexes were created by multiplying the proportion of responding PMNL by the corresponding MFI: [index = (positive %) $\times$ (MFI)], as greater index values indicate greater phagocytic and oxidative burst activities (MCCARTHY et al., 2016). To determine the expression of the adhesion molecule L-selectin, 50 μ L of EDTA blood samples were mixed with 50 μ L of PBS containing 1 μ g of a monoclonal antibody mouse IgG1-isotype (catalog number: BOV2046, clone: BAQ92A; Veterinary Microbiology and Pathology Monoclonal Antibody Center, Pullman, WA). After a 1 h incubation in an ice bath, erythrocytes were hypotonically lysed. After centrifugation, the leukocyte pellet was resuspended in a 50 μ L solution of fluorescein-labeled secondary antibody at a 1:400 dilution

(F(ab')₂ anti-mouse IgG:FITC; AbD Serotec Raleigh, NC) and incubated for 1 h in an ice bath. After a PBS wash, samples were analyzed using single-color flow cytometry. The PMN population was gated as previously described, and the MFI for L-selectin was gathered using BL-1. Data were analyzed using Attune Cytometric software (Life Technologies, Thermo Fisher Scientific Inc.).

Body weight was assessed at enrollment and 28 days later at the end of study. Through the practical and reliable technique, body weight was estimated using calibrated weight tapes measuring heart girth circumference (HEINRICHS; ROGERS; COOPER, 1992). These measurements were used to calculate the body weight average daily gain (ADG) during study period (final body weight –initial body weight / 28 days).

To respiratory-screening process, a calf scoring system modified for calves in group pens was used according to McGuirk and Peek (2014). This group pen respiratory scorer is available at <https://www.vetmed.wisc.edu/fapm/svm-dairy-apps/group-pen-respiratory-scorer/>. The categorization was made scoring nasal discharge, ocular discharge, ear tilt, and coughing. This modified scoring system identify diseases of the group instead of individual animals. If the group/pen is classified into having more than 25% of the animals with abnormal in 2 or more categories scored, this group would be classified as a high risk to develop respiratory disease. Abnormal scores are stated as score 2 or 3 from 0 to 3-point scale. This tool is used in farm's calf health-screening program to obtain early diagnostic of respiratory diseases in calves (MCGUIRK; PEEK, 2014).

The effect of ultra-diluted treatment on PMNL function, cells count, body weight, and growth (ADG) was performed using the MIXED procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). The model included the fixed effects of treatment and random effect of pen. The random effect of pen was used to recognize the experimental unit for treatment group. In addition, repeated observations within a pen were modeled using a compound symmetry residual variance-covariance structure. Respiratory scores were modeled using a categorical multinomial distribution fitted with a cumulative logit link function in GLIMMIX procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). Least square means and proportions are reported for all parameters evaluated. For all the analyses, differences detected at $P \leq 0.05$ were considered significant.

RESULTS

There was no effect of prophylactic ultra-diluted treatment on PMNL function, nor it had effect on neutrophils and lymphocytes count and its ratio ($P > 0.05$; Table 1). In addition, the ultra-diluted product did not affect body weight and ADG ($P > 0.05$; Table 2). Further, no effect was observed in respiratory scores throughout the study period ($P > 0.05$).

DISCUSSION

Several management changes are imposed to the animals over the heifer-raising period. The most cited changes reported are introduction to a new feeding and housing system, regrouping, social isolation, and diet modifications. These changes require extensive behavioral resiliency of the animals to acclimate to them (DE PAULA VIEIRA; VON KEYSERLINGK; WEARY, 2012). A study about a profile of calves' management practices in the U.S. related that about 80% of farms move calves into group pens around 8 weeks of age. Further, large dairies have moved their calves to group pens later than small operations in order to decrease the spread of pathogenic microorganisms in milk-feeding period (NAHMS, 2007). In an excellent review, Bøe and Færevik (2003) reported that grouping of unfamiliar animals increases aggression, social stress, and locomotor behavior in calves. Additionally, this practice may negatively impact feed intake, body weight ADG, and immune functionality of dairy calves after weaning (HULBERT; BALLOU, 2012).

We did not detect any effect of ultra-diluted supplementation on immune function and blood cells count of weaned dairy calves after grouping. Additionally, even though any effect was detected on BW ADG, satisfactory gains was detected between the groups (1.0 and 0.99 kg/d for controlled and ultra-diluted treated group, respectively). Differently of Hulbert and Ballou (2012), our findings may be explained through the absence of stress effects on neutrophil functionality at this life-time of the calves, similarly as mentioned by Leblanc (2020), who reviewed the immune function of transition dairy cows under social stress. This author reported that the association between stress (acute or chronic) and impaired immune function has not been confirmed in controlled trials, concluding that competitive displacement at the feed bunk in overcrowded groups may have no effect alone on immune function (phagocytosis and oxidative burst) of transition dairy

cows. To date, there is no well-designed studies verifying the effects of ultra-diluted products on immune function of weaned dairy calves to perform relevant discussion among ultra-diluted strategies.

Respiratory disease is the most diagnosed illness in weaned dairy calves. About 93.3% of respiratory disease diagnosed in U.S. dairies were treated with an antibiotic in a survey published in 2007 (NAHMS, 2007). Additionally, respiratory disease was the single largest cause of weaned heifer deaths (46.5%). Lundborg et al. (2005) and Svensson and Liberg (2006) studies indicate that size of the group after commingling matters, whereas calves housed in large group pens had higher risk for respiratory disease. In this study, prophylactic supplementation of an ultra-diluted complex did not alter the respiratory scores in weaned dairy calves after grouping. As mentioned earlier, to date, there is no randomized, double-blind, placebo-controlled trial reporting the effect of an ultra-diluted medicine on heifer health. Additionally, low quality studies have been reported for livestock (DOEHRING; SUNDRUM, 2016) and humans (MATHIE et al., 2017).

CONCLUSIONS

In conclusion, prophylactic use of an ultra-diluted complex did not alter PMNL function, growth, nor it had effect on respiratory score of weaned growing calves during 28 d immediately after grouping.

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Table 1. Blood measurements of weaned growing heifers supplemented with ultra-diluted complex (n=18) or control (n=18).

Item	Treatment		SEM	P-value
	Control	Ultra-diluted		
Phagocytosis, %	74.6	76.7	3.92	0.72
Phagocytosis, MFI	599.2	601.1	26.2	0.96
Phagocytosis index	449.5	467.8	40.7	0.77
Oxidative burst, %	56.4	57.2	4.76	0.91
Oxidative burst, MFI	1067.7	1153.2	65.3	0.41
Oxidative burst index	611.1	680.5	81.1	0.58
L-selectin, MFI	386.3	437.8	26.2	0.24
Neutrophil count, cells x 10 ⁶ /μL	3.8	4.1	0.61	0.62
Lymphocyte count, cells x 10 ⁶ /μL	6.2	6.1	0.44	0.93
Neutrophil : Lymphocyte	0.64	0.67	0.24	0.78

Table 2. Body weight (BW) and BW average daily gain (ADG) of weaned growing heifers supplemented with ultra-diluted complex (n=18) or control (n=18).

Item	Treatment		SEM	P-value
	Control	Ultra-diluted		
Initial BW, kg	105.1	106.1	2.56	0.78
Final BW, kg	130.8	130.3	2.94	0.89
BW ADG, kg/d	1.0	0.99	0.12	0.91

Figure 1. Estimated cumulative probabilities of respiratory scores (ocular, nasal, ear, and cough parameters) in weaned growing heifers during 4 weeks after grouping. Animals were receiving either ultra-diluted or control treatments. Respiratory scores were modeled using a categorical multinomial distribution fitted with a cumulative logit link function. No difference was detected between the groups among the different scores parameters (Treatment: $P > 0.05$; Week: $P > 0.05$; and Treatment x Week: $P > 0.05$, for all the variables).

REFERENCES

ALHUSSIEN, M. N.; DANG, A. K. Interaction between stress hormones and phagocytic cells and its effect on the health status of dairy cows: A review. **Veterinary World**, v. 13, n. 9, p. 1837–1848, 2020.

ARLT, S. et al. Efficacy of homeopathic remedies as prophylaxis of bovine endometritis. **Journal of Dairy Science**, v. 92, n. 10, p. 4945–4953, 2009. Available on: <<http://dx.doi.org/10.3168/jds.2009-2142>>.

BØE, K. E.; FÆREVIK, G. Grouping and social preferences in calves, heifers and cows. **Applied Animal Behaviour Science**, v. 80, p. 175–190, 2003.

BOLT, S. L. et al. Pair housing of dairy calves and age at pairing: Effects on weaning stress, health, production and social networks. **PLoS ONE**, v. 12, n. 1, p. 1–18, 2017.

COBB, C. J. et al. Improved performance and heightened neutrophil responses during the neonatal and weaning periods among outdoor group-housed Holstein calves. **Journal of Dairy Science**, v. 97, n. 2, p. 930–939, 2014. Available on: <<http://dx.doi.org/10.3168/jds.2013-6905>>.

DE PAULA VIEIRA, A.; VON KEYSERLINGK, M. A. G.; WEARY, D. M. Presence of an older weaned companion influences feeding behavior and improves performance of dairy calves before and after weaning from milk. **Journal of Dairy Science**, v. 95, n. 6, p. 3218–3224, 2012. Available on: <<http://dx.doi.org/10.3168/jds.2011-4821>>.

DOEHRING, C.; SUNDRUM, A. Efficacy of homeopathy in livestock according to peer-reviewed publications from 1981 to 2014. **Veterinary Record**, v. 179, n. 24, p. 1–13, 2016.

EBERT, F. et al. Randomized, blinded, controlled clinical trial shows no benefit of homeopathic mastitis treatment in dairy cows. **Journal of Dairy Science**, v. 100, n. 6, p. 4857–4867, 2017.

HAHNEMANN, S. **Organon der rationellen Heilkunde**. Dresden, Germany: Arnold, 1810. v. 1

HEINRICHS, A. J.; ROGERS, G. W.; COOPER, J. B. Predicting Body Weight and Wither Height in Holstein Heifers Using Body Measurements. **Journal of Dairy Science**, v. 75, n. 12, p. 3576–3581, 1992. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(92\)78134-X](http://dx.doi.org/10.3168/jds.S0022-0302(92)78134-X)>.

HULBERT, L. E. et al. Effects of changing milk replacer feedings from twice to once daily on Holstein calf innate immune responses before and after weaning¹. **Journal of Dairy Science**, v. 94, n. 5, p. 2557–2565, maio 2011.

HULBERT, L. E.; BALLOU, M. A. Innate immune responses and health of individually reared Holstein calves after placement into transition-pens 23 d after weaning. **Journal of Dairy Research**, v. 79, n. 3, p. 333–340, 2012.

KHAN, M. A.; WEARY, D. M.; VON KEYSERLINGK, M. A. G. Invited review: Effects of milk

ration on solid feed intake, weaning, and performance in dairy heifers. **Journal of Dairy Science**, v. 94, n. 3, p. 1071–1081, 2011. Available on: <<http://dx.doi.org/10.3168/jds.2010-3733>>.

LEBLANC, S. J. Review : Relationships between metabolism and neutrophil function in dairy cows in the peripartum period. **Animal**, v. 14, n. S1, p. s44–s55, 2020.

LUNDBORG, G. K.; SVENSSON, E. C.; OLTENACU, P. A. Herd-level risk factors for infectious diseases in Swedish dairy calves aged 0-90 days. **Preventive Veterinary Medicine**, v. 68, n. 2–4, p. 123–143, 2005.

MATHIE, R. T. et al. Randomised, double-blind, placebo-controlled trials of non-individualised homeopathic treatment: Systematic review and meta-analysis. **Systematic Reviews**, v. 6, n. 1, 2017.

MCCARTHY, M. M. et al. Associations between the degree of early lactation inflammation and performance, metabolism, and immune function in dairy cows. **Journal of Dairy Science**, v. 99, n. 1, p. 680–700, 2016. Available on: <<http://dx.doi.org/10.3168/jds.2015-9694>>.

MCGUIRK, S. M.; PEEK, S. F. Timely diagnosis of dairy calf respiratory disease using a standardized scoring system. **Animal Health Research Reviews**, v. 15, n. 2, p. 145–147, 2014.

MOORE, D. A. et al. Dairy Calf Housing and Environment: The Science Behind Housing and On-Farm Assessments. **Washington State University Introduction**, p. 1–53, 2012.

NAHMS NATIONAL ANIMAL HEALTH MONITORING SYSTEM. **Dairy 2007 Heifer Calf Health and Management Practices on U.S. Dairy Operations, 2007USDA:APHIS:VS. USDA**. Available on: <https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_ir_CalfHealth_1.pdf>.

SELLERS, M. D.; HULBERT, L. E.; BALLOU, M. A. Technical note: Determination of preanalysis storage temperature and time allowances for ex vivo innate immune responses. **Journal of Dairy Science**, v. 96, n. 1, p. 384–389, 2013. Available on: <<http://dx.doi.org/10.3168/jds.2012-5750>>.

SVENSSON, C.; LIBERG, P. The effect of group size on health and growth rate of Swedish dairy calves housed in pens with automatic milk-feeders. **Preventive Veterinary Medicine**, v. 73, n. 1, p. 43–53, 2006.

WEBER, P. S. D. et al. Mechanisms of glucocorticoid-induced down-regulation of neutrophil L-selectin in cattle: evidence for effects at the gene-expression level and primarily on blood neutrophils. **Journal of Leukocyte Biology**, v. 75, n. 5, p. 815–827, 2004.

CHAPTER 4 - Effect of injectable trace mineral supplementation on peripheral polymorphonuclear leukocyte function, oxidative stress, health, and performance of dairy cows in semi-arid conditions

PAPER SUBMITTED TO JOURNAL OF DAIRY SCIENCE (ATTACHMENT C)

INJECTABLE TRACE MINERALS IN DAIRY COWS

Effect of injectable trace mineral supplementation on peripheral polymorphonuclear leukocyte function, oxidative stress, health, and performance of dairy cows in semi-arid conditions

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ABSTRACT

The objective of this study was to evaluate the effect of 3 subcutaneous injections of 15 mg/mL of Cu, 5 mg/mL of Se, 60 mg/mL of Zn, and 10 mg/mL of Mn at dry-off, 20 days prior to expected day of calving, and 35 days postpartum, on immunity, oxidative stress, health and performance of dairy cows undergoing the transition period in high temperature humidity index (THI). The study was conducted on 2 commercial Holstein dairy farms located in west Texas. A total of 923 multiparous cows were randomly allocated into 1 of 2 treatment groups: injectable trace mineral supplement (ITMS) or control. Blood samples were collected at enrollment, and at 3 ± 1 , 7 ± 1 , 10 ± 1 , and 35 ± 3 DIM to evaluate hematology, polymorphonuclear leukocyte (PMNL) function, circulating biomarkers of oxidative stress, antioxidant enzymes, and circulating haptoglobin (Hp). Trace mineral supplemented cows had a lower incidence of metritis compared to control group (ITMS = 8.6% vs. control = 12.6%). Additionally, ITMS-treated cows had lower incidence of stillbirth compared to control cows (ITMS = 1.5% vs. control = 3.7%). However, ITMS did not influence milk yield, reproductive performance, and culling. Blood analysis revealed that ITMS did not influence the white blood, neutrophil, and lymphocyte counts, but neutrophil to lymphocyte ratio was greater for ITMS-treated cows in comparison to control cows. Additionally, ITMS cows had improved PMNL function during the post-partum period due to increased proportion of PMNL that performed phagocytosis and oxidative burst and increased the intensity of oxidative burst. Conversely, control cows had increased L-Selectin expression on PMNL surface. Concentration of serum antioxidant enzymes and oxidative stress biomarkers were not affected by ITMS treatment, but ITMS treatment tended to increase Hp concentration at 10 d after calving. In conclusion, ITMS improved PMNL function and reduced incidence of metritis and stillbirth parturition in dairy cows undergoing the transition period in high THI conditions. However, these findings did not translate into improved milk yield, reproductive performance, and survivability.

Key Words: trace minerals, dairy cows, immune function, health

INTRODUCTION

During the periparturient period, dairy cows experience a substantial increase in oxygen consumption that results in elevated production of reactive oxygen species (ROS; Sordillo, 2016). Therefore, an imbalance between the production of ROS and the availability of antioxidants defenses may expose these animals to oxidative stress (SORDILLO; AITKEN, 2009). Additionally, heat stress has been reported to aggravate this scenario, whereas cows can experience higher degrees of feed intake depression (ADIN et al., 2009), increased degree of oxidative stress (BERNABUCCI et al., 2002; SAFA et al., 2019), and immunosuppression (LACETERA et al., 2005; LECCHI et al., 2016; SAFA et al., 2019). Therefore, high proportion of puerperal disorders have been observed in dairy cows undergoing the transition period in heat stress conditions (GERNARD et al., 2019). Heat stress in dairy cows is particularly important dairy herds located in semi-arid regions. For instance, in the Southwest region of the High Plains in the United States or Northern Mexico, where the dairy industry is an important part of the local economy, the average Temperature Humidity Index (THI) during the summer is above 68, commonly used as the threshold for stress in dairy cows (ZIMBELMAN et al., 2009).

Providing shade and soaking lines are strategies commonly adopted to improve the performance and immune status of postpartum dairy cows undergoing heat-stress (DO AMARAL et al., 2011). Also, supplementation of trace minerals has been studied as alternative strategies to alleviate heat stress' detrimental effects on immunity, antioxidant status, and performance of chicken (ABD EL-HACK et al., 2017; RAJKUMAR et al., 2017), and sheep (ALHIDARY et al., 2015). Hence, it is plausible that trace mineral supplementation can also help to mitigate the burden of heat stress in dairy cows. The use of injectable trace mineral supplementation (ITMS) with Zn, Mn, Cu, and Se improved health (MACHADO et al., 2013) and antioxidant status (MACHADO et al., 2014) of dairy cows supplemented at dry-off, 30 days prior to calving, and during the second month of lactation. However, postpartum polymorphonuclear leukocyte (PMNL) function, milk yield, and reproductive performance were not influenced by ITMS. Those studies were performed in dairy herds located in NY, during cold weather conditions. Hence, the use of ITMS in cows in semi-arid conditions, with added stressful conditions such as increased THI during summer months, could lead to different outcomes. For example, during a stressful even (aflatoxin challenge) ITMS decreased inflammation and oxidative stress in dairy cows (PATE; CARDOSO, 2018).

Therefore, the objective of this study was to evaluate the effect of an injectable trace mineral supplementation containing copper, selenium, zinc, and manganese at dry-off, 20 days prior to expected day of calving, and 35 days postpartum on incidence of postpartum diseases, milk yield, reproductive performance, culling, hematological parameters, PMNL activity, concentrations of oxidative stress markers, and circulating haptoglobin in dairy cows undergoing the transition period in high THI.

MATERIALS AND METHODS

Farms, management, and environmental data

All experimental procedures were approved by the Texas Tech University Institutional Animal Care and Use Committee (#19045-05). This study was conducted on 2 commercial Holstein dairy farms (Farm A and Farm B), located in west Texas. Farms were selected based on their geographical location (near Lubbock, TX), and willingness to participate in the study. Cows were enrolled from 30 April until 23 July 2019; the follow-up period continued until 13 November 2019. The enrollment period was selected to maximize the number of days with THI > 68 during the transition period. The environmental information was assessed from the Lubbock Airport's meteorological station because its short geographic distance from both dairies (67 and 48 kilometers from farms A and B, respectively). Daily temperature and relative humidity data were downloaded from April 30th through November 13th, 2019. The THI throughout the study period was calculated from the equation developed by the National Research Council (NRC, 1971) below as indicated by Dikmen & Hansen (2009):

$$\text{THI} = (1.8 \times T^{\circ}\text{C} + 32) - [(0.55 - 0.0055 \times \text{RH}\%) \times (1.8 \times T^{\circ}\text{C} - 26)],$$

where T = ambient dry bulb temperature in °C, and RH% = relative humidity. Meteorological data are presented in Figure 1.

Farm A milked 2,700 Holstein cows 2 times per day in a double 30-stall parallel milking parlor. The cows were housed in dry-lot pens, consisting of non-vegetated open lots (i.e., corrals) with shaded areas. Farm B milked 3,700 Holstein cows three times daily in a 70-stall rotary milking parlor. During the dry period, the animals were housed in dry-lot pens, consisting of non-vegetated open lots. After calving, the cows were housed in free-stall barns, with concrete stalls and bedded with manure solids. At both farms, the animals were fed a total mixed ration (TMR) *ad libitum* with free access to water. According to herd characteristics, the diets were formulated to meet or exceed the NRC (2001). Composite total mixed ration samples from pre-fresh and lactation diets were submitted to a commercial laboratory (Cumberland Valley Analytical Services, Inc.). Dry matter, crude protein, acid detergent fiber, neutral detergent fiber, and macro and micro minerals were analyzed by wet chemistry methodology. Nutrient contents of the diets are described in Table 1.

Sample size calculation

Based on previously reported data (Machado et al., 2013), we assumed that ITMS would decrease the incidence of clinical mastitis from 26% to 18%. Considering a significance level of $\alpha=0.05$ and a power of 80%, at least 420 cows would have to be enrolled in each treatment group. Accounting for 10% attrition, 923 animals were enrolled in the study ($n = 449$ and $n = 474$ for control and ITMS group, respectively). With this sample size, we were able to detect a milk production difference between the groups of 1.1 kg/d with a S.D. of 6 kg/d.

Inclusion criteria, treatment allocation, case definitions, and data collection

Dry cows were included in the study based on expected calving date (between July and September). A completely randomized clinical study blocked by farms was performed. A total of 923 multiparous cows (from 2 dairies) were randomly allocated into 1 of 2 treatment groups: injectable trace mineral supplement (ITMS) or control. Randomization was completed in Excel (Microsoft) and imported into the farm's management software program. Cows that were randomly assigned to the ITMS group received 3 subcutaneous injections of 15 mg/mL of Cu, 5 mg/mL of Se, 60 mg/mL of Zn, and 10 mg/mL of Mn (Multimin 90, Multimin North America, Fort Collins, CO). A blanket dose (7 ml) was administered for all the animals enrolled in ITMS group. We followed the manufacturer's recommendation, administering for cattle over 2 years of age, at dry-off (208 ± 3 days of gestation), 260 ± 3 d of gestation, and at 35 ± 3 days postpartum. Pregnant heifers were not enrolled in the study because ITMS was not beneficial to cows entering the lactating herd (Machado et al., 2013).

Postpartum diseases were diagnosed and treated by trained farm personnel, who were blinded to treatments. Metritis was defined as the presence of fetid, watery, red-brown uterine discharge (SHELDON et al., 2006). Clinical mastitis was defined by the diagnosis of abnormal changes in the udder and/or milk (LAGO et al., 2011). Retained fetal membranes was defined as the failure to release fetal membranes within 24 h of calving (KELTON; LISSEMORE; MARTIN, 1998). Stillbirth was defined as the death of a calf occurring just before, during, or within 48 h of parturition (PHILIPSSON et al., 1979). Data regarding health traits, reproduction outcomes, milk yield, and survivability were extracted from Bovisynch (Dairy LLC, WI) and DairyComp 305 (Valley Agricultural Software, Tulare, CA) database on farms A and B, respectively.

Blood collection and analysis

For a subset of 134 cows from Farm A, blood samples were collected for hematology purposes, PMNL function, circulating biomarkers of oxidative stress, antioxidant enzymes, and circulating haptoglobin (Hp). Blood samples were collected at enrollment, and at 3 ± 1 , 7 ± 1 , 10 ± 1 , and 35 ± 3 DIM by puncture of the coccygeal vessels using a vacutainer tube without anticoagulant, a vacutainer tube with lithium heparin, a vacutainer tube with potassium EDTA, and a 20-gauge x 2.54-cm vacutainer needle (Becton, Dickinson and Company, Franklin Lakes, NJ). After collection, the heparinized blood was stored in an ice chest with no ice at ambient temperature, to preserve their phagocytic and oxidative burst capacity (SELLERS; HULBERT; BALLOU, 2013). The EDTA and coagulated blood samples were immediately placed on ice. Samples were analyzed or processed within 3 h after collection. The blood samples without anticoagulants were centrifuged at $2000 \times g$ for 15 min at 4°C for serum separation, and frozen at -80°C . The heparinized and EDTA blood samples were processed in the same day of collection for measures of hematology and ex vivo PMN responses.

Leukocyte count and differentials were performed using a hematology analyzer (IDEXX Procyte DX, Westbrook, ME), and the variables of interest were white blood cells, monocytes, neutrophils, lymphocytes, and neutrophil to lymphocyte ratio. Flow cytometry was used to determine the phagocytosis and oxidative burst capacity of PMNs and the quantification of their adhesion molecule L-selectin (CD62 L) as previously described with minor protocol modifications (HULBERT et al., 2011). To measure the phagocytic and oxidative burst capacity, 100 μL of heparinized whole blood were incubated for 15 min in an ice bath. After this initial incubation, 20 μL of a 100 μM solution of dihydrorhodamine (Invitrogen, Carlsbad, CA), and 20 μL of a 10^9 cfu/mL propidium iodide labeled *E. coli* suspension were added to the blood and then incubated in a 38.5°C water bath for 10 min (negative controls were incubated in an ice bath for 10 min). Then, samples were immediately placed on an ice bath for 5 min, and erythrocytes were hypotonically lysed, and washed with PBS. Dual-color flow cytometry was performed using an Attune flow cytometer (Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA). The PMN population was gated using forward and side scatter plots. The mean fluorescence intensity (MFI) and proportion of PMNs that performed phagocytosis and oxidative burst were acquired using the optical filters BL3 (excited by a 488 nm laser on a 695/40 filter) and BL1 (excited by a 488 nm laser on a 530/30 filter), respectively. Negative controls were used to determine negative

and positive signals on the BL1 by BL3 scatterplot used to assess PMNs that performed phagocytosis and oxidative burst. To determine the expression of the adhesion molecule L-selectin, 50 μ L of EDTA blood samples were mixed with 50 μ L of PBS containing 1 μ g of a monoclonal antibody mouse IgG1-isotype (catalog number: BOV2046, clone: BAQ92A; Veterinary Microbiology and Pathology Monoclonal Antibody Center, Pullman, WA). After a 1 h incubation in an ice bath, erythrocytes were hypotonically lysed. After centrifugation, the leukocyte pellet was resuspended in a 50 μ L solution of fluorescein-labeled secondary antibody at a 1:400 dilution (F(ab')₂ anti-mouse IgG:FITC; AbD Serotec Raleigh, NC) and incubated for 1 h in an ice bath. After a PBS wash, samples were analyzed using single-color flow cytometry. The PMN population was gated as previously described, and the MFI for L-selectin was gathered using BL-1. Data were analyzed using Attune Cytometric software (Life Technologies, Thermo Fisher Scientific Inc.).

Commercial kits (Cayman Chemical, Ann Arbor, MI) were used to assess the following markers of oxidative stress: Glutathione Peroxidase (#703102), Superoxide Dismutase (#706002), and Thiobarbituric Acid Reactive Substances (#10009055). Serum Hp levels concentration was determined using a colorimetric assay via quantification of the haptoglobin/hemoglobin complex by the estimation of differences in peroxidase activity (MAKIMURA; SUZUKI, 1982). Assays were performed in 16 $\times$ 100 borosilicate tubes. Briefly, 5 μ L of serum sample or deionized water (blank) were added to 7.5 mL of a solution containing 0.6 g/L of O dianisidine, 13.8 g/L of sodium phosphate monobasic, and 0.5 g/L EDTA (pH = 4.1). Immediately, 25 μ L of 0.3 g/L bovine hemoglobin solution was added to each assay, followed by a water bath incubation at 37 °C for 45 min. After incubation, 100 μ L of freshly prepared 156 mM hydrogen peroxidase solution was added to each assay. Samples were incubated at room temperature for 60 min. Then, 200 μ L of each assay was transferred to a 96-well polystyrene flat-bottom microplate. Optical density at 450 nm was measured on the Epoch2 Microplate Spectrophotometer (BioTek). Finally, the final OD of each assay was subtracted by the blank assay OD. Optical density data was converted to a concentration unit (μ g/mL) using standard curves generated by serial dilutions of a sample of known concentration determined by a commercially available ELISA kit following the manufacturer's instructions (Life Technologies) as previously described (COOKE; ARTHINGTON, 2013). The intra and inter-assay coefficient of variation for serum Hp were 1.8 % and 5.9 %, respectively.

Statistical analysis

Descriptive statistics analysis was undertaken in SAS using the FREQ procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). The odds of clinical metritis, clinical mastitis, retained placenta, stillbirth, and pregnancy per AI (P/AI) at first service was assessed by fitting the data using the GLIMMIX procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). Variables offered to the models included treatment (which was forced into all the models), parity (2, >2), twins, and previous days carried calf. Additionally, the variable farm was included in all models as a random effect. The independent variables and their respective interactions were kept when $P < 0.10$.

The effect of treatment on reproduction and survival was analyzed by Cox's Proportional Hazard using the proportional hazard regression procedure in SAS (v.9.4, SAS Institute Inc., Cary, NC). For analysis of reproduction, cows were right-censored if not diagnosed pregnant before culling, death, or the end of the data-collection period. Variables offered to the models included treatment, parity (2, >2), twins, and previous days carried calf. The independent variables and their respective interactions were kept when $P < 0.10$.

The effect of ITMS on monthly milk yield during the first 180 DIM was evaluated through a repeated measures model fitted by multiple mixed linear models using the MIXED procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). The model included the fixed effects of treatment, time, parity (2, >2), twins, previous days carried calf. The independent variables and their respective interactions were kept when $P < 0.10$. The farm variable was included as a random effect. To account appropriately for within-cow correlation, the error term was modeled by a compound symmetry (CS) covariance structure due to its smallest Bayesian Information Criterion (BIC) value.

To evaluate the effect of ITMS on cell blood count, PMN phagocytosis and oxidative burst activity, SOD, GPx, TBARS, and Hp throughout the five blood collection days (-72, 3, 7, 10, and 35 days relative to calving), repeat measures models, ordered by time, were fitted by multiple mixed linear models using the MIXED procedure of SAS (v.9.4, SAS Institute Inc., Cary, NC). The experimental unit was the cow. The normality of the residuals was analyzed with normal probability and box plots visualization. To account appropriately for within-cow residues correlation, a spatial (power) covariance structure (SP[POW]) was applied for all models. This variance-covariance structure is indicated for unequally spaced data collection and assumes

correlations decline as a function of time. For multivariate models above, independent variables and their respective interactions were kept when $P < 0.10$. The variable treatment, time, and their interaction were forced into all statistical models even in the absence of statistical significance. Days of gestation at enrollment, parity (2,>2), and the own variable value from -72 days relative to calving, were offered to all models as a covariate to control confounding effects. For all the analyses, differences detected at $P \leq 0.05$ were considered significant, and differences at $0.05 < P < 0.10$ were considered a tendency toward statistical significance.

RESULTS

Descriptive statistics

A total of 956 animals were enrolled in the study. From these, 33 cows were excluded due to loss to follow-up, with 24 cows being from Farm A (control group = 11 and ITMS = 13) and 9 cows being from Farm B (control group = 2 and ITMS group = 7). A total of 923 cows from initial 956 remained in the study. Descriptive statistics regarding the number of animals enrolled in parity 2 and >2 and average gestation length (days) at enrollment are presented in Table 2.

Effect of ITMS on health, reproductive performance, milk yield, and culling

The effect of treatment on health and P/AI at first service is presented in Table 3. Briefly, supplementing cows with injectable trace minerals did not improve the odds of mastitis ($P = 0.38$) and retained placenta ($P = 0.66$) compared with non-supplemented cows. However, trace mineral supplemented cows had a lower incidence of metritis compared to control group (OR = 0.68, 95% CI = 0.4 – 1.0, $P = 0.05$). Additionally, ITMS-treated cows had lower incidence of stillbirth compared to control cows (OR = 0.41, 95% CI = 0.17-1.01, $P = 0.05$).

Injectable trace mineral supplementation did not influence milk yield, reproductive performance, and culling. The average milk yield during the first 6 months of lactation for cows enrolled in control and ITMS group was 43.9 kg/d and 43.4 kg/d, respectively ($P = 0.17$), and there was no interaction between treatment and month of lactation ($P = 0.92$). Additionally, no effect of ITMS was detected during the follow-up period. The treatment did not improve P/AI at first service (OR = 1.03, 95% CI = 0.76 – 1.39, $P = 0.87$) or hazard of pregnancy up to 150 DIM compared to control cows (HR = 0.99, 95% CI = 0.88 – 1.17, $P = 0.89$). Regarding survivability, the hazard of culling up to 300 DIM was similar between the groups (HR = 1.23, 95% CI = 0.93 – 1.62, $P = 0.14$).

Effect of ITMS on hematology, PMNL function, oxidative stress biomarkers and haptoglobin

The effect of ITMS on white blood cells, monocyte, neutrophil, and lymphocyte counts, neutrophil to lymphocytes ratio are presented in Table 4. The ITMS treatment did not influence the white blood, neutrophil, and lymphocyte counts during the study. However, neutrophil to

lymphocyte ratio was greater for ITMS-treated cows in comparison to control cows throughout the study ($P = 0.03$).

The ITMS treatment improved the innate immunity during the post-partum period due to increased proportion of PMNL that performed phagocytosis ($P < 0.01$) and oxidative burst ($P < 0.01$) and increased the intensity of oxidative burst ($P = 0.05$; Figure 2A, B, D). Conversely, the expression of the L-Selectin on PMNL surface was increased in control cows ($P = 0.04$; Figure 2E).

Although we observed treatment differences in some health outcomes, ITMS did not influence serum antioxidant enzymes concentration and oxidative stress biomarkers concentration after calving ($P > 0.05$; Table 4). However, control cows tended to present higher serum haptoglobin concentration at 10 d after calving ($P = 0.09$; Figure 2F).

DISCUSSION

The objectives of this study were to evaluate the effect of ITMS on the health, performance, and biomarkers of postpartum immune and antioxidative status in dairy cows undergoing their transition period with THI > 68. The meteorological analysis presented in Figure 1 demonstrates that THI was greater than 68 for most days when study cows were undergoing their transition period (3 weeks before and 3 weeks after calving). Additionally, all cows were housed in dry-lot pens during the dry-period, being more exposed to environmental changes in the weeks before calving. Hence, we believe that cows experienced, at least partially, heat-stress conditions during the transition period.

In the present study, ITMS decreased the odds of cows having metritis and stillbirth parturitions, but no differences were observed in RP and mastitis incidence. These findings are in partial agreement with a previous report on the effect of ITMS on the health of dairy cows (MACHADO et al., 2013). While Machado et al. (2013) reported that ITMS improved udder health by decreasing the odds of clinical mastitis and reduced SCC linear scores throughout the first 6 months of lactation, herein we reported that udder health was not impacted by ITMS, because a reduction in clinical mastitis incidence was not observed. In the present study, we did not evaluate the effect of ITMS on SCC linear scores because of this information was not available in the farm's database. Although we did not observe an effect of ITMS on udder health, supplementation decreased the incidence of metritis. In previous studies, ITMS have also improved postpartum uterine health. Although Machado et al. (2013) reported that ITMS did not reduce the odds of metritis, they reported a lower incidence of clinical endometritis for cows that received ITMS. Furthermore, Machado et al. (2012) reported that ITMS reduced the presence of putative intrauterine pathogens, which can partially explain the beneficial impact of ITMS on uterine health. Herein, we also reported that ITMS reduced the incidence of stillbirth, which has also been previously observed (MACHADO et al., 2013). Stillbirth is a known risk factor for metritis (BICALHO et al., 2012), and the ITMS effect on stillbirth can partially explain the decreased odds of metritis in supplemented cows observed here.

Although herein ITMS did not improved the antioxidant status of cows (at least based on the biomarkers assessed), we hypothesize that the reduction in stillbirth parturitions might be due to improved antioxidant capacity in the last month of lactation (MACHADO et al., 2014). In sows,

high total antioxidant capacity has been associated with a low incidence of stillbirth (WANG et al., 2019). Sciorsci et al. (2020) showed that blood ROS levels undergo an expressive increase from 180-210 days of pregnancy onwards. These reactive oxygen species may compromise the physiology of the fetal-maternal unit, making it more susceptible to bacterial infection. Murray et al. (2008) reported that nutritional degenerative myopathy due to low uptake of selenium and vitamin E was associated with myocardial necrosis found in stillborn fetuses. The authors explain that the complex selenium/vitamin E, which increases GPx levels, helping to protect the fetus and placenta against external ROS damage. However, it is important to highlight that we did not observe the effect of ITMS on antioxidant enzymes and oxidative biomarkers. Likewise, Bicalho et al. (2014) did not observe differences in serum SOD and GPx concentrations. On the other hand, Machado et al. (2014) detected higher SOD levels in dairy cows during the transition period and subsequent lactation when supplemented with injectable trace minerals. Additionally, TBARS was not influenced by ITMS in our study. Plasma TBARS have been used as indicative of lipid peroxidation and oxidative stress (AOKI; OSHITA; SAKAGUCHI, 2008) in dairy cows, but others have questioned the value of this biomarker. Bernabucci et al. (2002) suggested that erythrocyte markers are a more appropriate and sensitive model to assess the oxidative status of moderate heat-stressed transition dairy cows than plasma markers. Further, Abuelo et al. (2013) consider 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.

Another plausible explanation for the beneficial impact of ITMS on uterine health observed herein is that ITMS also improved the PMNL function in the early postpartum period. Immunosuppression during the postpartum period is thought to be one of the major causes of increased incidence of uterine disease in dairy cows (KIM; NA; YANG, 2005; HAMMON et al., 2006; SORDILLO, 2013), and the higher proportion of phagocytic and oxidative burst PMNs coupled with improved oxidative burst intensity in ITMS-treated cows may partially explain the lower incidence of metritis in ITMS observed in the present study. Regardless of uterine disease signs, the uterus of about 90% of cows becomes contaminated by foreign microorganisms during the first 2 weeks after calving (SHELDON; DOBSON, 2004). Neutrophils are the first line of immune defense to be recruited from the bloodstream to the uterine lumen after a microbial attack (KIM; NA; YANG, 2005). Although Machado et al., (2014) reported that ITMS did not improve PMNL function at 10 DIM, others have observed supplementation benefits to PMNL activity.

Studies in beef and dairy cattle have demonstrated the benefit of using ITMS concurrently with bovine respiratory disease vaccines on humoral and cell mediated immune responses (PALOMARES et al., 2016; ROBERTS et al., 2016; BITTAR et al., 2018, 2020). Additionally, pre-weaned calves that received ITMS at 3 days of life had increased PMNL phagocytic activity at 14 days of life (TEIXEIRA et al., 2014). The positive effect of trace mineral supplementation on PMNL activity may be linked to trace mineral action on intracellular enzymatic modulation, such as cytosolic superoxide dismutase (Cu and Zn-dependent) and mitochondrial superoxide dismutase (Mn and Zn-dependent; Sordillo, 2016; Suttle, 2010), but these variables were not evaluated herein and more research is needed to test this hypothesis.

The results of the current study suggest that trace mineral supplementation resulted in a more robust innate immune system in the early postpartum period, which led to lower incidence of metritis. This resulted in a reduction in the inflammatory status of ITMS cows at 10 DIM, evidenced by the reduced PMNL expression of the pro-inflammatory adhesion molecule L-selectin (CD62L) and the observed tendency in the reduction of circulating concentration of Hp at 10 DIM. Initial steps in the recruitment process of PMNL to the infection sites involves rolling and attachment to the endothelium near the inflammation site. This process is dependent on the interaction between L-selectin protein adhesion receptors on the PMNL surface and its ligand on endothelial cells. Hence, we suggest that the lower expression of L-selectin in PMNL from ITMS at 10 DIM is evidenced that inflammatory response to uterine infection was regulated more rapidly in ITMS treated cows. Corroborating, Jacometo et al. (2015) results indicate that calves from dams fed with organic trace minerals (Zn, Cu, Mn, and Co) during the last 30 d of gestation had a trend for lower L-selectin microRNA expression compared to inorganic supplementation. Additionally, Hp is an acute phase protein that has been associated with metritis severity (HUZZEY et al., 2009). Similar to the L-selectin results, lower Hp at 10 DIM indicates that regulation of inflammation occurred sooner in ITMS cows. Conversely, greater neutrophil to lymphocyte ratio was observed among the ITMS cows. Generally, neutrophil to lymphocyte ratio has been associated with chronic and acute inflammatory processes due to margination, redistribution, and accelerated apoptosis of lymphocytes (ZAHOREC, 2001). In the context of the other results observed herein, neutrophil to lymphocyte ratio among ITMS cows could represent greater inflammatory potential, which could have contributed to greater capacity to eliminate the uterine pathogens.

Metritis and stillbirth parturition have been related to impair milk yield, reproductive performance, and culling rate in dairy cows (BICALHO et al., 2007, 2008; DUBUC et al., 2011; WITTROCK et al., 2011). Accordingly, in this present study, by decreasing those diseases, we expected to observe an improvement in those economically important outcomes. However, the positive effects of ITMS on metritis and stillbirth parturition herein found did not translate into better results. Likewise, Machado et al. (2013) found similar results in performance of dairy cows that received ITMS, whereas milk yield, reproductive outcomes, and culling rate did not differ between ITMS and control cows. Additionally, Springman et al. (2018) and Vanegas et al. (2004) reported no beneficial effect of ITMS treatment on reproduction outcomes in beef heifers and dairy cows, respectively.

CONCLUSIONS

Injectable trace mineral supplementation improved PMNL function and reduced incidence of metritis and stillbirth parturition in dairy cows undergoing the transition period in hot-months. However, these positive findings related to immunity and reproductive disorders did not translate into improved milk yield, reproductive performance, and survivability.

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CONFLICT OF INTEREST

None of the authors have stated any conflicts of interest.

Table 1. Chemical composition of pre-fresh and fresh cow diets (% of DM, otherwise stated).

Item	Farm A		Farm B	
	Pre-fresh	Fresh	Pre-fresh	Fresh
Dry matter, %	59.2	47.8	47.2	47.8
Crude protein	14.8	18.7	13.1	17.6
Soluble protein, % CP	43.8	34.1	32.6	34.6
Acid detergent fiber	23.5	20.6	28.5	17.4
Neutral detergent fiber	35.2	31.4	39.2	27.7
Non-fiber carbohydrates	37.8	36.4	34.1	41.9
Starch	24.9	23.9	24.5	27.0
Calcium	1.33	0.93	0.73	0.99
Phosphorus	0.34	0.44	0.3	0.37
Magnesium	0.42	0.37	0.28	0.35
Potassium	1.7	1.53	1.09	1.62
Sodium	0.17	0.34	0.13	0.74
Sulfur	0.25	0.3	0.25	0.22
Chloride	1.78	0.69	0.5	0.73
Iron, mg/kg	437	366	978	469
Zinc, mg/kg	103	57	49	104
Copper, mg/kg	27	15	8	18
Manganese, mg/kg	92	51	49	57
Selenium, mg/kg	0.62	0.43	0.39	0.62

Pre-fresh diets were fed from 3-week prepartum through parturition, and fresh diets were fed from parturition through week 35 postpartum.

Table 2. Descriptive statistics of treatment groups.

	Control	ITMS
Farm A		
Total number of enrolled animals (%)	253 (50)	252 (50)
Enrolled animals on parity 2 (%)	81 (48)	87 (52)
Enrolled animals on parity >2 (%)	172 (51)	165 (49)
Average days of gestation at enrollment ($\pm$ SE)	207.8 (4.8)	208.2 (4.8)
<i>Subset of animals for blood evaluation</i>		
Total number of enrolled animals (%)	74 (52)	68 (48)
Enrolled animals on parity 2 (%)	26 (55)	21 (45)
Enrolled animals on parity >2 (%)	48 (51)	47 (49)
Average days of gestation at enrollment ($\pm$ SE)	208.3 (1.6)	208.1 (2.0)
Farm B		
Total number of enrolled animals (%)	196 (47)	222 (53)
Enrolled animals on parity 2 (%)	92 (45)	114 (55)
Enrolled animals on parity >2 (%)	104 (49)	108 (51)
Average days of gestation at enrollment ($\pm$ SE)	209.6 (5.2)	209.2 (5.2)
Total enrolled animals (%)	449 (49)	474 (51)
Enrolled animals on parity 2 (%)	173 (46)	201 (54)
Enrolled animals on parity >2 (%)	276 (50)	273 (50)

Table 3. Incidence (%) of peripartum diseases, reproduction outcomes, and culling rate of cows treated with injectable trace mineral supplement (ITMS, n=474) and control (CTR, n=449).

Item	Treatments		OR ¹ (95% CI)	P-value
	Control (%)	ITMS (%)		
Metritis	12.6	8.6	0.63 (0.40 - 1.0)	0.05
Mastitis	30.4	32.0	1.14 (0.85 - 1.53)	0.38
Retained placenta	8.7	7.7	0.89 (0.54 - 1.47)	0.66
Stillbirth	3.7	1.5	0.41 (0.17 - 1.01)	0.05
First-service conception	35.4	36.0	1.03 (0.76 - 1.39)	0.87

¹Odds Ratio

Table 4. Effect of treatment on cells blood count, antioxidant enzymes, and thiobarbituric acid reactive substances.

Variable	Treatments			P-value		
	Control	ITMS	SEM	Treat ⁵	Day ⁶	Treat*Day
WBC, cells x10 ⁶ /mL ¹	10.8	11.3	0.316	0.30	<0.0001	0.31
Monocytes, cells x10 ⁶ /mL	2.22	2.23	0.087	0.98	0.02	0.20
Neutrophil, cells x10 ⁶ /mL	1.84	2.04	0.134	0.27	<0.0001	0.46
Lymphocytes, cells x10 ⁶ /mL	6.99	7.15	0.324	0.69	<0.0001	0.51
Neutrophil : lymphocyte	0.39	0.49	0.033	0.03	0.0181	0.87
GPx, nmol/min/mL ²	125.2	125.5	4.79	0.97	<0.0001	0.57
SOD, U/mL ³	3.52	3.63	0.076	0.32	<0.0001	0.25
TBARS, μ M ⁴	0.89	0.92	0.042	0.56	<0.0001	0.83
Haptoglobin, mg/mL	139.9	122.7	9.682	0.21	<0.0001	0.73

¹White blood cells; ²Glutathione Peroxidase; ³Superoxide dismutase; ⁴Thiobarbituric acid reactive substances;

⁵Treatment (Control vs. ITMS); ⁶Days relative to calving (-72, 3, 7, 10, 35 d).

Figure 1. Daily mean environmental ambient temperature ($^{\circ}\text{C}$), relative humidity (%), and temperature-humidity index (THI) throughout the study from April 30 to November 13, 2019, in west Texas. Dotted lines indicate THI threshold (ZIMBELMAN et al., 2009).

Figure 2. Effect of injectable trace mineral supplementation on phagocytosis percentage (A), oxidative burst percentage (B); phagocytosis intensity (C), oxidative burst intensity (D), L-selectin intensity (E) of PMNL, and serum haptoglobin concentration (F). An asterisk (*) indicates a $P \leq 0.05$; Two asterisks (**) indicate a $P \leq 0.10$.

REFERENCES

- ABD EL-HACK, et al. Alleviating the environmental heat burden on laying hens by feeding on diets enriched with certain antioxidants (vitamin E and selenium) individually or combined. **Environmental Science Pollution Research**, v. 24, p. 10708–10717, 2017. doi:10.1007/s11356-017-8690-5.
- ABUELO, A. et al. 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.
- ADIN, G. et al. Effects of cooling dry cows under heat load conditions on mammary gland enzymatic activity, intake of food and water, and performance during the dry period and after parturition. **Livestock Science**, v. 124, n. 1–3, p. 189–195, set. 2009.
- ALHIDARY, I. A. et al. Effects of selenium and vitamin E on performance, physiological response, and selenium balance in heat-stressed sheep. **Journal of Animal Science**, v. 93, n. 2, p. 576–588, 2015.
- AOKI, M.; OSHITA, T.; SAKAGUCHI, M. The comparison of Thiobarbituric Acid Reactive Substances (TBARS) concentrations in plasma and serum from dairy cattle. **Journal of Veterinary Medical Science**, v. 70, n. 1, p. 107–110, 2008.
- BERNABUCCI, U. et al. 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.
- BICALHO, M. L. S. et al. Effect of trace mineral supplementation on selected minerals, energy metabolites, oxidative stress, and immune parameters and its association with uterine diseases in dairy cattle. **Journal of Dairy Science**, v. 97, n. 7, p. 4281–4295, 2014.
- BICALHO, M. L. S. et al. Association between virulence factors of *Escherichia coli*, *Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows. **Veterinary Microbiology**, v. 157, p. 125–131, 2012. doi:10.1016/j.vetmic.2011.11.034.
- BICALHO, R. C. et al. Effect of stillbirths on dam survival and reproduction performance in holstein dairy cows. **Journal of Dairy Science**, v. 90, p. 2797–2803, 2007. doi:10.3168/jds.2006-504.
- BICALHO, R. C. et al. Stillbirth parturition reduces milk production in Holstein cows. **Preventive Veterinary Medicine**, v. 84, p. 112–120, 2008. doi:10.1016/j.prevetmed.2007.11.006.
- BITTAR, J. H. J. et al. Effects of injectable trace minerals on the immune response to *Mannheimia haemolytica* and *Pasteurella multocida* following vaccination of dairy calves with a commercial attenuated-live bacterin vaccine. **Professional Animal Scientist**, v. 34, p. 59–66, 2018. doi:10.15232/pas.2017-01695.

BITTAR, J. H. J. et al. Immune response and onset of protection from Bovine viral diarrhea virus 2 infection induced by modified-live virus vaccination concurrent with injectable trace minerals administration in newly received beef calves. **Veterinary Immunology and Immunopathology**, v. 225, p. 1–11, 2020.

COOKE, R. F.; ARTHINGTON, J. D. Concentrations of haptoglobin in bovine plasma determined by ELISA or a colorimetric method based on peroxidase activity. **Journal of Animal Physiology and Animal Nutrition**, v. 97, n. 3, p. 531–536, jun. 2013.

DIKMEN, S.; HANSEN, P. J. Is the temperature-humidity index the best indicator of heat stress in lactating dairy cows in a subtropical environment? **Journal of Dairy Science**, v. 92, n. 1, p. 109–116, 2009.

DUBUC, J. et al. Effects of postpartum uterine diseases on milk production and culling in dairy cows. **Journal of Dairy Science**, v. 94, p. 1339–1346, 2011. doi:10.3168/jds.2010-3758.

DO AMARAL, B. C. et al. Heat stress abatement during the dry period influences metabolic gene expression and improves immune status in the transition period of dairy cows. **Journal of Dairy Science**, v. 94, n. 1, p. 86–96, jan. 2011.

GERNAND, E.; KÖNIG, S.; KIPP, C. Influence of on-farm measurements for heat stress indicators on dairy cow productivity, female fertility, and health. **Journal of Dairy Science**, v. 102, n. 7, p. 6660–6671, 1 jul. 2019.

HAMMON, D. S. et al. Neutrophil function and energy status in Holstein cows with uterine health disorders. **Veterinary Immunology and Immunopathology**, v. 113, n. 1–2, p. 21–29, 15 set. 2006.

HULBERT, L. E. et al. Effects of changing milk replacer feedings from twice to once daily on Holstein calf innate immune responses before and after weaning¹. **Journal of Dairy Science**, v. 94, n. 5, p. 2557–2565, maio 2011.

HUZZEY, J. M. et al. Short communication: Haptoglobin as an early indicator of metritis. **Journal of Dairy Science**, v. 92, n. 2, p. 621–625, 2009. Available on: <<http://dx.doi.org/10.3168/jds.2008-1526>>.

JACOMETO, C. B. et al. Maternal consumption of organic trace minerals alters calf systemic and neutrophil mRNA and microRNA indicators of inflammation and oxidative stress. **Journal of Dairy Science**, v. 98, n. 11, p. 7717–7729, 2015. Available on: <<http://dx.doi.org/10.3168/jds.2015-9359>>.

KELTON, D. F.; LISSEMORE, K. D.; MARTIN, R. E. Recommendations for Recording and Calculating the Incidence of Selected Clinical Diseases of Dairy Cattle. **Journal of Dairy Science**, v. 81, n. 9, p. 2502–2509, 1998. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(98\)70142-0](http://dx.doi.org/10.3168/jds.S0022-0302(98)70142-0)>.

KIM, I. H.; NA, K. J.; YANG, M. P. Immune responses during the peripartum period in dairy cows with postpartum endometritis. **Journal of Reproduction and Development**, v. 51, n. 6, p. 757–764, 2005.

LACETERA, N. et al. Lymphocyte functions in dairy cows in hot environment. **International Journal of Biometeorology**, v. 50, n. 2, p. 105–110, nov. 2005.

LAGO, A. et al. The selective treatment of clinical mastitis based on on-farm culture results: I. Effects on antibiotic use, milk withholding time, and short-term clinical and bacteriological outcomes. **Journal of Dairy Science**, v. 94, n. 9, p. 4441–4456, 2011. Available on: <<http://dx.doi.org/10.3168/jds.2010-4046>>.

LECCHI, C. et al. In vitro assessment of the effects of temperature on phagocytosis, reactive oxygen species production and apoptosis in bovine polymorphonuclear cells. **Veterinary Immunology and Immunopathology**, v. 182, p. 89–94, 2016. Available on: <<http://dx.doi.org/10.1016/j.vetimm.2016.10.007>>.

MACHADO, V. S. et al. Investigation of postpartum dairy cows' uterine microbial diversity using metagenomic pyrosequencing of the 16S rRNA gene. **Veterinary Microbiology**, v. 159, p. 460–469, 2012.

MACHADO, V. S. et al. Effect of an injectable trace mineral supplement containing selenium, copper, zinc, and manganese on the health and production of lactating Holstein cows. **Veterinary Journal**, v. 197, p. 451–456, abr. 2013.

MACHADO, V. S. et al. The effect of injectable trace minerals (selenium, copper, zinc, and manganese) on peripheral blood leukocyte activity and serum superoxide dismutase activity of lactating Holstein cows. **Veterinary Journal**, v. 200, n. 2, p. 299–304, 2014.

MAKIMURA, S.; SUZUKI, N. Quantitative determination of bovine serum Haptoglobin and its elevation in some inflammatory diseases. Nihon juigaku zasshi. **The Japanese Journal of Veterinary Science**, v. 44, n. 1, p. 15–21, fev. 1982.

MURRAY, R. D.; WILLIAMS, A. J.; SHELDON, I. M. Field investigation of perinatal mortality in Friesian cattle associated with myocardial degeneration and necrosis. **Reproduction in Domestic Animals**, v. 43, n. 3, p. 339–345, 2008.

NRC. National Research Council. **Nutrient Requirements of Dairy Cattle**. 4th. ed. Washington DC.: National Academy Press, 1971.

NRC. **Requirements of Dairy Cattle** Seventh Revised Edition , 2001. Seventh ed.

PALOMARES, R. A. et al. Effects of injectable trace minerals on humoral and cell-mediated immune responses to Bovine viral diarrhea virus, Bovine herpes virus 1 and Bovine respiratory syncytial virus following administration of a modified-live virus vaccine in dairy calves. **Veterinary Immunology and Immunopathology**, v. 178, p. 88–98, 2016. doi:10.1016/j.vetimm.2016.07.003.

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. **Journal of Dairy Science**, v. 101, p. 8532–8543, 2018. doi:10.3168/jds.2018-14447.

PHILIPSSON, J. et al. Sire evaluation standards and breeding strategies for limiting dystocia and stillbirth. Report of an E.E.C./E.A.A.P. working group. **Livestock Production Science**, v. 6, n. 2, p. 111–127, 1979.

RAJKUMAR, U. et al. Effect of supplemental trace minerals on hsp-70 mRNA expression in commercial broiler chicken. **Animal Biotechnology**, p. 1–6, 2017. doi:10.1080/10495398.2017.1287712.

ROBERTS, S. L. et al. Effect of injectable trace mineral administration on health, performance, and vaccine response of newly received feedlot cattle. **Professional Animal Scientist**. 32:842–848, 2016. doi:10.15232/pas.2016-01543.

SAFA, S. et al. Heat stress abatement during the postpartum period: Effects on whole lactation milk yield, indicators of metabolic status, inflammatory cytokines, and biomarkers of the oxidative stress. **Journal of Animal Science**, v. 97, n. 1, p. 122–132, 2019.

SCIORSCI, R. L. et al. Oxidative status along different stages of pregnancy in dairy cows. **Large Animal Review**, v. 26, p. 223–228, 2020.

SELLERS, M. D.; HULBERT, L. E.; BALLOU, M. A. Technical note: Determination of pre-analysis storage temperature and time allowances for ex vivo innate immune responses. **Journal of Dairy Science**, v. 96, p. 384–389, 2013. doi:10.3168/jds.2012-5750.

SHELDON, I. M. et al. Defining postpartum uterine disease in cattle. **Theriogenology**, v. 65, n. 8, p. 1516–1530, 2006.

SHELDON, I. M.; DOBSON, H. Postpartum uterine health in cattle. **Animal Reproduction Science**, v. 82–83, p. 295–306, 2004.

SORDILLO, L. M. Selenium-Dependent Regulation of Oxidative Stress and Immunity in Periparturient Dairy Cattle. **Veterinary Medicine International**, p. 15045, 2013.

SORDILLO, L. M. Nutritional strategies to optimize dairy cattle immunity. **Journal of Dairy Science**, v. 99, n. 6, p. 4967–4982, 1 jun. 2016.

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

SPRINGMAN, S. A. et al. Effects of injectable trace minerals on reproductive performance of beef heifers in adequate trace mineral status. **Professional Animal Scientist**, v. 34, p. 649–652, 2018. doi:10.15232/pas.2018-01752.

SUTTLE, N. F. **Mineral Nutrition of Livestock**, 4th Edition. 4th. ed. Oxfordshire, UK: CAB International, 2010.

TEIXEIRA, A. et al. Effect of an injectable trace mineral supplement containing selenium, copper, zinc, and manganese on immunity, health, and growth of dairy calves. **Journal of Dairy Science**, v. 97, p. 4216–4226, 2014.

VANEGAS, J. A.; REYNOLDS, J.; ATWILL, E. R. Effects of an injectable trace mineral supplement on first-service conception rate of dairy cows. **Journal Dairy Science**, v. 87, p. 3665–3671, 2004. doi:10.3168/jds.S0022-0302(04)73505-5.

WANG, H. et al. Unraveling the association of fecal microbiota and oxidative stress with stillbirth rate of sows. **Theriogenology**, v. 136, p. 131–137, 2019.

WITTROCK, J. M. et al. Short communication: Metritis affects milk production and cull rate of Holstein multiparous and primiparous dairy cows differently. **Journal of Dairy Science**, v. 94, p. 2408–2412, 2011. doi:10.3168/jds.2010-3697.

ZAHOREC, R. Ratio of neutrophil to lymphocyte counts--rapid and simple parameter of systemic inflammation and stress in critically ill. **Bratislavské lekárske listy**, v. 102, n. 1, p. 5–14, 2001.

ZIMBELMAN, R. B. et al. A Re-evaluation of the Impact of Temperature Humidity Index (THI) and Black Globe Humidity Index (BGHI) on Milk Production in High Producing Dairy Cows. In: Proc. Southwest Nutr. Man. Conf., January 2017, Tempe, AZ. **Proceedings...** Tempe, AZ: University of Arizona, Tucson, 2009.

CHAPTER 5 - Associations between circulating levels of natural antibodies, total serum immunoglobulins, and polymorphonuclear leukocyte function in early postpartum dairy cows

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**Associations between circulating levels of natural antibodies, total serum immunoglobulins,
and peripheral neutrophil function in early postpartum dairy cows**

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ABSTRACT

The objective of this study was to investigate the associations between natural antibodies (NAbs) and total serum immunoglobulins concentrations (both from IgG and IgM isotypes), with variables of peripheral neutrophil function of dairy cows in early postpartum period. Seventy-five healthy postpartum dairy cows at 2 ± 1 days in milk were enrolled in a cross-sectional study. Natural and total antibodies in serum samples were measured by ELISA. Flow cytometry was used to determine the phagocytosis and oxidative burst capacity of peripheral neutrophils and the quantification of the adhesion molecule L-selectin. Leukocyte count and differentials, and plasma haptoglobin were also measured. A moderate positive correlation between NAbs_{IgM} and total serum IgM and a weak positive correlation between NAbs_{IgM} and NAbs_{IgG} were found. Before performing the associations between circulating immunoglobulin concentrations and neutrophil function variables, the cows were categorized into having low, medium or high circulating antibodies based on their NAbs_{IgM} and NAbs_{IgG} , total IgM and total IgG serum levels. None of the neutrophil parameters assessed differed between low, medium, and high cows for both NAbs_{IgM} and NAbs_{IgG} . While association between neutrophil function and total IgG were not observed, some associations between total IgM levels and neutrophil activity were found. Cows with low total serum IgM presented similar phagocytic intensity and phagocytic index to medium and high cows, but the latter differed between each other. Finally, the proportion of neutrophils that performed oxidative burst and neutrophil surface expression of L-selectin intensity was greater in high total IgM group when compared to medium and low groups. results. In conclusion, association between NAbs, and neutrophil activity variables were not observed, but total serum IgM was associated to some neutrophil function parameters in early post-partum dairy cows.

Key Words: natural antibodies, immunology, oxidative burst, phagocytosis

INTRODUCTION

About 40 to 70% of dairy cows develop metabolic or infectious diseases in the first month of lactation, depending on different management systems and milk production (SANTOS et al., 2010; RIBEIRO et al., 2013). The occurrence of disease during the transition period is associated with a state of immune dysregulation, when exacerbated or inadequate inflammatory responses can lead to poor health outcomes (LEBLANC, 2014). Cows that have inadequate immune responses against pathogens, often characterized by suppression of polymorphonuclear leukocyte (PMN) function (KEHRLI; NONNECKE; ROTH, 1989; HOEBEN et al., 2000; HAMMON et al., 2006) or decreased circulating immunoglobulin (Ig) concentrations (VAN KNEGSEL et al., 2007; HERR; BOSTEDT; FAILING, 2011), have increased risk for metritis, endometritis, retained placenta and mastitis in early lactation (CAI et al., 1994; KIMURA et al., 2002; HAMMON et al., 2006).

There is a growing interest in the assessment of immunocompetence around parturition. Such information has been used to determine genetic merit for improved immunity (BANOS et al., 2013; MALLARD et al., 2015) and disease risk of postpartum cows (AMADORI et al., 2015). Inadequate immune status around parturition is often assessed using variables related to the cellular branch of the immune system, such as PMN phagocytic and oxidative burst activity (MATEUS et al., 2002; HAMMON et al., 2006; MARTINEZ et al., 2012). Others have used humoral components of the immune system, including natural antibodies (NAbs) and total immunoglobulins (Igs) as markers of immunocompetence of dairy cows (HERR; BOSTEDT; FAILING, 2011; BANOS et al., 2013). To understand if these different immune variables are associated among themselves during the early postpartum period is crucial for the development of economic and practical manners of assessing the immunocompetence of postpartum dairy cows. For instance, it would be more practical to measure serum Igs concentrations, than PMN function which has been performed in fresh samples. Also, in this period of immune dysregulation, it is likely that measuring all these variables could be redundant.

Therefore, the objective of this study is to investigate the associations between NAbs and total serum Igs concentrations, both from IgG and IgM isotypes, with several variables of peripheral PMN function in early postpartum dairy cows. The association between these variables have been eluded in previous studies. For instance, NAbs and total Igs concentration in blood were

reported to be positively correlated in goats (CECCHINI; RUFRANO; CAPUTO, 2019). Additionally, circulating NAbs concentration and PMN function have similar relationship with disease risk and metabolic imbalance during the transition period (VAN KNEGSEL et al., 2007; THOMPSON-CRISPI; MIGLIOR; MALLARD, 2013; MACHADO et al., 2014b), and circulating leukocyte counts have been correlated with circulating total Igs concentrations around parturition (HERR; BOSTEDT; FAILING, 2011). Hence, we hypothesize that NAbs, total Igs, and PMN function variables measured in the early postpartum period of dairy cows will be associated to each other.

MATERIALS AND METHODS

All activities performed in this study were reviewed and approved by the Texas Tech University Institutional Animal Care and Use Committee (#17097-11).

Sample size calculation

Sample size calculation was undertaken in MedCalc version 18.11.6 software (MedCalc Software, Mariakerke, Belgium) based on significant expected differences in the proportion of PMNs that could perform oxidative burst from cows during the first week postpartum (NIGHTINGALE; SELLERS; BALLOU, 2015). We expected that the proportion of PMNs that could perform oxidative burst would be 10 percent points greater in cows classified in a high Ig group compared to cows in a low Ig group. Assuming a standard deviation of 12 percent points and considering $\alpha = 0.05$ and a power of 80%, 24 cows in each Ig group would be needed. Considering that we would categorize cows based on serum Ig concentrations in three groups (terciles), a total of 72 cows would be needed. A convenience sample of 75 cows were enrolled in the study.

Farm and management, inclusion criteria, and blood collection

Study cows were kept in a commercial dairy farm located in Eastern New Mexico that milks 2,800 Holstein cows 3 times per day in a double 40-stall parallel milking parlor. Cows are housed in dry-lot corrals with shaded areas and are fed a TMR ad libitum consisting of approximately 55% forage and 45% concentrate and have free access to water. After parturition,

cows are moved to a fresh-cow pen where they are kept until 20 days in milk (DIM). The chemical composition of the diet fed to the fresh cows is presented in Table S1. This pen is monitored daily by trained farm personnel who follow a specific diagnostic and treatment protocol designed by veterinarians. The average mature equivalent 305-day milk production (ME305) for the herd is 12,838 kg.

A total of 75 postpartum cows between 1 and 3 days in milk (DIM) were enrolled in a cross-sectional study from February 22 to March 15, 2018. To be included in the study, cows had to be in their first, second, or third DIM. Additionally, cows were visually inspected for inflamed quarters, retained fetal membranes and fetid vaginal discharge, and had their rectal temperature assessed using a digital thermometer (GLA M900, GLA Agriculture Electronics, San Luis Obispo CA) equipped with a 10 cm angle probe. Cows with any of these visual signs of disease or fever (rectal temperature $> 39.5^{\circ}\text{C}$) were not included in the study. Body condition score (BCS) was evaluated at enrollment using a 5-point scale as previously described (EDMONSON et al., 1989).

Blood samples were collected from each cow at enrollment by puncture of the coccygeal vessels using a vacutainer tube without anticoagulant, a vacutainer tube with lithium heparin, a vacutainer tube with potassium EDTA, and a 20-gauge x 2.54-cm vacutainer needle (Becton, Dickinson and Company, Franklin Lakes, NJ). After collection, the heparinized blood was stored at in an ice chest without ice, to preserve their phagocytic and oxidative burst capacity (SELLERS; HULBERT; BALLOU, 2013). The EDTA and coagulated blood samples were transported to the laboratory on ice. Samples were analyzed or processed within 4 hours after collection. The blood samples without anticoagulants were centrifuged at $2,000 \times g$ for 15 min at 4°C , and the serum was harvested and frozen at -80°C until further analyses were conducted. The heparinized and EDTA blood samples were processed in the same day of collection for measures of hematology and ex vivo PMN responses.

Hematology and *ex vivo* PMN function

Leukocyte count and differentials were performed using a hematology analyzer (IDEXX Procyte DX, Westbrook, ME), and the variables of interest were neutrophil count, lymphocyte count, and neutrophil to lymphocyte ratio. Flow cytometry was used to determine the phagocytosis and oxidative burst capacity of peripheral PMNs and the quantification of the adhesion molecule L-selectin (CD62L) as previously described by Hulbert et al. (2011), with minor protocol

modifications. To measure the phagocytic and oxidative burst capacity, 100 μL of heparinized whole blood were incubated for 15 min in an ice bath. After this initial incubation, 20 μL of a 100 μM solution of dihydrorhodamine (Invitrogen, Carlsbad, CA), and 20 μL of a 10^9 cfu/mL propidium iodide labeled *E. coli* suspension were added to the blood, and then incubated in a 38.5°C water bath for 10 min (negative controls were incubated in an ice bath for 10 min). Then, samples were immediately placed on an ice bath for 5 min, and erythrocytes were hypotonically lysed, and washed with PBS. Dual-color flow cytometry was performed using a BD Accuri™ C6 Plus (BD Biosciences, San Jose, CA). The PMN populations were gated using forward and side scatter plots (LENO et al., 2017; MCDUGALL; LEBLANC; HEISER, 2017). The mean fluorescence intensity (MFI) and proportion of PMNs that performed phagocytosis and oxidative burst were acquired using the optical filters FL3 (excited by a 640 nm laser on a 670 LP filter) and FL1 (excited by a 488 nm laser on a 533/30 filter), respectively. Negative controls were used to determine negative and positive signals on the FL1 by FL3 scatterplot used to assess PMNs that performed phagocytosis and oxidative burst. Phagocytosis and oxidative burst indexes were created by multiplying the proportion of responding PMNs by the corresponding MFI, as greater index values indicate greater phagocytic and oxidative burst activities (MCCARTHY et al., 2016).

To determine the expression of the adhesion molecule L-selectin, 50 μL of EDTA blood samples were mixed with 50 μL of PBS containing 1 μg of a monoclonal antibody mouse IgG1-isotype (catalog number: BOV2046, clone: BAQ92A; Veterinary Microbiology and Pathology Monoclonal Antibody Center, Pullman, WA). After a 1 h incubation in an ice bath, erythrocytes were hypotonically lysed. After centrifugation, the leukocyte pellet was resuspended in a 50 μL solution of fluorescein-labeled secondary antibody at a 1:400 dilution (F(ab')₂ anti-mouse IgG:FITC; AbD Serotec Raleigh, NC) and incubated for 1 h in an ice bath. After a PBS wash, samples were analyzed using single-color flow cytometry. The PMN population was gated as previously described, and the MFI for L-selectin was gathered using FL-1. All flow cytometry data were analyzed using FlowJo software (Ashland, OR).

Determination of serum natural antibodies, total immunoglobulin M and G, and haptoglobin

Quantifications of the serum levels of natural antibodies isotypes G (NAb_{SIgG}) and M were performed via indirect ELISA as previously described by Machado et al. (2014). Clear polystyrene flat bottom microplates (Greiner Bio-One, Monroe, NC) were coated with 200 μL of 0.1 M

carbonate buffer pH 9.6 containing 1 $\mu\text{g}/\text{mL}$ of keyhole limpet hemocyanin (KLH; Sigma Aldrich, St. Louis, MO) and incubated overnight at 4°C. Wells were then washed three times with 300 μL of PBS containing 0.1% Tween-20 (PBS-T). Blocking of non-specific binding sites was done by adding 200 μL of PBS containing 3% of fish gelatin (Sigma Aldrich) followed by a 3 h incubation at room temperature. Plates were washed as previously described, and 200 μL of 1:40 dilutions of serum samples were added to wells; serum samples were diluted in PBS containing 0.5 M of NaCl and 0.05% of Tween-20. Plates were then incubated overnight at 4°C. After plates were washed as previously described, the serotype-specific antibody bound to the antigen was detected with either 200 μL of PBS-T containing 1:20,000 dilution of anti-bovine IgG or anti-bovine IgM antibodies (Bethyl Laboratories, Montgomery, TX). Plates were incubated for 1 h at room temperature, then washed as previously described. One hundred microliters of the substrate 3,3',5,5'-tetramethylbenzidine at 1mg/mL (Thermo Fisher Scientific, Waltham, MA) were added to each well. After incubation of 20 min at room temperature in the dark, 50 μL of stop solution (0.16 M sulfuric acid; Thermo Fisher Scientific) were added to each well. Immediately, optical density (OD) was quantified at 450 nm using the Epoch2 Microplate Spectrophotometer (BioTek, Winooski, VT). The amount of color produced was indicative of the quantity of natural antibody bound to the antigens bound to the wells. Assays were performed in duplicates. One additional well was utilized for each sample to serve as a blank, to minimize variations due to unspecific binding, such as binding of sample antibodies to the block. Unlike the duplicates, the blank wells were not coated with the antigen; 0.1 M carbonate buffer pH 9.6 without KLH was used in the blank wells. For each sample, data was reported as OD at 450 nm (OD@450), which was a result of the average OD of the duplicates subtracted by the OD of their respective blanks. The intra and inter-assay CVs were 8.4% and 9.2% for NAb_{IgM}, respectively. The intra and inter-assay CVs were 6.7% and 10.2% for NAb_{IgG}, respectively.

Total serum IgM and IgG were measured using commercially available kits following manufacturer's instructions in a 96-well microplate method (Life Diagnostics, West Chester, PA). The intra and inter-assay CVs were 3.4% and 5.3% for IgM, respectively. The intra and inter-assay CVs were 4.2% and 6.6% for IgG, respectively.

Serum Hp concentration was determined using a colorimetric assay via quantification of the haptoglobin/hemoglobin complex by the estimation of differences in peroxidase activity (MAKIMURA; SUZUKI, 1982). Assays were performed in 16 x 100 borosilicate tubes. Briefly,

5 μ L of serum sample or deionized water (blank) were added to 7.5 mL of a solution containing 0.6 g/L of O dianisidine, 13.8 g/L of sodium phosphate monobasic, and 0.5 g/L EDTA (pH = 4.1). Immediately, 25 μ L of 0.3 g/L bovine hemoglobin solution was added to each assay, followed by a water bath incubation at 37°C for 45 min. After incubation, 100 μ L of freshly prepared 156 mM hydrogen peroxidase solution was added to each assay. Samples were incubated at room temperature for 60 min. Then, 200 μ L of each assay was transferred to a 96-well polystyrene flat-bottom microplate. Optical density at 450 nm was measured on the Epoch2 Microplate Spectrophotometer (BioTek). Finally, the final OD of each assay was subtracted by the blank assay OD. Optical density data was converted to a concentration unit (μ g/mL) using standard curves generated by serial dilutions of a sample of known concentration determined by a commercially available ELISA kit following the manufacturer's instructions (Life Technologies) as previously described (COOKE; ARTHINGTON, 2013). The intra and inter-assay CV for serum Hp were 6.9% and 7.7%, respectively.

Statistical analyses

To assess the correlation between circulating levels of total IgM and NAb_SIgM, total IgG and NAb_SIgG, and NAb_SIgM and NAb_SIgG, three scatter plots of linear regression were generated using MedCalc. To facilitate data analysis and interpretation, cows were classified in low, medium, or high Igs groups based on the serum levels of total IgM, total IgG, NAb_SIgM and NAb_SIgG. For those variables, cows with serum levels in the first, second, and third tercile were categorized as low, medium, and high, using the distribution function of JMP 14 (SAS Institute Inc., Cary, NC). Classification of cows in different Ig groups (low, medium and high) was performed for each type of Ig analyzed (total IgM, total IgG, NAb_SIgM and NAb_SIgG). For instance, a cow could be in the low total IgG group, but in the high total IgM group. Descriptive statistics analysis regarding parity, DIM at enrollment, and BCS of cows classified in each Ig group was undertaken using the chi-square and ANOVA functions of JMP 14.

To evaluate the association between serum Ig levels and markers of PMN function and activation, four groups of mixed ANOVA models were fitted to the data using the MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Immunoglobulin level (low, medium, and high) for NAb_SIgM, NAb_SIgG, total IgM, and total IgG, were the fixed effects for the first, second, third, and fourth groups of mixed ANOVA models, respectively. The dependent variables analyzed

were proportion of PMNs that performed phagocytosis, phagocytosis MFI, phagocytosis index, proportion of PMNs that performed oxidative burst, oxidative burst MFI, oxidative burst index, l-selectin MFI, neutrophil count, lymphocyte count, neutrophil to lymphocyte ratio, and haptoglobin concentration. Date of sampling was included in all models as a random variable. Parity, DIM at enrollment, and BCS were offered to all models as independent variables and were kept when $P < 0.10$. Dependent variables were log-transformed when necessary (Phagocytosis MFI, Phagocytosis Index, Oxidative Burst MFI, Oxidative Burst Index, and Lymphocyte count). Data reported were not back transformed after analysis. For all models described above, visual evaluation of the distribution plot of the studentized residuals was used to confirm that the residuals were normally distributed.

RESULTS

The correlations between total IgM and NAb_SIgM, total IgG and NAb_SIgG, and NAb_SIgM and NAb_SIgG are presented in Figure 1. While the circulating levels of NAb_SIgM had a positive correlation with total serum IgM of postpartum dairy cows ($r = 0.47$, $P < 0.001$, Figure 1A), NAb_SIgG and total serum IgG were not correlated ($r = 0.02$, $P = 0.83$, Figure 1B). Additionally, we observed a positive correlation between the circulating levels of NAb_SIgM and NAb_SIgG ($r = 0.25$, $P = 0.03$, Figure 1C).

Serum NAb_SIgM levels for cows classified as low, medium, and high for NAb_SIgM were < 1.79 , $1.79 - 2.17$, and > 2.17 OD@450, respectively. Cows with circulating levels of NAb_SIgG < 2.49 , $2.49 - 3.00$, and > 3.00 OD@450 were categorized as low, medium, and high for NAb_SIgG, respectively. Cows with total IgM < 2.30 , $2.30 - 3.51$, and > 3.51 mg/ mL were classified as low, medium and high for total IgM. Finally, total serum IgG concentration for cows classified as low, medium, and high for total IgG was < 28.64 , $28.64 - 36.81$, and > 36.81 mg/mL, respectively. Descriptive statistics of parity, DIM at enrollment, and BCS for cows classified as low, medium and high for NAb_SIgM, NAb_SIgG, total IgM and total IgG serum levels are presented in Table 1.

The association of circulating levels of NAb_SIgM and NAb_SIgG with PMN response variables are presented in Table 2. None of the variables assessed differed between low, medium, and high cows for NAb_SIgM and NAb_SIgG. The association of total serum IgM and IgG, with PMN function variables are shown in Table 3. For total IgM, a few variables differed within low, medium, and

high cows. The intensity of phagocytic activity of PMNs, evident by greater phagocytosis MFI, was greater for high cows, in comparison to cows classified in the medium group ($P = 0.05$); phagocytic intensity did not differ between low cows and cows in the medium ($P = 0.69$) and high ($P = 0.23$) groups. Additionally, the phagocytic index, which considers the proportion of PMNs performing phagocytosis and their phagocytic intensity, was greatest for total IgM high group when compared to medium group cows ($P = 0.01$) and tended to be greater compared to cows in the low group ($P = 0.07$); phagocytosis index was not different among the cows in the medium and low groups ($P = 0.72$). The proportion of PMNs that performed oxidative burst was elevated for the total IgM high group in comparison to the medium and low groups ($P = 0.04$ for both comparisons), but it was not different between cows in the low and medium groups ($P = 0.99$). Lastly, the PMN surface expression of l-selectin MFI was greater in the high group for total IgM when compared to cows in the medium and low groups ($P = 0.01$ and 0.02 , respectively), but it did not differ among cows in the medium and low groups ($P = 0.76$). No differences were observed among total IgM low, medium, and high groups in any of the other variables assessed. Additionally, there were no differences in the variables assessed across low, medium, and high cows for total IgG.

DISCUSSION

Natural antibodies are found in the serum of healthy, non-immunized individuals (HERZENBERG et al., 1986; MADI et al., 2009). They are produced spontaneously by the long-lived, self-renewing B1 B-lymphocytes, in the absence of an apparent antigenic stimulation (HAMILTON; LEHUEN; KEARNEY, 1994). In this study, we used KLH to measure NAbs because keyhole limpet hemocyanin is a metalloprotein derived from the *Megathura crenulata*, a giant keyhole limpet that lives up to 33 meters deep into the Pacific Ocean. Therefore, it is assumed that dairy cattle are naïve to KLH and only non-specific Igs, such as NAbs could recognize it (STAR et al., 2007). Natural antibodies are essentially formed from IgM isotype (BOES, 2000). However, protective roles of other NAbs isotypes such as IgG and IgA have been suggested (MACHADO et al., 2014b; ROTHSTEIN, 2016; PALMA et al., 2018; CECCHINI; RUFRANO; CAPUTO, 2019).

In the present study, we observed that the circulating levels of total IgM has a positive correlation with NAb_SIgM, but total IgG is not associated with NAb_SIgG. We also observed a positive correlation between circulating levels of NAb_SIgM and NAb_SIgG. A positive correlation between NAb_SIgM and total IgM concentration was also observed in goats (CECCHINI; RUFRANO; CAPUTO, 2019). In dairy cattle, positive associations between serum NAb_SIgM titers and circulating concentrations of antibodies against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium were reported (VAN ALTENA et al., 2016). In chickens, Cecchini et al. (2016) showed a positive correlation between Nab_IIgM and anti-human- γ -globulins specific antibodies in laying hens immunized with human- γ -globulins. Likewise, (Parmentier et al. (2004) showed that chickens selected for high antibody response against sheep red blood cells had greater concentrations of NAbs when compared to chickens selected for low antibody responsiveness.

It was reported that the variation in NAb_SIgG is greater than NAb_SIgM among cows, likely because the heritability for circulating concentrations of NAb_SIgM is greater than NAb_SIgG (THOMPSON-CRISPI; MIGLIOR; MALLARD, 2013; DE KLERK et al., 2015). Additionally, it was observed that the dynamics of circulating levels of total IgG and IgM around parturition are different. Total IgG blood concentration reaches its nadir around parturition, due to selective translocation of IgG into the udder for colostrum synthesis, but the same does not occur to total IgM (HERR; BOSTEDT; FAILING, 2011). Additionally, NAb_SIgM are the first antibodies produced by B lymphocytes, but due to antigenic challenge and consequent activation of T lymphocytes, B lymphocytes may switch their Ig production from IgM to IgG and IgA (MARKET; PAPAVALASIOU, 2003). Further, total serum IgG are produced stimuli-dependently of NAb_SIgM and total IgM concentrations (BAUMGARTH et al., 2000; EHRENSTEIN; NOTLEY, 2010). Thus, both total IgG and NAb_SIgG concentrations are more affected by environmental and genetic factors than total serum IgM and NAb_SIgM.

Others have suggested that NAbs could be markers of overall competence of the innate immune system. Banos et al. (2013) observed fewer mastitis and lameness episodes in low concentrate/high forage group of cows, which had elevated NAbs concentrations, and they interpreted those results as a positive relationship between high concentrations of NAbs and better capacity of the innate immune system to respond to a pathogenic challenge. Machado et al. (2014b) found higher concentrations of NAb_SIgG around parturition in healthy cows in comparison to cows that developed metritis and endometritis. However, they could not conclude that NAbs have an

active role in the bovine uterus defense but could merely be a marker of overall immunocompetence. Conversely, we did not find any significant associations between circulating concentrations of NAb_{SIgM} or NAb_{SIgG} and variables of PMN function. Although Nabs can activate the complement system, complement killing of bacteria such as *E. coli* and *S. enterica* serovar Typhimurium was not different between serum containing high and low concentrations of NAb_{SIgM} in dairy cows (VAN ALTENA et al., 2016). There are other manners that NAbs could be promoting health that have not been studied in cattle yet. In mice that underwent an acute peritonitis model, NAb_{SIgM} played a role in the inflammatory response, mediating the production of TNF- α and PMN recruitment, lowering the bacterial load in the peritoneum and circulating levels of endotoxin and pro-inflammatory cytokines (BOES et al., 1998). More research is warranted to better understand how NAbs are promoting health in dairy cattle.

Herr, Bostedt, and Failing (2011) reported positive correlations between total serum Igs and lymphocyte counts around parturition, suggesting that low concentrations of IgG and IgM around parturition could be indicative of an “unstable immune system”. In our study, we did not observe any association between total serum Igs and lymphocyte counts. However, total IgM was associated positively associated with some PMN activity variables, but total serum IgG was not. Based on our data, we cannot explore mechanisms that could explain why total IgM is associated with greater PMN responses. As mentioned earlier, because of colostrum formation and other environmental factors, the total IgG levels are more variable than total IgM levels around parturition (HERR; BOSTEDT; FAILING, 2011). Therefore, we suggest that total IgM is a better biomarker of the innate immunocompetence of dairy cows in the early postpartum period.

We acknowledge that our data provide insights solely into a temporal association between the analyzed immune variables, and that mechanistic pathways explaining how these variables are related was not explored in our study. Additionally, these associations were only assessed in the early postpartum period (between 1 and 3 DIM) of cows that did not present signs of disease. The concentration of Igs and PMN phagocytosis and oxidative burst function varies during different times of the transition period and disease status of dairy cows (CAI et al., 1994; HERR; BOSTEDT; FAILING, 2011; MACHADO et al., 2014b). Therefore, it is possible that these relationships could be different if evaluated during the pre-partum period, later in lactation, or in diseased animals. In this pilot study, we had budgetary limitations and decided to focus our efforts to collect data during the early postpartum period, which is known to be the nadir of circulating

Igs concentration and PMN function. More research is needed to elucidate how these variables are associated in these different conditions. Additionally, causative mechanisms of these relationships remain elusive, and further research is needed to understand how NAbs are associated with improved health of dairy cows.

CONCLUSIONS

In conclusion, circulating concentrations of NAb_{sIgM} and total IgM were positively correlated, but NAb_{sIgG} and total IgG were not associated. Additionally, no association was found between NAbs from IgM or IgG isotypes and PMN activity variables in early post-partum dairy cows. Conversely, total serum IgM, but not total serum IgG, was positively associated to PMN phagocytosis, oxidative burst, and surface expression of L-selectin, and can be a useful tool to assess PMN function when blood samples cannot be processed in a timely manner. It is important to highlight that we have only sampled cows within the first 3 days postpartum, and these conclusions can only be made for cows during this period. It is possible that different results could be obtained if cows in different stages of lactation are included in the analysis. Therefore, more research is needed to completely elucidate the associations between circulating NAbs, total serum Igs, and PMN function.

DECLARATION OF COMPETING INTEREST

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Table 1. Descriptive statistics on parity, DIM at enrollment, and BCS of the 75 cows enrolled in the study. Cows were classified into low, medium, and high groups based on the serum levels of natural antibodies or total immunoglobulins.

		Parity, n (%)		<i>P</i>	Cows enrolled in different DIM, n (%)			<i>P</i>	BCS (SEM)	<i>P</i>
		Primiparous	Multiparous		1	2	3			
NAb _{SIgM} ¹	low	4 (16)	21 (84)	0.26	7 (28)	8 (32)	10 (40)	0.71	3.23 (0.07)	0.95
	medium	9 (36)	16 (64)		10 (40)	7 (28)	8 (32)		3.24 (0.07)	
	high	6 (24)	19 (76)		9 (36)	6 (24)	6 (24)		3.21 (0.07)	
NAb _{SIgG} ²	low	6 (24)	19 (76)	0.93	6 (24)	8 (32)	11 (44)	0.26	3.26 (0.06)	0.07
	medium	7 (28)	18 (72)		9 (36)	7 (28)	9 (36)		3.11 (0.06)	
	high	6 (24)	19 (76)		11 (44)	10 (40)	4 (16)		3.31 (0.06)	
Total IgM ³	low	6 (24)	19 (76)	0.61	10 (40)	7 (28)	8 (32)	0.81	3.18 (0.06)	0.38
	medium	5 (20)	20 (80)		7 (28)	9 (36)	9 (36)		3.30 (0.06)	
	high	8 (32)	17 (68)		9 (36)	9 (36)	7 (28)		3.20 (0.06)	
Total IgG ⁴	low	6 (24)	19 (76)	0.93	8 (32)	10 (40)	7 (28)	0.89	3.21 (0.06)	0.72
	medium	6 (24)	19 (76)		10 (40)	8 (32)	7 (28)		3.27 (0.06)	
	high	7 (28)	18 (72)		8 (32)	7 (28)	10 (40)		3.20 (0.06)	

¹NAb_{SIgM}: low < 1.79, medium = 1.79 – 2.17, high > 2.17 (OD@450)

²NAb_{SIgG}: low < 2.49, medium = 2.49 – 3.00, high > 3.00 (OD@450)

³Total IgM: low < 2.30, medium = 2.30 – 3.51, high > 3.51 (mg/ml)

⁴Total IgG: low < 28.64, medium = 28.64 – 36.81, high > 36.81 (mg/ml)

Table 2. Association between circulating levels of natural antibodies (IgM and IgG) and immune variables measured at DIM 2 ± 1 .

Variable	NAbs _{IgM} ¹			SEM ⁴	<i>P</i>	NAbs _{IgG} ²			SEM	<i>P</i>
	low	medium	high			low	medium	high		
Phagocytosis, %	58.9	60.2	63.4	6.07	0.25	59.5	60.3	62.6	6.27	0.52
Phagocytosis, MFI ^{3*}	9.74	9.84	9.85	0.19	0.76	9.74	9.86	9.83	0.19	0.47
Phagocytosis Index*	9.20	9.32	9.35	0.27	0.34	9.20	9.32	9.34	0.28	0.47
Oxidative burst, %	34.9	34.6	38.5	3.08	0.19	34.9	36.7	36.6	3.14	0.77
Oxidative burst, MFI*	11.32	11.26	11.35	0.23	0.51	11.25	11.34	11.35	0.23	0.41
Oxidative burst index*	10.25	10.16	10.36	0.30	0.32	10.16	10.29	10.31	0.31	0.50
L-selectin, MFI	654	624	755	60.4	0.13	681	708	643	54.70	0.63
Neutrophil count, cells x 10 ⁶ /mL	4.42	4.09	4.74	0.58	0.72	4.59	4.21	4.44	0.59	0.90
Lymphocyte count, cells x 10 ⁶ /mL*	1.53	1.44	1.70	0.15	0.43	1.38	1.72	1.55	0.16	0.28
Neutrophil: lymphocyte	0.97	1.07	0.97	0.15	0.88	1.09	0.78	1.14	0.15	0.20
Haptoglobin, mg/mL	116	175	232	48.2	0.24	139	162	223	49.7	0.46

¹NAbs_{IgM}: low < 1.79, medium = 1.79 – 2.17, high > 2.17 (OD@450)²NAbs_{IgG}: low < 2.49, medium = 2.49 – 3.00, high > 3.00 (OD@450)³MFI = mean fluorescence intensity⁴SEM: Largest standard error of the mean

*data was log-transformed for normality of model residuals

Table 3. Association between total serum IgM and IgG, and immune variables measured at DIM 2 ± 1.

Variable	Total IgM ¹			SEM ⁴	<i>P</i>	Total IgG ²			SEM	<i>P</i>
	low	medium	high			low	medium	high		
Phagocytosis, %	58.6	59.3	64.6	5.63	0.06	57.9	62.0	62.5	6.02	0.17
Phagocytosis, MFI ³ *	9.79 ^{a, b}	9.72 ^a	9.94 ^b	0.17	0.05	9.77	9.85	9.82	0.18	0.67
Phagocytosis Index*	9.24 ^{a, b}	9.16 ^a	9.45 ^b	0.25	0.01	9.18	9.36	9.32	0.26	0.20
Oxidative burst, %	34.1 ^a	34.0 ^a	39.9 ^b	2.52	0.02	33.9	36.8	37.1	2.90	0.32
Oxidative burst, MFI*	11.33	11.27	11.34	0.22	0.64	11.30	11.32	11.32	0.22	0.98
Oxidative burst index*	10.23	10.16	10.38	0.28	0.22	10.19	10.29	10.28	0.29	0.71
L-selectin, MFI	622 ^a	604 ^a	775 ^b	50.72	<0.01	641	713	664	48.22	0.49
Neutrophil count, cells x 10 ⁶ /mL	3.82	4.72	4.70	0.57	0.45	4.35	4.22	4.67	0.58	0.85
Lymphocyte count, cells x 10 ⁶ /mL*	1.53	1.48	1.64	0.16	0.73	1.62	1.65	1.38	0.16	0.31
Neutrophil: lymphocyte	0.87	1.15	0.98	0.15	0.41	1.06	0.91	1.03	0.15	0.75
Haptoglobin, mg/mL	100	193	231	48.28	0.15	157	196	171	49.20	0.85

¹Total IgM: low < 2.30, medium = 2.30 – 3.51, high > 3.51 (mg/ml)²Total IgG: low < 28.64, medium = 28.64 – 36.81, high > 36.81 (mg/ml)³MFI = mean fluorescence intensity⁴SEM: Largest standard error of the mean^{a, b}Different superscripts within a row indicates *P* < 0.05

*data were log-transformed for normality of model residuals.

Table S1. The chemical composition of fresh cow diet

Chemical composition	
Dry matter (DM), %	62.2
Crude protein (CP), % of DM	17.7
Rumen undegradable protein, % of CP	36.2
Ether extract, % of DM	6.1
ADF, % of DM	21.8
NDF, % of DM	32.8
NFC, % of DM	36.8
Calcium, % of DM	0.83
Phosphorus, % of DM	0.43
Potassium, % of DM	1.26
Magnesium, % of DM	0.36

Figure 1. Scatter plot of the correlation between (A) total IgM serum concentration and circulating levels of IgM natural antibodies (NAb_{IgM}), (B) total IgG serum concentration and circulating levels of IgG natural antibodies (NAb_{IgG}), and (C) circulating levels of NAb_{IgM} and NAb_{IgG}.

REFERENCES

- AMADORI, M. et al. Disease risk assessment by clinical immunology analyses in periparturient dairy cows. **Research in Veterinary Science**, v. 102, p. 25–26, 2015. Available on: <<http://dx.doi.org/10.1016/j.rvsc.2015.07.002>>.
- BANOS, G. et al. Identification of Immune Traits Correlated with Dairy Cow Health, Reproduction and Productivity. **PLoS ONE**, v. 8, n. 6, 2013.
- BAUMGARTH, N. et al. B-1 and B-2 Cell-Derived Immunoglobulin M Antibodies Are Nonredundant Components of the Protective Response to Influenza Virus Infection. **The Journal of Experimental Medicine**, v. 192, n. 2, p. 271–280, 2000.
- BOES, M. et al. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. **Journal of immunology** (Baltimore, Md. : 1950), v. 160, n. 10, p. 4776–87, 1998. Available on: <<http://www.ncbi.nlm.nih.gov/pubmed/9590224>>.
- BOES, M. Role of natural and immune IgM antibodies in immune responses. **Molecular Immunology**, v. 37, 2000. Available on: <www.elsevier.com/locate/molimm>.
- CAI, T. Q. et al. Association between neutrophil functions and periparturient disorders in cows. **American Journal of Veterinary Research**, v. 55, n. 7, p. 934–943, 1994.
- CECCHINI, S. et al. Evaluation of the effects of dexamethasone-induced stress on levels of natural antibodies in immunized laying hens. **Veterinary Immunology and Immunopathology**, v. 177, p. 35–41, 2016.
- CECCHINI, S.; RUFRANO, D.; CAPUTO, A. R. Natural antibodies and their relationship with total immunoglobulins and acquired antibody response in goat kid (*Capra hircus*, L. 1758) serum. **Veterinary Immunology and Immunopathology**, v. 211, p. 38–43, 2019.
- COOKE, R. F.; ARTHINGTON, J. D. Concentrations of haptoglobin in bovine plasma determined by ELISA or a colorimetric method based on peroxidase activity. **Journal of Animal Physiology and Animal Nutrition**, v. 97, n. 3, p. 531–536, jun. 2013.
- DE KLERK, B. et al. Phenotypic and genetic relationships of bovine natural antibodies binding keyhole limpet hemocyanin in plasma and milk. **Journal of Dairy Science**, v. 98, n. 4, p. 2746–2752, 2015.
- EDMONSON, A. J. et al. A Body Condition Scoring Chart for Holstein Dairy Cows. **Journal of Dairy Science**, v. 72, n. 1, p. 68–78, 1989.
- EHRENSTEIN, M. R.; NOTLEY, C. A. The importance of natural IgM: Scavenger, protector and regulator. **Nature Reviews Immunology**, 2010.
- HAMILTON, A. M.; LEHUEN, A.; KEARNEY, J. F. Immunofluorescence analysis of B-1 cell ontogeny in the mouse. **International Immunology**, v. 6, n. 3, p. 355–361, 1994.

HAMMON, D. S. et al. Neutrophil function and energy status in Holstein cows with uterine health disorders. **Veterinary Immunology and Immunopathology**, v. 113, n. 1–2, p. 21–29, 2006.

HERR, M.; BOSTEDT, H.; FAILING, K. IgG and IgM levels in dairy cows during the periparturient period. **Theriogenology**, v. 75, n. 2, p. 377–385, 2011. Available on: <<http://dx.doi.org/10.1016/j.theriogenology.2010.09.009>>.

HERZENBERG, L. A. et al. The LY-1B Cell Lineage. **Immunological Reviews**, v. 93, n. 1, p. 81–102, 1986.

HOEBEN, D. et al. Chemiluminescence of bovine polymorphonuclear leucocytes during the periparturient period and relation with metabolic markers and bovine pregnancy-associated glycoprotein. **Journal of Dairy Research**, v. 67, n. 2, p. 249–259, 2000.

HULBERT, L. E. et al. Effects of changing milk replacer feedings from twice to once daily on Holstein calf innate immune responses before and after weaning¹. **Journal of Dairy Science**, v. 94, n. 5, p. 2557–2565, 2011.

KEHRLI, M. E.; NONNECKE, B. J.; ROTH, J. A. Alterations in bovine neutrophil function during the periparturient period. **American Journal of Veterinary Research**, v. 50, n. 2, p. 207–214, 1989. Available on: https://lib.dr.iastate.edu/vmpm_pubs/191

KIMURA, K. et al. Effects of mastectomy on composition of peripheral blood mononuclear cell populations in periparturient dairy cows. **Journal of Dairy Science**, v. 85, n. 6, p. 1437–1444, 2002. Available on: <[http://dx.doi.org/10.3168/jds.S0022-0302\(02\)74211-2](http://dx.doi.org/10.3168/jds.S0022-0302(02)74211-2)>.

LEBLANC, S. J. Reproductive tract inflammatory disease in postpartum dairy cows. **Animal**, v. 8 Suppl 1, p. 54–63, 2014.

LENO, B. et al. Short communication: Relationship between methods for measurement of serum electrolytes and the relationship between ionized and total calcium and neutrophil oxidative burst activity in early postpartum dairy cows. **Journal of Dairy Science**, v. 100, p. 1–9, 2017.

MACHADO, V. S. et al. Short communication: Relationship between natural antibodies and postpartum uterine health in dairy cows. **Journal of Dairy Science**, v. 97, n. 12, p. 7674–7678, 26 set. 2014.

MADI, A. et al. Organization of the autoantibody repertoire in healthy newborns and adults revealed by system level informatics of antigen microarray data. **Proceedings... National Academy of Sciences of the United States of America**, v. 106, n. 34, p. 14484–14489, 2009.

MAKIMURA, S.; SUZUKI, N. Quantitative determination of bovine serum Haptoglobin and its elevation in some inflammatory diseases. **The Japanese journal of Veterinary Science**, v. 44, n. 1, p. 15–21, fev. 1982.

MALLARD, B. A. et al. Genetic selection of cattle for improved immunity and health. **Japanese Journal of Veterinary Research**, v. 63, p. S37–S44, 2015.

MARKET, E.; PAPAVALILIOU, F. N. V(D)J recombination and the evolution of the adaptive immune system. **PLoS Biology**, v. 1, n. 1, p. 24–27, 2003.

MARTINEZ, N. et al. Evaluation of periparturient calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. **Journal of Dairy Science**, v. 95, n. 12, p. 7158–7172, 2012. Available on: <<http://dx.doi.org/10.3168/jds.2012-5812>>.

MATEUS, L. et al. Blood and intrauterine leukocyte profile and function in dairy cows that spontaneously recovered from postpartum endometritis. **Reproduction in Domestic Animals**, v. 37, n. 3, p. 176–180, 2002.

MCCARTHY, M. M. et al. Associations between the degree of early lactation inflammation and performance, metabolism, and immune function in dairy cows. **Journal of Dairy Science**, v. 99, n. 1, p. 680–700, 2016. Available on: <<http://dx.doi.org/10.3168/jds.2015-9694>>.

MCDUGALL, S.; LEBLANC, S. J.; HEISER, A. Effect of prepartum energy balance on neutrophil function following pegbovigrastim treatment in periparturient cows. **Journal of Dairy Science**, v. 100, n. 9, p. 1–15, 2017. Available on: <<http://dx.doi.org/10.3168/jds.2017-12786>>.

NIGHTINGALE, C. R.; SELLERS, M. D.; BALLOU, M. A. Elevated plasma haptoglobin concentrations following parturition are associated with elevated leukocyte responses and decreased subsequent reproductive efficiency in multiparous Holstein dairy cows. **Veterinary Immunology and Immunopathology**, v. 164, n. 1–2, p. 16–23, 2015. Available on: <<http://dx.doi.org/10.1016/j.vetimm.2014.12.016>>.

PALMA, J. et al. Natural antibodies – Facts known and unknown. **Central European Journal of Immunology**, v. 43, n. 4, p. 466–475, 2018.

PARMENTIER, H. K. et al. Different levels of natural antibodies in chickens divergently selected for specific antibody responses. **Developmental and Comparative Immunology**, v. 28, n. 1, p. 39–49, 2004.

RIBEIRO, E. S. et al. Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. **Journal of Dairy Science**, v. 96, n. 9, p. 5682–5697, 5 jul. 2013.

ROTHSTEIN, T. L. Natural antibodies as rheostats for susceptibility to chronic diseases in the aged. **Frontiers in Immunology**, v. 7, n. APR, 7 abr. 2016.

SANTOS, J. et al. Applying nutrition and physiology to improve reproduction in dairy cattle. In: **Reproduction in Domestic Ruminants VII**. Nottingham University Press, 2010. p. 387–404.

SELLERS, M. D.; HULBERT, L. E.; BALLOU, M. A. Technical note: Determination of preanalysis storage temperature and time allowances for ex vivo innate immune responses. **Journal of Dairy Science**, v. 96, n. 1, p. 384–389, 2013. Available on: <<http://dx.doi.org/10.3168/jds.2012-5750>>.

STAR, L. et al. Natural humoral immune competence and survival in layers. **Poultry Science**, v. 86, n. 6, p. 1090–1099, 2007. Available on: <<http://dx.doi.org/10.1093/ps/86.6.1090>>.

THOMPSON-CRISPI, K. A.; MIGLIOR, F.; MALLARD, B. A. Incidence Rates of Clinical Mastitis among Canadian Holsteins Classified as High, Average, or Low Immune Responders. **Clinical and Vaccine Immunology**, v. 20, n. 1, p. 106–112, jan. 2013.

VAN ALTENA, S. E. C. et al. Bovine natural antibodies in antibody-dependent bactericidal activity against *Escherichia coli* and *Salmonella Typhimurium* and risk of mastitis. **Veterinary Immunology and Immunopathology**, v. 171, p. 21–27, 1 mar. 2016.

VAN KNEGSEL, A. T. M. et al. Natural Antibodies Related to Energy Balance in Early Lactation Dairy Cows. **Journal of Dairy Science**, v. 90, n. 12, p. 5490–5498, 16 nov. 2007.

Overall Conclusions

Chapter 2 - Prophylactic use of ultra-diluted complex did not improve performance of weaned Holstein calves, nor it had an impact on the heifers-raising period and subsequent first-lactation. However, reduced proportion of digestive problems, fewer cumulative days for tick-borne disease, and a lower culling rate in the heifer-raising period were detected in ultra-diluted treated group. In addition, in specific days after enrollment, systemic inflammation status of calves was improved by ultra-diluted complex based on circulating biomarkers of inflammation and stress. More research is needed to prove the effectiveness of ultra-diluted products on farm animals since there is a lack of well-designed studies in the literature.

Chapter 3 - Prophylactic use of an ultra-diluted complex did not alter PMNL function, growth, nor it had effect on respiratory score of weaned growing calves during 28 d immediately after grouping.

Chapter 4 - Injectable trace mineral supplementation improved PMNL function and reduced incidence of metritis and stillbirth parturition in dairy cows undergoing the transition period in hot-months. However, these positive findings related to immunity and reproductive disorders did not translate into improved milk yield, reproductive performance, and survivability.

Chapter 5 - Circulating concentrations of NAb_{sIgM} and total IgM were positively correlated, but NAb_{sIgG} and total IgG were not associated. Additionally, no association was found between NAbs from IgM or IgG isotypes and PMN activity variables in early post-partum dairy cows. Conversely, total serum IgM, but not total serum IgG, was positively associated to PMN phagocytosis, oxidative burst, and surface expression of L-selectin, and can be a useful tool to assess PMN function when blood samples cannot be processed in a timely manner. It is important to highlight that we have only sampled cows within the first 3 days postpartum, and these conclusions can only be made for cows during this period. It is possible that different results could be obtained if cows in different stages of lactation are included in the analysis. Therefore, more research is needed to completely elucidate the associations between circulating NAbs, total serum Igs, and PMN function.

Final Considerations

According to findings herein presented on ultra-diluted medicines, we must consider this formulation use in order to reduce diseases such as tick-borne disease and digestive problems at the post-weaning period immediately after grouping. However, there are few well-designed studies published in the literature demonstrating the effectiveness of these products on the livestock health and performance. Therefore, the producers must be cautious when using formulations that have not been studied yet.

Regarding injectable trace minerals, we detected lower metritis incidence and lower stillbirth parturitions when animals were injected with trace mineral supplement containing Copper, Selenium, Zinc, and Manganese. In addition, the expressive impact of these trace minerals on innate immune system activity may suggest that such strategy may improve health and performance of dairy cows undergoing the transition period under heat stress conditions. All the events occurring in the transition period such as lower dry matter intake coupled to the high fatty acid mobilization and so on, may impair the immune system functionality. Moreover, the oscillation often found in dairy cows' dry matter intake during this period, may compromise the balance of antioxidant intake and free-radicals production by the body, getting the oxidative stress worse. Bringing this information to the Brazilian conditions, this strategy may help the most milk producers since the most dairies have passed by heat stress issues.

The associations of immune system cells and immunoglobulins herein detected, allowing us to focus the dairy cows' immune system studies in less variables, since some of them are redundant. For instance, total serum IgM, but not total serum IgG, was positively associated to PMN phagocytosis, oxidative burst, and surface expression of L-selectin, and can be a useful tool to assess PMN function when blood samples cannot be processed in a timely manner. It is really

important in order to reduce the costs involved in these types of analysis, enabling the researchers to explore other variables instead of spend time and energy in analysis already answered by a simple variable such as total serum IgM.

Attachment A

Submission Confirmation

Print

Thank you for your submission

Submitted to

Journal of Dairy Science

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Title

Prophylactic use of an ultra-diluted complex on health, metabolism, and performance of weaned Holstein calves and its carryover impacts up to first-lactation

Authors

Silva, Thiago

Guimarães, Iuli

Martins, Mellory

Saran Netto, Arlindo

Date Submitted

17-Jan-2021

Attachment B

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Thank you for your submission

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Title

Prophylactic use of an ultra-diluted complex on polymorphonuclear leukocyte function and respiratory scores of weaned Holstein calves immediately after grouping

Authors

Silva, Thiago

Guimarães, Iuli

Saran Netto, Arlindo

Date Submitted

15-Jan-2021

Attachment C

This manuscript will be submitted as soon as possible to the journal of dairy science. The delay is justified through the high number of animals enrolled in the study. Thus, to ensure high quality of the results presented, several screenings have been performed by Dr. Machado's team.

Attachment D

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Research paper

Associations between circulating levels of natural antibodies, total serum immunoglobulins, and polymorphonuclear leukocyte function in early postpartum dairy cows

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ABSTRACT

The objective of this study was to investigate the associations between natural antibodies (NABs) and total serum immunoglobulins (Igs) concentrations (both from IgG and IgM isotypes), with variables of polymorphonuclear leukocytes (PMN) function of dairy cows in the early postpartum period. Seventy-five healthy postpartum dairy cows at 2 ± 1 days in milk were enrolled in a cross-sectional study. Natural and total antibodies in serum samples were measured by ELISA. Flow cytometry was used to determine the phagocytosis and oxidative burst capacities of PMN and the quantification of the adhesion molecule L-selectin. Leukocyte count and differentials, and serum haptoglobin were also measured. A positive correlation between NABs_{IgM} and total serum IgM and between NABs_{IgM} and NABs_{IgG} were found. Before performing the associations between circulating Igs concentrations and PMN function variables, cows were categorized into having low, medium or high circulating antibodies based on their NABs and total Igs serum concentrations. None of the PMN variables assessed differed between low, medium, and high cows for both NABs_{IgM} and NABs_{IgG}. While associations between PMNs function and total IgG were not observed, some associations between total IgM concentrations and PMN activity were found. Cows with high serum IgM had greater phagocytic activity compared to cows in the low IgM group. Finally, the proportion of PMN that performed oxidative burst and PMN surface expression of L-selectin intensity was greater in high total IgM group when compared to medium and low groups. In conclusion, association between NABs, and PMN activity variables were not observed, but total serum IgM was associated to some PMN function variables in early post-partum dairy cows.

1. Introduction

About 40–70% of dairy cows develop metabolic or infectious diseases in the first month of lactation, depending on different management systems and milk production (Santos et al., 2010; Ribeiro et al., 2013). The occurrence of disease during the transition period is associated with a state of immune dysregulation, when exacerbated or inadequate inflammatory responses can lead to poor health outcomes (LeBlanc, 2014). Cows that have inadequate immune responses against pathogens, often characterized by suppression of polymorphonuclear leukocyte (PMN) function (Kehrli et al., 1989; Hoeben et al., 2000; Hammon et al., 2006) or decreased circulating immunoglobulin (Ig) concentrations (van Knegsel et al., 2007; Herr et al., 2011), have increased risk for metritis, endometritis, retained placenta and mastitis in early lactation (Cai et al., 1994; Kimura et al., 2002; Hammon et al., 2006).

There is a growing interest in the assessment of immunocompetence around parturition. Such information has been used to determine genetic merit for improved immunity (Mallard et al., 2015; Banos et al., 2013) and disease risk of postpartum cows (Amadori et al., 2015). Inadequate immune status around parturition is often assessed using variables related to the cellular branch of the immune system, such as PMN phagocytic and oxidative burst activity (Hammon et al., 2006; Martinez et al., 2012; Mateus et al., 2002). Others have used humoral components of the immune system, including natural antibodies (NABs) and total immunoglobulins (Igs) as markers of immunocompetence of dairy cows (Herr et al., 2011; Banos et al., 2013). To understand if these different immune variables are associated among themselves during the early postpartum period is crucial for the development of economic and practical manners of assessing the immunocompetence of postpartum dairy cows. For instance, it would be more practical to measure serum Igs concentrations, than PMN function which has been performed in fresh

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