LAILA MIYURI MORITA

Development of innate immune response in healthy Holstein calves from birth until weaning

São Paulo

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

Department:

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Advisor:

Prof. Viviani Gomes Ph.D.

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CERTIFICADO

Certificamos que a proposta intitulada "DESENVOLVIMENTO DA RESPOSTA IMUNE INATA EM BEZERRAS HOLANDESAS SAUDÁVEIS DURANTE A FASE DE ALEITAMENTO", protocolada sob o CEUA nº 5324110219 (ID 006348), sob a responsabilidade de **Viviani Gomes** *e equipe; Laila Miyuri Morita* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 27/03/2019.

We certify that the proposal "Development of Innate immune response in healthy Holstein calves from birth until weaning.", utilizing 177 Bovines (177 females), protocol number CEUA 5324110219 (ID 006348), under the responsibility of **Viviani Gomes** *and team; Laila Miyuri Morita* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 03/27/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 03/2019 a 08/2019 Área: Clínica Médica Veterinária

Origem:	Animais de proprietários						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	1 a 89 dias	N:	177
Linhagem:	Holandesa			Peso:	30 a 90 kg		

Local do experimento: Fazenda Colorado localizada em Araras - SP. Coordenadas geográficas da cidade de Araras: latitude 22o21□25□□S; longitude 47o23□03□□W. Altitude 629m. Esta fazenda é reconhecida como a maior produtora de leite do Brasil, com um rebanho de aproximadamente 1.980 vacas em lactação, produção média/vaca ao redor de 39kg, o que resulta na produção de 80.000 kg de leite ao dia/propriedade.

São Paulo, 07 de dezembro de 2019

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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EVALUATION FORM

Author: MORITA, Laila Miyuri

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Date: _____/_____/_____

Committee Members

Prof. _____

Institution:_____ Decision:_____

Prof. _____

Institution:_____ Decision:_____

Prof. _____

Institution:_____ Decision:_____

DEDICATION

To my parents, Lourdes and Pedro, for setting as an example to me and for teaching that persistence is the way to success.

To my fiancé Danilo for never stop believing in me, for his partnership and for acting as the voice to reason whenever I needed.

To my advisor Viviani Gomes and to the team at Gecria for the hard work, for never quitting and for the inspiration in team work and cooperation, always.

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I would like to thank my advisor, Professor Dr. Viviani Gomes, for her patience, trust and major participation in conducting and in the conclusion of this work. You are an example for me and for the entire team of Gecria of a hard-working person, dedicated and motivate everybody around to not be afraid of overcoming challenges or obstacles. Thank you for never giving up and for your constant support!

To Fazenda Colorado for agreeing to open the doors and allowing us to perform the experimental step with the animals there. I would like to thank the Veterinary Sérgio Soriano for the support, attention and affection demonstrated during all study period. I would also like to thank the team of employees of Fazenda Colorado that made possible to execute each procedure with animals, for facilitating our work and making our routine more flexible with our activities.

To the main people responsible for conducting the study and that lived at Fazenda Colorado, therefore: Karen Nascimento da Silva and Karinne Ávila Bosco. Without the girls the presentation of this work would not be possible, thank you for no measuring efforts to make each step work. To Karen Nascimento da Silva, my special thanks. Thank you for making this commitment to lead such complex study with care, dedication and efficiency. I admire your will, strength and energy to do things the best way possible and with joy and always with a friendly smile. I am thankful for our friendship, you have great potential, always believe on yourself!

To Gecria team and all colleagues that have dedicated part of their time for this work to happen, specially to Camila Cecilia Martin, Camila Costa Baccili, Daniela Castro Tardón and Jean Silva Ramos. Thank you for the commitment, for dedicating and engaging so much during laboratorial phase. For the consecutive days of hard work, I greatly appreciated.

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To Professor Dr. Alice Maria Melville Paiva Della Libera and her team for the encouragement and great help in sharing the *Mannheimia haemolytica* bacteria marking technique, an important step that was part of executing this work.

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“Once we accept our limits, we go beyond them”

Albert Einstein

RESUMO

MORITA, L.M. **Desenvolvimento da resposta imune inata em bezerras holandesas saudáveis durante a fase de aleitamento.** 2020. 72 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

O objetivo geral desse estudo foi avaliar o perfil hematológico e imune inato de bezerras holandesas expostas naturalmente aos microorganismos na fase de aleitamento. Este estudo transversal incluiu 175 bezerras, distribuídas em 3 grupos: G1 - 1 a 7 dias de vida (n=65), G2 - 30 a 40 dias de vida (n=52) e G3 - 60 a 89 dias de vida (n=58), distribuídos em 2 subgrupos, saudáveis e doentes, de acordo com os achados do exame clínico. Foram obtidas amostras sanguíneas em tubos sem e com os anticoagulantes EDTA e heparina, para a determinação dos marcadores bioquímicos, realização do hemograma e leucograma no equipamento ADVIA 2120 e ensios imunológicos. A técnica citômetro de fluxo foi usada para a avaliação da fagocitose das bactérias marcadas *Escherichia coli, Staphylococcus aureus* e *Mannheimia haemolytica* pelas células sanguíneas mononucleares (MN) e polimorfonucleares (PMN). Espécies reativas de oxigênio (ERO's) (AFU) foram mensuradas pelo uso do corante fluorescente Dihidrorodamina 123 por meio de fluorescência a partir de leucócitos assim calculado o response ratio (RR) para comparação entre os grupos/subgrupos. No subgrupo doente, a diarreia (46%) predominou no G1, enquanto a Doença Respiratória Bovina (DRB) foi à doença mais frequente no G2 (63%) e G3 (59%). As diferenças etárias para os parâmetros hematológicos foram encontradas contrastando-se os grupos G1 com o G2 e G3, observando-se em neonatos a tendência à diminuição dos parâmetros do RBC e ferro sérico e perfil pró-inflamatório mediante desafio natural. A maioria dos parâmetros de fagocitose (MN e PMN) mostrou-se independente da idade ou estado de saúde, no entanto a quantidade de ERO's (AFU) diminui com o avançar da idade. Os doentes do G1 apresentaram menor Brix (%) e proteínas totais com diminuição de eritrócitos, leucocitose por neutrofilia, linfopenia e maior produção de ERO´s (AFU) contra *M. haemolytica* em relação ao subgrupo G1 saudável. Doentes G2 apresentaram discretas alterações nas respostas hematológicas e imune inata, além de menor Brix (%) e proteína total em relação ao demais grupos de idade e menor resposta à produção de ERO's (AFU) e response ratio (RR) para todos os antígenos em relação

ao G2 saudável. G3 doentes em relação aos saudáveis apresentaram maior número de monócitos e ambos PMN e MN foram mais responsivos a fagocitose, ERO's e RR, especialmente contra a *M. haemolytica*. Este estudo evidenciou que os neonatos apresentaram maior intensidade da resposta imune inata contra a exposição natural aos patógenos, enquanto que o grupo de bezerras da fase intermediária (G2) apresentaram-se menos responsivas imunologicamente aos antígenos associada à alta frequência de DRB. O grupo G3 apesar de enfrentar os desafios associados à fase final do aleitamento apresentou resposta imune mais efetiva em relação aos antígenos. Acredita-se que as diferenças relatadas estão associadas com a concentração dos fatores maternos do colostro que agem intensificando a resposta imune inata nos neonatos, com queda gradual no G2 e subsequente desenvolvimento da imunidade adaptativa nas novilhas do grupo G3.

Palavras-chave: Hemácias. Leucócitos. Neutrófilos. Fagocitose. Espécies Reativas do Oxigênio (EROs).

ABSTRACT

MORITA, L.M. **Development of innate immune response in healthy Holstein calves from birth until weaning**. 2020. 72 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2020.

The general aim of this research was to evaluate the hematological and innate immune profile in Holstein calves naturally exposed to microorganisms during pre-weaning phase. It was performed a transversal study including 175 dairy calves distributed in three age groups: Group 1 - 1 to 7 days of life (n=65), Group 2 - 30 to 40 days of life (n=52) and Group 3 - 60 to 89 days of life (n=58) and also distributed in 2 subgroups, healthy and unhealthy, according to clinical examination findings. Blood samples were harvested in tubes with and without EDTA anticoagulant and heparin for Red Blood Cells (RBC) and White Blood Cell (WBC) count determination by using ADVIA 2120 system, biochemical markers and for immune response assays. The flow cytometry technique was used to assess polymorphonuclear (PMN) and mononuclear (MN) cells phagocytosis of pre labeled *Escherichia coli, Staphylococcus aureus* and *Mannheimia haemolytica*. Production of Reactive Oxygen Species (ROS) was assessed in leucocytes by use of the fluorescent dye dihydrorhodamine 123 and then the response ratio (RR) was calculated to compare data between the groups/subgroups. The predominant disease detected in unhealthy neonate calves (G1) was diarrhea (46%), while Bovine Respiratory Disease (BRD) was the most important illness in G2 (63%) and G3 (59%). Regard to age physiological response, the most differences were observed contrasting G1 with G2 and G3 observing in neonates a decrease trend of RBC and serum iron also a pro inflammatory profile under natural challenges. Most of phagocytosis parameters (MN and PMN) were independent of age or healthy groups, although the amount of ROS (AFU) decreased through advance of age. G1 unhealthy subgroup presented low concentration of total solids and total protein associated with low number of RBC and leukocytosis due to neutrophilia and lymphopenia compared with healthy subgroup. Production of ROS (AFU) for *M. haemolytica* was higher in unhealthy than healthy neonates. G2 unhealthy subgroup presented subtle changes in hematological and innate immune response, however the concentration of total solids, protein and ROS production and RR for all antigens were lower in the unhealthy group. G3 unhealthy animals presented increase of MN cells in WBC count, and both

MN and PMN cells were more phagocytic responsive for all antigens, especially *M. haemolytica,* also in ROS assay, compared with healthy calves*.* This research has evidences that neonate had a high innate immune response intensity against natural exposition to pathogens, while the intermediate heifers (G2) presented immunologically less responsive to antigens associated with the high rate of BRD. G3 group seen to be more prepared to face the challenges presented during pre-weaning phase as the immune response was more effective against the antigens. The assumption is that the differences reported are associated with the concentration of colostrum maternal factors that act enhancing the innate immune response of neonate calves, with a gradual decrease of immune response in animals with 30 to 40 days of life (G2) and subsequently development of the active adaptative response in G3.

Keywords: Red Blood Cells. White Blood Cells. Neutrophils. Phagocytosis. Reactive oxygen species (ROS).

SUMMARY

1. GENERAL INTRODUCTION

The Brazilian herd contains approximately 218 million heads of cattle and it is responsible for the production of 33.5 billion of milk and 9.5 billion tons of carcass per year (FAO, 2017). It is estimated that the export of Brazilian beef will grow 2.15% per year, and by 2020 the expectation is to supply 44.5% of the world market. Faced with this scenario, the potential of developing the calf raising system is well appreciate in order to restore the production chain (DOS SANTOS; BITTAR, 2015).

The calf raising is a challenge by the high susceptibility of neonate during the first months of age due to the immune system status at birth. While all organs and immune cells are presenting in neonates at birth, they are unable to initiate a successful immune response due to immaturity of protective mechanisms and the time delay to initiate and produce these necessary mechanisms to generate the humoral and cell mediated immune response (CORTESE, 2009).

Once the newborn calves are immunologically naïve at birth and unable to initiate and produce necessary mechanisms to generate humoral and cell-mediated immunity, an immunologic assistance through maternal immunoglobulins and other colostrum factors can provide an transitory and immunologic protection to neonate during the first weeks of life (BARRINGTON; PARISH, 2001; MOREIN; ABUSUGRA; BLOMQVIST, 2002; CHASE; HURLEY; REBER, 2008).

A successful colostrum management program requires producers to consistently provide calves with a sufficient volume of clean, high-quality colostrum within the first few hours of life (GODDEN, 2008). Among other management procedures, the main factors that influence the successful passive transfer program is the quality, quantity and how quickly the colostrum is provided to the calf. The concentration of IgG in colostrum has traditionally been considered the hallmark for evaluating colostrum quality, with high quality defined as IgG levels greater than 50 g/L (GODDEN; LOMBARD; WOOLUMS, 2019). Regarding the volume of colostrum, it is recommended that calves be fed 10% to 12% of their body weight (BW) of colostrum at first feeding that corresponds 3 to 4 L for a Holstein calf (GODDEN; LOMBARD; WOOLUMS, 2019). Moreover, the colostrum must be delivered quickly to calves in order to be absorbed efficiently the immunoglobulins. The term open gut refers to the unique ability of the neonatal enterocyte to nonselectively absorb intact large molecules, such as Ig, by pinocytosis (BROUGHTON; LECCE, 1970). In a process

referred to as closure, the absorption of Ig across the intestinal epithelium decrease linearly with time from birth to completely close at approximately 24 hours (WEAVER et al., 2000).

The IgG is recognized as a main immunological component of bovine colostrum and IgG1 is the main predominant antibody which may be responsible for 65 to 90% of the total colostrum immunoglobulin content (LARSON; HEARY; DEVERY, 1980). After the ingestion of colostrum, the serum levels of immunoglobulins reach significant concentrations in few hours (ADEREM; UNDERHILL, 1999).

Calves at birth have some level of innate function and calves immediately receiving colostrum can be supplied by the “targeting” capacity of transferred IgG1 to make the innate response work better. Calves that do not rapidly receive colostrum can still mount innate responses but have a much greater risk of disease during the neonatal period (Besser and Gay, 1994). Once the calf ingests colostrum, the IgG1 rapidly crosses the gut epithelial cells, facilitated by the transient presence of specialized Fc receptors, and enters the circulation. IgG1 antibody is the key element in immediate immune targeting for protection against microbial agents from the local environment. IgG1 antibody, because of its structural flexibility, readily enters the tissue spaces to enhance the speed and efficacy of the response by macrophages, monocytes (and the cells that differentiate from monocytes) and neutrophils by orders of magnitude. This enhancement is the core reason that failure of passive transfer is associated with markedly increased infection, disease and death in young calves (ADEREM; UNDERHILL, 1999; SMITH; METRE; PUSTERLA, 2019).

It is a fortune that transfer of antibody from colostrum is dependent primarily on the presence of a high level of IgG1, and the fact that almost any source of bovine IgG1 can be used to replace the IgG1 from the mother (QUIGLEY et al., 2001). Yet, as long as the IgG1 is transferred quickly, it will still provide the antibody “targeting” function that amplifies the tissue level innate responses needed to drive immediate protection (SMITH; METRE; PUSTERLA, 2019).

However, the immunoglobulins are not the only components involved in the immune response, the cytokines and cells transferred passively has an important role in the immune response development protecting neonatal from pathogens (DONOVAN et al., 2007a). Barrington and Parrish (2001) described that PMN leukocytes are important for cytoplasmic transport of immunoglobulins to the bloodstream of newborn calves, protecting them from enzymatic digestion during its passage through the

gastrointestinal tract. The high proportion of macrophages in colostrum of first milking has intrigued researchers and recent research has shown that these cells may be the key to the activation of specific immune system of calves, by the production of cytokines and presentation of antigens to immature lymphocytes located in secondary lymphoid organs (REBER et al., 2008). Proinflammatory cytokines IL-1 beta, IL-6, TNF-alpha and IFN-gamma are also present in maternal colostrum, believing in the possibility of its absorption by the intestinal mucosa of calves like the immunoglobulins form, reaching highest concentrations in newborns bloodstream in the first 72 h of life (MADUREIRA, 2011).

Despite of the importance of the innate immune system to protect calves from natural exposure to antigens after birth, few longitudinal studies have been done to evaluate these responses in healthy and unhealthy dairy calves. As far as I'm concerned, there are any publications about ex vivo response against *M. haemolytica* in Holstein calves during the pre-weaning phase, with exception to Batista et al. (2015) study. In addition, there are few studies published with calves' blood parameters analysis performed by ADVIA 2120 system.

2. LITERATURE REVIEW

2.1. GENERAL ASPECTS OF THE INNATE IMMUNE RESPONSE

The first defense mechanism of organism is the physical barriers that impede the penetration and invasion of microorganisms as the body skin, oral cavity, the mucosal surfaces of gastrointestinal and respiratory tract. Those pathogens that are eventually capable of overcoming physical barriers will be confronted by cellular mechanisms, through the innate immune response, which will grant initial resistance to infection (TIZARD, 2008). So, the second line of defense it's the innate immune which consists in cellular and chemical mechanisms that are capable of destroying microorganisms (TIZARD, 2008). The main types of cells involved in the innate response are neutrophils, dendritic cells (DCs), natural killer cells (NK) and macrophages that recognize, phagocyte and kill pathogenic agents and, simultaneously to this attack, orchestrate responses of hosts through the synthesis of a great variety of inflammatory mediators and cytokines (ADEREM; UNDERHILL, 1999; TIZARD, 2008). A common feature of these cell populations is their ability to

recognize and destroy foreign organisms or infected cells without previous encounter with the antigen (KAMPEN et al., 2006). However, for this to efficiently occur the immune system need to develop mechanisms to differentiate antigens potentially harmful from microbiota (KELLY; COUTTS, 2000).The innate cells recognize foreign antigens based on patterns found on the surface of foreign cells that are not present on host cells (JANEWAY, 2007). Due to the general nature of these receptors, innate cells have no “memory” and cannot distinguish specific organisms. They simply determine whether an encountered cell is “self” or “nonself” cells (JANEWAY, 2007).

The major cell blood leucocyte is the polymorphonuclear neutrophil granulocyte, otherwise called the neutrophil (TIZARD, 2008). Neutrophils constitute about 60% to 75% of the blood leucocytes in most carnivores but only about 20% to 30% in cattle. Neutrophils are the first line of innate immune cells arriving at the site of bacterial inoculation, where they exert diverse antimicrobial mechanisms to prevent pathogen dissemination to normally sterile sites through a process named phagocytosis. The neutrophils of animals when stimulated by pathogenic agents leave the circulation, adhere to the vessels and enter the epithelium through the diapedesis mechanism, thus reach the infection sites and are able to perform their phagocytose functions (TIZARD, 2008).

After chemotaxis, the neutrophil must adhere to the bacterium which requires that the microorganism be coated with opsonins in order to promote the phagocytosis. Antibodies and complement components usually act synergistically in the opsonization of particles. Receptors for IgG and complement are the best characterized receptors involved in recognition and phagocytosis, but receptors for IgE and IgA may also serve as opsonin receptors (HIEMSTRA; DAHA, 1998).

During this phagocytosis, the release of intracellular enzymes and cytokines occurs and production of free radicals and reactive oxygen species, which have the function of killing the pathogenic microorganisms (ADEREM; UNDERHILL, 1999). Phagocytosed organisms will be digested within the lysosome resulting in multiple antigenic particles which can then be presented by macrophages and dendritic cells to the B and T cells to initiate cell replication and production of receptors specific for the presented antigen (MOSTOV, 1994). The macrophages also act in the production of anti-inflammatory cytokines that regulate the systemic response (TIZARD, 2008). Macrophages and dendritic cells recognize different patterns expressed in different families of microorganisms upon recognition of a foreign antigen, Pathogen-Associated

Molecular Patterns (PAMPs) are derived from microorganisms and are recognized by Pattern Recognition Receptors (PRRs), which are found on innate immune cells as well as many epithelial cells (JANEWAY, 2007). The initial response of activated macrophages after antigen recognition is the production of proinflammatory cytokines IL-1α, IL-6, IFN-γ and TNFα, which are responsible for recruiting and activating more innate cells (neutrophils and monocytes) for circulation which intensify the inflammatory response in conjunction with the activation of other antimicrobial molecular factors ((JANEWAY, 2007).

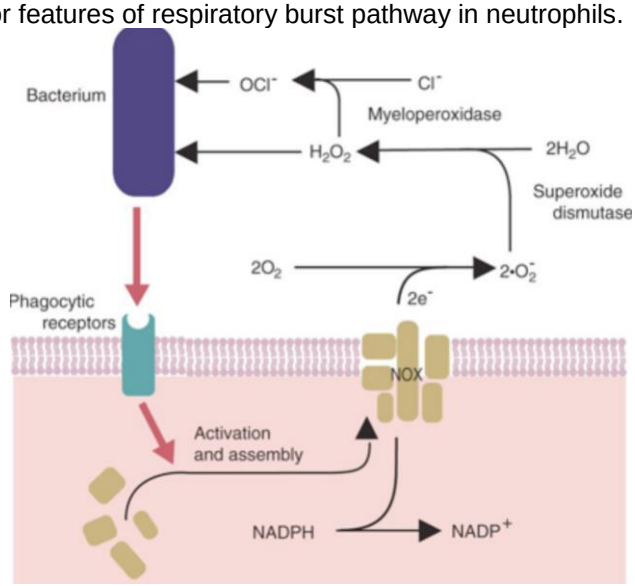
The process by which neutrophils kill invading pathogens depends on two primary mechanisms: production of highly toxic reactive oxygen species (ROS) in the pathogen-containing vacuole and fusion of neutrophil granules, containing various antimicrobial mediators to the vacuole (TIZARD, 2008). Coincident with phagocytosis of bacteria, neutrophils produce an oxidative burst that is triggered by cytokines such as TNFα or by binding of opsonized bacteria to phagocytic receptor (Figure 01). This process results in the rapid release of high levels of bactericidal reactive chemical species under the catalyzation of NADPH oxidase (NOX), myeloperoxidase (MPO), or nitric oxide (NO) synthetase (JANEWAY, 2007). NADPH oxidase is responsible for the generation of ROS, such as superoxide anion (O^{-}_{2}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (HO) (JANEWAY, 2007). Superoxide anion interacts spontaneously (dismutate) to generate one molecule of H_2O_2 under the influence of the enzyme superoxide dismutase (SOD) (TIZARD, 2008):

$$2O_2 + NADPH \xrightarrow{NOX} 2O_2^{-} + NADP^{+} + H^{+}$$

$$2O_2^{-} + 2H^{+} \xrightarrow{SOD} H_2O_2^{-} + O_2$$

Because this reaction occurs so rapidly, superoxide anion does not accumulate but H_2O_2 does. The hydrogen peroxide is converted to bactericidal compounds through the action of myeloperoxidase, the most significant respiratory burst enzyme in neutrophil granules. Myeloperoxidase catalyzes the reaction between hydrogen peroxide and intracellular ions to produce bactericidal radicals as hypocloride ions (OCl^{-}) (TIZARD, 2008).

Figure 01: The major features of respiratory burst pathway in neutrophils.



Source: TIZARD, 2008.

While many bacteria are killed by oxidation, the phagosomes continue to mature. Pathogens sequestered by neutrophils are trafficked to and fused with the phagosome in a process called degranulation, leading to the killing of invading pathogens in a process involving the release and action of proteinases and peptidases (TIZARD, 2008).

After inactivation of pathogens by neutrophils phagocytosis, the respiratory burst and the excessive number of neutrophils leads to host tissue injury damaging the surrounding tissues. To prevent senescent neutrophils from releasing their toxic contents, these cells become apoptotic and are then recognized, engulfed and cleared by professional phagocytes such as tissue macrophages (SERHAN; SAVILL, 2005). In infected tissues, their apoptosis can be delayed both by microbial constituents and by pro-inflammatory stimuli (COLOTA et al., 1992). Generally, the tissue neutrophils die in apoptosis; however, if the infection is serious enough, some undergo necrosis or other styles of cell death as autophagy.

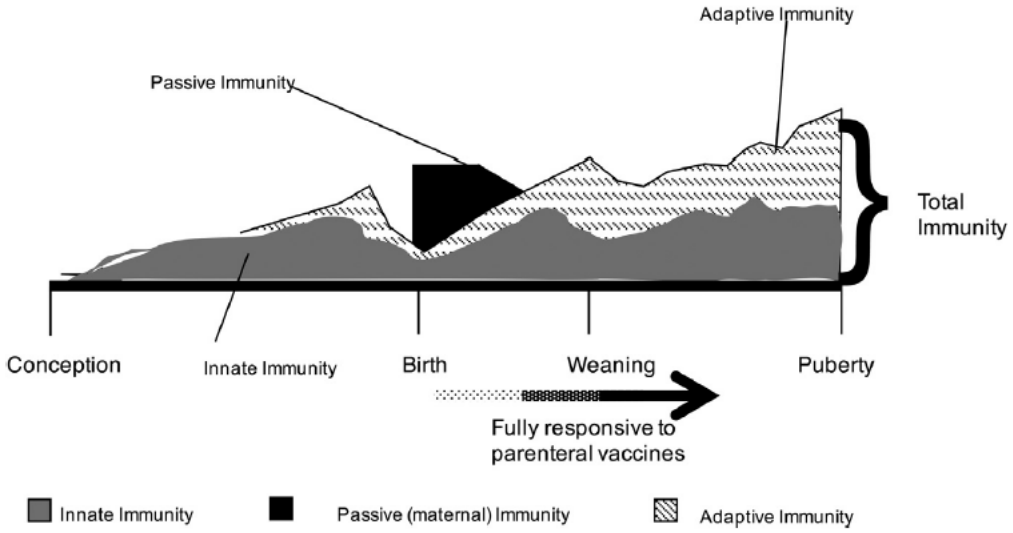
2.2 PARTICULARITIES OF THE INNATE IMMUNE RESPONSE IN CALVES

The sinepitheliochorial placenta type of bovines does not allow the transference of maternal antibodies to fetus during pregnancy leading to birth of agammaglobulinemic calves (TIZARD, 2008). The protection to fetus is granted by innate immune system in sterile uterine environment (Figure 2). The acquired immune response is composed by all of cells components as antibodies, memory lymphocytes

and effector cells and it is present in fetal calves (BARRINGTON; PARISH, 2001; CHASE; HURLEY; REBER, 2008).

When born, neonates emerge from a sterile uterus to an environment, where they are immediately exposed to uncountable challenges and microorganisms (BARRINGTON; PARISH, 2001). Regardless the fact of neonate calves are born with an immune system developed during gestation, they are considered functionally immature and unable to initiate and produce a successful immune response to generate humoral and cell-mediated mechanisms (BARRINGTON; PARISH, 2001). The development of specific immune system is a gradual process, and it will be becoming similar to the adult animal around the sixth to the eighth month of life (KAMPEN et al., 2006).

Figure 2 – Development of immune response in the calf from conception until puberty



Source: Chase, 2018.

By late gestation, the innate immune response mediated by phagocytic cells as neutrophils and macrophages is active, but not fully developed. After birth, phagocytic efficiency increases rapidly, although the time course of this increase is not known exactly. This increase of polymorphonuclear cells phagocytic activity after birth is supported by the ingestion of colostrum.

At the end of pregnancy and during calving, cows in the pre-birth period produce estrogen in response to fetal cortisol, both hormones have immunosuppressive effect in the dams and neonate. During the birth process, calves also produce high levels of cortisol that remain elevated during the first week of life (NOVO et al., 2015).

Cortisol is a stress hormone, and it induces changes in leukocyte profile. Specifically, the changes brought on by stress or glucocorticoids treatment increase the numbers of neutrophils (neutrophilia) and decrease lymphocytes numbers (lymphopenia or lymphocytopenia). Moreover, since numbers of neutrophils and lymphocytes are affected by stress in opposite directions, researches have often considered the ratio of one to the other, that is, the relative proportion of neutrophils and lymphocytes (N:L) in mammals (DAVIS; MANEY; MAERZ, 2008). Cortisol stimulates the release of segmented neutrophils from the bone marrow compartment to the circulation. In addition, cortisol reduces the expression of adhesion molecules and decreasing the connection of neutrophils and endothelial cells during the migration process, which results in neutrophilia and reduction the number of neutrophils in marginal compartment. Other hypothesis that could explain the neutrophilia would be the stimulation of stem cells by the increase of colony-stimulating factor of granulocytes (SCHALM, 2010).

Mallard et al. (1998) discussed the association with the immunological alterations in cow peripartum and its implications in calves. These authors showed some evidences that demonstrate reduction of innate immunization mechanisms in calves three weeks before birthing up to three weeks after birth according to numerous studies evaluated by them. For example, it’s described the influence of glucocorticoids that have a suppress effect on immune responses during the peripartum period. In addition, the study describes the importance to maximize the response to vaccination choosing an interval of 3 weeks precalving in order to offer an appropriate protective response for calves. The authors concluded that peripartum immune dysfunction has ramifications for the health of both cow and calf (MALLARD et al., 1998).

The birth process has a great impact in the immune system of the newborn calf due to the release of corticosteroids during this process. The type of labor which the calves are submitted, the intensity of obstetric maneuvers and health of the cow are also related to the release of corticoids (BENESI et al., 2012a). Neutrophilic profile has been reported previously in Holstein calves by Rocha et al. (2010), Benesi et al. (2012b), Novo et al. (2015), Baccili et al. (2018).The alterations present in the leucogram of newborn calves are compatible with the stress leucogram, being characterized by neutrophilia with lymphopenia, monocytopenia and low eosinophil counts (ROCHA et al., 2010).

Benesi et al. (2012b) observed a greater count of total leucocytes in animals with 0 to 8 hours of life, with the neutrophils responsible for this increase. A posterior reduction occurred of the number of leucocytes up to its minimum value with 4 days of life returning to the normal values between 12 and 14 days after birth. Zwahlen and Roth (1990) found higher total white cell counts, due to the increase of neutrophils, in the blood of calves with 48 hours of life when compared to adult animals (ZWAHLEN; ROTH; WYDER-WALTHER, 1990).

Novo et al. (2015) evaluated the hematological profile of Holstein calves during first month of life and observed a leukocytosis due to neutrophilia and eosinopenia during first days of life. A gradual increase of lymphocytes with increase of age was also observed. Guarantee a good management that allow the post-natal adaptation can favors the reverse the neutrophilic to lymphocytic pattern in calves after birth, and it can be rapidly happen in the 3rd or 4th days of life (NOVO et al., 2015).

The hematological profile of calves from birth to 6 months of age was evaluated by Baccili et al. (2018) as well. The results in their study showed a hemoconcentration right after birth and a gradual decrease in hematimetric rates until 6 months. Regarding leukogram, neonatal presented a stress profile with neutrophilia with an inversion to lymphocytic profile after with 4 days of life.

Brun-Hansen et al (2006) evaluated age-related changes in hematological values in calves from birth to 6 months of age using ADVIA 120 hematology system. They have found interindividual variation in RBC (red blood cells) count, hematocrit, hemoglobin levels, MCHC, and monocyte and eosinophil counts were larger the first 5 to 8 weeks of life than later. The MCV, RDW, and neutrophil, lymphocyte, and platelet counts showed most changes during the same early time period and tended to stabilize thereafter. These results showed that the transition from a fetal to a neonatal environment involves large physiologic changes (BRUN-HANSEN et al., 2006).

Despite a transient increase in number of polymorphonuclear leukocytes (PMN) in the early of life, there is decrease of their functional cellular activity, affecting the rates of phagocytosis and bactericidal capacity due to the low capacity to produce ROS (COSTA; 2017).

The macrophages and dendritic cells of newborn are also present in low number with reduced functional activity, observing low capacity of antigen presentation in the early of life (CHASE, 2018). According to Kelly and Coutts (2000) the main neonatal

limitation for triggering a suitable immune response refers mainly to deficiency in the presentation of antigens, especially in the function of cells responsible for this function.

The low rate of phagocytosis by polymorphonuclear (neutrophils) and mononuclear cells (macrophages and dendritic cells) reported for neonate in the early of life can be influenced by the low concentration of opsonins immediately after birth. Calves are agammaglobulinemic and these animals also are deficient in some components of the complement system, resulting in a poor opsonization activity that is reflected in an increase in susceptibility to infection (TIZARD, 2008). Cortese (2009) suggests that the complement system of newborn only reaches the levels of adult animal animals at 6 months of age, having effectiveness ranging from 12 to 60% in relation to adult animals.

In addition to the limitations presented in this section considering the innate immune response, the newborns have a greater number of suppressor T cells (CORTESE, 2009). Kampen et al. (2006) described in their study that the T-cell subpopulations are present in peripheral blood of calves at levels comparable with adult values, while the B-cell population increases significantly with age.

The functional deficit of immunologic response lasts for some time after birth, leaving the newborn vulnerable to bacterial and viral attack (KELLY; COUTTS, 2000). Therefore, no discussion on bovine neonatal immunology is complete without considering an important component of the newborn calf's defense mechanism, colostrum. (CORTESE, 2009).

2.3 POTENTIATION OF INNATE IMMUNE RESPONSE MEDIATED BY MATERNAL COLOSTRUM COMPONENTS

In order to survive, the newborn animal will have to receive an immunologic support that is provided by maternal colostrum. The colostrum is mainly consisted by immunoglobins, cytokines and other immune factors and become essential to newborn calves for providing an immunology protection during the first 2 to 4 weeks of life of calves (CHASE; HURLEY; REBER, 2008; CORTESE, 2009).

It is estimated that bovine colostrum has from $1x10^6$ to $3x10^6$ cells/mL, among which 32% are viable (CHASE; HURLEY; REBER, 2008; GOMES et al., 2011; SILVA, 2014). The population of leucocytes in colostrum consists in granulocytes and mononuclear cells, including neutrophils, macrophages and lymphocyte and epithelial

cells (BARRINGTON; PARISH, 2001). Watson (1980) suggested that colostrum-derived leukocytes could be transferred to the neonatal calf. The passage of cells through the gastrointestinal epithelium has been reported in several species (including cattle) (SHELDRAKE; HUSBAND, 1985; TUBOLY et al., 1988; WILLIAMS, 1993; REBER et al., 2006). Fluorescent maternal leukocytes were detected in the blood, Peyer's patch and lymph nodes in newborn calves (LIEBLER-TENORIO et al., 2002; REBER et al., 2006; ALDRIDGE et al., 1998). These cells have been shown to circulate in the neonatal calf (REBER et al., 2006), and to alter both innate and specific immune responses in the calf during the first few days of life (RIDDEL-CASPARI, 1993; REBER et al., 2005; DONOVAN et al., 2007a; COSTA et al., 2017; NOVO et al., 2017a; NOVO et al., 2017b;).

The cytokines present in the colostrum are important for the immune development of neonatal. The origin of these cytokines is not fully explained, being passively secreted in mammary gland, produced by leucocytes present in the colostrum, or both (CHASE; HURLEY; REBER, 2008). The main cytokines present in bovine colostrum has pro-inflammatory profile such as lL 1β, lL-6, TNFα e IFNγ (GOTO et al., 1997; HAGIWARA et al., 2000; HAGIWARA et al., 2008). These cytokines probably are transferred from gut lumen to the neonate blood, because it is undetectable before colostrum intake observing a transitory increase of its concentration immediately after colostrum feeding on D3 (YAMANAKA et al., 2003a; SHECAIRA, 2013). Researchers believe that these cytokines intensify the innate immune response in the perinatal period by the increase of the phagocytosis, which also influences in the antigen presentation and subsequent development of the specific immune response. Hagiwara et al. (2001) reported an increase in the neutrophils ROS production and lymphocytes proliferation after oral treatment of neonate calves with IL-1β. Yamanaka et al. (2003b) also reported that the pro-inflammatory cytokines increase the proliferation of mononuclear cells expressing CD25 and IL-2.

Finally, the third component of colostrum is the immunoglobulins. *Colostrogenesis* is the process whereby antibodies are transferred to prepartum from the maternal circulation into the mammary secretions. In cattle, this process begins several weeks before parturition and ends abruptly at the moment of parturition. During this period, up to 500 g/week of IgG are transferred into mammary secretions (BRANDON et al., 1971) which make this immunoglobulin the hallmark of colostrum formation (PORTER, 1972). IgG can activates complement proteins, and it is readily

transferred across various membranes within the body (MOSTOV, 1994). In addition to complement activation, IgG can also neutralize antigens and mark them for phagocytosis by macrophages and neutrophils thereby increasing the efficiency of the innate immune response(ADEREM; UNDERHILL, 1999).

The colostrum ingestion is capable to influence the immune response of calves as demonstrated by Costa et al. (2017). The research aimed to evaluate the influence of maternal cells from colostrum on the development and function of innate immune response of Holstein calves. Through phagocytosis and radical oxygen species assessment it was possible to conclude that calves who received fresh colostrum appeared to have somewhat enhanced neonatal innate immune response against natural exposure to diarrhea compared to the group that received frozen colostrum (COSTA et al., 2017). Menge et al. (1998) analyzed characteristics of blood phagocytes from calves' groups colostrum deprived or not. Colostrum ingestion was accompanied by an increase in the percentage of phagocytizing polymorphonuclear leucocytes and monocytes, phenomena absent in colostrum deprived calves (MENGE et al., 1998).

The neutrophil function in calves during the first 6 months of life was evaluated by Kampen et al. (2006). Neutrophil phagocytosis, respiratory burst and bactericidal activity were measured in fifteen calves. The results of the assay showed that the neutrophil function is intact and functional from the first week of life. Furthermore, the proportion of phagocytosing granulocytes stayed quite similar in all age groups (KAMPEN et al., 2006). On the other hand, Batista et al. (2015) evaluated the phagocytic function of neutrophils of twelve Holstein calves during the first 90 days of life. It was observed fewer phagocytes activity from 40 days of life (BATISTA et al., 2015). Menge et al. (1998) estimated the functional maturity of the phagocytic defense in neonatal calves as well. Neonate calves without colostrum supplementation presented lower phagocytic activity of polymorphonuclear leucocytes stimulated with *Escherichia coli*. However, the oxidative burst activity was higher in newborn calves.

3. OBJECTIVE

The innate immune system of calves is extremely important, and it is independent of prior antigenic exposure and immune maturation. Despite of the importance of the components of innate immune response on the protection of calves

during their immune maturation, few longitudinal studies have been done to evaluate innate immune response comparing unhealthy and healthy calves. Furthermore, there are few studies published with cattle blood parameters analysis performed by ADVIA 2120 system.

The hypothesis of this research is that the blood parameters and innate immune response are influenced by the age and health status of calves. So, the general aim of this study was to evaluate the hematological profile and the innate immune response in Holstein calves from birth up to weaning-phase. In addition to age evaluation, this research also investigated the immune response against *Escherichia coli* (*E. coli*), *Staphylococcus aureus (S. aureus)* and *Mannheimia haemolytica (M. haemolytica)* in face to natural/environmental challenges during the first three months of life.

4 HEMATOLOGICAL PROFILE AND INNATE IMMUNE RESPONSE IN HOLSTEIN CALVES NATURALLY EXPOSED TO PATHOGENS IN THE PRE-WEANING PERIOD

4.1 INTRODUCTION

Newborn calves are born with a complete and developed immune system, however it is still a naïve and immature. The prerequisite for the developing of the adaptative immune response is prior exposure to antigens that will happen over time, from birth until puberty. So, the innate immune response independently of the previous antigens' presentation will be primordial to calves survive in the first months of life (CHASE; HURLEY; REBER, 2008).

Maternal factors transfer to calves from colostrum intake are essential to enhance the innate immune response against the natural challenges in the early of life such as antibodies, cells and other factors in colostrum (CORTESE, 2009). IgG1 is the main colostrum bioactive factor and it is a small molecule readily transferred across various membranes to the tissues within the body (MOSTOV, 1994). This protein can activate complement system, besides neutralizing and marking antigens for phagocytosis by macrophages and neutrophils, increasing the efficiency of the innate immune response (ADEREM; UNDERHILL, 1999).

Colostrum also contains around 1-2 millions of different types of leukocytes, and the most of them are macrophages and epithelial cells. These cells are specialized to

antigen-presentation and pro-inflammatory cytokines production such as IL 1β, IL-6, TNFα e IFNγ (GOTO et al., 1997; HAGIWARA et al., 2008; HAGIWARA et al., 2000). Researchers believe that these cytokines intensify the innate immune response in the perinatal period by the increase of the phagocytosis, which also influences in the antigen presentation and subsequent development of the specific immune response (HAGIWARA et al., 2001; Yamanaka et al., 2003b). Also, as reported by Costa et al (2017), the colostrum ingestion is capable to influence the immune response of neonatal calves that presented an innate immune response more quickly and efficiently after natural exposure to pathogens after receiving colostrum compared to the group that received frozen colostrum.

In our concern, there are few studies that have evaluated the innate immune response in calves in pre-weaning calves. Additionally, conflicting data exist regarding the functional responsiveness of neutrophils against Gram-positive and Gram-negative bacteria in dairy calves according to age. Menge et al. (1998) reported decreased neutrophils phagocytic activity against E*. coli* in neonatal calves compared to older calves (3–9-week-old), whereas the respiratory burst activity has been shown to be higher. On the other hand, , Batista et al. (2015) reported higher rate of phagocytosis of *E. coli* by granulocytic cells in calves with one week of life than occurring in older animals with no differences for production of reactive oxygen species over time. Kampen et al. (2006) evaluated the neutrophil function of calves during the first 6 months of life. They have performed phagocytosis and respiratory burst assays using the flow cytometer and showed that neutrophilic granulocytes are functional and able to mount an effective response in young calves from the first week of life. Also, many of the parameters showed the most obvious changes during the first 5–8 weeks of life.

The intense and uncontrolled innate immune response can turn on an inflammatory status. In this context, red blood cells (RBC) and write blood cells (WBC) parameters represent as important tool to detect the inflammatory profile in dairy calves (KNOWLES et al., 2000, BENESI et al., 2012a; NOVO et al., 2015; BACCILI et al., 2018). However, it could be interpretative with caution due to the physiological age-variation in the first months of life and the standardizing of the technique used to establish the reference values. The ADVIA hematology system is widely used nowadays, however the hematological reference parameters values for calves determined for this equipment have been reported only by Brun-Hansen et al (2006) with measurements in Norwegian Red calves from 1 week up to 6 months and

Panousis et al (2018) with focus in hematologic profile of Holstein calves during neonatal period.

Despite of the importance of the innate immune response to protect dairy calves in the early of life against a high range of pathogens, the literature about this subject is scarce. The most of previous researches have used health calves and did not report the capacity immune system response against natural pathogen challenge.

A description of neutrophil responses in calves with different health status followed over time is lacking. Furthermore, there is relatively few information regarding the roles and function of blood phagocytes.

The hypothesis of this research is that the blood parameters and innate immune response is influenced by the age and health status of calves. So, the general aim of this study was to evaluate the hematological profile and the innate immune response in Holstein calves from birth up to weaning-phase. In addition to age evaluation, this research also investigated the immune response against *Escherichia coli, Staphylococcus aureus* and *Mannheimia haemolytica* in face to natural/environmental challenges during the first three months of life.

4.2 MATERIALS AND METHODS

This research used clinical and laboratory data for convenience previously collected from a major research project developed by the team denominated “Use of immunostimulant to prevent BRD in dairy Holsteins calves in individual housing: Influence on health, performance, hematological and immune response”. This research has approval of the Animal Use Ethics Committee of FMVZ-USP, certificate CEUA No. 5324110219.

4.2.1 Farm

In this research data were selected arising from 226 calves provided from a commercial herd located in Araras city, west region of the State of São Paulo, with the following geographic coordinates: latitude 22° 21’ 25” S; longitude 47° 23’ 03” W; and Altitude 629 m. This farm is recognized as the greatest milk producer in Brazil, with a herd of approximately 1,980 lactating cows, average/cow production a day equivalent to 39 kg, which results in a daily production of 80,000 kg of milk.

4.2.2 Calves and management

In this project, 175 Holsteins calves were included between the 1st and the 89th day of life. All animals that were included were tested for success of passive transfer of maternal immunoglobulin through serum total solids (minimum of 8.4%) and serum total protein (minimum of 5.5 g/dL) (QUIGLEY et al., 2013; DEELEN et al., 2014). The heifers were distributed into three experimental groups, according to the age: Group 1 (G1) - 1 to 7 days of life, Group 2 (G2) - 30 to 40 days of life and Group 3 (G3) - 60 to 89 days of life. In addition to age segregation, in each age group the animals were also distributed according to their health status: G1 healthy = 26 and unhealthy= 39; G2 healthy = 28 and unhealthy= 24; G3 healthy = 36 and unhealthy = 22.

The healthy status of calves was established after the general clinical examination and respiratory and diarrhea diseases were identified following the Calf Health Scoring Criteria of Wisconsin University (McGUIRK, 2008; POULSEN; McGUIRK, 2009) and RBC parameters. The fecal score was classified according to the following score: 0= normal consistency; 1= pasty, semi formed; 2= pasty with largest amount of water; 3= liquid with fecal content adhered in the perineum and tail. The animals were considered with diarrhea when presented score 2 or 3. The respiratory disease score is composed by the following parameters: rectal temperature, cough, nasal or ocular discharge and head/ear positioning. The classification is based on intensity of this findings with a score that goes from 0 to 3. The sum of the total score above or equal than 5 was considered as bovine respiratory disease.

The navel score after umbilicus evaluation was determined and any abnormality (presence of pain, bigger size than normal, evidence of swelling) was registered.

Anemia was determined when the animals presented number of erythrocytes lower than 6 $x10^6/\mu L$ or packed cell volume lower than 18% (JEZEK et al., 2011) or when during the clinical exam it was observed that more than 2 mucosal examinations (ocular, oral or vulvar) was found pallid.

The pink eye was detected by clinical exam only in animals with 60 – 89 days of life. Some abnormalities described as pink eye signs (ANGELOS et al., 2015) as increase of lacrimation, epiphora, edema and hyperpigmentation of ocular mucosa were considered during clinical examination.

4.2.3 Feed management of animals

The calves were nursed with seven liters of waste milk up to their 24th day of life, divided in two meals. As from the 25th day old, the calves started to take milk substitute and received eight liters of milk replacer/day up to the beginning of weaning which took place between the 69 and 80 days. The solids of the waste milk were corrected to reach 14%. On the 1st day of weaning the animals were supplied with a reduced milk volume from eight to five liters of milk in two meals (3 days), afterwards, the volume was reduced to four (3rd day) and three liters (5th day) of milk until the complete weaning. During this phase, water and ration were available *ad libitum*. In the farm routine, the heifers were selected weekly for weaning, according to a visual analysis of the calves' sizes. The animals from G3 were included in this study during this adaptative phase of weaning.

Calves received two doses of intranasal vaccine (Inforce®, Zoetis) on the 1st week of live and between D30 and D40. The dehorning with iron was made one week before starting the weaning. After the dehorn procedure, the collaborators apply iodide at 5% in the lesions and tetracycline in powder. As from the weaning, the calves received two doses of injectable commercial vaccine for BRD (Cattle Master Gold FP5®, Zoetis). The calves received preventive dose of Baycox® (Bayer) for Eimeriosis with 10 days after the weaning, moment in which the weight of the calves was estimated with the use of thoracic weight tape.

4.2.4 Blood samples

Blood samples for Hemogram, Leucogram and reactive oxygen species (ROS) by leucocytes analysis were obtained from 3 tubes with Ethylenediamine Tetracetic Acid (EDTA) by external jugular vein puncture with the vacutainer system and 2 tubes with heparin were used for ROS production and phagocytosis analysis, respectively. After sampling, the blood was transported from farm to the College of Veterinary Medicine and Animal Science of the University of São Paulo in the maximal interval of three hours.

4.2.4.1 Blood Cell Count

Complete blood count (RBC) was performed with an automated hematology system (ADVIA 2120i, Siemens Healthcare Diagnostics, Tarrytown NY, USA) that combines classic hematological variables with individual cell indices. The variables evaluated were hematocrit value (HCT), hemoglobin concentration (HGB), cellular hemoglobin content (CH), cellular hemoglobin content of mature red blood cells (CHm), red blood cells count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), corpuscular hemoglobin concentration mean (CHCM), hemoglobin concentration distribution width (HDW) and RBC distribution width (RDW).

WBC count and differential WBC counts were also performed. In ADVIA 2120, the peroxidase method was used to evaluate WBC (WBCP). In the peroxidase method, the RBCs are lysed, and peroxidase reagents are used to distinguish between peroxidase-positive cells, such as neutrophils, eosinophils, and monocytes, and peroxidase-negative cells, which include lymphocytes, basophils and “large unstained cells” (LUCs). This channel allowed also to determine the lobularity index (LI), which is the relationship between the number of cells producing high light scattering (PMN with higher lobularity) and cells with lower light scattering (mononuclear, immature granulocytes and blasts) (BRAGA, 2014). Platelet indices were analyzed and included platelets count (PLT) and mean platelet volume (MPV).

In addition to the above-mentioned variables, we evaluated the following reticulocyte indices: absolute reticulocyte count (Ret $x10^9$/L), percentage of reticulocytes (Ret %), cellular hemoglobin content of mature red blood cells (CHm) and cellular hemoglobin content of reticulocytes (CHr).

4.2.5 Serum Iron

The serum iron determination was performed with a commercial kit IRON/UIBC (Randox®), following the manufacturer instructions.

4.2.6 Inflammation monitoring

Inflammation was monitored by determination of total serum protein, total serum solids and haptoglobin. Plasma total protein (g/dL) was estimated by using an optical

refractometer[1] with a range of 0 to 12 g/dL. The percentage of solids in blood plasma was measured by using a Brix refractometer[2].

The concentration of haptoglobin was assessed by turbidometry assay, considering the principle of haptoglobin and meta-hemoglobin binding according to the method descripted by Jones and Mold (1984) and Bastos et al. (2013). The standard curve was prepared using a serial dilution of control serum. The determination of serum haptoglobin concentration was calculated by interpolation of the linear regression of the standard curve for each assay after reading of the absorbance with a microplate reader at a wavelength of 450 nm.

4.2.6 Phagocytosis assessment

The phagocytosis of bacteria *S. aureus, E. coli* and *M. haemolytica* was determined by the flow cytometry technique using tubes made of polypropylene that are suitable for flow cytometer.

To perform the phagocytosis assay, 100µL of heparinized blood were incubated with 12 µL *Staphylococcus aureus*[3] and 12 µL *Escherichia coli*[4] pre labelled and *M. haemolytica* was labeled with R-Phycoeritrin according to previously description made by Hasui et al. (1989) modified by Batista et al. (2018).The volume of *M. haemolytica* solution used in the phagocytosis assays was 125 µL (BATISTA et al., 2018). The final volume of all assays was adjusted for 300 µL by the addition of 300 µL of supplemented with 10% of bovine fetal serum[5]. The mixture was incubated for one hour in an incubator of CO_2 at 37 °C. The assay was stopped by addition of 2 mL of cold 3 mM EDTA per tube, following by the centrifugation at 1200 rpm for 5 minutes. Red blood cells were lysed by using 1mL of hypotonic solution followed by 1 mL of PBS 2× concentrated. In the final process, the pellet was suspended in 300 µL of PBS (Phosphate buffered solution) for flow assessment.

The assessment was performed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Diego, CA). For each sample, 20,000 events were acquired in the granulocyte forward angle and 90 light scatter gate using

[1] Model EEQ9042, *Astral Científica*, *Curitiba, Brazil*
[2] Model: Q767-1, Precision & Instrument, Shangai, China
[3] Phrodo S. aureus bioparticles conjug – batch 1834968, Life Technologies do Brasil
[4] Conjugado E.coli pHrodo BioParticles – batch 1875872
[5] Hyclon, Logan, UT n° cat. G6392

the CellQuest® software (Becton Dickinson Immunocytometry Systems, San Diego CA).

The recorded data were analyzed using Flow Jo software, version 7.6.1 for Windows (Tree Star Inc., Ashland, OR). The mononuclear (monocytes and lymphocytes) and granulocyte gated was established in samples containing cells only, and the gate was copied to the other assays from the same animal for consistency. Similar process was established the threshold to determine the frequency (%) of positive cells for fluorescence FL2.

4.2.7 Reactive Oxygen Species (ROS) assessment

Production of ROS was assessed by use of the fluorescent dye dihydrorhodamine 123 (batch 1861629, Life Techonologies do Brasil) as described by Donovan et al. (2007b) and Nace et al. (2014), with certain modifications. Initially, 3 mL of blood were added in silicon tubes of 15 mL sterile. Initially the blood was lysed by the addition of 6 mL of Tris-chloride ammonia lysis solution, incubated at 37 °C for 30 minutes. After the incubation, the tubes were centrifuged in 290xg for 10 minutes, afterwards, the cells were washed in 5 mL of salt buffered solution twice at 290xg for 5 minutes. After the last wash, the supernatant was discarded, and the leucocytes diluted in 1 mL of RPMI1640 cell culture medium without red phenol[6] supplemented with 10% of fetal serum inactivated by heat and 2 mM L-glutamine[7]. The concentration and viability of leucocytes was determined by Trypan Blue exclusion. For this, 50 µL of cell suspension was diluted in 450 µL of Trypan Blue 0.04%. From this mixture, 10 µL was pipetted in Newbauer chamber, counting the number of living and dead cells in a quadrant of the chamber specific for counting leucocytes. Finally, the cell suspension was adjusted to $3x10^6$ cells/mL.

The leucocytes suspension (100 µL) was plated in flat bottom cell cultivation plates, being afterwards diluted in 100 µL of supplemented cell cultivation medium (none stimulated leucocytes) or in a cell cultivation medium containing specific treatment (stimulated leucocytes). During method validation of ROS assay, the higher was the concentration tested of bacteria, more activated was the response for ROS production. The cells were stimulated with PMA, *Escherichia coli* 1:10, *Staphylococcus*

[6] Cat no.: 7509, Sigma-Aldreich, St Louis, MO
[7] Cat no.:21051-024, Gibco®, Brazil

aureus 1:5, *Mannheimia haemolytica* and *Mannheimia haemolytica* 1:5 (prepared in School of Veterinary Medicine and Animal Science of the University of São Paulo) and Phorbol Myristate Acetate[8]. The bacteria dilutions used were determined in previous pilots in which was obtained the maximum production of ERO's after the incubation of leucocytes with the stimulus. Finally, 10 µL of subtract DHR with final concentration of 10 µM was added in each well, except for the negative control containing only the medium (medium background).

The plates were incubated for two hours in CO_2 incubator at 37 °C. The fluorescent reading was performed in Fluoroskan Ascent FL (Thermo Scientific), excitation 485 nm and emission 538 nm. The fluorescence was obtained with the arbitrary fluorescence unit (AFU). In each test, the PMA was used as a maximum positive control and RPMI-1640 medium was used as negative control. Production of ROS at each concentration of each stimulus is presented as a response ratio (RR), calculated as follows:

$$\text{Response ratio (RR)} = \frac{\text{AFU value for stimulated cells}}{\text{FU value for ustimulated cells}}$$

4.3 STATISTICAL ANALYSIS

All data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). The effects of groups and healthy status were estimated by the PROC GLM. Data were tested for residue normality and variance homogeneity. Whenever necessary, variables that did not comply with these statistical premises were subjected to transformations in order to obey these statistical assumptions (e.g. log10, square root, inverse). The statistical system generated for each parameter the values considered as outliers. Some outliers were removed from the statistical analysis when biologically justified. Differences between groups and healthy status were analyzed using a parametric test with the general linear model procedure for each factor separately. Differences between age groups were analyzed using the Tukey's test and to detect differences between unhealthy and healthy animals it was used Student t-test. Results were reported as untransformed means ± standard error of the mean (S.E.M). A probability value of $P \leq 0.05$ was considered significant.

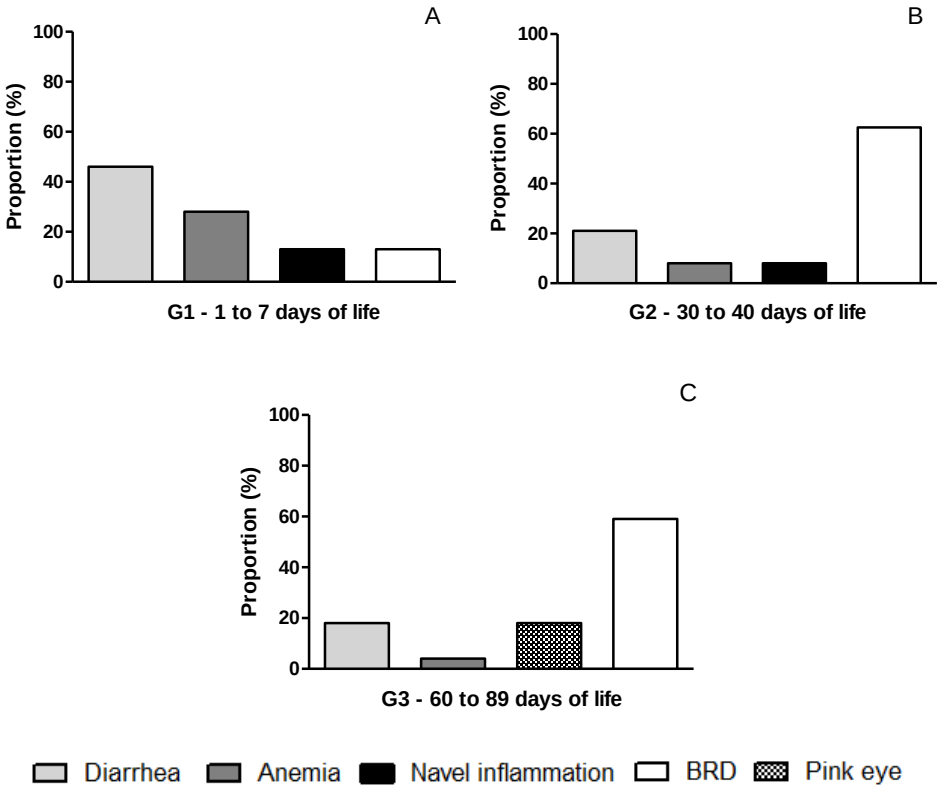
[8] PMA - P8139, Merck Milipore, Darmsdtadt, Germany, Sigma

5. RESULTS

5.1 INCIDENCE OF DISEASES IN UNHEALTHY ANIMALS

The proportion of diseases detected in each age group in unhealthy dairy calves' subgroup is illustrated on Figure 3A, 3B and 3C. The most prevalent disease detected in dairy calves from 1 up to 7 days of life was diarrhea (46%) followed by anemia (28%), navel inflammation (13%) and Bovine Respiratory Disease (BRD) (13%). For unhealthy animals in G2, the incidence of diseases was: Bovine Respiratory Disease (BRD) (63%), diarrhea (21%), navel inflammation (8%) and anemia (8%). In G3, the unhealthy animals presented more Bovine Respiratory Disease (BRD) (59%), pink eye (18%), diarrhea (18%) and anemia (4%).

Figure 3: Proportion (%) of diseases detected in unhealthy Holstein calves according to age group. (A) G1- 1 to 7 days of life; (B) G2 - 30 to 40 days of life; (C) G3 - 60 to 89 days of life



Source: MORITA, 2020.

5.2 RED BLOOD CELL PARAMETERS

The physiological variations according to age in healthy animals, as well as the influence of diseases on RBC and platelets parameters are shown on Table 1.

In relation to age, physiological variation was considered only for healthy animals, despite to the similar age profile observed in unhealthy. It was possible to observe lowest values for RBC count (P<.0001), hemoglobin concentration (HGB) (P<.0001), Packed Cell Volume (PCV) (P<.0001), Cell Hemoglobin Concentration Mean (CHCM) (P<.0001), Mean Corpuscular Hemoglobin Concentration (MCHC) (P<.0001), Red Cell distribution Width (RDW) (P<0.0001) and Hemoglobin Concentration distribution Width (HDW) (P=0.0002) in neonate calves from health group (G1) compared with other age groups (G2 and G3). On the other hand, the following parameters were significantly higher in health neonates than the other age groups: Mean Corpuscular Volume (MCV) (P<.0001), Mean Corpuscular Hemoglobin (MCH) (P<.0001), Cellular hemoglobin Content (CH) (P<.0001), Mean Platelet Volume (MPV) (P=0.038) and Cellular Hemoglobin content of mature RBC (CHm) (P=0.0003).

Health calves from 30 up to 40 days of life (G2) presented lower values for MCH and CH than those numbers observed in neonates (G1) and heifers with 60 to 89 days of life (G3). The G3 groups was differentiate from neonate (G1) and intermediate age (G2) for presenting the lowest number of platelets (P=0.0002).

No significant differences were detected for reticulocytes count and Mean Hemoglobin content of reticulocytes (CHr) (P>0.05).

The comparison of RBC and platelets parameters according to health status did not showed many differences between the healthy and unhealthy subgroups in each age groups (Figure 4). Neonate calves (G1) manifesting diseases had low number of RBC (P=0.05) (Figure 4A) and sick heifers from G3 (60 to 89 days of life) presented low values for Hemoglobin concentration Distribution Width – HDW (P=0.004) (Figure 4B) and Mean Platelet Volume - MPV (P=0.01) (Figure 4C). G2 heifers (30 to 40 days of life) did not present differences on RBC or platelets parameters between healthy and unhealthy subgroups (P>0.05).

Table 1- Red blood cells and platelets parameters (mean±S.E.M) in Holstein calves with different ages and healthy status

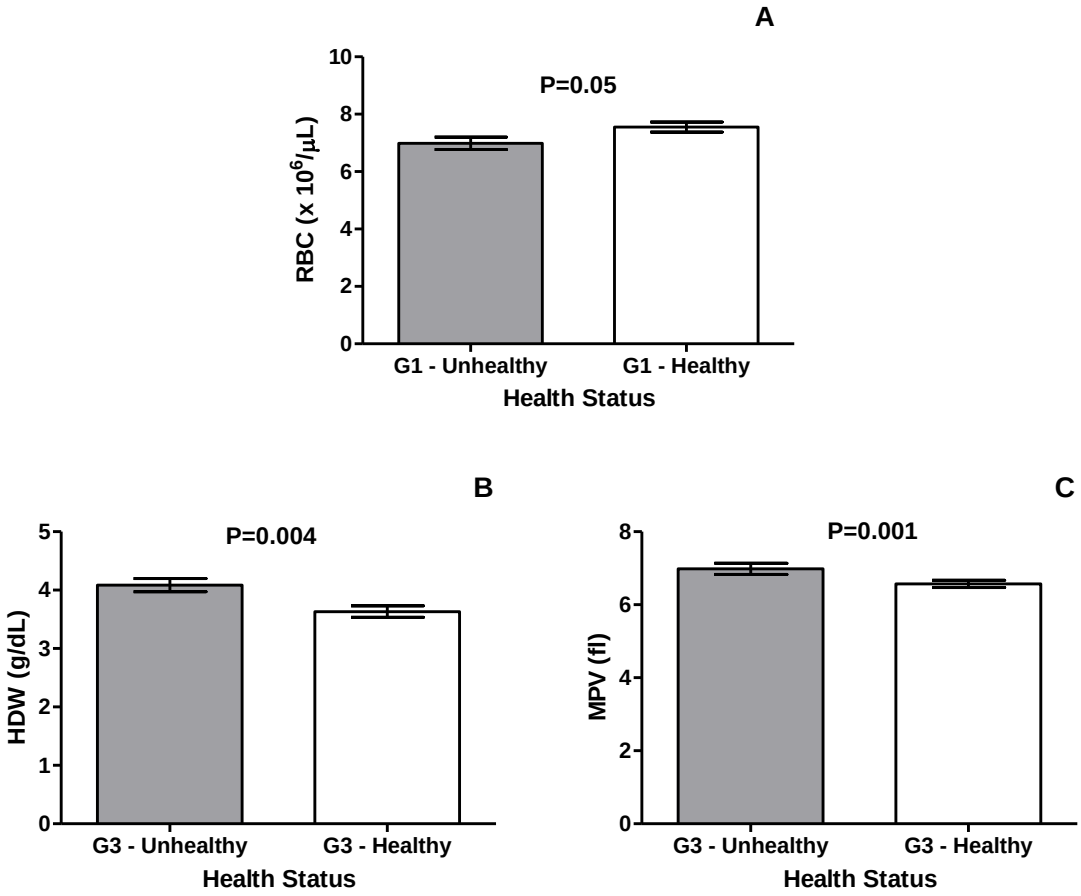
Parameters	G1	G2	G3	G1	G2	G3
	Unhealthy calves			Healthy calves		
Red Blood Cells count (x10^6/μL)	6.98 ± 0.22^{a*}	9.45 ± 0.30^{b}	9.88 ± 0.32^{b}	7.55 ± 0.18^{a*}	9.75 ± 0.19^{b}	10.05 ± 0.27^{b}
Hemoglobin (g/dL)	8.65 ± 0.32^{a}	9.68 ± 0.63^{ab}	10.93 ± 0.19^{b}	9.39 ± 0.30^{a}	9.23 ± 0.33^{a}	11.34 ± 0.15^{b}
PCV- Packed Cell Volume (%)	29.12 ± 1.01^{a}	33.11 ± 1.23^{b}	36.03 ± 0.65^{b}	30.37 ± 0.88^{a}	32.83 ± 0.93^{a}	36.63 ± 0.54^{b}
MCV- Mean Corpuscular Volume (fL)	43.37 ± 0.38^{a}	34.99 ± 0.52^{b}	36.35 ± 0.59^{b}	43.80 ± 0.44^{a}	33.90 ± 0.72^{b}	37.28 ± 0.71^{c}
MCH- Mean Corpuscular Hemoglobin (pg)	12.31 ± 0.16^{a}	9.84 ± 0.22^{b}	10.96 ± 0.22^{c}	12.40 ± 0.17^{a}	9.48 ± 0.27^{b}	11.20 ± 0.23^{c}
CH-Cellular Hemoglobin Content (pg)	12.27 ± 0.13^{a}	10.29 ± 0.22^{b}	11.48 ± 0.16^{c}	12.36 ± 0.14^{a}	9.89 ± 0.25^{b}	11.48 ± 0.19^{c}
MCHC - Mean Corpuscular Hemoglobin Concentration (g/dL)	28.36 ± 0.19^{a}	28.12 ± 0.24^{a}	30.13 ± 0.22^{b}	28.28 ± 0.20^{a}	27.85 ± 0.22^{a}	30.37 ± 0.14^{b}
CHCM- Cell Hemoglobin Concentration Mean (g/dL)	28.44 ± 0.17^{a}	29.71 ± 0.27^{b}	31.74 ± 0.19^{c}	28.33 ± 0.21^{a}	29.48 ± 0.20^{b}	31.33 ± 0.14^{c}
RDW- Red Cell Distribution Width (%)	20.62 ± 0.51^{a}	26.09 ± 0.56^{b}	24.10 ± 0.49^{b}	19.83 ± 0.35^{a}	26.62 ± 0.65^{b}	23.39 ± 0.42^{c}
HDW - Hemoglobin Distribution Width (g/dL)	3.70 ± 0.07^{a}	4.13 ± 0.10^{b}	4.09 ± 0.11^{b*}	3.51 ± 0.07^{a}	4.10 ± 0.11^{b}	3.63 ± 0.10^{a*}
Platelets (x10^3/uL)	841.15 ± 43.75^{a}	705.78 ± 55.60^{ab}	570.55 ± 36.41^{b}	824.80 ± 71.61^{a}	754.31 ± 53.89^{a}	542.14 ± 26.04^{b}
MPV - Mean Platelet Volume (fL)	7.16 ± 0.09	6.78 ± 0.15	6.98 ± 0.15*	6.94 ± 0.13^{a}	6.55 ± 0.14^{b}	6.57 ± 0.10^{ab*}
Reticulocytes (%)	0.56 ± 0.41	0.21 ± 0.02	0.12 ± 0.02	0.15 ± 0.02	0.25 ± 0.06	0.11 ± 0.02
Reticulocytes(x10^9/L)	9.32 ± 1.83	17.17 ± 2.38	11.78 ± 2.25	11.22 ± 1.50	26.48 ± 7.06	11.23 ± 1.43
CHr- Mean hemoglobin content of reticulocytes (pg)	18.35 ± 0.80	17.76 ± 0.45	18.46 ± 0.32	18.93 ± 0.82	16.97 ± 0.30	19.30 ± 0.38
CHm - Cellular hemoglobin content of mature red blood cells(pg)	13.23 ± 0.17^{a}	11.44 ± 0.21^{b}	12.54 ± 0.09^{c}	13.10 ± 0.17^{a}	11.63 ± 0.18^{b}	12.27 ± 0.28^{b}

Source: MORITA, 2020.

G1: animals from 1 up to 7 days of life, G2: animals from 30 up to 40 days of life, G3: animals from 60 up to 89 days of life.

^{a,b} Different lowercase letters on the same row indicate significant differences by Tukey's test (p≤0.05) between age groups of the same healthy status. *indicates a significant difference by Student t-test (p≤0.05) between unhealthy and healthy animals from the same age group.

Figure 4: Variations detected for Red Blood Cells (RBC) and platelets parameters in unhealthy and healthy Holstein calves with different ages. A: RBC= Red Blood Cell count; B: RDW= Red Cell Distribution Width; C: MPV: Mean Platelet Volume



Source: MORITA, 2020.
G1: animals from 1 up to 7 days of life; G3: animals from 60 up to 89 days of life. No differences observed in G2. Significant differences obtained by Student t-test (p≤0.05).

5.3 WHITE BLOOD COUNT AND SERUM BIOCHEMICAL MARKERS

The physiological variations according to age in healthy animals, as well as the influence of diseases on White Blood Count (WBC) parameters are shown on Table 2.

The comparison of results between different ages in healthy heifers showed an increase on WBC count according to animals' age. White Blood Cells count was lower (P=0.02) in neonate than calves from other age groups (G2 and G3). The absolute number of lymphocytes also increased according to age, observing higher values in G3 than G1 and G2 (P<0.0001). The profile for WBC parameters in sick calves was a quite different from healthy heifers, observing higher proportion (%) of neutrophils in G1 than the other groups (G2 and G3) (P<0.0001). On the other hand, relative (P<0.0001) and absolute number (P<0.0001) of lymphocytes and the absolute number

of monocytes (P=0.003) were lower in unhealthy neonates than other unhealthy ages groups (G2 and G3).

The myeloperoxidase intracellular index (MPXI) was high in neonates, both unhealthy (P<0.0001) and healthy (P<0.0001) subgroups, when compared with other age groups. The relative and absolute number of basophils were lower in neonate group (G1) than G2 and G3 (P<0.0001) in both unhealthy and healthy animals.

No significant differences were observed in eosinophils count, large unstained cells count, lobularity index, WBC count from the Peroxidase channel and Mean hemoglobin content of reticulocytes (CHm) for both subgroups (unhealthy and healthy) when parameters mean were compared between ages group (P>0.05).

For neonate heifers (G1), the proportion (%) of neutrophils (P=0.01) was higher in unhealthy than healthy calves (Figure 5A), associated with low percentage of lymphocytes (P=0.04, Figure 5B). It was no possible to detect difference between healthy and unhealthy calves at 30 to 40 days of life for WBC parameters (P>0.05). For G3 (calves with 60 – 89 days of life), the percentage of monocytes was significantly higher in unhealthy calves compared to healthy animals (P=0.02) (Figure 5C).

The results from biochemical biomarkers measured in this research are also shown in Table 2. In relation to age physiological variations, it was detected lower percentage of plasma total protein in healthy (P<0.0001) and unhealthy (P=0.0031) heifers from G2. Related to serum total solids (%) the animals with 30 – 40 days of life (G2) presented lower values compared to other age groups for unhealthy (P=0.0016). And for healthy animals, all groups presented significant difference between them (P<0.0001). Neonates calves with a good status of health presented lowest serum iron concentration (P<0.0001). No differences were obtained for haptoglobin values when different ages of animals were compared (P>0.05).

It was possible to detect lowest serum total solids (P=0.01) and plasma total protein g/dL (P=0.04) and in unhealthy than healthy neonate calves. No differences were obtained when unhealthy and healthy animals were compared for serum iron and haptoglobin parameters (P>0.05).

Table 2- White blood cells (WBC) parameters and serum biochemical markers (mean±S.E.M) used to evaluate the inflammatory response in Holstein heifers according to age and healthy status

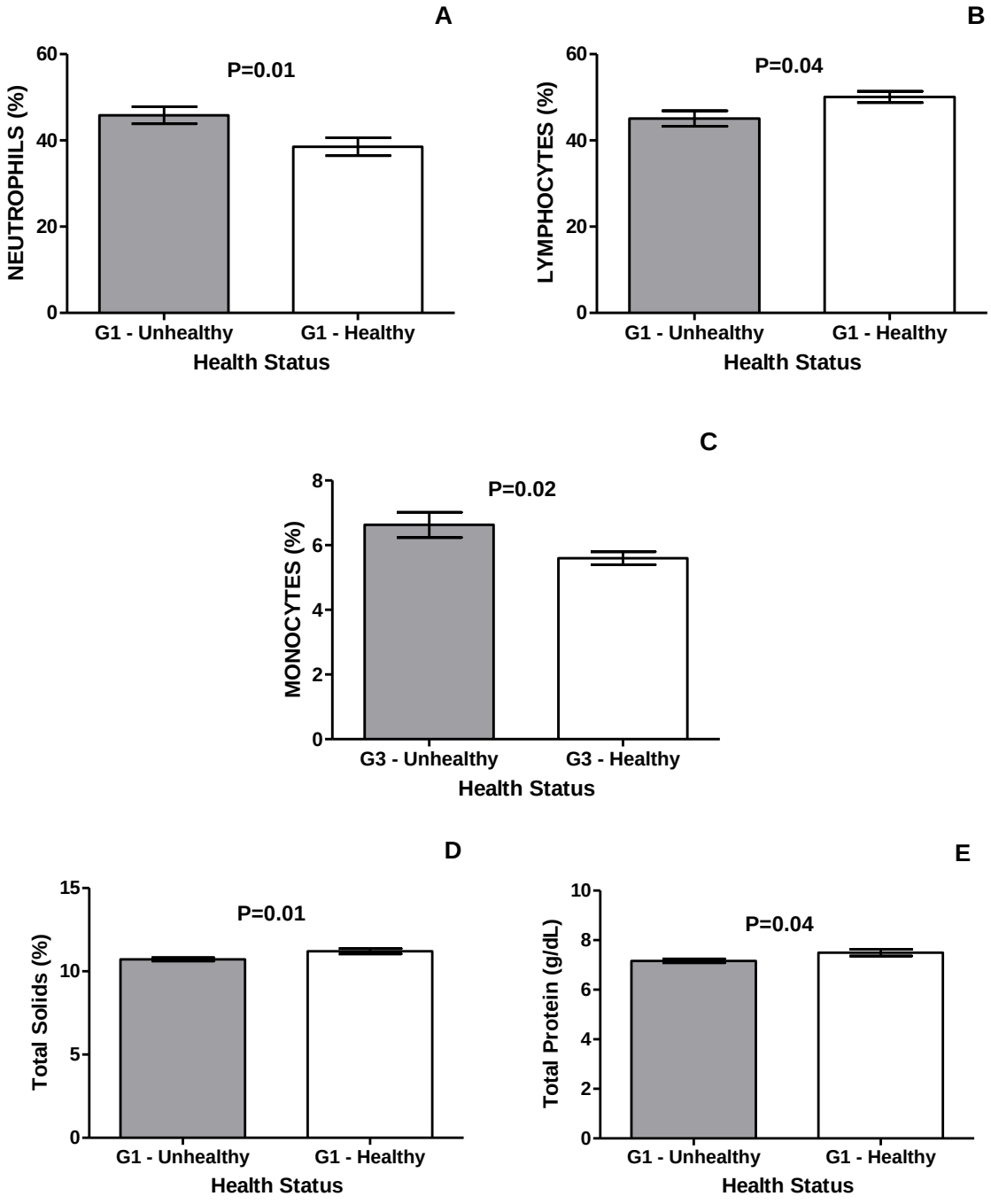
Parameters	G1	G2	G3	G1	G2	G3
	Unhealthy calves			Healthy calves		
White Blood Cells count (x10³/uL)	9.25 ± 0.58	10.78 ± 0.79	11.36 ± 0.82	9.37 ± 0.39[a]	10.39 ± 0.64[ab]	11.72 ± 0.56[b]
Neutrophils (x10³/uL)	4.51 ± 0.46	4.45 ± 0.58	3.84 ± 0.46	3.80 ± 0.25	4.53 ± 0.48	4.23 ± 0.36
Neutrophils (%)	45.84 ± 1.99[a]*	36.62 ± 2.80[b]	31.68 ± 2.12[b]	38.55 ± 2.08*	40.27 ± 2.44	34.16 ± 1.73
Lymphocytes (x10³/uL)	4.28 ± 0.32[a]	5.48 ± 0.24[b]	6.52 ± 0.40[b]	4.66 ± 0.20[a]	4.98 ± 0.26[a]	6.47 ± 0.21[b]
Lymphocytes (%)	45.07 ± 1.79[a]*	53.25 ± 2.58[b]	58.83 ± 1.91[b]	50.07 ± 1.30*	49.90 ± 2.27	55.69 ± 2.27
Monocytes (x10³/uL)	0.56 ± 0.04[a]	0.76 ± 0.08[b]	0.75 ± 0.06[b]	0.64 ± 0.07	0.63 ± 0.04	0.67 ± 0.05
Monocytes (%)	6.43 ± 0.46	7.04 ± 0.52	6.63 ± 0.39*	6.52 ± 0.53	6.38 ± 0.46	5.60 ± 0.20*
Eosinophils (x10³/uL)	0.14 ± 0.01	0.18 ± 0.03	0.17 ± 0.03	0.14 ± 0.02	0.20 ± 0.04	0.24 ± 0.04
Eosinophils (%)	1.55 ± 0.16	1.67 ± 0.19	1.59 ± 0.25	1.51 ± 0.17	1.84 ± 0.32	2.04 ± 0.31
Basophils (x10³/uL)	0.038 ± 0.005[a]	0.062 ± 0.006[b]	0.084 ± 0.009[b]	0.043 ± 0.004[a]	0.075 ± 0.009[b]	0.094 ± 0.006[b]
Basophils (%)	0.45 ± 0.09[a]	0.60 ± 0.05[b]	0.72 ± 0.05[b]	0.45 ± 0.04[a]	0.72 ± 0.06[b]	0.80 ± 0.04[b]
LUC- Large Unstained Cells (x10³/uL)	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.004	0.06 ± 0.004
LUC- Large Unstained Cells (%)	0.67 ± 0.10	0.60 ± 0.06	0.56 ± 0.05	0.56 ± 0.08	0.51 ± 0.05	0.49 ± 0.03
LI- Lobularity Index	2.32 ± 0.06	2.12 ± 0.08	2.18 ± 0.07	2.35 ± 0.07	2.20 ± 0.07	2.15 ± 0.06
MPXI- Myeloperoxidase Intracellular Index	-6.84 ± 0.56[a]	-3.18 ± 0.66[b]	-3.79 ± 0.64[b]	-7.42 ± 0.62[a]	-2.73 ± 0.57[b]	-4.15 ± 0.55[b]
WBPC - WBC count from the Peroxidase channel (x10³/uL)	8.67 ± 0.59	9.97 ± 0.81	9.99 ± 0.72	8.65 ± 0.35	9.57 ± 0.59	10.32 ± 0.53
Total Solids (%)	10.72 ± 0.10[a]*	10.07 ± 0.13[b]	10.59 ± 0.18[a]	11.21 ± 0.16[a]*	10.15 ± 0.11[b]	10.75 ± 0.12[c]
Total Protein (g/dL)	7.17 ± 0.08[a]*	6.68 ± 0.11[b]	7.15 ± 0.16[a]	7.50 ± 0.13[a]*	6.76 ± 0.08[b]	7.37 ± 0.15[a]
Iron (µMol/L)	12.87 ± 2.74[a]	23.04 ± 4.13[ab]	27.16 ± 1.99[b]	10.13 ± 1.65[a]	22.02 ± 3.54[b]	30.36 ± 1.96[b]
Haptoglobin(g/dL)	0.04 ± 0.01	0.03 ± 0.004	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.003	0.03 ± 0.004

Source: MORITA, 2020.

G1: animals from 1 up to 7 days of life, G2: animals from 30 up to 40 days of life, G3: animals from 60 up to 89 days of life.

[a,b] Different lowercase letters on the same row indicate significant differences by Tukey's test ($p \leq 0.05$) between age groups of the same healthy status. *indicates a significant difference by Student t-test ($p \leq 0.05$) between unhealthy and healthy animals from the same age group.

Figure 5: Variations detected for White Blood Cells (WBC) and serum biochemical markers in unhealthy and healthy Holstein calves with different ages. A: Relative count of neutrophils (%); B: Relative count of lymphocytes (%); C: Relative count of monocytes (%); D: Total solids (%); E: Total protein (%)



Source: MORITA, 2020.
G1: animals from 1 up to 7 days of life, G3: animals from 60 up to 89 days of life. No differences observed in G2. Significant differences obtained by Student t-test (P≤0.05).

5.4 PHAGOCYTOSIS

The flow cytometry analysis was used to assess the mononuclear and polymorphonuclear phagocytic rate (%) and the mean fluorescence intensity (MFI) of pre-labelled *E. coli*, *S. aureus* and *M. haemolytica* (Table 3).

Only few differences were observed for cellular activity between ages in the healthy and unhealthy subgroups. Overall, the MN cells under antigens stimulus presented a similar variation of phagocytosis: *E.* coli (15.33% to 19.49%), *S. aureus* (22.71% to 28.00%) and *M. haemolytica* (26.49% to 35.11% for unhealthy animals and 21.69% to 28.93%). Related to results of phagocytosis of PMN cells, it was possible to observe high levels of activity for all antigens tested and the values were similar for groups/subgroups. The following variation according to each bacterium was obtained: *E.* coli (60.30% to 67.47%), *S. aureus* (86.25% to 92.11%) and *M. haemolytica* (94.62% to 97.45% for unhealthy animals and 92.33% to 96.41%).

In the healthy animals, MN cells stimulated with *M. haemolytica* presented higher intensity of phagocytosis (MFI) in G2 compared to other age groups (P= 0.0007).

The proportion (%) of blood mononuclear cells was lower in unhealthy than healthy neonate heifers (P= 0.01) (Figure 6A). As presented in Figure 6B, unhealthy calves from 30 up to 40 days of life presented higher mononuclear phagocytosis intensity (MFI) cells when stimulated by *E. coli* healthy calves (P=0.02). Unhealthy heifers from G3 presented higher mononuclear cells phagocytosis rate (%) and the avidity measured by fluorescence intensity (MFI) against *M. haemolytica* (P=0.02 and P=0.01, respectively) compared with values measured in the whole blood from healthy calves (Figure 6C and 6D). Additionally, PMN cells stimulated with *M. haemolytica* in unhealthy heifers presented higher rate (%) of phagocytosis (P=0.05) and fluorescence avidity (MFI) (P=0.04) than healthy animals (Figure 6E and 6F). For the rest of parameters, it was not possible to detect difference between animals with different healthy status for G1, G2 or G3 (P>0.05).

Table 3: Phagocytosis and mean fluorescence intensities consistent values (mean±S.E.M) of Holstein calves with different ages and healthy status

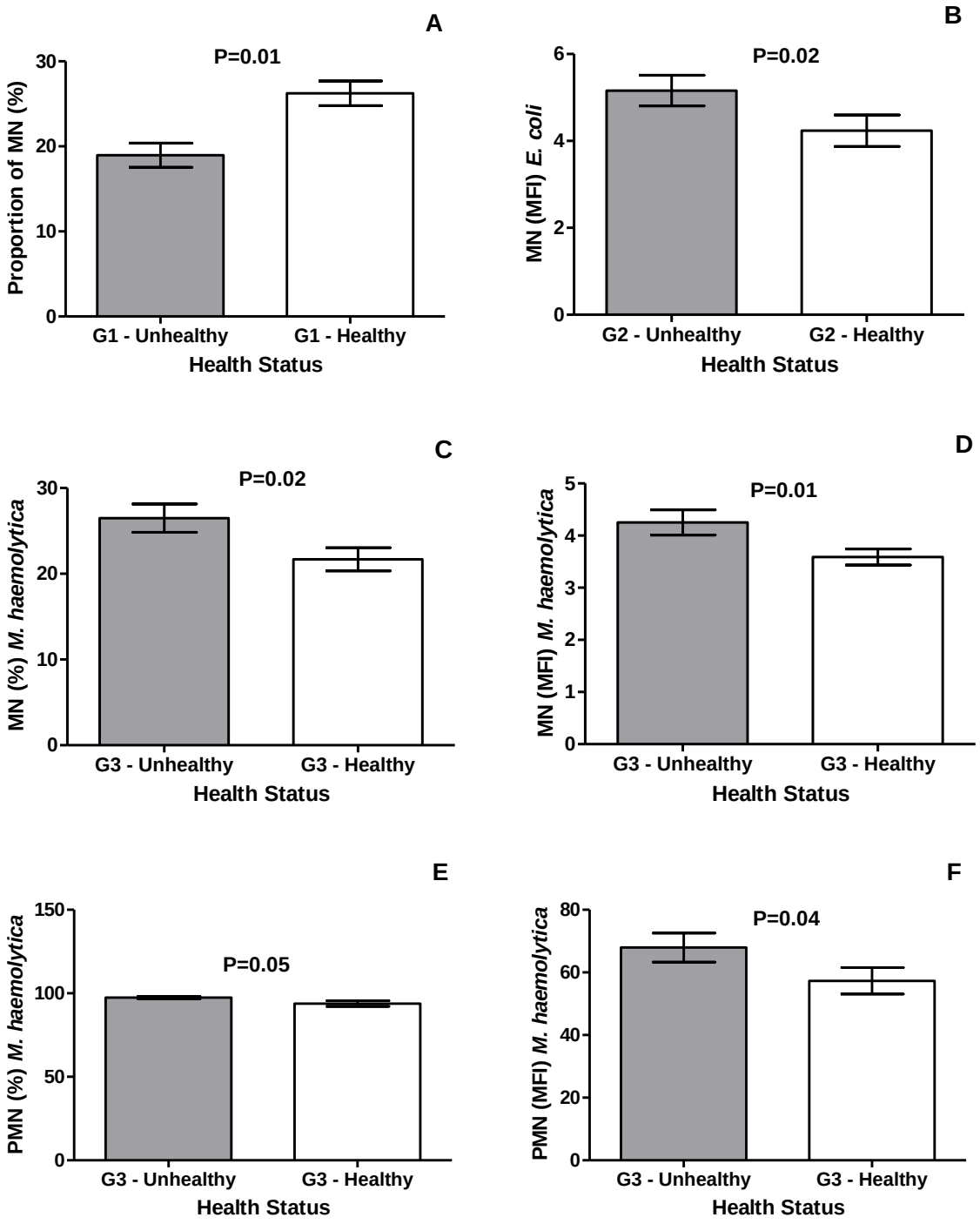
Type of cells	Parameters	G1	G2	G3	G1	G2	G3
		Unhealthy calves			Healthy calves		
Mononuclear (MN) cells	**Proportion (%)**	18.97 ± 1.42[a]*	29.46 ± 1.63[b]	28.34 ± 2.69[b]	26.24 ± 1.46*	27.19 ± 1.83	32.80 ± 2.29
	Phagocytosis rate (%) of *E. coli*	17.84 ± 1.30	19.49 ± 1.47	16.34 ± 1.17	17.02 ± 1.02	17.57 ± 1.74	15.33 ± 0.81
	Relative uptake of *E. coli* (MFI)	5.06 ± 0.37	5.16 ± 0.35*	4.24 ± 0.22	4.53 ± 0.27	4.23 ± 0.36*	3.86 ± 0.13
	Phagocytosis rate (%) of *S. aureus*	24.42 ± 1.46	28.00 ± 1.70	25.58 ± 1.53	23.26 ± 1.89	27.21 ± 2.60	22.71 ± 1.46
	Relative uptake of *S. aureus* (MFI)	6.45 ± 0.60	6.71 ± 0.55	5.41 ± 0.34	5.41 ± 0.52	7.78 ± 1.01	5.09 ± 0.33
	Phagocytosis rate (%) of *M. haemolytica*	27.27 ± 2.87	35.11 ± 3.04	26.49 ± 1.65*	24.98 ± 3.63	28.93 ± 2.04	21.69 ± 1.36*
	Relative uptake of *M. haemolytica* (MFI)	4.67 ± 0.42	5.61 ± 0.53	4.25 ± 0.24*	4.50 ± 0.81[a]	5.35 ± 0.45[b]	3.59 ± 0.15[a]*
Polymorphonuclear (PMN) cells	**Proportion (%)**	34.40 ± 2.08	37.61 ± 3.04	29.37 ± 3.17	34.24 ± 2.10	40.42 ± 2.99	35.58 ± 2.29
	Phagocytosis rate (%) of *E. coli*	65.27 ± 2.64	60.30 ± 2.59	61.79 ± 2.39	67.47 ± 3.85	61.98 ± 3.43	63.04 ± 2.41
	Relative uptake of *E. coli* (MFI)	89.92 ± 13.43	67.50 ± 9.61	59.32 ± 6.82	100.22 ± 15.89	71.77 ± 10.10	71.91 ± 9.17
	Phagocytosis rate (%) of *S. aureus*	92.11 ± 0.78	90.78 ± 1.02	88.95 ± 1.61	86.25 ± 3.87	87.72 ± 1.54	89.87 ± 1.09
	Relative uptake of *S. aureus* (MFI)	97.43 ± 6.76	89.28 ± 6.21	96.22 ± 8.67	87.44 ± 7.75	78.39 ± 6.16	95.69 ± 6.32
	Phagocytosis rate (%) of *M. haemolytica*	96.28 ± 0.92	94.62 ± 1.37	97.45 ± 0.75*	96.41 ± 1.18	92.33 ± 1.92	93.87 ± 1.65*
	Relative uptake of *M. haemolytica* (MFI)	58.22 ± 6.23	55.79 ± 4.02	67.94 ± 4.63*	46.48 ± 3.86	54.99 ± 5.50	57.33 ± 4.23*

Source: MORITA, 2020.

G1: animals from 1 up to 7 days of life, G2: animals from 30 up to 40 days of life, G3: animals from 60 up to 89 days of life.

[a,b] Different lowercase letters on the same row indicate significant differences by Tukey's test ($p \leq 0.05$) between age groups of the same healthy status. *indicates a significant difference by Student t-test ($p \leq 0.05$) between unhealthy and healthy animals from the same age group.

Figure 6: Variations detected for phagocytosis assay of mononuclear (MN) and polymorphonuclear (PMN) cells in unhealthy and healthy Holstein calves with different ages. A: Basal phagocytosis of MN (%) in G1; B: Relative uptake (MFI) of MN for *E. coli* in G2; C: Phagocytosis (%) of MN for *M. haemolytica* in G3; D: Relative uptake (MFI) of MN for *M. haemolytica* in G3; E: Phagocytosis (%) of PMN for *M. haemolytica* in G3; F: Relative uptake (MFI) of PMN for *M. haemolytica* in G3



Source: MORITA, 2020.
G1: animals from 1 up to 7 days of life, G2: animals from 30 up to 40 days of life, G3: animals from 60 up to 89 days of life.
Significant differences obtained by Student t-test ($p \leq 0.05$).

5.6 REACTIVE OXYGEN SPECIES (ROS)

The reactive oxygen species (ROS) endogenous and after leukocytes stimulation with Phorbol Myristate Acetate (PMA), *E. coli* 1:10, *S. aureus* 1:5*, M. haemolytica* and *M. haemolytica* 1:5 is presented in Table 4. Moreover, the results regard the comparison between unhealthy and healthy animals is shown in Figure 7, 8 and 9.

In relation to physiological variations detected by age variations, it was not possible to observe significant differences to detect for the number of total leukocytes ($x10^6$) isolated from 3mL of whole blood and viability (%) ($P>0.05$).

The intensity of the fluorescence released by blood cells (AFU) was influenced by age. A decrease in ROS production by using bacteria stimulation according to age was observed. Healthy neonates from G1 presented higher values of AFU when cells were stimulated by *E. coli* 1:10 (P=0.0018) and *M. haemolytica* 1:5 (P=0.04) than in G2 and G3. As it was seen in healthy neonates, the unhealthy animals from G1 presented higher amount of ROS produced by blood cells stimulated with PMA (P=0.007), *E. coli* 1:10 (P=0.0002), *S. aureus* 1:5 (P=0.02), *M. haemolytica* (P=0.002) and *M. haemolytica* 1:5 (P=0.0002).

The response ratio (RR) for healthy animals presented some significant differences between age groups. It was possible to observe high values for response ratio when phagocytes were stimulated by *E. coli* from in G1 compared with G3 (P=0.03). RR for *M. haemolytica* and *M. haemolytica* diluted 1:5 was higher in G2 (P=0.01 and P=0.03, respectively) than G1 and G3. Related to response ratio (RR) in unhealthy animals, heifers from G2 presented low values compared to one or both age groups when cells were stimulated with PMA (P=0.004), *E. coli* (P=0.0002), *S. aureus* (P=0.01), *M. haemolytica* (P=0.01) and *M. haemolytica* 1:5 (P=0.0006).

Regarding to neonate calves (G1) with good health status, there were no differences for the number of leukocytes ($x10^6$) obtained from 3mL of blood, viability (%), ROS producing cells (AFU) for all antigens, except for *M. haemolytica* 1:5. The mean production of ROS when stimulated by *M. haemolytica* 1:5 and the response ratio for the same antigen were higher in unhealthy than healthy neonate calves (P=0.04 and P=0.02, respectively) as it is outlined in Figure 8.

For G2, RR of cells producing ROS for PMA (P=0.01), *E. coli* (P=0.02)*, S. aureus* (P=0.02)*, M. haemolytica* (P=0.006) *and M. haemolytica* 1:5 (P=0.002) were

lower in unhealthy than healthy animals (Figure 9). The other parameters did not present any significant difference. In contrast with what was observed in G2, unhealthy animals with 60 – 89 days of life (G3) presented higher response ratio of cells producing ROS for PMA (P=0.05), *E. coli* 1:10 (P=0.05), *S. aureus* 1:5 (P=0.02), *M. haemolytica* (P=0.01) and *M. haemolytica* 1:5 (P=0.02) than healthy heifers (Table 4 and Figure 10). For the rest of parameters, it was not found any difference (P>0.05).

Table 4 -Reactive Oxygen Species (ROS) and response ratio (RR) consistent values (mean±S.E.M) of Holstein calves with different ages and healthy status

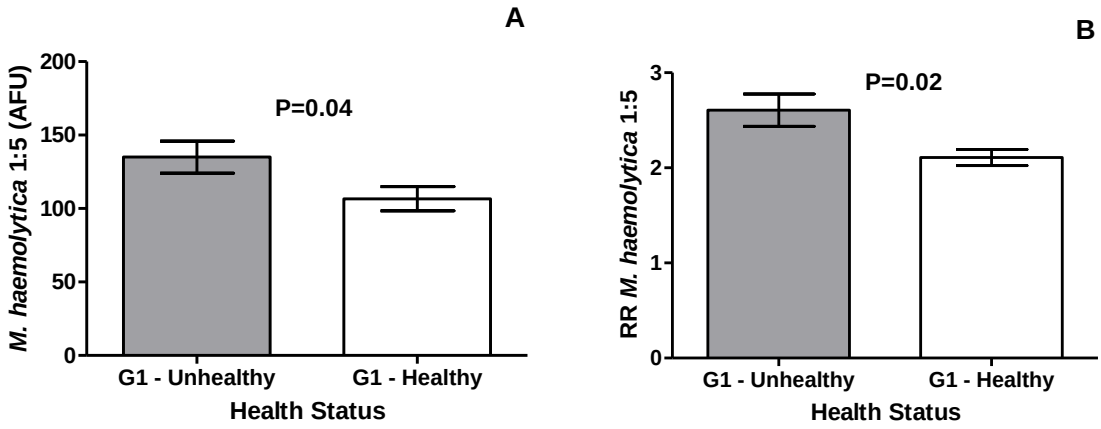
	G1	**G2**	**G3**	**G1**	**G2**	**G3**
	Unhealthy calves			**Healthy calves**		
Cells/mL (x10^6)	14.03 ± 1.27	15.21 ± 1.42	15.37 ± 1.75	12.90 ± 0.87	14.65 ± 1.23	16.54 ± 1.04
Viability (%)	92.73 ± 0.76	92.84 ± 0.67	93.25 ± 0.89	91.99 ± 0.76	93.27 ± 0.86	93.52 ± 0.49
Basal ROS producing cells (AFU)	53.02 ± 2.84	61.62 ± 10.70	35.46 ± 4.33	52.64 ± 4.75	38.56 ± 2.62	46.21 ± 3.55
ROS producing cells + PMA (AFU)	272.79 ± 11.55^{a}	229.18 ± 20.62^{ab}	204.37 ± 17.17^{b}	252.05 ± 12.21	212.52 ± 12.17	231.15 ± 13.17
Response Ratio PMA	5.48 ± 0.25^{ab}	4.70 ± 0.35^{a*}	6.41 ± 0.40^{b*}	5.34 ± 0.30	5.77 ± 0.27*	5.50 ± 0.27*
ROS producing cells + *E. coli* 1:10 (AFU)	141.92 ± 10.28^{a}	106.93 ± 14.62^{b}	79.79 ± 9.18^{b}	123.41 ± 9.89^{a}	83.51 ± 5.21^{b}	88.97 ± 5.75^{b}
Response Ratio *E. coli* 1:10	2.73 ± 0.15^{a}	1.92 ± 0.10^{b*}	2.33 ± 0.12^{a*}	2.47 ± 0.14^{a}	2.27 ± 0.13^{ab*}	2.05 ± 0.09^{b*}
ROS producing cells + *S. aureus* 1:5 (AFU)	188.45 ± 9.52^{a}	171.79 ± 13.9^{ab}	144.20 ± 11.75^{b}	179.51 ± 8.94	154.76 ± 7.29	153.62 ± 7.64
Response Ratio *S. aureus* 1:5	3.73 ± 0.17^{a}	3.55 ± 0.27^{a*}	4.57 ± 0.27^{b*}	3.82 ± 0.23	4.30 ± 0.24*	3.78± 0.22*
ROS producing cells + *M. haemolytica* (AFU)	150.28 ± 7.55^{a}	128.56 ± 12.62^{ab}	105.78 ± 9.40^{b}	129.41 ± 7.88	119.00 ± 6.34	108.44 ± 5.74
Response Ratio *M. haemolytica*	2.95 ± 0.13^{ab}	2.63 ± 0.26^{a*}	3.30 ± 0.19^{b*}	2.67 ± 0.15^{a}	3.30 ± 0.19^{b*}	2.70 ± 0.19^{a*}
ROS producing cells + *M. haemolytica* 1:5 (AFU)	135.01 ± 10.87^{a*}	102.77 ± 14.16^{b}	79.11 ± 8.11^{b}	106.62 ± 8.24^{a*}	88.00 ± 5.45^{ab}	84.12 ± 4.57^{b}
Response Ratio *M. haemolytica* 1:5	2.61 ± 0.17^{a*}	1.89 ± 0.13^{b*}	2.43 ± 0.17^{a*}	2.11 ± 0.08^{ab*}	2.42 ± 0.13^{a*}	2.05 ± 0.12^{b*}

Source: MORITA, 2020.

G1: animals from 1 up to 7 days of life, G2: animals from 30 up to 40 days of life, G3: animals from 60 up to 89 days of life.

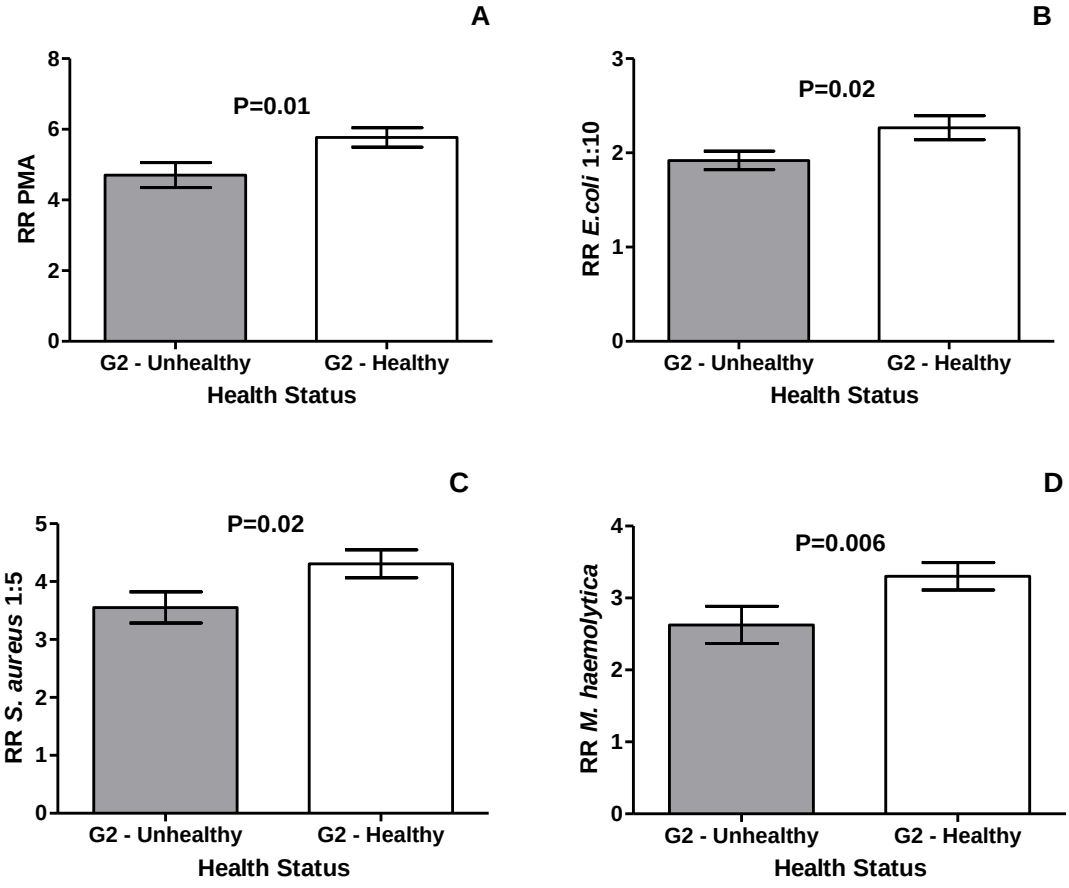
^{a,b} Different lowercase letters on the same row indicate significant differences by Tukey's test (p≤0.05) between age groups of the same healthy status. *indicates a significant difference by Student t-test (p≤0.05) between unhealthy and healthy animals from the same age group. Response ratio: AFU value for stimulated cells/ AFU cell value not stimulated.

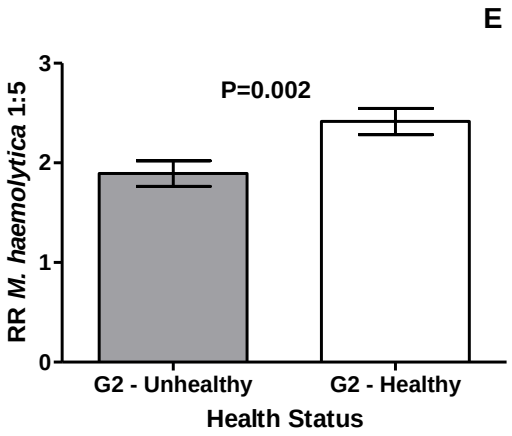
Figure 8 – Variations detected in AFU and response ratio (RR) of blood leukocytes stimulated with M. haemolytica 1:5 in unhealthy and healthy Holstein calves in G1. A: ROS producing cells with *M. haemolytica* 1:5 (AFU); B: Response Ratio *M. haemolytica* 1:5



Source: MORITA, 2020.
G1: animals from 1 up to 7 days of life.
Significant differences obtained by Student t-test (p≤0.05).

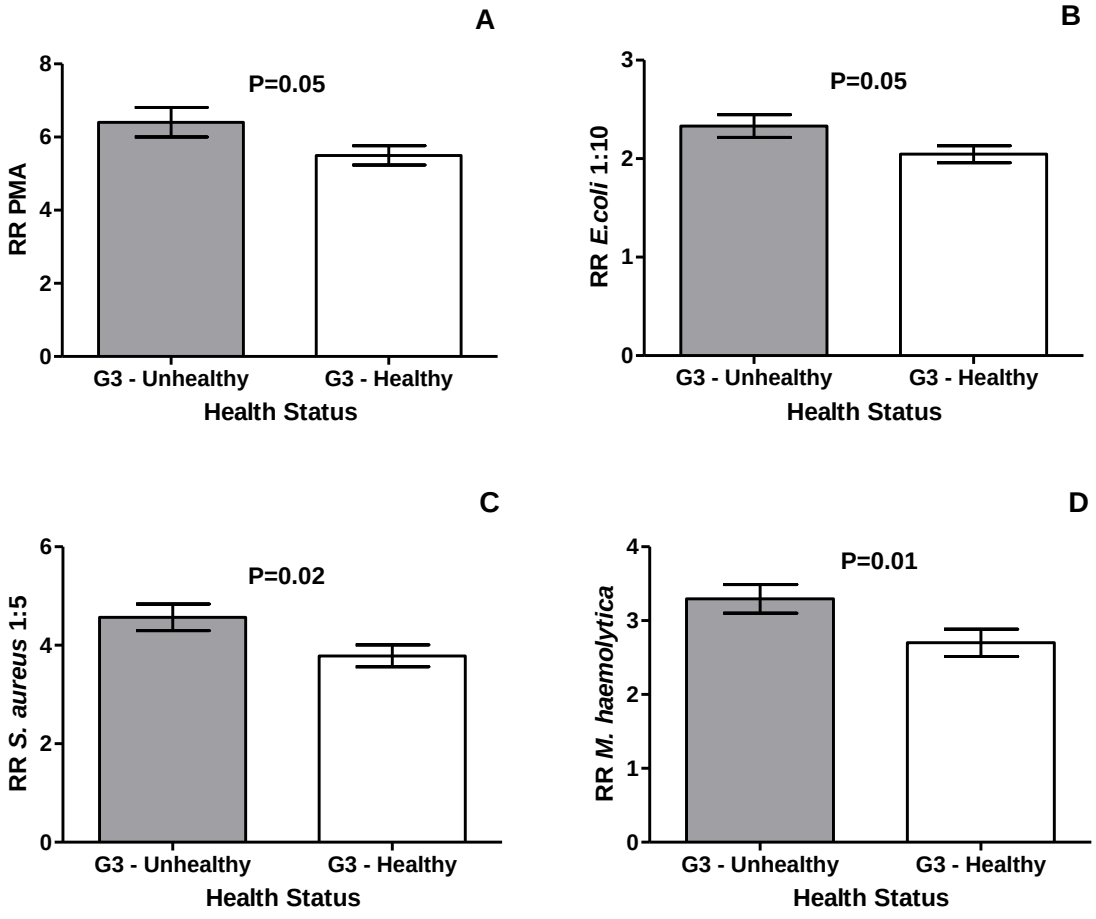
Figure 9 – Variations detected for response ratio of blood leukocytes stimulated with different species and concentration of bacteria in unhealthy and healthy Holstein calves in G2. A: Response Ratio PMA; B: Response Ratio *E. coli* 1:10; C: Response Ratio *S. aureus* 1:5; D: Response Ratio *M. haemolytica;* E: Response Ratio *M. haemolytica* 1:5

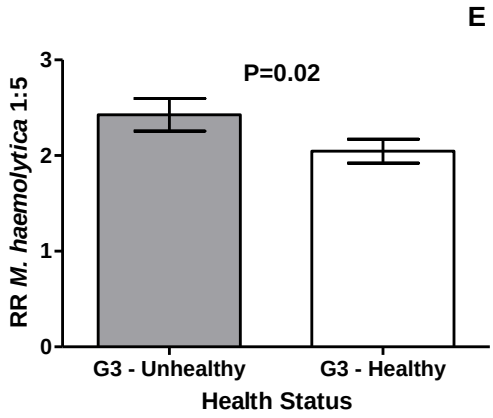




Source: MORITA, 2020.
G2: animals from 30 up to 40 days of life.
Significant differences obtained by Student t-test (p≤0.05).

Figure 10 – Variations detected in response ratio of blood leukocytes stimulated with different species and concentration of bacteria in unhealthy and healthy Holstein calves in G3. A: Response Ratio PMA; B: Response Ratio *E. coli* 1:10; C: Response Ratio *S. aureus* 1:5; D: Response Ratio *M. haemolytica;* E: Response Ratio *M. haemolytica* 1:5





Source: MORITA, 2020.
G3: animals from 60 up to 89 days of life.
Significant differences obtained by Student t-test (p≤0.05).

6. DISCUSSION

Holstein calves with one week until 89 days of age and different health status under natural challenge of pathogens were evaluated for hematological and innate immune response by phagocytosis and reactive oxygen species assays and presented differences according to their groups/subgroups.

The most prevalent disease screened in G1 was diarrhea (46%), while G2 (63%) and G3 (59%) had a high incidence of BRD. The occurrence of diarrhea in young neonate calves and respiratory disease during the first months in heifers it's well known by literature. Neonatal calf diarrhea and bovine respiratory disease (BRD) are the most common causes of morbidity and mortality in young dairy cattle (WINDEYER et al., 2014). The major enteric pathogens known to cause calf diarrhea are *E. coli, Salmonella enterica, Clostridium perfringes* and *Cryptosporidium parvum* as bacteria pathogens (CHO; YOON, 2014) and the it occurs mainly during the 2 weeks of calves' life. BRD is an infectious respiratory disease of cattle with multifactorial causes including *Mannheimia haemolytica, Pasteurella multocida, Histophilus sommi* and *Mycoplasma bovis* as important bacterial pathogens (GRIFFIN et al., 2010).

RBC, hemoglobin and PCV had a gradual increase through the age groups, observing lower values of these parameters in neonate calves (G1) compared to other age groups in both unhealthy and healthy subgroups. The decline of RBC, PCV and hemoglobin in neonates has been considered in the past as a direct result of plasma volume expansion after colostrum consumption and dilution of solid blood components (REID et al., 1948). Some studies (MOHRI et al., 2007; NOVO et al., 2015; PANOUSIS

et al., 2018) also reported a gradual increase trend for the RBC parameter over time, but not followed by the same pattern for hemoglobin and PCV parameters that presented a tendency to decrease (MOHRI et al., 2007) or to stabilize the values between groups with the same age as this study (NOVO et al., 2015). Benesi, (2012a) reported no changes in erythrogram components (RBC, hemoglobin and PCV) in calves from birth up to 30 days of life. Baccili et al. (2018) also did not find difference for RBC, hemoglobin and PCV values between animals with 2 days until 80 days of age.

In agreement to other scientific literature, the results for Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH) of this study showed the similar profile with high levels in neonates and decreasing according to age's calf (BAGLIONI et al. 1956, NOVO et al. 2015). That profile coincides with the disappearance of fetal hemoglobin that occurs after 2 months of life (FREI, PERK, DANON, 1963).

The Cellular Hemoglobin Content (CH) is a result provided from optical measurement (based on light scatter) of intact RBC by some hematology analyzers, as ADVIA. This is the "optical equivalent" of the MCH and it's considered more accurate (ECLINPATH). CH values decreased according to age animals, presenting a reference interval of 9.89 ± 0.25 to 12.36 ± 0.14, following the same decrease of MCH results.

Values of MCHC (Mean Corpuscular Hemoglobin Concentration) and CHCM (Cell Hemoglobin Concentration Mean) showed an increase trend from neonates to the oldest group (60 – 89 days of life). The MCHC is the mean hemoglobin concentration in a specific volume of red blood cells or is the percentage of the red blood cell that contains hemoglobin. However, the ADVIA hematology analyzer provides both a calculated MCHC and a directly measured CHCM which is based on the ability of hemoglobin to scatter laser light sideways (high angle light scatter) as intact red blood cells pass through a laser beam. Thus, it is obtained a calculated MCHC (from measured hemoglobin after lysing RBC) and a directly measured CHCM (from internal complexity of intact RBC) (ECLINPATH). In accordance with other studies, MCHC showed an increase according to age (KNOWLES et al., 2000; BRUN-HANSEN et al., 2006; MOHRI et al., 2007; PANOUSIS et al., 2018). There is not data available of CHCM for cattle or calves in literature. Our study obtained a reference interval of 28.33 ± 0.21 to 31.33 ± 0.14. However, Ventrella et al. (2017) reported CHCM results obtained by ADVIA 2120 system from piglets with different ages and

showed differences between animals with 5 days and 30 days of age. They have described an increase of values for animals with 30 days compared to neonate group.

The Red Cell Distribution Width (RDW) is an index that describes variation in RBC size or anisocytosis (BRAGA, 2014). This parameter showed a tendency to increase values from neonates to older animals in this study. Similar increasing of RDW was observed by Panousis et al. (2018) from animals of 1 to 9 days of life and they described a reference interval of 18.6% to 27.6%, in accordance with mean values obtained in G1 (19.83 ± 0.35). RDW continues to increase up to 3 weeks of age and tended to stabilize thereafter (up to 27 to 29 weeks of age) based on Brun-Hansen et al. (2006) study. In this study, for healthy G2, RDW mean values was 26.62 ± 0.65 and for healthy G3 was 23.39 ± 0.42.

As far as is known, there are no studies reporting values of Hemoglobin Distribution Width (HDW) in cattle or calves. HDW indicates the heterogeneity of intraerythrocytic hemoglobin concentration (anisochromia). In this study, HDW increased according to animal´s age, presenting differences between mean values of G1 and G3. The same difference occurred with hemoglobin concentration (g/dL).

The results of the present study showed a decreasing of platelets numbers according to age's group in contrast with other studies conducted by Mohri et al. (2007) and Knowles et al. (2000) which demonstrated an increase of platelets around 2 weeks of age compared to newborn calves and a tendency to stabilize thereafter. Also, it was possible to observe a decrease of Mean Platelet Volume (MPV) values comparing neonates (G1) to animals with 60 to 89 days of life (G3). Mean platelet volume (MPV) is a measure of the average size of platelets and it's important in determining the cause of thrombocytopenia or thrombocytosis (BRAGA, 2014). There's only one study available (PANOUSIS et al., 2018) in calves that described MPV values from animals with 1 to 9 days of life. They associated a decrease of MPV according to advance of age with a compensatory mechanism for increasing number of platelets which could not be observed in this present study. Although, according to Braga (2014), MPV increases together with the number of platelets as a sign of young platelets being released to blood circulation.

Reticulocytes are precursors of RBCs and correspond to immature RBCs in the bloodstream (BRAGA, 2014). The number and relative values of reticulocytes did not present significant difference between groups with different animal´s age, independently of health status. Benesi et al. (2012a) evaluated the leukogram of dairy

calves since birth up to 30 days of life and observed differences in reticulocytes values with an increase in calves with 3 to 5 days of life.

Mean hemoglobin content of reticulocytes (CHr) measures the amount of hemoglobin in reticulocytes and cellular hemoglobin content of mature red blood cells (CHm) is the mean of the RBC hemoglobin concentration for the red blood cell population. To my knowledge, the physiological variation according to age and the influence of health in CHr and CHm is described for the first time in this study. CHr did not show difference by age, but CHm presented higher measure in neonates compared to G2 and G3. Ventrella et al. (2017) evaluated this parameter in piglets and detected higher values in neonates (5 days of life) than older piglets (30 days of life).

Moreover, the comparison between animals with different healthy status in each age group resulted in significant differences. Unhealthy neonates (G1) showed lower number of RBC than healthy animals. The assumption is that sick animals were presenting an anemic profile, as 28% of these animals were presenting anemia, presumably originated by an inflammatory process. Unhealthy animals with 60 to 89 days presented higher values of HDW and MPV. Thus, they were producing more erythrocytes with an unequal hemoglobin content among the red blood cells and more platelets with variation of average size compared to healthy heifers.

In this present study, white blood cell count (WBC) values presented lower values in neonates (1 to 7 days of life) when compared to older healthy animals (G3). In contrast, other studies described high levels of WBC at birth with a gradual decrease with increase of age (MOHRI et al., 2007; BENESI et al., 2012b; NOVO et al., 2015).

In agreement with previous reports (MOHRI et al., 2007; BENESI et al., 2012b; NOVO et al., 2015; PANOUSIS et al., 2018) the mean neutrophil rate (%) was higher than the mean lymphocyte rate (%) in unhealthy neonates whereas in older animals (G2 and G3) this was reversed. The high neutrophil count profile in calves was not observed in healthy neonates instead. But an increase of lymphocyte count according to increase of age in healthy animals was observed in this study and reported on the same studies above. The stress leukogram profile was followed by unhealthy animals due to high natural stimulus of microorganism which complies with an inflammatory response mainly due to diarrhea (46% of incidence in G1). A stress leukogram is characterized by neutrophilia, lymphocytopenia, eosinopenia, and occasionally monocytosis (KLINKON; ZADNIK, 1999).

In this study, the basophil absolute and relative counts (%) increased with age together with an increase (but not significant) of eosinophils up to 60 – 89 days of life. According to Eberhart and Patt (1971), Tennant et al. (1974) and Adams et al. (1992), eosinophils and basophils are not often observed at birth, possibly due to the increased cortisol concentration in the circulation. However, these two cell types are present in small numbers in the blood of both young calves and adult animals (BENESI et al., 2012b).

Large unstained cells (LUC) include peroxidase-negative monocytes, large lymphocytes and blast cells (TVEDTEN et al., 2000). In unhealthy and healthy animals, LUC count presented no difference between age groups in accordance with Brun-Hansen et al. (2006) results.

Myeloperoxidase (MPO) is an enzyme produced by neutrophil leukocytes and released upon activation, killing pathogens and causing tissue damage (NIKULSHIN et al., 2015). Our methodology used the hematological analyzer ADVIA 2120i (Siemens AG), while routinely assessing neutrophil count, measures their MPO content by means of myeloperoxidase index (MPXI). The MPXI has been shown to be a measure of systemic neutrophil activation (NIKULSHIN et al., 2015). No data in calves for MPXI has been published. For interpretation matter, a negative MPXI value means that the sample cells contain less peroxidase than the ideal normal population; a positive value means higher MPO content compared to the ideal normal population. In this study, it was possible to observe that neonates presented lower levels of MPXI compared to older animals (G2 and G3). As neutrophils in neonates are functionally immature and the subsequent maturation process could be reflected the low level of cellular MPO content in neonate calves. Similar results were presented by Nikulshin et al. (2015) who showed highly significantly lower levels of MPXI in neonates 0–28-day old in comparison with older children.

There were no influence of age or health status in lobularity index (LI) and WBC count from Peroxidase channel (WBCP). Both of parameters are being reported in this study for the first time in calves. LI is the ratio between the number of cells producing high light scattering (PMN with higher lobularity) and cells with lower light scattering (mononuclear, immature granulocytes and blasts) (BRAGA, 2014). This channel also provides valuable information about the degree of maturity of each WBC's nucleus by measuring its lobularity and density (HARRIS et al., 2005). WBCP is the WBC count in peroxidase channel where peroxidase reagents are used to distinguish between

peroxidase-positive cells, such as neutrophils, eosinophils, and monocytes, and peroxidase-negative cells, which include lymphocytes, basophils, and LUC (HARRIS et al., 2005).

Related to differences between animals with different health status on each group, it was possible to observe that unhealthy neonates presented higher percentage of neutrophils than healthy calves which is an indication of response to an inflammatory process due to more exposition of pathogens and natural challenges that sick animals were going through. In this present study, most of sick neonates (G1) were presenting diarrhea. Hematological changes in neonatal diarrheic calves were evaluated by Brar et al. (2015). They have reported that neonatal calves with 1 to 7 days of life presenting diarrhea showed higher number of neutrophils and lower number of lymphocytes as the present study.

The higher numbers of monocytes in unhealthy animals compared to healthy animals indicate that mononuclear cells in sick animals of G3 were more activated than heifers with a healthy status, presumably due to respiratory disease influence. The alveolar and intravascular macrophages do function in generation of cell-mediated, humoral, and regulatory (inhibitory) responses during respiratory disease infection (ACKERMANN; DERSCHEID; ROTH, 2010).

It was possible to observe lower values of total solids and total protein in G2 compared to other age groups. Even if the mean values did not indicate failure of passive transfer (minimum of 8.4% and 5.5 g/dL, respectively) these results demonstrated a susceptibility of G2 immune system which are consistent with a decline in colostral immunity at approximately 30 days of age when the calf immune response is not completely functional yet. In G3 the animals were able to produce their own immunoglobulins once levels of total solids and total protein increased and then they were capable in developing the acquired immunity better than younger animals. A further analysis of IgG level of the animals can support this hypothesis.

The unhealthy neonates showed lower levels of total protein and total solids than healthy animals. This finding complies with the health status of these animals once serum total protein may be used as a rough estimative of colostrum transfer in ruminants which indicates the immune status of calves in field conditions.

In this study, serum iron concentration in animals of G1 differed with G3 which agrees with Mohri et al. (2007) that identified a significative difference between iron concentration of animals with 1 – 2 days of age compared to animals with 60 to 84

days of life. Also, a significant increase of iron levels from neonates to animals with 60 to 89 days of age was observed and by the previous authors as well. Low serum iron concentration in newborns can impact in erythropoiesis, thus, decreasing values of RBC, Packed Cell Volume and Hemoglobin (MOHRI et al., 2004).

The present study did not show any influence of health status in serum iron. Iron requirement for domestic animals are influenced by age, growth rate, availability of dietary iron source, and the criteria of adequacy. The iron requirements of ruminants are not well established and most recommendations are estimated (SMITH, 1989). It is generally accepted, however, that the iron requirements of young animals are higher than those of mature ruminants are and thought to be about 100 ppm. Therefore, neonatal calves can easily become iron deficient as they grow, additionally the calves were allocated in individual suspended cages without any contact with the ground. These conditions can be associated with the lower levels of serum iron on these animals (MOHRI; POORSINA; SEDAGHAT, 2010). On this way, the hypothesis is that the neonates were anemic due to an inflammatory process. The high levels of total protein found in neonates corroborates with the inflammatory profile of these group of animals. Also, RBC results were lower in unhealthy animals than healthy calves.

There were no statistically significant differences between age groups in haptoglobin levels nor an influence of health status on this parameter. Ramos (2018) evaluated 216 calves with 7 up to 41 days of age in terms of haptoglobin variation in animals presenting different diseases and he could obtain significant difference according to health status and between age groups. Murray et al. (2014) also reported increase of haptoglobin for unhealthy animals evaluating 1,365 calves since 1 up to 120 days of life. However, Knowles et al. (2000) investigated changes in 14 calves from birth to 83 days of age of hematological parameters, including haptoglobin. Their study presented no variation of haptoglobin values. No differences were obtained as well in Tóthová et al. (2012) in terms of haptoglobin between animals with or without diarrhea (n=10). Thus, one hypothesis that could explain the absence of difference in this present study is the low number of animals and the studies described above are in consistency with that.

To determine the innate immune response from calves with different ages and different health status, the phagocytic activity of MN (mononuclear cells) and PMN (polymorphonuclear cells) under bacteria (*E. coli, S. aureus* and *M. haemolytica*) stimulation were evaluated. It was not possible to detect many differences of

phagocytosis rate or avidity (MFI) between animals according to ages or health status. Despite the few differences between ages or health status, the rate of PMN phagocytosis showed an interesting pattern with high percentage independently of the antigen tested. So, there's a need to take in consideration that our methodology to perform the immune response assays followed the instructions of pre labeled bacteria (*E. coli and S. aureus*) suppliers and to do so we adapt the phagocytosis assay for *M. haemolytica* from what is described by Batista et al. (2018).

The activity of phagocytosis determined by MN cells was measured and analyzed by Flow Jo software. Neonates presented lower phagocytosis rate by their MN cells compared to activity of G2 and G3. Also, when comparing the phagocytosis activity between health status, unhealthy neonates was lower than healthy calves from the same group age. The lower levels of MN phagocytosis in neonates are corroborated by lower levels of lymphocytes (relative and absolute numbers) and monocytes (absolute number) obtained in WBC. Thus, it's assumed the hypothesis that a decrease phagocytosis capacity of mononuclear cells is correlated to a characterized low stimulation of inducing the adaptative immune system on these animals and it's supported by other studies (BARRINGTON; PARISH, 2001; CHASE; HURLEY; REBER, 2008; CORTESE, 2009).

Additionally, for healthy animals, G2 presented higher MFI phagocytosis of *M. haemolytica* compared to G1 and G3. A study was conducted by Batista (2015) and it was used by the author in her PhD thesis. This work described the methodology of labelling *M. haemolytica* which originated the paper where the technique is validated (Batista et al. 2018) when the first data describing the conjugation of bacteria with R-phycoerythrin (R-PE) was published. Nevertheless, there's no data available regarding development of calves innate immune system by influence *M. haemolytica* until now. Regarding the results from the present study, the animals from G2 may showed a high MFI response of *M. haemolytica* due to the determinant environmental challenge to bovine respiratory disease, as this group presented a great incidence of this disease inside this group and could be associated as a resistant sign of these animals.

In contrast to our findings, Batista et al. (2015) reported some differences of MN and PMN phagocytosis and MFI when these cells were stimulated with *S. aureus* and *E. coli* according to the different age of health calves. They assessed phagocytosis by flow cytometry as well, but *E. coli and S. aureus* were stained with propidium iodide (PI) instead of using pre labelled bacteria as in this study. Also, there were some

particularities between both of assays preparation (difference in incubation time). In general, their study showed lowest activity of phagocytes in calves in the intermediate phase with 31– 40 days of age with lower activity of MN and PMN phagocytes and MFI compared to neonates (first week of life). There was no difference in this present study for intensity of fluorescence (MFI) results for all antigens tested (MN and PMN). In Batista et al. (2015), MFI for MN cells with *S. aureus* did not present any differences according to age and neither for MFI of PMN cells with *E. coli*.

Menge et al. (1998) evaluated phagocytic activity by using a commercial kit with a labelled *E. coli*. They showed a greater phagocytosis capacity in health animals of 3 to 9 weeks of age compared to newborn calves. In accordance with our study, Kampen et al. (2006), who used the same assay methodology of Menge et al. (1998), showed that the PMN rate of *E. coli* phagocytosis maintained quite similar since first week of life until 27 – 29 weeks of age.

The MFI phagocytosis of *E. coli* in unhealthy calves of G2 was higher than healthy animals. These results obtained by group 2 were considered inconclusive by this author and perhaps were associated with the natural exposure of pathogens from the environment.

The unhealthy subgroup with 60 to 89 days of life presented higher phagocytosis (percentage and intensity) against *M. haemolytica* exposure compared to healthy animals of this age group, for MN and PMN cells. The high number of monocytes in unhealthy animals found in WBC evaluation corroborates the higher level of mononuclear phagocytosis of *M. haemolytica* for this group. Considering that unhealthy animals from G3 presented mainly BRD, it's possible associate with the high values of *M. haemolytica* phagocytosis, once this microorganism is the main responsible for respiratory disease in calves. According to many studies an interstitial cell derived from the circulating monocyte is considered as the immediate precursor of alveolar macrophage (PINKETT et al., 1966; BOWDEN et al., 1969; BOWDEN; ADAMSON, 1972). Using the tritiated thymidine in vivo labelling technique, AlIzzi et al. (1982) confirmed that in calf, alveolar macrophages were derived from peripheral blood monocytes. They also showed that, once in the alveolus, alveolar macrophages are essentially non dividing cells. It is known that the first line of defense against *M. haemolytica* is carried out by alveolar macrophages (ACKERMAN et al., 2010).

Evaluating the results of phagocytosis under different types of stimulus it was possible to observe that few differences were obtained when comparing animals with

distinct health status or ages. Neonates did not present relevant differences while G2 only presented major response in sick animals for MFI by *E. coli*. The unhealthy animals of G3 showed high response against *M. haemolytica*. Furthermore, there's a real lack of studies discussing the development of innate immune response in calves, specially oriented to *M. haemolytica* stimuli and comparing animals with different health status.

ROS production was measured in the leucocyte population using dihydrorhodamine-123 (DHR-123) and the assay was developed for this study but adapted of Donovan et al. (2007b) and Nace et al. (2014). Stimulants of ROS production included PMA, *Escherichia coli* 1:10, *Staphylococcus aureus* 1:5, *Mannheimia haemolytica* and *Mannheimia haemolytica* 1:5. Total ROS production was measured, and fluorescence data was obtained in cumulative AFU (arbitrary fluorescence units). Production of ROS at each concentration of each stimulus comparing to production of basal cells not stimulated is presented as a response ratio (RR).

The production of reactive oxygen species in unhealthy neonates was significantly higher for all antigens (PMA, *E. coli* 1:10*, S. aureus* 1:5*, M. haemolytica* and *M. haemolytica 1:5*) and for healthy neonates were higher for *E. coli* and *M. haemolytica 1:5* compared to other age groups. The results showed by this present study corroborates with Menge et al. (1998) and Kampen et al. (2006) which described highest levels of respiratory burst activity/reactive oxygen species in neonates compared to older animals. Both of previous studies performed the measurement of ROS using a commercial kit with unopsonized *E. coli* and data were collected by flow cytometry. Additionally, the oxidative burst activity evaluated in both studies considered the production of intracellular ROS and this study methodology took in consideration the intracellular and extracellular measurement of ROS. Even though the methodology of this study differed with Menge et al. (1998) and Kampen et al. (2006), our results showed comparative and consistency results with them which indicate a high biological effect. Also, unhealthy animals presented higher rates of neutrophils (%) compared to other age groups which comply with the fact that the production of ROS is mainly due to activation of PMN cells.

Batista et al. (2015) evaluated the ROS production of leucocytes using 2,7-dichlorofluorescein diacetate (DCFH-DA) and by flow cytometry. For MN cells, the lowest ROS production were found in animals with 31 – 40 days of life compared to

the group with 8 - 14 days. And for PMN cells, no difference for ROS production were found.

Due to the absence of data published of *M. haemolytica*-induced ROS production, it was not possible to compare the results for this microorganism. Although, the same significant increase occurred in neonates comparing to other age groups for this antigen.

At birth, an immunologically immature and naive calf is naturally confronted with a massive microbial exposure. Neonatal calves are dependent on the function of their innate immune activities directed by maternal antibody, cells and protein immune modulating factors transferred with colostrum. These interactions modulate the microbial colonization of the intestine and the lungs of calves and provide targeting and priming activation of innate responses in the neonate (GRIEBEL, 2009; LIANG ET AL., 2015). The immunoglobulins provided by colostrum can neutralize antigens and mark them for phagocytosis therefore increasing the efficiency of the innate immune response. Furthermore, the unhealthy neonates generated high percentages of phagocytosis when stimulated by all antigens and remarkably the granulocytes performed their role with greater efficiency producing higher levels of ROS (AFU). Interestingly, these results indicate that the neutrophilic granulocytes are functional and able to mount an effective response in neonate calves from the first week of life due to a positive influence of maternal colostrum.

For ROS analysis, the response ratio was calculated using the AFU value of stimulated cells (PMA, *E. coli* 1:10*, S. aureus* 1:5*, M. haemolytica* and *M. haemolytica 1:5*) versus AFU values of unstimulated cells. Stimulation of leucocytes with *E. coli* produced higher RR in neonates' healthy calves compared to G2 or G3. The most important disease in G1 was diarrhea (46%) and it's known that *E. coli* is one of the major enteric pathogens causing diarrhea in calves. So, the healthy animals from G1 were under pressure of the environment challenge for this pathogen and they respond accordingly producing higher ROS response for this microorganism.

Healthy animals with 30 – 40 days (G2) of life showed high response ratio by *M. haemolytica* and *M. haemolytica* 1:5 compared to G1 and G3. However, the same parameters in unhealthy animals were lower in G2 compared to other ages groups, likewise in all other antigens tested (PMA, *E. coli* 1:10*, S. aureus* 1:5). These results indicate that compared to basal cells AFU production, the ROS production of cells stimulated by all antigens in this group were less active for unhealthy animals, but for

M. haemolytica stimulus the healthy animals from G2 could perform an active and efficient response against this pathogen that was related to the main disease for this group (BRD). PMN generation of ROS is critical to their role in host defense and its impaired production is associated with increased susceptibility to infection (DINAUER, 1993). Results from unhealthy animals from G2 are consistent with a decline in colostrum immunity weeks after birth and the beginning of active immunity activation in calves characterizing a window of susceptibility during this phase. Most maternal antibody has a decay half-life of 16 to 28 days for the main respiratory (FULTON et al., 2004). During this transition, a window of susceptibility occurs when the immune response decrease reflecting a functional efficacy of certain cells but not their maximum capacity. At this stage, the animals are able to respond to a pathogen, although the response is weaker, slower and less efficient than that of animals with a mature immune system (CORTESE, 2009).

The unhealthy animals from G3 presented higher levels of response ratio for all antigen's stimulus cells (PMA, *E. coli* 1:10*, S. aureus* 1:5*, M. haemolytica* and *M. haemolytica 1:5*) compared to healthy animals. That fact indicates that these animals were more stimulated to produce more ROS than the animals with a good health status as they were under diseases' challenging. These results are in comply with phagocytosis rates of MN and PMN of *M. haemolytica* that were higher in unhealthy animals of G3 group as presented previously and they were functionally apt to respond against natural pathogens, mainly for bovine respiratory disease.

In general, ROS production results demonstrated the potential of innate immune response of G1 and G3. The performance of unhealthy and healthy neonates were likely influenced by colostrum factors support on innate immune system as they have presented high production of ROS (AFU) for the antigens tested when comparing with animals with other ages. The unhealthy animals from G3 could perform with efficiency the response against all antigens once they have presented higher response ratio than healthy animals. On the other hand, unhealthy animals from G2 were less responsive for ROS production (lower RR) than healthy heifers. Finally, this present study developed an adapted technique to measure ROS production that implicates in a limitation to compare the results with other studies also this study reported innovative data regarding development of immune response in calves with *M. haemolytica* stimuli and comparing animals with different health status.

7. CONCLUSION

Calves with different ages and health status under natural challenge to pathogens were evaluated for hematological and innate immune response by phagocytosis and reactive oxygen species assays and presented differences according to their groups/subgroups. The analytical methodology (ADVIA 2120) for hematologic measurements brought out results of parameters for the first time reported in calves by this study. The neonate group presented high frequency of diarrhea and able to mount an effective innate immune response against natural exposition to pathogens from the first week of life. The intermediate group with 30 to 40 days of age were mainly affected by Bovine Respiratory Disease and showed less responsive in terms of innate immune mechanisms likely due to reduction of maternal immunoglobulins and the lack of self-immunity during this phase. Animals with 60 to 89 days of life were efficient to respond against pathogens, mainly caused by respiratory disease. These findings were likely associated with the influence of colostrum maternal factors that acted enhancing the innate immune response of calves against natural pathogens challenges while the active adaptative response was under development. In conclusion, age and health status were intrinsically associated with changes in hematological and biochemical profile and innate immune response in Holstein calves.

8. REFERENCES

ACKERMAN, M.R.; DERSCHEID, R.; ROTH, J.A. Innate immunology of Bovine Respiratory Diseases. Veterinary Clinics of North America: Food Animal Practice, v.26, p.215-228, 2010.

ADAMS R.R., GARRY F.B., ALDRIBGE B.M. Haematologic values in newborn beef calves. **American Journal of Veterinary Research.** V. 53, p. 944-50, 1992.

ADEREM, A.; UNDERHILL, D. M. Mechanisms of Phagocytosis in Macrophages. **Annual Review of Immunology**, v. 17, n. 1, p. 593–623, 1999.

ALDRIDGE, B. M.; MCGUIRK, S. M.; LUNN, D. P. Effect of colostral ingestion on immunoglobulin-positive cells in calves. **Veterinary immunology and immunopathology**, v. 62, n. 1, p. 51-64, 1998.

AL-IZZI S.A., MAXIE M.G., VALLI V.E.O., WILKIE B.N., JOHNSON J.A. The kinetics of mononuclear phagocytes in normal calves and in calves given Corynebacterium parvum. Canadian Journal of Comparative Medicine and Veterinary. v. 46, p. 138-14, 1982.

ANGELOS, J. A. Infectious bovine keratoconjunctivitis (pinkeye). **Veterinary Clinics of North America - Food Animal Practice**, v. 31, n. 1, p. 61–79, 2015.

BACCILI, C. C. et al. Hematological and immunological development from birth to six months of age in Holstein calves. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 70, n. 6, p. 1823-1832, 2018.

BAGLIONI T., LOCATELLI A., QUARENGHI F. Il quadro ematologico nella prima settimana di vita del vitello. **La Clin. Vet**, v. 79, p. 289-297, 1956.

BARRINGTON, G. M.; PARISH, S. M. Bovine neonatal immunology. **The Veterinary clinics of North America. Food animal practice**, v. 17, n. 3, p. 463–476, 2001.

BATISTA, C. F. et al. Evolution of phagocytic function in monocytes and neutrophils blood cells of healthy calves. **Journal of Dairy Science**, v. 98, n. 12, p. 8882–8888, 2015.

BATISTA, C. F. **Dinâmica de fagócitos sanguíneos e alveolares em bezerros com mannheimiose**. São Paulo, 2015. 145f. Tese (Doutorado em Clinica Veterinária) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2015

BENESI F.J. 1992. **Hematologia de bezerros recém-nascidos. Influência da asfixia neonatal, do tipo do parto e da ingestão de colostro sobre a crase sanguínea**. São paulo, 1992. 126f. Tese (Doutorado em Clínica Veterinária) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 1992.

BENESI, F. J.; TEIXEIRA, C. M. C.; LEAL, M. L. R.; LISBOA, J. A. N.; MIRANDOLA, R. M. S.; SHECAIRA, C. L.; GOMES, V. Leukograms of healthy Holstein calves within the first month of life. **Pesquisa. Veterinária Brasileira**, v. 32, n. 4, p. 352-356, 2012b.

BENESI, F. J.; TEIXEIRA, C. M. C.; LISBOA, J. A. N.; LEAL, M. L. R.; BIRGEL JÚNIOR, E.H.; BOHLAND, E.; MIRANDOLA, R. M. S. Eritrograma de bezerros sadios, da raça Holandesa, no primeiro mês de vida. **Pesquisa Veterinária Brasileira**, v. 32, n. 4, p. 357-360, 2012a.

BESSER, T. E.; GAY, C. C. The importance of colostrum to the health of the neonatal calf. Vet. Clinics North America. v.10, p.107–117, 1994.

BOWDEN D.H., ADAMSON. I.Y.R., GRANTHAM W.G., WYATT J.P. Origin of the lung macrophage: evidence derived from radiation injury. Archives of Pathology & Laboratory Medicine. v. 88, p. 540-546, 1969.

BOWDEN D.H., ADAMSON I.Y.R. The pulmonary interstitial cell as immediate precursor of the alveolar macrophage. The American Journal of Pathology. v. 68, p. 521-528, 1972.

BRAGA, D. S. G. P. A. **Contagem globular automática: parâmetros avaliados significado clínico e causas de erro.** Julho de 2014, 68 f. Monografia do 2º ciclo de estudos conducente ao Grau de Mestre em Análises Clínicas – Faculdade de Farmácia Universidade do Porto, Porto, 2014.

BRANDON, M. R.; WATSON, D. L.; LASCELLES, A. K. The mechanism of transfer of immunoglobulin into mammary secretion of cows. **Australian Journal of Experimental Biology and Medical Science**, 49.6: p. 613-623, 1971.

BRAR, A. P. S. et al. Hematological changes in neonatal diarrheic calves of different age groups. **Indian Journal of Veterinary Pathology**, v. 39, n. 1, p. 73, 2015.

BROUGHTON, C. W.; LECCE J. G. Electron microscopic studies of the jejunal epithelium from neonatal pigs fed different diets. **The Journal of Nutrition**. v.100, p. 445–449, 1970.

BRUN-HANSEN, H. C.; KAMPEN, A. H.; LUND, A. Hematologic values in calves during the first 6 months of life. **Veterinary Clinical Pathology**, v. 35, n. 2, p. 182–187, 2006.

CHASE, C. C. L.; HURLEY, D. J.; REBER, A. J. Neonatal Immune Development in the Calf and Its Impact on Vaccine Response. **Veterinary Clinics of North America -**

Food Animal Practice, v. 24, n. 1, p. 87–104, 2008.

CHASE, C. C. L. Enteric Immunity: Happy Gut, Healthy Animal. **Veterinary Clinics of North America - Food Animal Practice**, v. 34, n. 1, p. 1–18, 2018.

CHO, Y.; YOON, K. An overview of calf diarrhea - infectious etiology , diagnosis , and intervention. Journal of Veterinary Science. v. 15, p. 1–17, 2014.

CORTESE, V. S. Neonatal Immunology. **Veterinary Clinics of North America - Food Animal Practice**, v. 25, n. 1, p. 221–227, 2009.

COSTA, J. F. D. R. et al. Innate immune response in neonate Holstein heifer calves fed fresh or frozen colostrum. **Research in Veterinary Science**, v. 115, p. 54–60, 2017.

DEELEN, S. M. et al. Evaluation of a Brix refractometer to estimate serum immunoglobulin G concentration in neonatal dairy calves. **Journal of Dairy Science**, v. 97, n. 6, p. 3838–3844, 2014.

DINAUER, M. C. The respiratory burst oxidase and the molecular genetics of chronic granulomatous disease. **Critical Reviews in Clinical Laboratory Sciences**. v. 30, p. 329–369, 1993.

DONOVAN, D. C.; REBER, A. J.; GABBARD, J. D.; ACEVES-AVILA, M.; GALLAND, K. L.; HOLBERT, K. A.; ELY, L. O.; HURLEY, D. J. Effect of maternal cells transferred with colostrum on cellular responses to pathogen antigens in neonatal calves. **American Journal of Veterinary Research**, v. 68, n. 7, p. 778-82, 2007a.

DONOVAN D. C., JACKSON C. A., COLAHAN P. T., NORTON N. N., CLAPPER J. L., MOORE J. N., HURLEY D. J. Assessment of exercise-induced alterations in neutrophil function in horses. **American Journal of Veterinary Research**. v. 68, n. 11, 2007b.

DOS SANTOS, G.; BITTAR, C. M. M. A survey of dairy calf management practices in some producing regions in Brazil. **Revista Brasileira de Zootecnia**, v. 44, n. 10, p. 361–370, 2015.

EBERHART R.J., PATT JR J.A. Plasma cortisol concentrations in newborn calves. **American Journal of Veterinary Research.** v. 32, p. 1921-1927, 1971.

ECLINPATH.COM. CORNELL UNIVERSITY, MCHC/CHCM. Disponível em: http://eclinpath.com/hematology/tests/mchc/. Acesso em: 03 Jan. 2020.

FREI YF, PERK K, DANON D. Correlation between osmotic resistance and fetal hemoglobin in bovine erythrocytes. **Experimental Cell Research**, v. 30, p. 561–568, 1963.

FULTON, R. W. et al. Maternally derived humoral immunity to bovine viral diarrhea virus (BVDV) 1a, BVDV1b, BVDV2, bovine herpesvirus-1, parainfluenza-3 virus bovine respiratory syncytial virus, Mannheimia haemolytica and Pasteurella multocida in beef calves, antibody decline . **Vaccine**, v. 22, n. 5–6, p. 643–649, 2004.

GODDEN, S. Colostrum Management for Dairy Calves. **Veterinary Clinics of North America - Food Animal Practice**, v. 24, n. 1, p. 19–39, 2008.

GODDEN, S. M.; LOMBARD, J. E.; WOOLUMS, A. R. Colostrum Management for Dairy Calves. **Veterinary Clinics of North America - Food Animal Practice**, v. 35, n. 3, p. 535–556, 2019.

GOMES, V; MADUREIRA, K. M; DELLA LIBERA, A. M. M. P.; ALVES, M.; BAPTISTELLA, F.; BENESI, F. J. Dinâmica da celularidade do colostro de vacas holandesas no pós-parto imediato. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v. 63, n. 5, p.1047-1053, 2011.

GOTO M., MARUYAMA M., KITADATE K., KIRISAWA R., OBATA Y., KOIWA M., IWAI H. Detection of Interleukin-1b in sera and colostrum of dairy cattle and in sera of neonates**. Journal of Veterinary Medical Science**. 59(6), p. 437-441, 1997.

GRIEBEL, P.J., Mucosal vaccination of the newborn: an unrealized opportunity. **Expert Rev. Vaccines**, v. 8, p. 1–3, 2009.

GRIFFIN, D. et al. Bacterial Pathogens of the Bovine Respiratory Disease Complex. v. 26, p. 381–394, 2010.

HAGIWARA, K.; ANDO, M.D.J. Bovine colostral CD8-positive cells are potent IFN-γ producing cells. **Veterinary Immunology and Immunophatology**, v.124, p.93-98, 2008.

HAGIWARA K, KATOKA S, YAMANAKA H, et al. Detection of cytokines in bovine colostrum. **Veterinary Immunology and Immunopathology**, v. 76, p. 183–90, 2000.

HARRIS, N. **The ADVIA 2120 Hematology System: Flow Cytometry – Based Analysis of Blood and Body Fluids in the Routine Hematology Laboratory**. n. January 2018, 2005.

HIEMSTRA, P.; DAHA, M. **Opsonization**. Delves, PJ (ed.) Encyclopedia of immunology. London: Academic Press, p. 1885–1888, 1998.

JAIN, N.C., 1986. **Schalm's Veterinary Hematology**, Fourth ed. Lea and Febiger, Philadelphia.

JANEWAY, C. A. **Imunobiologia**. 7th. ed. [s.l.] Artmed, 2007.

JEZEK, J. et al. **Age related chances and reference intervals of haematological variables in dairy calves.** Bull Vet Inst Pulawy, v.55, p. 471-478, 2011.

KAMPEN, A. H. et al. Lymphocyte subpopulations and neutrophil function in calves during the first 6 months of life. **Veterinary Immunology and Immunopathology**, v. 113, n. 1–2, p. 53–63, 2006.

KELLY, D.; COUTTS, A. G. P. Early nutrition and the development of immune function in the neonate. **Proceedings of the Nutrition Society**, v. 59, n. 2, p. 177–185, 2000.

KLINKON M., ZADNIK T. Dynamics of red and white blood picture in dairy cows during the periparturient period. Comparative Hematology International. v. 9, p. 156–161, 1999.

LARSON, B. K.; HEARY, H. K.; DEVERY, J. E. Immunoglobulin Production and Transport by the Mammary Gland**. Journal of Dairy Science**, v. 63, n. 2, p. 665-671, 1980

LIANG, G., MAMUTHUGE, N., GUAN LE, L., GRIEBEL, P. Model system to analyze the role of miRNAs and commensalmicroflora in bovine mucosal immune system development. **Molecular Immunology**, v. 66, p. 57–67, 2015.

LIEBLER-TENORIO, E. M.; RIEDEL-GASPARI, G.; POHLENZ, J. F. Uptake of colostral leukocytes in the intestinal tract of newborn calves. **Veterinary Immunology and Immunopathology**, v. 85, n.1-2, p. 33-40, 2002.

MADUREIRA, K. M. **Citocinas IL-1β, IL-6, TNF-α e IFN-γ no sangue e colostro de fêmeas bovinas da raça Holandesa. Importância na transferência de imunidade passiva.** São Paulo, 2011. 108f. Tese (Doutorado em Clínica Veterinária) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2011.

MALLARD, B. A. et al. Alteration in Immune Responsiveness during the Peripartum Period and Its Ramification on Dairy Cow and Calf Health. **Journal of Dairy Science**, v. 81, n. 2, p. 585–595, 1998.

MCGUIRK, Sheila M. Disease management of dairy calves and heifers. **Veterinary Clinics of North America**: Food Animal Practice, v. 24, n. 1, p. 139-153, 2008.

MEGANCK, V. **Neonatal diarrhea in dairy calves and the relevance of bovine colostral leukocytes**. Belgium, 2015. 241 f. Thesis (PhD Veterinary Sciences) - Faculty of Veterinary Medicine, Ghent University, Belgium, 2015.

MENGE, C. et al. Compensation of preliminary blood phagocyte immaturity in the newborn calf. **Veterinary Immunology and Immunopathology**, v. 62, n. 4, p. 309–321, 1998.

MOHRI, M.; SARRAFZADEH, F.; SELFI, H. A.; FARZANEH, N. Effects of oral iron supplementation on some haematological parameters and iron biochemistry in neonatal dairy calves. **Comparative Clinical Pathology**, v. 13, p. 39-42, 2004.

MOHRI M., POORSINA S., SEDAGHAT R. Effects of parenteral supply of iron on RBC parameters, performance, and health in neonatal dairy calves. Biological Trace Element Research. v. 136, p. 33–39, 2010.

MOHRI, M.; SHARIFI, K.; EIDI, S. Hematology and serum biochemistry of Holstein dairy calves: age related changes and comparison with blood composition in adults. **Research in Veterinary Science**, v. 83, p. 30-39, 2007.

MOREIN, B.; ABUSUGRA, I.; BLOMQVIST, G. Immunity in neonates. **Veterinary Immunology and Immunopathology**, v. 87, n. 3–4, p. 207–213, 2002.

MOSTOV, K. E. TRANSEPITHELIAL TRANSPORT The major class of immunoglobulin found in a wide variety of mucosal. **Cardiovascular Research**, n. 1, p. 63–84, 1994.

MURRAY, C. F. et al. Associations of serum haptoglobin in newborn dairy calves with health, growth, and mortality up to 4 months of age. **Journal of Dairy Science**, v. 97, n. 12, p. 7844–7855, 2014.

NACE, E. L., NICKERSON S. C., KAUTZ F. M., BREIDLING S., WOCHELE L. O., HURLEY D. J. Modulation of innate immune function and phenotype in bred dairy heifers during the periparturient period induced by feeding an immunostimulant for 60 days prior to delivery. **Veterinary Immunology and Immunopathology.** v. 161, p. 240–250, 2014

NIKULSHIN, S. et al. Intracellular neutrophil myeloperoxidase level in pediatric patients: Significant age and gender variability. **International Journal of Laboratory Hematology**, v. 37, n. 1, p. 120–124, 2015.

NOVO, S. M. Fontes et al. Hematological adaptation in Holstein calves during the neonatal period. **Brazilian Journal of Veterinary Research and Animal Science**, v. 52, n. 3, p. 212-216, 2015.

NOVO, S. M. F. et al. Effect of maternal cells transferred with colostrum on the health of neonate calves. **Research in veterinary science**, v. 112, p. 97-104, 2017a.

NOVO, S. M. F. **Efeito dos leucócitos do colostro materno na resposta imune de bezerros recém-nascidos**. São Paulo, 2017. 128f. Dissertação (Mestrado em Clínica Veterinária) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2017b.

PANOUSIS, N. et al. Hematology reference intervals for neonatal Holstein calves. **Research in Veterinary Science**, v. 118, n. January, p. 1–10, 2018.

PINKETT O., COWDREY C.R., NOWELL P.C. Mixed haematopoetic and pulmonary origin of alveolar macrophages as demonstrated by chromosome markers. The American Journal of Pathology. v. 48, p. 859-867, 1966.

PORTER, P. Immunoglobulins in bovine mammary secretions: quantitative changes in early lactation and absorption by the neonatal calf. **Immunology**, 23.2: 225, 1972

POULSEN, K. P.; MCGUIRK, S. M. Respiratory disease of the bovine neonate. **Veterinary Clinics of North America**: food animal practice, v. 25, p. 121–137, 2009.

QUIGLEY, J. D. et al. Evaluation of the Brix refractometer to estimate immunoglobulin G concentration in bovine colostrum. **Journal of Dairy Science**, v. 96, n. 2, p. 1148–1155, 2013.

RAMOS, J. S. **Haptoglobina como biomarcador de doenças em bezerras Holandesas.** São Paulo, 2018. 84f. Dissertação (Mestrado em Clínica Veterinária) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, 2018.

REID, J.T., WARD, G.M., SALSBURY, R.L., Mineral metabolism studies in dairy cattle. IV. Effects of mineral supplementation of the prepartal diet upon the composition of the blood of cows and their calves at parturition. **The Journal of Nutrition**, v. 56, p. 75–89, 1948.

REBER, A. J.; HIPPEN, A. R.; HURLEY, D. J. Effects of the ingestion of whole colostrum or cell-free colostrum on the capacity of leukocytes in newborn calves to stimulate or respond in one-way mixed leukocyte cultures. **American Journal of Veterinary Research**, v. 66, p. 1854–1860, 2005.

REBER, A. J.; LOCKWOOD, A.; HIPPEN, A. R.; HURLEY, D. J. Colostrum induced phenotypic and trafficking changes in maternal mononuclear cells in a peripheral blood leukocyte model for study of leukocyte transfer to the neonatal calf. **Veterinary Immunology and Immunopathology**, v. 109, n. 1-2, p. 139-50, 2006.

REBER A. J.; DONOVAN D. C.; GABBARD J.; GALLAND K.; ACEVES-AVILA M.; HOLBERT K. A.; MARSHALL L.; HURLEY D J. Transfer of maternal colostral leukocytes promotes development of the neonatal immune system Part II. Effects on neonatal lymphocytes. Veterinary Immunology and Immunopathology. v.123, p. 186-196, 2008.

ROCHA, T. G. et al. Hemograma e proteínas do soro sanguíneo de bezerros Canchim-Nelore e da raça Holandesa nos primeiros 30 dias de vida. **Arquivo Brasileiro de Medicina Veterinaria e Zootecnia**, v. 62, n. 5, p. 1250–1254, 2010.

SERHAN, C. N.; SAVILL, J. Resolution of inflammation: The beginning programs the end. **Nature Immunology**, v. 6, n. 12, p. 1191–1197, 2005.

SCHALM, O. W. **Veterinary hematology.** 2nd ed. Philadelphia, PA: Lea and Febiger, p. 307-312, 2010.

SCHECAIRA, C. L. **Avaliação da transferência de citocinas para o sangue de neonatos via ingestão de fêmeas bovinas Holandesas**. São Paulo, 2013. Dissertação (Mestrado em Clínica Médica) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2013.

SHELDRAKE, R.F., HUSBAND, A.J., Intestinal uptake of intact maternal lymphocytes byneonatal rats and lambs. **Research in Veterinary Science**. v. 39, p. 10–15, 1985.

SILVA, C. P. C. **Imunidade inata no sangue e secreção mamária de vacas Holandesas no período periparto.** São Paulo, 2014. 142 f. Dissertação (Mestrado em Clínica Veterinária) – Faculdade de Medicina Veterinária, Universidade de São Paulo, São Paulo, 2014.

SMITH J. E. Iron metabolism and its disease. In: Kaneko JJ (ed) Clinical biochemistry of domestic animals. Academic, San Diego, p. 262, 1989.

SMITH, B.; METRE, D. VAN; PUSTERLA, N. **Large Animal Internal Medicine**. 6th edition ed. [s.l.] Elsevier, 2019.

TENNANT B., HARROLD D., REINA-GUERRA M.B. Haematology of the neonatal calf: Erithrocyte and leukocyte values of normal calves. **Cornell Vet**. v. 64, p. 516-532, 1974.

TIZARD, I. R. **Imunologia Veterinária**. 8th. ed. Rio de Janeiro: Elsevier, 2008.

TÓTHOVÁ, C.; NAGY, O.; NAGYOVÁ, V.; KOVÁČ, G. Acute phase prteins in relation to various inflammatory diseases of calves. **Comparative Clinical Pathology**, v. 21, n. 5, p. 1037 – 1042, 2012.

TUBOLY, S., BERNÁTH, S., GLÁVITS, R., I. Intestinal absorption of colostral lymphoid cells in newborn piglets. **Veterinary Immunology and Immunopathology**. v. 20, p. 75–85. Medveczky, 1988.

TVEDTEN H, SCOTT M, BOON GD. **Interpretation of cytograms and histograms of erythrocytes, leukocytes and platelets**. In: Bonagura JD, ed. Kirk's Current Veterinary Therapy XIII. Philadelphia, PA: WB Saunders Co, p. 381–390, 2000.

VENTRELLA, D. et al. The biomedical piglet : establishing reference intervals for haematology and clinical chemistry parameters of two age groups with and without iron

supplementation. **BMC Veterinary Research**, p. 1–8, 2017

WATSON, D. L. Immunological functions of the mammary gland and its secretion comparative review. **Australian Journal of Biological Sciences**, v. 33, n. 4, p. 403-422, 1980.

WEAVER, D. M.; TYLER J. W.; VANMETRE D. C.; HOSTETLER D. E.; BARRINGTON G. M. Passive transfer of colostral immunoglobulins in calves. **Journal of Veterinary Internal Medicine.** v.14, p. 569–577, 2000.

WILLIAMS, P. P. Immunomodulating effects of intestinal absorbed maternal colostral leukocytes by neonatal pigs, **Canadian Journal of Veterinary Research**, v. 57, p.1-8, 1993.

YAMANAKA H., HAGIWARA K., KIRISAWA R., IWAI H. Proinflammatory cytokines in bovine colostrum potentiate the mitogenic response of peripheral blood mononuclear cells from newborn calves through IL-2 and CD25 expression. **Microbiology and Immunology**, v. 47(6), p. 461-468, 2003a.

YAMANAKA H., HAGIWARA K., KIRISAWA R., IWAI H. Transient detection of proinflamatory cytokines in sera of colostrums-fed newborn calves. **Journal of Veterinary Medical Science.** v. 65(7), p. 813-816, 2003b

YIN, J.; REN, W.; LIU, G.; DUAN, J.; YANG, G.; WU, L.; LI, T.; YIN, Y. Birth oxidative stress and the development of an antioxidant system in newborn piglets. **Free Radical Research**. v.47(12), p.1027–1035, 2013.

ZWAHLEN, R. D., ROTH, D. R., WYDER-WALTHER, M. In vitro aggregation of bovine neonatal neutrophils - A comparative study with adult cattle. **Inflammation**, v. 14, n. 4, p. 375–387, 1990.