FABRÍCIO DIAS TORRES

ASPECTOS CLÍNICOS E IMUNOLÓGICOS RELACIONADOS À INFECÇÃO PELO VÍRUS DA LEUCOSE ENZOÓTICA BOVINA (VLB), EM NOVILHAS NO PERÍODO DE TRANSIÇÃO

São Paulo

2021

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Tese apresentada ao Programa de Pós-Graduação em Clínica Veterinária da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutora em Ciências.

Departamento:
Clínica Médica

Área de concentração:
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Orientadora:
Profa. Dra. Viviani Gomes

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FMVZ USP

CERTIFICADO

Certificamos que a proposta intitulada " INFLUÊNCIA DA INFECCÃO PELO VÍRUS DA LEUCEMIA BOVINA (*BLV*) SOBRE O PERFIL METABÓLICO, IMUNIDADE E SANIDADE EM NOVILHAS LEITEIRAS NO PERÍODO DE TRANSIÇÃO", protocolada sob o CEUA nº 2188221018, sob a responsabilidade da Profa. Dra. Viviani Gomes ***e equipe; Fabricio Dias Torres***, 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 12/12/2018.

Procedência: Animais de proprietários
Espécie: Bovinos sexo: Fêmeas idade: 20 a 30 meses N: 30 Linhagem: holandesa e jersey Peso: 400 a 600 kg

São Paulo, 03 de maio de 2021.

Marcelo Bahia Labruna
Coordenador

Av. Prof. Dr. Orlando Marques de Paiva, 87
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Autor: TORRES, Fabrício Dias.

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Data: _____/_____/_____

Banca Examinadora

Prof. Dr._____

Instituição:_____ Julgamento:_____

Prof. Dr._____

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Instituição:_____ Julgamento:_____

DEDICATÓRIA

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Senhor, dai-me força para mudar o que pode ser mudado...
Resignação para aceitar o que não pode ser mudado...
E sabedoria para distinguir uma coisa da outra.

São Francisco de Assis

RESUMO

TORRES, F.D. **Aspectos clínicos e imunológicos relacionados à infecção pelo Vírus da Leucose Enzoótica Bovina (VLB), em novilhas no período de transição**. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

O objetivo da pesquisa foi avaliar o impacto do Vírus da Leucemia Bovina (*Bovine Leukemia Virus - BLV*) sobre o perfil hematológico, bioquímico, inflamatório e do metabolismo energético em novilhas leiteiras naturalmente infectadas no período de transição. O rebanho foi inicialmente triado por meio da prevalência de anticorpos específicos anti-BLV pela técnica de ensaio imunoenzimático (ELISA). Para tanto, 24 novilhas prenhas das raças Holstein e Jersey foram distribuídas em 2 grupos experimentais, composto por novilhas soropositivas BLV +, (n=12); e o grupo BLV – composto por novilhas soronegativas (n=12). Foi realizada ainda a pesquisa de DNA proviral por avaliação digital da reação da polimerase em cadeia (ddrPCR). Amostras de sangue foram colhidas semanalmente nos períodos -3, -2, -1, parto, +1, +2 e +3 semanas em relação ao parto previsto, e avaliadas para determinação de perfil hematológico, bioquímico hepático (AST, GGT) Inflamatório (Fibrinogênio, HP) e energético (BHB, triglicerídeos e colesterol). Os animais foram monitorados diariamente por um sistema automático de avaliação de ruminação. Foram levantados os registros relativos à saúde, produção e qualidade do leite das novilhas.. De forma complementar, os animais foram ainda distribuídos em grupos com alta carga proviral e baixa carga proviral, para avaliação dos mesmos parâmetros previamente descritos. Para análise estatística, os dados foram linearizados e avaliados pelo modelo linear misto (Mixed model). O método de regressão logística foi usado para avaliação de inflamação, cetose e lesão hepática. A prevalência encontrada para anticorpos anti-BLV foi de 57.25% (351/613), sendo nas novilhas prenhes de 38.7% (76/124). A infecção por BLV alterou o perfil hematológico das novilhas para CHCM (P= 0.026), bioquímico com a elevação nas concentrações da enzima AST (P= 0.023), além de alterar o perfil cinético dos triglicerídeos entre os grupos (P= 0.023). A avaliação do perfil inflamatório revelou uma influência da infecção por BLV no fibrinogênio (P= 0.043), e aumento expressivo no risco de elevação da HP (OR= 4.959). Adicionalmente, as contagens leucocitárias de animais com alta carga viral foram

significativamente maiores no grupo com alta carga vira (P= 0.018), especialmente por linfocitose (P= 0.036). Conclui-se que a infecção pelo BLV pode alterar o perfil hematológico e bioquímico e o metabolismo energético de novilhas no período de transição; além de aumentar as chances de inflamação. A carga viral pode impactar nas contagens leucocitárias, especialmente por predisposição de linfocitose.

Palavras-chave: Leucose, Carga proviral, Inflamação, Leucocitose, Linfocitose

ABSTRACT

TORRES, F.D. **Clinical and immunological aspects related to bovine leukemia virus (BLV) infection in heifers, during the transition period.** Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

The aim of the study is to evaluate the impact of the Bovine Leukemia Virus (BLV) on hematological, biochemical, inflammatory and energetic metabolic profile, in naturally infected heifers during the transition period. Initially, herd was screened (n= 613) to determinate anti-BLV specific antibodies by using immunenzymatic assay (ELISA). Therefore, 24 pregnancy Holstein and Jersey heifers were divided in two experimental groups composed by seropositive BLV+ (12); and seronegative BLV- (12) heifers. Proviral DNA was also evaluated through the Droplet digital Polymerase chain reaction (ddPCR). Whole blood and serum were weekly sampled in interval -3, -2, -1, 0, calve, +1, +2, +3 weeks, and evaluated to determination of hematological, hepatic biochemistry (AST and GGT), Inflammatory (Fibrinogen, Haptoglobin), and energetic (BHB, triglycerides and cholesterol). Animals were daily monitored with automatic rumination monitoring system, and clinically by the farm veterinaries assistance. Complementarily. Complementarily, animals were also divided in groups with high and low proviral load, to evaluation of the same parameters previously described. For the statistical analyses, data were linearized and evaluated according to Mixed Model. Regression logistic model were used to evaluate inflammation, ketosis and hepatic damage. Herd antibodies prevalence was 57.25% (351/613), with heifer's prevalence of 38.7% (76/124). BLV infection altered heifer's hematological profile for MCHC (P= 0.026), Biochemical by increasing AST enzymatic activity (P= 0.023), and altered the triglycerides kinetic among groups (P= 0.023). Inflammatory profile evaluation revels an influence of BLV infection on fibrinogen (P= 0.043), and an expressive increase risk for HP elevation (OR= 4.959) in BLV+ animals. Moreover, leukocytes count was significantly increased in group with high proviral load (P= 0.018), mainly by lymphocytosis (P= 0.036). In conclusion, BLV infection altered the hematological, biochemical and energetic metabolic profile of heifers during the transition period. Moreover, proviral load may increase leukocytes count especially due to lymphocytosis.

Key word: Leukosis, Proviral Load, Inflammation, Leukocytosis, Lymphocytosis

SUMÁRIO

1 INTRODUCTION

Bovine Leukemia Virus (BLV) is a beef and dairy cattle retrovirus, spread in bovine herds worldwide, associated with Lymphosarcoma and especially disbalance of immune system leading in immunosuppressive state (GILLET et al., 2007).

Direct mensurable appears as the less problem, and indirect involved impact is difficult to measure. Those, real monetary losses are poor estimated. Herds infected estimate losses of $ 3.500,00 per cow/year, associated to mortality or early culling, veterinary assistance (JOHNSON et al., 1985; SORENSEN; BEAL, 1979;), and accentuate drop milk production (PELZER, 1997). Moreover, estimated directed impact BLV clinical infection on productivity on dairy industry and consumers close to $500 million yearly (OTT, et al. 2003), and the net losses duo to lymphoma are estimated to be $412 each case (RHODES et al., 2003). Other study predicted losses by Lymphosarcoma and carcasses condemnation in US, an order of U$ 16 million per year (PELZER, 1997).

According to Kuczewski, et. al, 2018, a model considering a 40% prevalence into a herd with size of 146-adult cow herd. For all options tested in models, net benefic far exceed the cost according to following actions adoption: Can$1,315/year for management strategies (freezering colostrum); Can$1,243/year for management strategies (pasteurizer colostrum); Can$785/year for management strategies (powdered colostrum); Can$1,028 for some management strategies; Can$1,592 for test and cull; and Can$1,594 for test and segregate. Understand host-BLV interaction and the impairment in homeorhetic and homeostasis statement is key point to frame BLV as a productive disease.

Duo the considerable productive and economic impact, BLV was controlled and even eradicated from over than 20 countries, manly in Europe (ANUAL EU REPORT, 2018). This status limits trades from countries or regions positives to negative by impose of barriers. Commercial implications and drop in productive makes more controversially that BLV is still worldwide spread and, especially in American continent, has a high increased incidence (BARTLEET, 2020). This increase of occurrence may be explain due to intensively production and adoption of biotechnologies, especially reproduction management (POLAT, 2017, BARTLETT, 2014).

In Brazil, BLV is an endemic high prevalent disease (FLORES et al., 1989; BIERGEL JUNIOR et al., 1995). Local research groups were very efficient to evaluate

virus interaction/damage in immune system of cows naturally infected (SOUZA, 2011; DELLA LIBERA 2012; DELLA LIBERA, 2015; GOMES, 2017). However, field impact is unknown since very few studies aiming to access the real losses associated to virus infection in herds. Moreover, most of prevalence description has more than 10 years, and due to demand of high productivity, incidences of transmission are probably increasing nowadays.

Is clear that BLV infection act as an important player in immune disbalances, leading in other disease occurrence (DELLA LIBERA, 2012). Recently, a research showed that BLV infected animals during the transition period, shows a market disbalance in immune functions; and in blood cellularity (WISNIESKI et al., 2020). Transition period is defined as the peripartum between 3 weeks before calving and 3 weeks later. During this stage, dams suffered huge metabolic and physiological changes aiming to support calve requirement, preparing to delivery moment and further lactation (DRACKLEY et al., 1999). To cross the periparturient period storm is determinant to health status, leading productivity, and profitability. Thus, evaluation of nutrition and management of dairy cows during the transition period over the last three decades had provide more skills (DRANKLEY et al., 2005), and is still a frontier that need more understanding (REF 2021 Chase). However, challenges on energetic metabolism, biochemistry and hematological, and relationship between this disbalances and diseases is still in evidence (Le BLANC, 2010, REIS et al 2016; GOMES et et al, 2017).

Very few studies evaluating the impact of BLV in transition period, especially in hematological, biochemical and health status are available in literature. Moreover, BLV immunosuppressive is fully associated with cows, rather that heifers. Motivation for our thesis came from the light of curious in access the main hematological, biochemical and metabolic energetic profile of heifers, during the obscure and challenge periparturient period. Additionally, we intended to evaluate proviral load of heifers and possible relationship with those parameters.

2 LITERATURE REVIEW

This topic will be present some aspects of BLV: etiopathogenesis and clinical signs; main feature involving in transmission and challenges in control disease; possible zoonotic potential; and review the hematological and biochemical trends on heifers and cows, especially and transition period.

2.1 BOVINE LEUKEMIA VIRUS (BLV) – WORLDWIDE OVERVIEW SITUATION.

Eradicated in over 20 countries/regions, Bovine Leukemia virus (BLV) can still be a consider a high prevalent worldwide pathogen; and Bovine Enzootic Leukosis (BEL) have defined as a chronic endemic disease associated with lymphomas and persistence lymphocytosis (PL). Countries like German, Italy, Austria, Belgian, Netherlands, UK and others, are classified as free areas for BLV (ANUAL EU REPORT, 2018). In other hand, in most regions/countries, especially outside Europe, the BLV prevalence is still high or increases mainly in dairy herds.

The most of epidemiology data available are outdated, and more recent studies evaluating prevalence are missing. However, an US inquiry found prevalence of 46,5% for dairy cattle level, and 82,5% of positive herds. This data suggests an increase in the prevalence of BLV infection over the 5 last decades, according to previous studies (LADRONKA et al., 2018). Moreover, in this same study was asked about risk perception for BLV prevalence as a problem. Only 11,2% recognizes BLV as a significant or a big problem; while 88,8% assume problem is not a problem or is unsignificant.

Europe and US are diametrically opposed in terms of plan to control BEL. Some sporadic and isolated actions aiming to control agent in US farms are find in literature; while in Europe most countries adopted a joint effort to eradicate BEL. In north America, there are studies and inquires accessing prevalence's and incidences (BARTLETT et al., 2020); evaluations of productivity and economic impact; but probably duo to hard control/eradication, opted to coexist with the agent. Latin American countries are probably some steps back compare to north America and Europe. The prevalence's of BLV in Latin America countries are shown in the table 1.

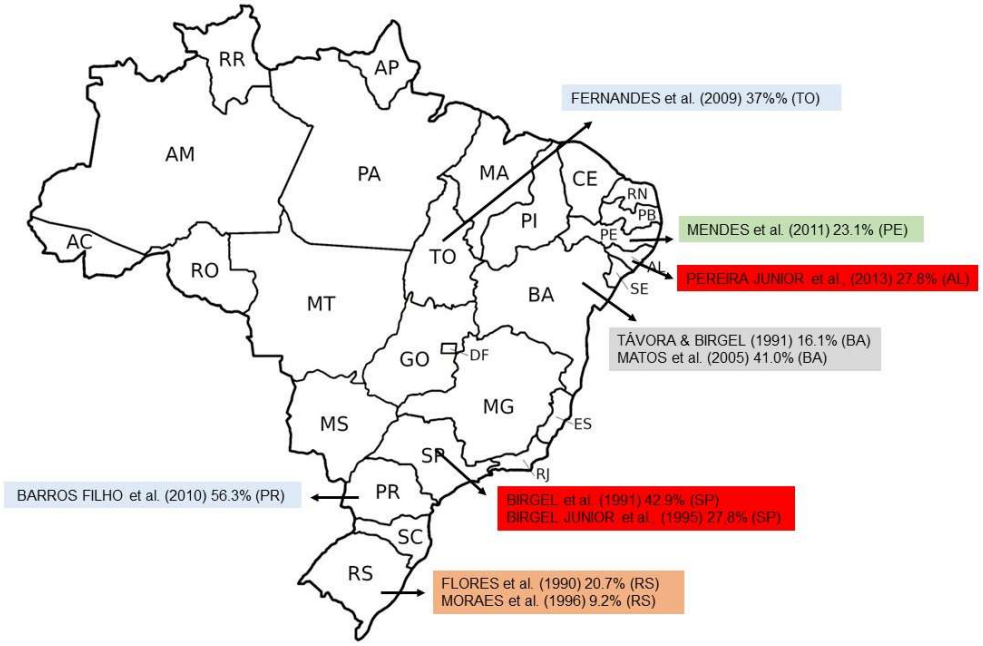
Tabela 1 - BLV Prevalence's on Latin America countries.

Country	Region	Prevalence	Study
Argentina	Buenos Aires	77.4% at individual and 90.9% at herd level, 2007	Polat et al., 2016
Argentina	Multiple regions	32.85% at individual and 84% at herd level, 1998–1999	Trono et al., 2001
Chile	Southern region	27.9% at individual level, 2009	Polat et al., 2016
Bolivia	Multiple regions	30.7% at individual level, 2008	Polat et al., 2016
Peru	Multiple regions	42.3% at individual level, 2008	Polat et al., 2016
	Multiple regions	31.0% at individual level, 1983	Ch, 1983
Venezuela	Nationwide	33.3% at individual level, 1978	Marin et al., 1978
Uruguay	Multiple regions	77% at individual level	Furtado et al., 2013
Paraguay	Asuncion	54.7% at individual level, 2008	Polat et al., 2016
Colombia	Narino	19.8% at individual level, 2013	Benavides et al., 2013

Adapted and updated from Polat, 2017.

Studies evaluation clinical and economic losses are rare, and Brazil is included in this scenery indeed. Some classical serological studies found herd prevalence around 42 to 100%, and prevalence ranging 6,3 to 65% within each property (FLORES et al., 1988; BIRGEL et al., 1995). Figure 1 shows main studies with BLV prevalence by states in Brazil based on Pereira Junior et al (2013) published data.

Figure 1 – Bovine Leukemia virus prevalence in Brazil according to region.



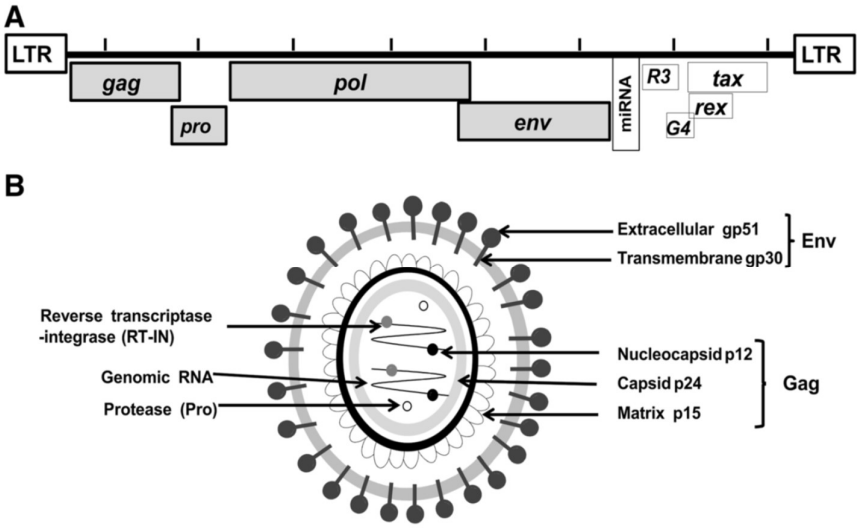
Most studies in Brazil have more than 10 years, and used agar gel immune diffusion (AGID), a less sensitivity test (BAPTISTA FILHO et al., 2019). Thus, as well as in other countries/regions, studies may not reveal the true prevalence's of the agent, and diseases impact is completely unknown on Brazilian herds. Moreover, probably veterinaries ignore the presence of the agent, and most of farmers has unfamiliarity with the disease, escaping from unknown.

2.1 ETIOPATHOGENESIS

Bovine Leukemia virus (BLV), is an enveloped RNA virus, belongs to *Retriviridae* family, genus *Deltaretrovirus.* BLV shares high similarity with Human T Cell Lymphotropic Virus (HTLV-1 e HTLV -2) and others retroviruses (GILLET, et al., 2007). Viral genome and proteins are showed in figure 2.

Surfaces proteins like gP51 and gP30 encoded by enzymatic gene Env are highly immunogenic; wile Gag gene encodes are capsids protein p12, p24 and matrix p15. Into nucleocapsid find encoded genomic RNA, reverse transcriptase and protease. Retrovirus integrates provirus in host genome and, once integrated, the genome is propagated via mitosis cell. Expression of BLV proviral genes occurs through endogenous transcription and translation mechanisms. Thereby, an infected animal became a persistent infected reservoir of the virus (RODRÍGUEZ et al., 2011; POLAT; TAKESHIMA; AIDA, 2017)

Figure 2 – BLV genome structure and encoded proteins.



Source: Polat, et. al.2017. (A)Schematic representations of the BLV genome structure. (B) Viral particle.

BLV Lymph tropism infection leads to immunodeficiency, duo to a severe change in host immune response (OTTA; JOHNSON; WELLS, 2003). Surface cells protein allow understanding virus-cell interaction by using marked monoclonal antibodies, and provide a valorous knowledge about cells proliferation, balance and function. Virus B cells tropism is the main responsible by LP, with rising marked CD21, CD5 and CD11b. This are membrane cells markers expressed by B-1 cells, and increases occurs due to the modulation of apoptosis in these cell (SCHWARTZ et al., 1994; SOUZA et al., 2011). B-lymphocyte population is classically divided into B-1 ($CD5^+$ B-1a, $CD5^-$ B-1b), and “conventional” $CD5^-$ B-2 lymphocytes. B-1 and B-2 cells differs in many properties. B-2 subpopulation is mainly depending on T cells modulation; and B-1 present a capacity for self-renewal, less specific affinity. Thus, B-1 as target cell is very convenient to virus thrive (CHEVALLIER et al., 1998)

Although rare, the BLV can infect other subpopulations of lymphocytes, and changes in immune functions are not restrict to B cells (LAVANYA et al., 2008). T cells that even with no altered in MHCII and CD25 surfaces expression, increase expression of LAG3, a down regulator for T cells activation (SCHWARTZ et al., 1994; DELLA LIBERA et al., 2012; BLAGITZ et al., 2017). This explains unbalance between T and

B cells. As a summary, virus can increase the number of B cells (a virus factory); and decrease function of other players of the immune responses.

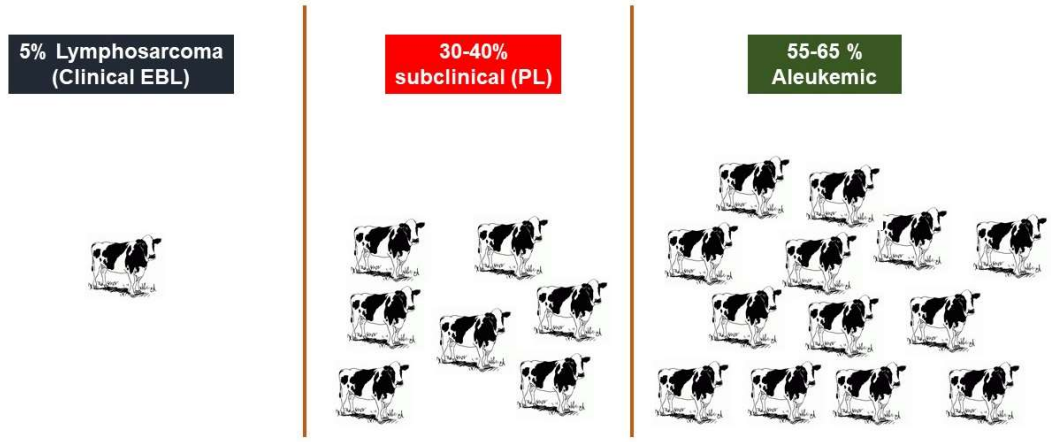
Beside Lymphocytes, nonspecific immunity is also affected by BLV infection. Blagitz et a., 2017 showed a dysfunction of blood monocytes and neutrophils, with lower of cells in animals BLV +, especially in animals PL. Additional, Neutrophil and monocytes phagocytic capable were decreased; and of reactive oxygen species (ROS) neutrophil production was decreased in neutrophil, and increased by monocytes. Moreover, innate immune responses in milk neutrophil equal had lower phagocytosis and ROS production (DELLA LIBERA et al., 2015).

In practice, BLV imply in litter host responses following challenges. BLV positive heifers primed vaccinated against Foot and Mouth Disease (FMD) had a lower Synectic of seroconversion for IgM and IgG-1 (PUENTES et al., 2016a), as well as dairy cows BLV infected had lower antibody response against to J5 antigen *E. coli* bacterin post immunization (ERSKINE et al., 2011).

2.2 CLINICAL MANIFESTATIONS

There are three clinical possibilities due to BLV infection. Figure 3 represents a herd, whereas about 30% of infected animals has a PL characterized by polyclonal nonmalignant lymphocytes, and less than 5% ongoing to lymphoma (GILLET et al., 2007). Iceberg theory is widely used to define EBL, since few animals get sick and major are subclinical or aleukemic.

Figure 3 – Distribution of BLV in a herd, according the manifestation and or Lymphocytosis/aleukemic



Lymphosarcoma's are the apsis of clinical disorders, and occurrence is more frequent in animals older than 4 years. Most of clinical manifestations observed depends on the tumoral location. Superficial adenomegalies may be evident, but hyperplasia's are more common in visceral tissues. Spinal cord neoplasia's may carry on neurological disturbs, observing pelvic member incoordination. Gastrointestinal track invasion is find (especially abomasal) leading to the obstructive picture manifested by clinical anorexia, recurrent bloat and weight loss; as well as lymphosarcoma's on myocardium are also observed (JOHNSON; KANEENE, 1991). Tumors in abdominal cavity, uterus and kidney are likewise find especially during rectal palpation. However, the diagnosis may be harmed if tumors are limited to lymph nodes and organs with difficult assess, In eyes, the tumor results in exophthalmia. Clinical signs are gradually getting worse, and tumors are frequent diagnosed during the slaughter house inspection (MILLER; VAN DER MAATEN, 1982).

Further on lymphosarcoma, PL and immune-disruption associated to BLV infection predispose in inapparent disorders. BLV infection predisposing mastitis and its increase of clinical severity (WATANABE et al., 2019), and raising hoof disorders (EMANUELSON; SCHERLING; PETTERSSON, 1992). Still controversially, BLV had being also associated to the reduction on reproductive performance in positive herds (EMANUELSON; SCHERLING; PETTERSSON, 1992). Moreover, conception rates in positive animals were up to 27% lower (PUENTES, 2016b), and also is observed a

high number of inseminations to achieve the pregnancy (KALE et al., 2007). The truth is that clinical BLV is rarely, and secondary infections duo to immune disbalance are the main productive impact.

2.3 HEMATOLOGY AND BIOCHEMICAL

To determine a pathological event, the very first beginning is to know what is physiologically normal. Hematology and biochemistry are complementary diagnosis methods that can offer a relatively noninvasive, with a great cost-benefit method of assessing the health status of a number of organ systems. It allows clinician to assess many disorders like inflammation and anemia and, in combination with biochemistry, may provide a diagnosis or differential diagnosis and/or suggest other complementary test (COCKCROFT, 2015)

Classical bovine hematological parameters were recent revised, with a mean neutrophil significantly counts increased. Opposite, an important lymphocyte, monocyte, and eosinophil counts, as well as hemoglobin decreased its concentration. Consequently, proportion between Neutrophil and Lymphocyte significantly increased to 1.17. Thereby, bovine hematology reference intervals could lead to misinterpretation taken old parameters, especially use of neutrophil: Lymphocyte as an inflammation evidence (GEORGE; SNIPES; LANE, 2010).

Moreover, differences in cows and heifers hematological and biochemical parameters must be carefully taken. Hematological, biochemical, immunophenotypes and cytokines involved in transition period of heifers and cows showed several significant differences. Hematological most prominent differences were observed exactly in white blood cell count (WBC), within increased neutrophils a lymphocyte counts in heifers (JONSSON et al., 2013). Take by differences finding in literature, hematological parameters should be carefully analyzing in periparturient, especially in the leukocytes count. This huge variation might influence by environmental conditions, breed, diet, infectious disease, among others (SOUZA et al., 2011; JONSSON et al., 2013; REIS et al., 2016; GOMES et al., 2017; BÜNEMANN et al., 2020).

Recognized as indispensable tool to evaluate physiopathology of system like urinary and hepatic by analyzing enzymatic concentration, biochemistry has providing answers and guiding veterinaries in face of challenges. Mots of research focus on

evaluate enzymatic activity related to liver function and muscular reservoir, given hepatic involvement in energetic balance and inflammation during transition period.

Aspartate Aminotransferase (AST) is a non-specific hepatic/muscular enzyme that had being associated with hepatocytes, and liver steatosis and necrosis, also related to muscular tissue injury (HERDT, 2000; COCKCROFT, 2015). There is no consensus in the literature about AST enzymatic activity behavior during peripartum. Some authors suggest an increasing on AST enzymatic activity's immediately after calving, due to muscular contraction and influenced by season (ALVARENGA et al., 2015; MOREIRA et al., 2015); wile other's revels no significance variation in AST rates peripartum (BIRGEL JUNIOR et al., 2003; DEPAULA 2010). Negative energetic balance may also increase the AST enzymatic activity secondary to muscular catabolism involved in the gluconeogenesis (HERDT, 2000).

Enzyme Gamma-glutamyl transferase (GGT) is a specific hepatic enzyme involved in aminoamides transport trough cellular surface and protein synthesis. GGT concentration abnormalities mainly secondary to acute and chronic liver disease, Plant toxicities, crop diets, and is also elevated in neonatal (COCKCROFT, 2015). Just as AST enzymatic activity, some authors found discrepancies on GGT behavior concentration during the transition period, with possible elevation on rates (FEITOSA E BIRGEL 2000; BIRGEL JUNIOR et al., 2003) or no influence of the peripartum on average concentration (GREGORY et al, 1999; (BUSATO, 2002). Moreover, season of the transition period may also influence on GGT concentration (MOREIRA et al., 2015).

In instance to clearance hepatic enzymes concentration behaviors during the transition period, is important to underline that according to most of authors oscillation may occurs but without outlier the physiological limits (FEITOSA E BIRGEL 2000; BIRGEL JUNIOR et al., 2003; MOREIRA et al., 2015).

2.4 BLV AND ZOONOTIC POTENTIAL

Association of BLV as a potential risk factor for breast cancer in humans is as brand-new as delicate. The supposition comes from studies that investigated BLV genome integrated into host tumoral cells, as well as antibodies presence in patients. Evidences based on serology seems to be weakness compared to proviral integrated

(BUEHRING et al., 2015; SCHWINGEL et al., 2019). Possible routs of infection are contact with raw bovine products like milk and meat (BARTLETT, et al., 2020)

A recent metanalyses analyzing BLV, HTLV-1, BLV-Cancer links, and more using Google Scholar and PubMed, looking for Key search terms including: “BLV”, “BLV causes Breast Cancer”, and “BLV oncogenic mechanisms”, shows climb subject interesting. Papers from countries like US (BUEHRING et al. 2015), Brazil (SCHWINGEL, et al. 2019), Argentina (CERIANI et al., 2018), Australia (BUEHRING et al., 2017) and Colombia (GIOVANNA et al., 2013) were carefully checked comments by the. Even if some positives correlations between find proviral by PCR, Immunohistochemistry, ELISA and sequencing, evidences must be thoroughly cautiously confirmed (GAO; KOUZNETSOVA; TSIGELNY, 2020)

2.5 GENERAL ASPECTS OF TRANSITION PERIOD

Apart from infectious disease, some life productive moments challenge the homeostasis in high productive dairy cows. Transition period is marked by a several adaptations in front of gestation, colostrogenesis, calving and lactogenesis, characterized by an increase in the nutrients demands, having a high demand of energy and a decrease of dry-matter intake in the last of gestation. Dairy cows will present at least a transitory negative energy balance in the first week of lactation, and immunosuppressive status (BERNABUCCI et al., 2005; HAMMON et al., 2006).

The reason for immunodepression during the transition period is multifactorial, and can be related to endocrinological factors (IGF-I and cortisol), and metabolic. In negative energetic balance (NEB) state, a drop in plasmatic glucoses leads in mobilize body reserves to access energy needs. This process is responsible for upper plasmatic concentration of non-esterified fatty acids (NEFA) by recruited during homeorhetic mechanism that leads in lipidosis as well as a high concentration of Beta-hydroxybutyrate (BHB), and, consequently, an increase of the oxidative stress (BERNABUCCI et al., 2005; HAMMON et al., 2006; REIS et al., 2016; BALDICIM et al., 2018).

There are few studies evaluating heifers during transition period. In general, dairy cow present a leucogram of stress due to the high cortisol (partum related), represented by leukocytosis and lymphopenia. Lymphopenia is linked to lymphocytes influx into tissues and changes on interleukin 12 genetic transcription, responsible for

stimuli lymphocytes proliferative response (DAVIS et al., 2008). This mechanism is associated to the decrease of T helper (CD4+) and T cytotoxic (CD8+) lymphocytes, around parturition (VAN KAMPEN; MALLARD, 1997; KIMURA et al., 2002). B cells lymphocytes levels (CD21+) present in blood during transition period has not a standard, can be above baseline (MEGLIA et al., 2005); below the normal (OHTSUKA et al., 2010); or with similar pattern of lactations (VAN KAMPEN; MALLARD, 1997; KIMURA et al., 2002;) However, unbalance of Lymphocyte subpopulations in cows' positives to BLV with pronounced climb of cells $CD21^{+}$; as well as possible BLV infection as an increases risk to peripartum disease (GOMES et al., 2017).

Innate immunity plays a central part of the immune response, and polymorphonuclears leukocytes (PMNL) are essential to the microbiological clearance of placenta and defense against bacterial agents. Also, unbalance of proportions and migrations of the subpopulations of lymphocytes can affect both local and systemic immune responses, making animals more susceptible to inflammatory and infectious diseases during immediate postpartum, especially metritis and mastitis (VAN KAMPEN; MALLARD, 1997; MALLARD et al., 1998; KIMURA et al., 2002). Additional to peripartum event of immune disequilibrium, Della Libera, et al., (2015) showed that, neutrophil in milk from positive animals has decreased phagocytosis capable and less ROS production than non-infected animals.

Acute-phase protein (APPs) are a class of protein, expressed in responses to injury by local inflammatory cells (neutrophil granulocytes and macrophages) and inflammation (NIGHTINGALE; SELLERS; BALLOU, 2015). By definition, APPS concentration in healthy animals are low, rapidly increased under clinical disorders and backing to basal after homeostasis restore. Recently, explored the possibility of bovine major APPs, such as haptoglobins (Hp) produced by hepatocytes, had being successfully used as biomarkers to calves' inflammation (MARTIN et al., 2021), respiratory disease (MOISÁ et al., 2019), mastitis (WOLLOWSKI et al., 2021), among others clinical disorders, and also to predict the inflammatory disease in dairy cows during the periparturient period (REIS et al., 2016). In healthy cows Hp production has a low level and its production increases in cows diagnosed with metritis at first week post-partum, and it depends on the severity of the disease (HUZZEY et al., 2009). In an experiment that aimed to evaluate APPs behavior in cows diagnosed with uterine infection during the transition period, concentration Hp also increased first week post-

partum for multiparous uterine infected cows compared to healthy cows (SCHNEIDER; CORRÊA; BUTLER 2013).

Most studies evaluating periparturient period exclude BLV positive animals. A recent study evaluated serological BLV antibody profile, neutrophil and lymphocytes in cows between dry-off and earlier calving. Seroprevalence increased significantly from dry-off (38.9%) to closeup (43.6%); and slightly decreased from delivery to 7 to 10 d after calving (43.0%). Moreover, Lymphocyte counts were significantly higher in ELISA positive animals, but only among second and third or greater parity animals, instead of heifers (WISNIESKI et al., 2020). In instance, the study is an outstanding evaluation of BLV infected animals, taken that only few researches are available, especially in heifers; and an important number of productive cows worldwide positive to BLV are at time facing the challenges of transition period.

Hypothesis of the research is that heifers BLV positive has changes in the hematological and biochemical profile, are more inflamed during transition period compared to negative heifers; and proviral load has a direct impact in this scenery.

3 GENERAL OBJECTIVES

The aim of the study is to evaluate the influence of BLV on hematological, biochemical and inflammatory dynamic in in dairy heifers through the transition period; and if the proviral viral load influences in the same aspects as well.

3.1 Specific objectives – evaluation:

- Determinate if BLV infection can impact in hematological and biochemical profile of dairy heifers along transition period in dairy heifers;
- Possible influence of BLV on energetic balance inflammatory markers and healthy.
- Access the proviral load of BLV+, and its possible effects on hematological and biochemical; energetic balance and inflammatory parameters.

4 BOVINE LEUKEMIA VIRUS INFECTION AND PROVIRAL LOAD INFLUENCES ON HEMATOLOGICAL, BIOCHEMICAL AND INFLAMATORY PROFILE OF HEIFERS DURING THE TRANSITION PERIOD

4.1 INTRODUCTION

Bovine leukemia virus (BLV) is *Retroviridae* family member, belong genus *Deltaretrovirus*, and is genetically and antigenically similar to the Human T-lymphotropic virus (HTLV-1 and 2) (GILLET, et al. 2007). Vertical transmission of BLV can occurs in utero or during delivery from infected dams, and by administration of infected fresh colostrum/raw milk (RUIZ et al., 2018). Although, the main way of BLV transmission is iatrogenic by the transfer of infected cell, especially through sharing needle, palpation sleeves, dehorning and tattooing (RUGGIERO; BARTLETT, 2019; BARTLETT et al., 2020). The risk of transmission increase proportionally of the proviral load (JULIARENA; GUTIERREZ; CERIANI, 2007). The most BLV infected bovine have subclinical disease, approximately 30 to 40% of these animals have persistent lymphocytosis (PL), and less than 5% develop malignant lymphosarcoma (SCHWARTZ et al., 1994). Animals presenting or not persistent lymphocytosis can have high proviral load (HPL). The proviral load might be associated with genetic factors (MIRSKY et al., 1998; JULIARENA, 2008), and cows showed pronounced lymphocytosis during the transition period, instead of heifers (WISNIESKI et al. 2020).

BLV infection alters the mechanism of apoptosis and proliferation of infected immune cells, especially the main virus target - B lymphocytes, disrupting function and distribution of lymphocytes subsets in the blood (FARIAS et al., 2018). Rates of B:T cells in positive animals are often unproportionally, with a discernible bias toward B population, and Innate immune response is also compromised by the BLV (BLAGITZ et al., 2017). Della Libera et al. (2015) reported that BLV dairy cows presented reduced milk neutrophil function marked by the reduction on phagocytosis and reactive oxygen species production (ROS), as well as decreased viability of B cells presented in milk, especially in PL animals. In consequence, infected animals are more susceptible to clinical disorders and infection occurrence is related to drop in dairy production and leading weak animals (EMANUELSON et al., 1992; BARTLETT et al., 2013), as well as an impairment follow the immunization against other pathogens (PUENTES et al., 2016b, FRIE et al., 2018)

Transition period is critical for dairy cattle, due to the pro-inflammatory and immunosuppression status, which results in high incidence of infectious diseases. This scenario can be worst in BLV positive herd, due to the negative impact of virus on the immune response. Recently research reported an increase of the lymphocyte counts between dry-off and close to calving in BLV + cows (WISNIESKI; NORBY; BYREM, 2020). Gomes et. al. (2017) evaluated BLV positive multiparous cows during the transition period, and found variations along time in leucogram, lymphocytosis between weeks -3 up to partum, followed by a decrease in leucocytes, especially lymphocytes, along 3 weeks after partum. Furthermore, BLV antibodies positive rates significantly increased from dry-off (38.9%) to deliver (43.6%). Otherwise, no differences were observed for heifers confronting of serological status and lymphocytes count (WISNIESKI; NORBY; BYREM, 2020). Although very confident and useful as biomarker and prediction of inflammation, APPs concentration should be interpreted with carful since it is not specific and range of variation can oscillate (REF)

In our concern, there are few studies evaluation the profile of heifers during the transition period, especially considering the BLV status of the herd (WISNIESKI et al., 2020). Moreover, the most of BLV studies did not evaluate the proviral load, that seems to be the main marker for the immune disbalance and induction on LP (BLAGITZ et al., 2017).

The hypothesis of this study was that heifers BLV positive shows altered homeorhetic profile of heifers during transition period, and the high proviral load is directly related to LP. So, the aim of this research was to evaluate hematological, biochemical and acute phase proteins; access proviral load and possible relationship with LP of heifers BLV+ during the transition period.

4.2 METHODOLOGY

This research was conducted between October 2018 to September 2019. All procedures were carried out in agreement with the guidelines of Ethical Principles in Animal Research adopted by the Ethic Committee on the Use of Animals of the School of Veterinary Medicine and Animal Science from São Paulo University (Protocol number 2188221018). Written informed consent was obtained from the farm responsible.

4.2.1 Farm, animals and management

This study was performed in a commercial dairy farm located in Rio Grande do Sul State, Brazil (latitude 29°28'14.0"S longitude 51°33'53.1"W, altitude 484 meters). Herd was composed by a total of 648 Holstein and Jersey bread animals, being 230 in lactation, housed in a freestall barn and milked twice a day, with a production of 6.100L/day (average 26,2L per cow). Calves were maintained in individual cages from birth until weaned with about 75 days. Heifers were then transferred to collective grouped by age, kept in native grass with common drinking water line, and a bunker with free choice calf starter diet (Nutron, São Paulo, Brazil). Between 10-11 months of age heifers kept in pasture were included into the standard reproductive protocols for 13-14 months of age. Each heifer received a dose of prostaglandin (MSD Animal Health, Montes Claros, Minas Gerais) weekly, and were inseminated when they demonstrated they were inheat. Pregnancy diagnosis using ultrasound (Mindray, Shenzhen, China) was performed every 14 days. Animals were managed in field up to 2 months from estimated partum.

Heifers Included were housed -60 days from estimated partum in freestall barn, aiming behavior acclimation, and are transferred -30 days before estimated calving to a compost barn maternity. After calving, animals returned to the freestall with mattresses bedding, covered with wonder with automatic forced ventilation and sprinklers to avoid heat stress.

Main nutritional information about formulation and chemical composition are listed on table 1. All feed formulation is based on software Dairy Max (Cargill, São Paulo, Brazil) and use internal database as standard to determinate diet.

Tabela 1 - Ingredients and chemical analyses of diet of dairy heifers pre- and post-partum.

Ingredients	Pre-Partum Prescription	Post Partum Prescription
Corn Silage	18 kg	33 kg
seed husk	2,5kg	2,5 kg
azeven hay	2,5kg	6,5 kg
prepartum feed	2,8 kg	
lactation feed		10,5

Total	25,8 kg	52,5 kg
Diet analyses	**Pre-partum diet**	**Post-partum diet**
Dry Matter	50,24%	47,17%
Crude Protein	14,58%	15,87%
Fat	2,60%	2,81%
Neutral detergent fiber (NDF)	25,07%	19,87%
Non protein nitrogen	0,23%	1,22%
Ash	8,35%	6,92%
Adj total starch	15,84%	27,01%
Calcium	0,75%	0,77%
Phosphorus	0,30%	0,39%

4.2.2 Initial screening and inclusion criteria into experimental groups

All animals from herd with more than 90 days old (n=613) were submitted to an initial serological screening for BLV by using enzyme linked immunosorbent assay – blocking ELISA (Ingenasa – Ingezim BLV COMPACT 2.0®), aiming to access the general prevalence in herd. Animals less than 3-month-old were not tested to avoid the interference of passive immunity transfer in the results.

After initial serological screening, 24 close-to-calve pregnant Holstein (n=12) and Jersey (n=12) heifers between 23 and 26-month-old were included in the experimental. Animals were distributed into two groups: BLV positive (BLV+, n=12) and BLV negative (BLV-, n=12). To be add as positive, animal should have tested specific antibodies at time -3, 0 and +3; and digital PCR (ddPCR) at -3 and +3 positive. Same criteria for BLV negatives heifers, all should have negatives results in both tests at same check times.

Positive and negative heifers were matched in pairs, following the birth, breed and partum criteria. A total of 16 animals (8 pairs) were sampled from October 2018 and February 2019, comprising spring/summer season; and 8 animals (4 pairs) were sampled between July and September 2019, comprising winter/spring. The multiparous cows were excluded from experiment due to the difficult to find animals older than 36 months negative for BLV. From the beginning, 9 heifers were taken out from experiment because of abortion (n = 1), positive result in the PCR and negative for serology (2), anticipated calving (n=3) and consequence mismatched (n=3).

4.2.3 Blood samples

Heifers included from both groups were weekly sampled at time -3, -2, -1, 0 (calving), 1, 2, 3-week time. Whole blood and serum were harvest in 3 tubes containing K3 EDTA and 2 tubes containing clot activator (Vacutube, LABOR IMPORT – China), by coccygeal venipuncture using 25x8mm needle (BD Vacutainer – Brazil) in vacuum system, after antisepsis by using alcohol 70°. Serum and plasma were obtained by centrifuge clot activator and K3 EDTA plasma respectively, at 1000 xg por 10 min, and were stored in 2ml microtubes at -20°C.

4.2.4 Droplet digital PCR for BLV quantification

Droplet digital PCR (ddPCR) was performed using the QX200 Droplet Digital PCR System (Bio-Rad, California, USA) as per the manufacturer's protocol. A HEX probe and primer sequences were designed to amplify a partial segment of the BLV env gene. The primer sequences were: F: 5- 'CAG TGA CTG GGT TCC CTC TGT C-3', R: 5'-AGG GCG AGR CCG GGT CCA GAG-3' and the probe was: HEX 5'-CCC TCC CTG GGC TCC CGA RA-3'BHQ1. Briefly, 1 µl of DNA was mixed with 1 µl of each primer (10µM) + 0.5µl of HEX probe (10µM) + Bio-Rad 2 × Supermix were emulsified and the droplets transferred to a 96-well plate (Eppendorf, Hauppauge, NY). The PCR was performed in a C1000 TOUCH CYCLER w / 96W FSRM thermocycler (BioRad, Hercules, CA) with the following parameters: initial denaturation of 10 min at 95°C, then 40 cycles of 30 sec at 94°C and 10 min at 58°C, and a final deactivation of the enzyme for 10 min at 98°C. Finally, the presence of fluorescent droplets determined the number of resulting positive events that were analyzed in the QuantaSoft version 1.7.4 software (BioRad, Hercules, CA), using dot charts. The fluorescence detection threshold was determined manually, according to negative controls.

The BLV proviral load was determined as copies/µL of genomic bovine DNA and each sample was performed on duplicate. The result was expressed as the average of the two measurements. A Median was traced to determinate animals with low/high proviral load.

4.2.5 Hematology

Hematological cells and compounds were evaluated using an automatic hematological reader (Mindray BC 2800 vet). Absolute red blood cell (RBC) count, hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) and absolute total leukocyte count (WBC) were obtained using an automatic counter; and differentiation of leucocytes subsets was performed by using microscopy blood smear giemsa stain (Laborclin®). Hematocrit (Hct) was determined via microcentrifuge method, utilizing standard table reader and small hematocrit capillary tubes at 12000 x g for 5 minutes.

4.2.6 Biochemical biomarkers

Serum AST (Labtest® ref. 109) and GGT (Labtest® - ref. 105) were measured using an automated wet chemistry analyzer (Smart 200 – biotécnica®) and commercial kits (LABTEST®), according to the manufacturer's standard protocol. The measurements of serum BHB (Randox® – ref. RB1008), cholesterol (Randox® - ref. CH2655) and triglycerides (Randox® – ref. CH3810) were performed using another automated wet chemistry analyzer (Labtest LabMax 240 - Tokio Boeki). TPP were measured by using a total protein refractometer (Contec – Vet RHC-300 ATC).

4.2.8 Acute phase protein

Fibrinogen were evaluated at 7 moments (-3 to +3 week) from positive and negative animals included. Heat denaturation technical were used to determination fibrinogen concentration, according to Millar; Simpson; Stalker, (1971).

The concentration of haptoglobin from heifers included was assessed from time -3, 0 and +3, by using a turbidometric assay. Unfortunately, samples with minimum trace of hemolysis were not allow to be tested because of interferences, and total samples (72) had missed 7 and tested were 65.

Methodology used a standard curve prepared using a serial dilution of a known level control serum. The determination of serum haptoglobin concentration was calculated by interpolation a linear regression of the standard curve for each assay after collecting based on the absorbance value recorded with microplate reader (450 nm). Samples with haptoglobin concentration above 2.0 mg/dL were assigned as having a "high haptoglobin level". This was used as an indicator of an activated

inflammatory state as suggested by the reference values established by Eckersall and Bell (2010).

4.2.9 Electronic Rumination-Monitoring and Farm manager information System

For daily rumination movements evaluation, a day average was traced for animals per day to set up each group behavior. Animals were monitored with a neck tag (SenseHub™ SCR. Allflex SCR) received 60 days before estimated birth, when housing at free stall, allowing monitoring variations along rumination, using algorisms to evaluate if movements are above or below than expected, and predict diseases or clinical manifestation, especially metabolic diseases, like ketosis and hypocalcemia during transition period and lactation (SCHIRMANN et al., 2012, 2016; KAUFMAN et al., 2018). Heifers' productive information were collected by accessing the software Smartmilk (PRODAP, Minas Gerais, Brazil.), with follow-up of clinical profile, treatments, monthly milk control evaluating production, Somatic cell count (SCC), Fat and Protein.

4.2.10 Statistical Analyses

Program Statistical Package for the Social Sciences (SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp).) were used for statistical analyses. Data were initial submitted to distribution data analyses through histograms using, q-plot and homogeneity variances test. Variables with non-linear distribution were submitted to numeric transformation. After data linearizes, mixed model was used to evaluate BLV effects on hematological and biochemical parameters of heifers during transition period. Variable's time, group, and time x group interaction were used as time fixed effects along transition period, according to week -3, -2, -1, 0, +1, +2, +3. Time variable for lactation/production were categorized according to month of milk control (14 month of evaluation). For neck collar rumination parameters time used was daily evaluation between days -21 before birth, and +21 after. The unstructured, autoregressive and component symmetric covariant matrix were tested, and models were selected according to lower value of Akaike information criteria (AIC). EMEANS command comparisons with Bonferroni correction allowed to assess the general effect of the time and group. Comparisons between groups in each moment was realized using the T-test. Logistic regression model for repeated measures was realized

considering the groups (BLV+ and BLV-) as an independent variable and animals with inflammation (Hp > 2mg/dL), ketosis (BHB > 1.2 mmol/L) and hepatic damage (AST > 132 U/L) as a dependent variable (COCKCROFT, 2015).

R studio version 4.0.5 (Rstudio, Boston, USA) allowed to obtain the correlation between the viral load, hematological and biochemistry parameters at week-3 and week3. Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies.

4.3 RESULTS

4.3.1 Serological screening

Initial herd serology showed an average prevalence of 57.25% (n=351/613) in the analysis of the whole herd. Whitin pregnant heifers' group, the prevalence for serum positive animals for BLV was 38.7% (76/124).

4.3.2 Hematological profile

Red blood cells and white blood cells parameters from heifers positive and negative are shown in the table 2. The effect of time was detected for the number of red blood cell, hemoglobin concentration, hematocrit, eosinophil and platelet. Effect of BLV groups was not detected for the hematological parameters and the Interaction between time and BLV groups was observed only for the MCHC (P= 0.026). Cell's linage of metarubricytes, myelocytes, metamyelocytes, band and basophil were not identified in the WBC analysis. The descriptive results for each parameter in heifers during the transition period is shown on the supplementary table 1.

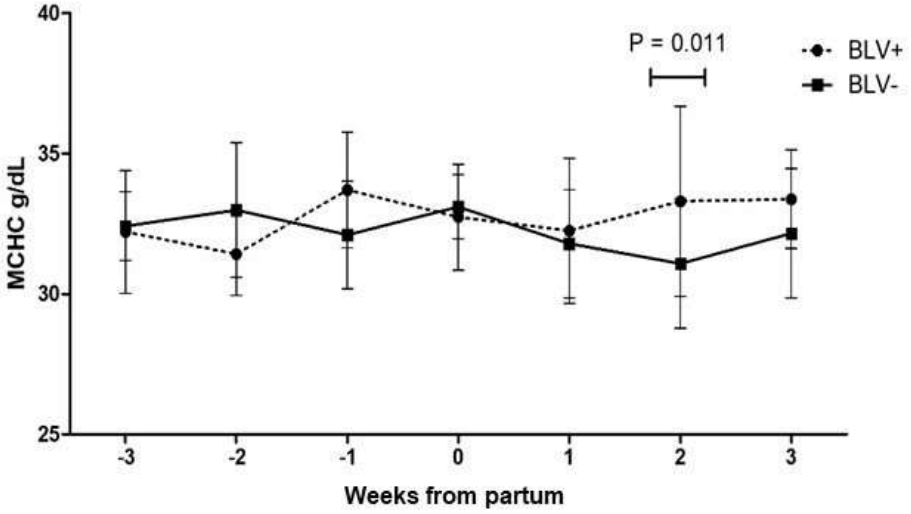
Table 2 – Effect of Bovine Leukemia Virus (BLV) on the red blood cells and white blood cells parameters in dairy heifers during the transition period by Linear mixed models.

	Groups		P-value		
	BLV+ (n=12)	BLV- (n = 12)	Time	Groups	Time x Groups
Red blood cell/µL	5.98±1.39	5.96±0.69	0.004	0.676	0.887
Hemoglobin g/dL	9.74±1.42	9.53±1.4	0.001	0.471	0.370
Hematocrit (%)	29.81±4.08	29.19±4.98	0.006	0.567	0.590
M.C.V.fL	50.7±5.73	49.71±3.88	0.219	0.324	0.492
MCHC.g/dL	32.72±2.31	32.23±1.99	0.537	0.232	0.026
R.D.W.(%)	18.04±11.02	16.52±0.93	0.001	0.507	0.224
Leukocytes x 10^3/µL	17.9±9.24	13.16±3.92	0.346	0.184	0.889
Neutrophil x 10^3/µL	5.36±3.24	4.75±2.42	0.258	0.673	0.740
Lymphocytes x 10^3/µL	11.56±8.1	7.81±2.6	0.644	0.392	0.310
Monocytes x 10^3/µL	0.20±0.3	0.17±0.20	0.410	0.646	0.978
Eosinophil x 10^3/µL	0.76±1.01	0.42±0.43	0.001	0.088	0.469
Platelets x 10^3/µL	368.7±174.67	302.89±114.91	0.001	0.134	0.986

Legend: M.C.V. = mean corpuscular volume; MCHC = Mean corpuscular hemoglobin concentration; RDW = Red Cell Distribution Width. Statistical difference was considered when the p-value was < 0.05 and the tendency when the p-value was between 0.05 and 0.1.

Interaction between group and time was detected for MCHC variable, so it was contrasted the mean values in each time point. The differences between groups were detected by comparisons with Bonferroni correction, observing high values on BLV+ group at week +2 (figure 1).

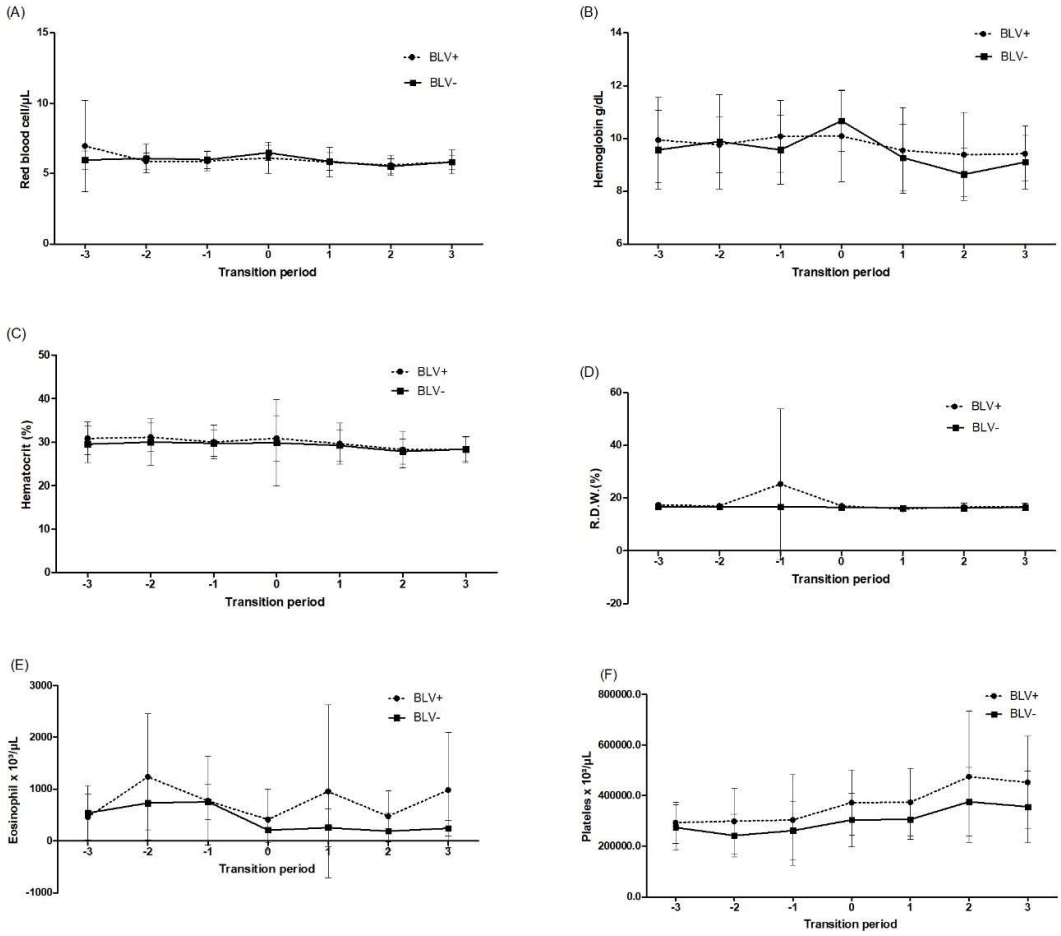
Figure 1 - Mean corpuscular hemoglobin concentration (MCHC) in dairy heifers BLV+ (n=12) and control group BLV- (n=12) during the transition period.



Legend: P = p-value; Test T-student allowed to obtained the difference between groups along the transition period. Differences between with groups was considered when the P-value was < 0.05 and trend between groups P > 0.05 and P < 0.1.

It was possible to identify variations for RBC count during the transition period. In general, the values for the RBC count, hematocrit and hemoglobin were high at calving in contrast with the post-partum period (weeks +2 and +3) (figure 2A, 2B and 2C). Red Cell Distribution Width (RDW) presented a variation along the transition period, observing decrease of the values from week -3 up to week +1, and return for the basal values in the subsequent moments (figure 2D). The number of eosinophils had an intense fluctuation during the study (figure 2E). The number of eosinophils were higher in the pre-partum than post-partum period, observing the lowest value in the calving time. Platelets had a gradual increase from pre-partum until week +3 (figure 2F).

Figure 2 – Time-analysis of the hematological parameters in dairy heifers BLV+ (n =12) and BLV- (n=12) during the transition period.



Legend: RDW = Red Cell Distribution Width. Differences between the moments was identified with the EMEANS time comparison with Bonferroni correction and contrasted the difference between the moments with different letters during the time.

4.3.3 Biochemical biomarkers profile

Linear mixed models' results from the biochemical analyses are shown on table 3. Time effect were observed for AST, GGT, BHB, Triglycerides, cholesterol, total plasmatic protein (TPP) and HP; while a group effect were observed only for AST and a tendency for HP. Interaction between time x group were detected for triglycerides and fibrinogen parameters. General description was reported in the supplementary table 2.

Table 3 – Linear mixed models to evaluate the effect of Bovine Leukemia Virus (BLV) on the biochemistry parameters in dairy heifers BLV+ (n =12) and BLV- (n=12), during the transition period

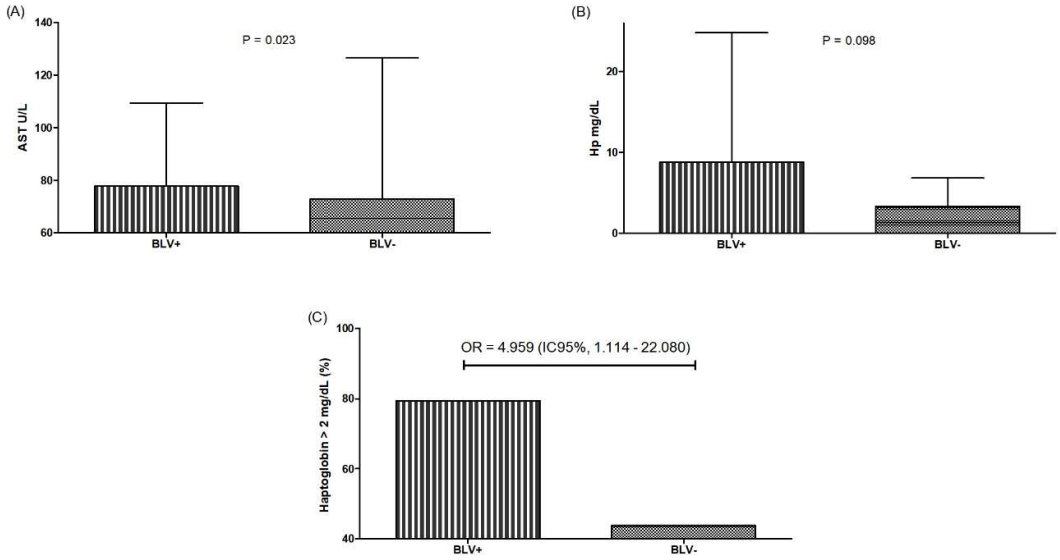
	Groups		P-valor		
	BLV+	BLV-	Time	Groups	Time x Group
AST U/L	77.73±31.57	72.87±53.71	0.012	0.023	0.215
GGT U/L	23.41±9.5	21±6.67	0.015	0.250	0.195
BHB (mmol/L)	0.45±0.19	0.45±0.19	0.001	0.834	0.718
Triglycerides	18.63±12.59	18.83±12.83	0.001	0.774	0.023
Cholesterol	78.89±18.8	71.52±17.75	0.001	0.178	0.758
T.P.P. g/dL	6.68±0.62	6.57±0.76	0.001	0.536	0.597
Haptoglobin (mg/dL)	8.81±15.96	3.35±3.49	0.001	0.098	0.956
Fibrinogen mg/dL	354.88±204.37	371.43±174.64	0.322	0.604	0.043

Legend, AST = Aspartate aminotransferase; GGT = Gamma-glutamyl transferase; BHB = β-Hydroxybutyrylation; T.P.P = Total plasma protein. Statistical difference was considered when the p-value was < 0.05 and the tendency when the p-value was between 0.05 and 0.1.

The effect of group was detected to AST, and the mean of this biomarker was higher for the BLV+ group 77.73±31.57, whereas the value for BLV- was 72.87±53.71 (figure 3A). Trend of difference was observed to Hp, and the values to the group BLV+ was higher (8.81±15.96) than the group BLV- 3.35±3.49, (figure 3B).

BLV+ group showed a high number of animals with the concentration of Hp > 2 mg/dL during the transition period 79.4% (27/34) in relation to the BLV- group with 43.8% (14/32), whereas the odds were 4.959 (IC95%, 1.114 – 22.080) for the inflammation at the group BLV+ in comparison with the BLV- group (figure 3C). In relation to the ketosis and hepatic damage, the association between the group (BLV+ and BLV-) was not observed in a logistic model since all animals stayed into acceptable range limits.

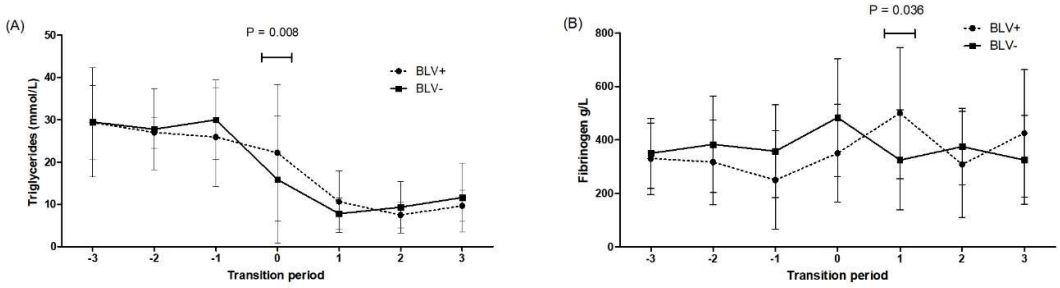
Figure 3 – Effect of the Bovine Leukemia Virus (BLV) on the AST activity and haptoglobin concentration and percentage of animals with haptoglobin > 0.02 mg/dL in dairy heifers BLV+ and BLV- during the transition period



Legend: AST = Aspartate aminotransferase; Hp = haptoglobin; OR = odds ratio; IC95% = confidence interval; P = p-value obtained using the emeans command comparisons with Bonferroni correction. Odds ratio was obtained using the logistic model for repeated measures. Statistical difference was considered when the p-value was < 0.05 and the tendency when the p-value was between 0.05 and 0.1.

It was observed interaction between group and time for the triglycerides and fibrinogen variables. Triglyceride's concentrations variated along time for both groups. BLV+ group showed significantly reduce on triglycerides one week after calving, while BLV- reduced at calving. Groups had differences at calving time (P = 0.008). During this moment, BLV+ group had 22.18±16.15, and BLV negative 15.89±15.04 (figure 4A). Regarding fibrinogen, significant variations were observed for BLV+ groups (P = 0.015), and a difference between groups was observed at week +1, whereas the concentration of fibrinogen was 500±244.95 for BLV+ group and the concentration of 325±186.47 for the BLV- group (figure 4B).

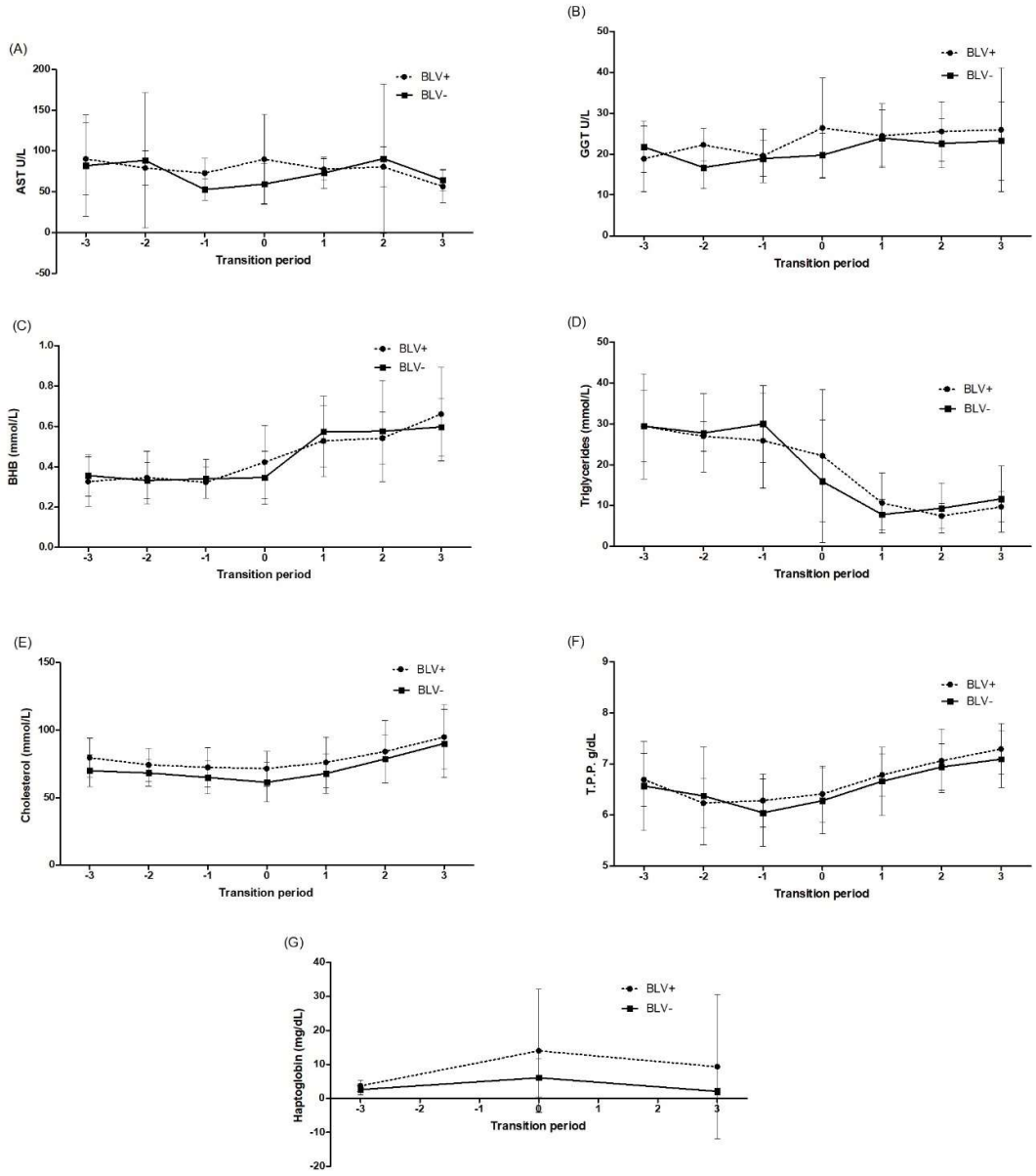
Figure 4 - Effect of Leucosis on the triglycerides and fibrinogen concentration in BLV + (n=12) and BLV- (n=12) dairy heifers during the transition period



Legend: Test T-student allowed to obtained the difference between groups along the transition period. Differences between with groups was considered when the P-value was < 0.05 and trend between groups P > 0.05 and P < 0.1.

The time-analysis by using EMEANS command comparison with Bonferroni correction detected some variations for the different biochemical biomarkers through the transition period. The lowest value for AST enzymatic activity was detected at calving time and week +3 (figure 5A). The activity of GGT increased through the transition period, observing high values after calving (figure 5B). BHB concentration increased from calving up to week +3 (figure 5C). Triglyceride's concentration presented an abrupt decrease from calving up to week +3 (figure 5D). On the other hand, the levels of cholesterol increased through the study. The cholesterol concentration was low at the weeks -2, -1, 0, +1 and had an increase at week 3 (figure 5E). TPP increased significantly concentration along transition period. At week +1, +2 and +3 TPP concentration was significantly greater than week -1 (figure 5F). Hp also showed variations, within increasing concentrations at calving, and significant dropping at week +3 (figure 5G). Supplementary table 3 shows the numerical concentration of HP in pairs BLV + and BLV-; the mean and standard deviation on the 3 checked points (weeks -3,0 and 3).

Figure 5 – Time-analysis of the biochemistry biomarkers parameters in BLV+ (12) and BLV- (n=12) dairy heifers during the transition period



Legend: AST = Aspartate aminotransferase; GGT = Gamma-glutamyl transferase; BHB = β-Hydroxybutyrylation; T.P.P = Total plasma protein; Differences between the moments were identified with the EMEANS time comparison with Bonferroni correction and contrasted the difference between the moments with different letters during the time.

4.3.4 Production, milk quality, and rumination-monitoring

Milk production, fat, protein, SCC, and daily pondered rumination showed variation during the transition period, however there were any group effect or interaction between time x groups (table 4).

Table 4 – Linear mixed models to evaluate the effect of Bovine Leukemia Virus on production, milk quality and time of rumination in BLV+ (12) and BLV- (n=12) dairy heifers during the transition period.

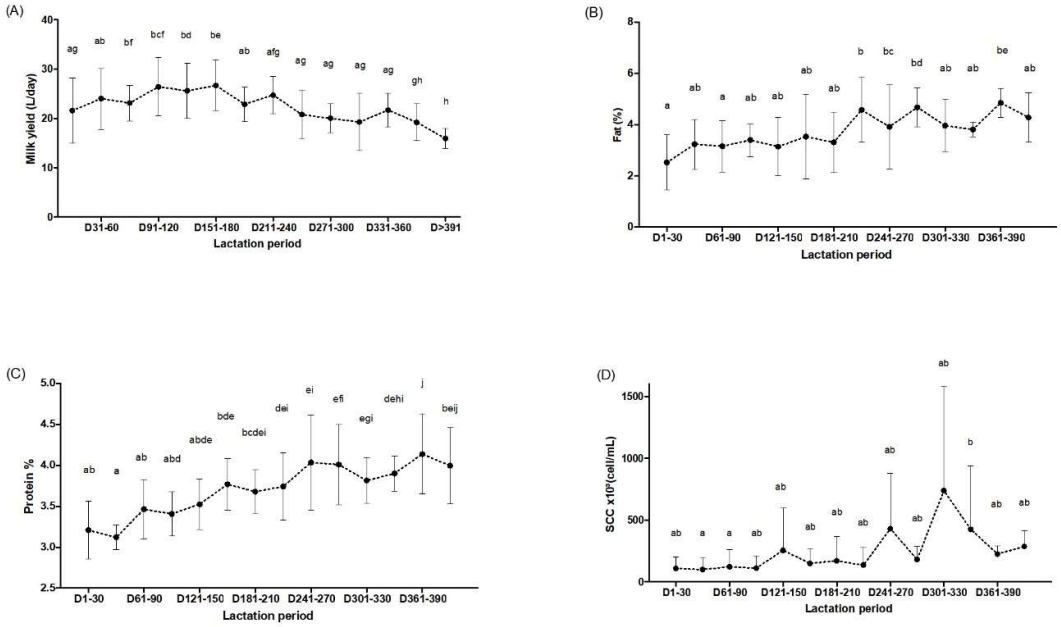
	Groups		P-value		
	BLV+ (n=12)	BLV- (n=12)	Time	Groups	Time x Groups
Milk production(/day)	24.35±7.27	21.55±5.94	0.001	0.220	0.738
Fat (%)	3.46±1.28	3.6±1.13	0.001	0.655	0.981
Protein (%)	3.61±0.48	3.69±0.46	0.001	0.449	0.510
SCC	354.38±600.48	290.01±596.63	0.022	0.425	0.400
Rumination by time	0.21±9.35	0.49±8.52	0.001	0.708	0.667

Legend: SCC = Somatic count cells. Statistical difference was considered when the p-value was < 0.05 and the tendency when the p-value was between 0.05 and 0.1.

The highest milk production was detected on D151-180, represented by a mean volume of 26.68± 5.11L/day, followed by a decrease through the lactation. The lowest milk production was detected in the final of lactation (> 391 days in milk), represented by a mean volume of 15.92±2.03 L/day (figure 6A). On the other hand, the percentage of fat increase during the lactation, observing the highest value at D361-390 DIM (4.85%±0.56). The lowest value (2.52%±1.07) for milk fat was observed in the early of lactation (D1-30 days in milk) (figure 6B). Total protein (%) in the milk had a similar profile of fat. The highest value (4.12±0.42 %) at D361-390, and the lowest concentration (3.12±0.15 %) was at D31-60 (figure 6C). Regarding the SCC, the number of somatic cells increased through the lactation. The highest value (739.00±842.67cells/mL) was detected at 301-330 days in milk (figure 6D).

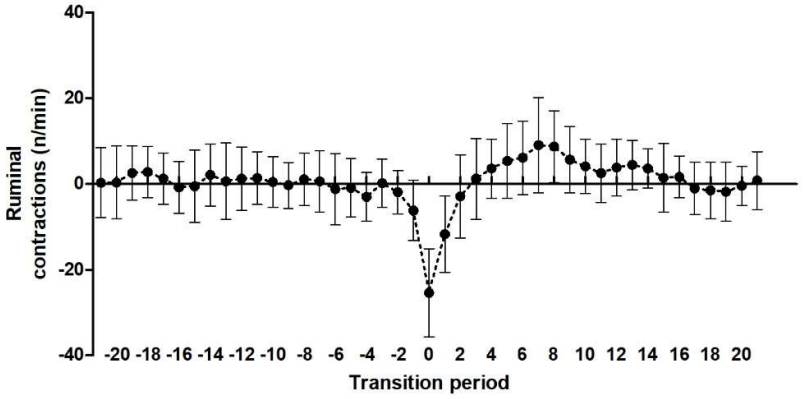
The rumination movements had a significant drop at calving, where it was observed the lowest value (-25.44±10.32) (figure 7).

Figure 6 – Time-analysis of production, fat, protein and SCC in BLV+ (12) and BLV- (n=12) dairy heifers during the lactation



Legend: SCC = Somatic count cells. Differences between the moments were identified with the EMEANS time comparison with Bonferroni correction and contrasted the difference between the moments with different letters during the time.

Figure 7 – Time-analysis of rumination in dairy heifers along the transition period (week-21 until week +21)



4.3.5 Effect of BLV with different viral loads

Separation between proviral load in HPL and LPL was performed by accessing the median (3.275 copies/μL of genomic bovine DNA). Table 5 shows the results of proviral load on week -3 and +3, separated between high and low proviral load.

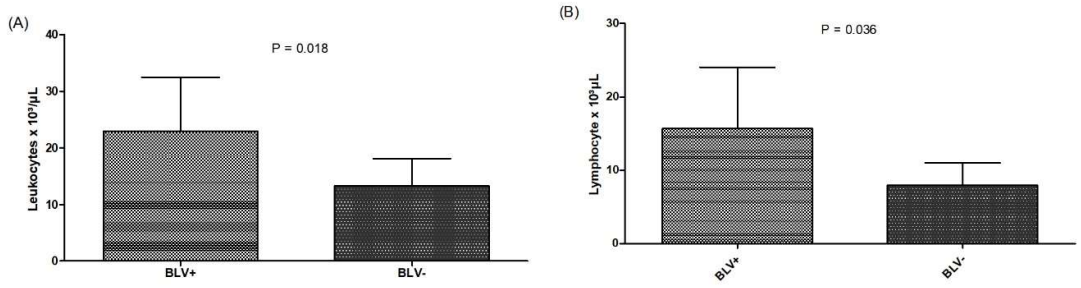
Table 5 – Proviral load of Bovine Leukemia Virus separated in high proviral load (n=6) in low proviral load (n=6) of dairy heifers during the transition period.

			Week	
	animal ID	Breed	-3	+3
Low Proviral load	475	Jersey	0,14	1,2
	917	Jersey	0,3	0,21
	946	Jersey	1,3	0,26
	511	Holstein	0,07	0,3
	641	Holstein	0,18	0,27
	694	Holstein	2,5	3,9
High Proviral load	716	Jersey	2,5	4,2
	844	Holstein	3,6	3,8
	688	Jersey	7,7	7,9
	714	Holstein	10,6	17,1
	517	Holstein	13,3	19
	858	Jersey	20,7	19,4

Legends : ID = identification. Results are expressed in copies/μL of genomic bovine DNA.

Analyzing proviral loads, heifers that had a high viral load showed increased total leukocytes (P = 0.018) and for lymphocytes (P = 0.036) compared to it corresponding BLV negative animals (figures 8A and 8B). Analysis of groups considering animals with high viral load revels a variations over the transition period for VG, RDW, monocytes, eosinophils, BHB, triglycerides, cholesterol, TPP, and HP; while the analysis of groups of animals with low viral load, variations were observed for red blood cells, hemoglobin, hematocrit, RDW, GGT, BHB, Triglycerides, PPT, BHB, PT and Hp (supplementary table 4). Variation along the time were showed in a general comparison between LEB+ and LEB- (supplementary table 1 and 2).

Figure 8– Effect of the group on the leukocytes and lymphocytes in cows BLV+ (high viral levels) and BLV- during the transition period



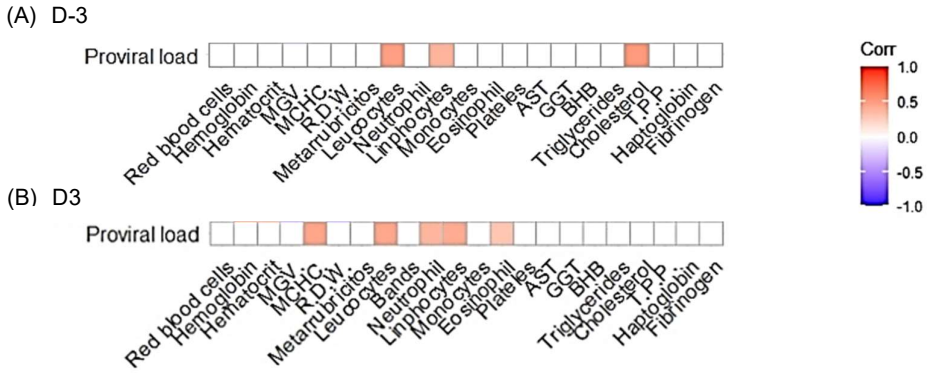
Legend: P = p-value obtained using the EMANS command comparisons with Bonferroni correction. Statistical difference was considered when the p-value was < 0.05 and the tendency when the p-value was between 0.05 and 0.1.

Production along lactation, comparing high/low proviral load with each respective control group, were evaluated according to time, group, and interaction time x group, with no statistical difference. Time effect were observed for protein and fat in both groups. SCC oscillated along time for animals with low proviral load. Rumination showed variations along time of observation for both groups (P <0,001).

4.3.7 Association between the viral charge, hematological and biochemistry parameters at week -3 and 3.

Proviral load showed a positive and significative correlation with the leukocytes, lymphocytes and cholesterol at -3 week (figure 9A). At week 3 the association between the proviral load was observed for MCHC, Leukocytes, neutrophils, lymphocytes and eosinophil (figure 9B). Regarding the hematological parameters, at week -3, we observed an association between red blood cells and MGV, hemoglobin with hematocrit and MCHC. CHCM was associated with the neutrophils. Metarubricytes was associated with the AST (positive correlation) and GGT (negative correlation).

Figure 9 – Correlation between the proviral load with the hematological and biochemistry parameter at pre parturition (D-3, figure A) and post parturition (D3, figure B) in heifers



Legend: M.C.V. = mean corpuscular volume; MCHC = Mean corpuscular hemoglobin concentration; RDW = Red Cell Distribution Width; AST = Aspartate aminotransferase; GGT = Gamma-glutamyl transferase; BHB = β-Hydroxybutyrylation; T.P.P = Total plasma protein.

4.4 DISCUSSION

Evaluation of heifers positive to BLV during the periparturient period is still obscure, as far as we know. Furthermore, very few studies accessing BLV infection and possible influences in this category are available. Taken this, our main results provide interesting updates in heifers during transition period, evaluate possible virus impact homeorhetic and homeostasis by analyzing BLV antibodies status and viral presence and proviral load and segregate the groups; and accessing the hematological parameters, biochemical and biomarkers and correlation with viral infection.

4.4.1 Herd serology

The investigated herd presented a serum prevalence of 57.25%. This high prevalence could be explained by the diverse ways of BLV transmission. Bloodborne transmission is the most recognized route of transmission, and, nowadays use of reproductive biotechnologies and veterinary practices managements in modern dairy facility's, increasing possible BLV transmission (BARTLETT et al., 2014; RUIZ et al., 2018). As well as most of Brazilian facilities, this dairy farm does not adopt any king of management to avoid BLV herd spread, with no attention on reproduction practices

equipment disinfection, and sharing needle between animals, increasing prevalence (HUTCHINSONA et al. 2020).

The prevalence is greater than the most of data presented in other Brazilian studies, in different country regions, that ranges between 9.2% and 56.3% (MORAES et al. 1996, FLORES et al., 1998 ; BIRGEL et al., 2005). The differences observed for prevalence's between studies could be explained by a gap of recent BLV prevalence's research. Moreover, another point to consider is the diagnosis methods used to screen animals for BLV. The most of the studies used agar gel immune diffusion (AGID), that is less sensitive than ELISA (BAPTISTA FILHO et al., 2019).

Taken by category, heifers had lower prevalence (38.7%) compared to cows. It seems to be logical in light of risk of transmission directly related to management, especially reproductive. By the analyses of prevalence in a Brazilian herd, we could find that the risk of infection increased especially after the reproductive management (between 12–24-month-old), becoming greater in older cows with more than 3 years old. (TORRES et al., 2017). The choice of heifers' pass throws the interesting in accesses BLV impact in young animals, and the lower prevalence that allowed to find negative animals to matched and compare with BLV+. Taken by animals with 2 or more lactation, difficulty to form pairs due to the absence of serum negative in this category (GOMES et al, 2017). An interesting result is that all the serological positive animals were ELISA positive along all weeks along experiment, in contrast to Wisnieski et al. (2019) that reported changes in serological status in the transition period.

4.4.2 Hematological and biochemical findings

Hematological finding showed that red blood cell, hemoglobin hematocrit and RDW suffered influence of the transition period. This is expected for period in accordance with Paiano et al. (2019), and shows no differences between cows and heifers (JONSSON, et al. 2013). However, no difference between groups as well as time group effect were observed, indicating that BLV infection has no inference on this paraments. Fattah et al., 2019, found no difference in red cells of heifers and cows positive to BLV, in comparison with the control group. MCHC parameter had a different profile within the hematological variables. It was not observed effect of time and groups, however it was detected an interaction between time x group. BLV + animals showed a dropped in time -2, becoming greater in week 2 post-partum. This

heterogenous behavior of the MCHC were previously related, and showed a marked variation (SAUT & BIRGEL JUNIOR 2012), or no influences along transition period (D'ANGELINO et al., 1997). To explain this interaction, we may take a look on step back on the main hemoglobin reservoir, and evaluate liver AST biomarket, that showed significantly differences among groups BLV+ and BLV - on week's -1 and 0 (supplementary table 2), and may reflects in MCHC time x group interaction, that occurs manly in week 2. Other interesting point is that Iron metabolism is essential to hemoglobin, that directly influence in MHCM (ROLAND; DRILLICH; IWERSEN, 2014). Inflammation status may reduce iron bioavailable, especially in liver. Although, iron was not evaluated, it would be dropped, especially by the acute phase's protein increased, mainly HP at birth, that also reveals an inflamed status of positive animals.

4.4.3 Biochemical Biomarkers

The effect of group was present only for the hepatic enzyme AST, observing high values in BLV+ animals. As mentioned before, the main differences on AST enzymatic activity occurs at weeks -1 (P=0,010) and 0 (P=0,045), being greater for BLV positive animals. Enzyme AST is an unspecific and highly sensitive biomarker of liver healthy and muscular catabolism (HERDT, 2000), and results showed an increased AST enzymatic activity for BLV+ animals along transition period. Our results are in accordance with Fattah et al., (2019), that found that BLV infection altered the concentration of AST, even without LP, and suggested a liver dysfunction. However, a important difference is that during all the transition period AST enzymatic activity variated into the acceptable physiological range, with no suggestion of liver damage.

Enlarging analyses, interaction between time and group was detected for triglycerides, with an increase in triglycerides concentration pro BLV+ group at week 0 (P=0,008). Taken together, triglycerides that are manly metabolized in liver, and AST, increased at same time pro BLV+ animals. However, the significant differences among groups showing an influence of BLV infection in AST enzymatic activity, and the interaction with an increased triglycerides at birth, both groups along the transition period stayed above the maximum limited, and oscillated its concentration within the physiological range expected for the transition period (MOREIRA, 2015; DJOKOVIC et al. 2019). Same authors also evidenced GGT and TPP concentration behavior with significant differences along time during the transition period in cows, in accordance

with our results. However, no differences between groups and interaction time group were observed and stayed into physiological limit. In contrast, Jonsson et al., 2013 had no differences in AST, GGT and TPP, in dairy heifers and cows.

The evaluation BHB, cholesterol and triglycerides are useful indicators for the evaluation of the energy profile (HERDT, 2000). Metabolic energetic profile allows to assist in monitoring mainly the intensity of mobilization of body reserves, and the negative energy balance, predicting clinical and metabolic alteration important to production (LEBLANC; LESLIE; DUFFIELD, 2005). Break of physiological balance mechanisms and adaptation in negative energy condition, and failure of hepatic gluconeogenesis to supply adequate glucose for lactation and body needs, may lead in Ketosis and fatty liver (HERDT, 2000). Analyzing the kinetic of BHB, cholesterol and triglycerides along time, all parameters variated according the expectation for the transition period (LEBLANC; LESLIE; DUFFIELD, 2005; MOREIRA, et al., 2015). Moreover, no differences among groups were observed predicting no influence of BLV infection on this parameter. Interaction between time x group were found for triglycerides. However, besides this interaction, behavior was in accordance to expectation along time (MOREIRA, et al., 2015).

Evaluation of inflammation cascade is a puzzle to be assemble. An important player involved in inflammation is the liver, that during physiological disbalance changes it approach from metabolism to defense, leading to the APPs response, that act in the immune system response (STRNAD ET AL., 2017). With different magnitudes, all periparturient dairy cows, even with no diseases, experience periparturient inflammation. However, the severity of the inflammation dictates the phenotypic outcomes and precedes clinical diagnosis of a disorder or disease (HORST; KVIDERA; BAUMGARD, 2021).

Fibrinogen behavior that became greater in BLV+ group during pre-partum until birth, and marked dropped first week after partum, opposite to BLV- reflects in a possible influence of viral infection on this inflammation biomarker. By the HP analyses side, one the most useful nonspecific acute phase protein for access bovine inflammation. HP showed to be an important tool aiming to analyses diarrhea in calves (MARTIN et al., 2021), respiratory disease (MOISÁ et al., 2019), mastitis (WOLLOWSKI et al., 2021), among others clinical disorders. Moreover, even in subclinical profile it can be altered (ALENIUS & WALLER 2007) as well as in different phase of production influenced, like transition period due to inflammation (REIS et al,

2016). In our results, differences between groups reveled a tendency of increasing, and was not significant probably due to the limited number of animals included. However, animals BLV+ showed more than 4 times greater chances to elevation of HP compared to BLV-, and this provide flag on BLV infection x inflammation.

Enlarging correlations between liver biomarkers and inflammation activity, HP is described as a mighty anti-inflammatory player that is involved in immunotolerances and maintenance of homeostasis of inflammatory responses (RAJU ET AL., 2019). It also provides more trends on other liver biomarkers modulation, like AST activity and synthesis of transferrin, involved in iron (STRNAD ET AL., 2017).

4.4.5 Milk Production and quality parameters

We herein analyzed mains productive parameters, and found no group effect, eighter interaction time and group. Time effect were observed for all parameters analyzed (Milk production, fat, protein, SCC). Norby et al. 2016 determine negative association between individual milk production and bovine leukemia virus (BLV). At cow level a significant decrease of milk production was observed, nevertheless an association with decreased 305-d mature-equivalent yields, especially among the older cows. Drop in productive parameters was observed by Watambe, et al. 2019, with mastitis predisposition. Evaluation of a possible impact of BLV on milk quality and production showed no differences between groups, or suffered no time x group effect. Only expected variations along a lactation was observed.

4.4.6 Rumination monitoring

Currently, access the rumination profile though the use electronic rumination-monitoring system by neck collar or ear tag, may provide valorous information on health status of dams during the transition period (KAUFMANN et al., 2018; SCHIRMANN et al., 2016). According to our results, an expected rumination kinetic, with an evident drop in rumination movements between 2 days before and after partum was observed. However, no differences among groups along transition period was found. Taken by the absence of ketosis angle that have negatively an effect on rumination movements (KAUFMANN et al. 2018), Our results are in accordance with evaluation of rumination profile of heifers during the transition period (SORIANI; TREVISI; CALAMARI, 2012). Moreover, due to the influence of inflammation on rumination behavior, it was expected the animals more inflamed can present an drop

in rumination activity urlier close-to-calve, and back to normal latter. However, probably due to the low number of animals we were not able confirm.

4.4.7 Proviral load

The ddPCR is a modern, high sensitivity, specific and quantitative tool available and its uses to investigate the BLV genetic material is nowadays increasing (HUTCHINSON et al., 2020; ISHIKAWA et a., 2020). This accuracy feature allows to trace the median and separate animals in HPL and LPL animals, with a half into each.

Although HPL is not restrict to animals with LP (JULIAREMA et al., 2016), herein the evaluation of proviral load provided a valorous information regarding differences in groups for leukocytes and lymphocytes. In the individual analyses of Leukocytosis mainly increased by the Lymphocytosis, we could observe a direct influence of the proviral load. By the perspective of the goal of the study, it was expected that animals BLV+ had leukocytosis mainly by lymphocytes increasing. However, even numerically, animals' positives showed differences comparing BLV+ and BLV-, however without statistical significances (Table 2). Overviewing separated animals by proviral load, animals LPL leukocyte and lymphocyte behavior were similar to negative group. Thus, in a future perspective, answer in BLV serology for heifers would be carefully analyzing and proviral load would be necessary on interpretation.

Unfortunately, we could not evaluate the proviral load at calve, and this makes a lack of answer, since animals BLV+ increase's antibodies detection and has more LP.

Since BLV infection induce upper expression of B lymphocyte subpopulations especially in LP (DELLA LIBERA, 2015); and showed a marked relationship with proviral load and RNA expression (CHEN et al., 2020). Moreover, Gomes et. al, 2017 showed an increasing marked $CD21^{+}$ during the transition period showing a disbalance in lymphocytes subpopulation during the transition period. Although viral load was not measured, Wisnieski et al., 2020 found a pronounced leukocytosis with lymphocytosis, in a bovine positive to BLV, especially those animals with seroconversion. Our results make sense with previously studies, and was capable to shows in this population that BLV, LP and HPL was close related. Moreover, Analyses of AST in LPL group revels a significant difference between groups. Although it is into the range expected for heifers in transition period (JONSSON et al. 2013).

4.5 CONCLUSION

Taken by results, BLV infection altered CHCM, however no differences among leukocytes were observed, especially in lymphocytes, that were the main expectation. BLV infected animals has more risk of inflammation due the Haptoglobin measurement and fibrinogen rising close to calving; and disturbed biochemical profile by increased AST enzymatic activity immediately postpartum. Furthermore, taken the low number of animals access it's possible to claim with safeguard that heifers with HPL showed a leukocytosis profile, mainly by increasing Lymphocyte count. Future studies evaluating proviral load along all transition period and lymphocytes subpopulation may clearance BLV impact in periparturient heifers.

Supplementary table 1. Mean and standard deviation values of hemogram exam in dairy heifers during transition period.

	Grupos	-3	-2	-1	0	+1	+2	+3	
Red blood cell/µL	1	6,96±3,23	5,87±0,6	5,88±0,68	6,11±1,12	5,8±1,06	5,59±0,72	5,83±0,85	0,519
	0	5,97±0,65ab	6,07±1,01ab	5,98±0,62ab	6,49±0,54a	5,85±0,62ab	5,51±0,55b	5,82±0,5ab	0,012
p-value		0,995	0,433	0,7424	0,2698	1,0000	0,8181	0,9497	
Hemoglobin	1	9,94±1,62	9,76±1,07	10,08±1,36	10,08±1,73	9,54±1,61	9,38±1,59	9,42±1,05	0,383
	0	9,57±1,48ab	9,88±1,78ab	9,57±1,32ab	10,67±1,16a	9,27±1,26ab	8,64±0,99b	9,1±1,01bc	0,001
p-value		0,405	0,606	0,314506248	0,221205556	0,562206614	0,224622126	0,539748676	
Hematocrit	1	30,8±3,77	31,08±3,26	30±3,91	30,83±5,24	29,62±4,69	28,25±4,14	28,25±3,02	0,091
	0	29,5±4,17	29,99±5,31	29,75±3,05	29,78±9,93	29,18±3,67	27,83±2,86	28,33±2,77	0,062
p-value		0,302	0,137	0,782004959	0,609915809	0,728081953	0,798606735	0,941583558	
MVG.	1	48,78±11,82	53,08±3,86	50,95±3,81	50,82±4,88	51,5±5,18	50,6±3,93	48,83±4,23	0,922
	0	49,42±3,7	49,59±4,37	49,84±3,4	49,71±3,63	49,9±3,91	50,67±4,3	48,84±4,48	0,078
p-valor		0,915	0,037	0,418682384	0,474786609	0,290892706	0,988063005	0,963871996	
MCHC.	1	32,2±2,19	31,41±1,46	33,7±2,07	32,73±1,88	32,24±2,58	33,29±3,39	33,37±1,76	0,228
	0	32,41±1,22	32,98±2,4	32,1±1,92	33,1±1,14	31,78±1,93	31,08±2,31	32,15±2,3	0,076
p- value		0,771	0,069	0,065570931	0,668168411	0,59614943	0,011176005	0,160832491	
R.D.W.	1	17,4±1,1a	17,03±0,92ab	25,32±28,58ab	17,04±1,1ab	15,9±0,81b	16,67±1,48ab	16,8±1,4ab	0,001
	0	16,69±0,9	16,74±1,07	16,66±1,03	16,59±0,97	16,24±0,87	16,23±0,83	16,5±0,89	0,146
p- value		0,215	0,475	0,468285879	0,334080994	0,277809944	0,476687011	0,651043805	
Leucocytes	1	16460±8817,05	19966,67±11315,42	18575±10752,52	16433,33±6301,42	18841,67±9853,15	17350±10346,32	17375±8142,61	0,723
	0	12133,33±2543,92	12258,33±2476,6	14333,33±4328,83	13900±4819,85	14908,33±6278,17	12258,33±2445,2	12333,33±2579,76	0,483
p- value		0,124	0,117	0,475644152	0,315929183	0,426939376	0,466398097	0,186351785	
Neutrophil	1	4218,9±2211,62	4730,58±3379,76	4494,25±2068,08	5801,67±3423,06	7005,33±4651,7	5430,83±3471,42	5674,08±2573,74	0,569
	0	3710,58±1389,93	3945,25±1544,29	5653,5±2480,54	5396,5±3696,4	5532,75±3165,92	4784,25±2020,88	4236±1326,76	0,358
p- value		0,650	0,715	0,256927414	0,531892033	0,803705668	0,902833709	0,305931375	
Linphocytes	1	11511,6±8178,86	13805,92±9793,79	13189,58±10674,95	10057,67±7172,73	10650,08±6865,49	11207,75±7655,87	10469,25±6634,12	0,205
	0	7771,33±2210,51	7424,67±1969,56	7724,33±2651,15	8186,33±2439,62	8851,92±4185,82	7099,75±1436,15	7628,33±2743,03	0,852
p- value		0,229	0,121	0,388142122	0,919186145	0,903845143	0,410451214	0,497072632	
Monocytes	1	265,7±565,12	192,33±229,05	115,83±175,75	162±236,11	229,08±269,78	223,5±365,45	248,92±427,56	0,849
	0	107,08±140,35	154,33±116,52	197,83±244,3	105,75±149,6	265,58±268,08	184,08±249,43	214,08±211,52	0,581
p- value		0,908	0,963	0,363813898	0,654779969	0,641270997	0,90957761	0,750298992	
Eosinophil	1	463,8±444,78	1237,83±1217,9	775,33±854,23	412±585,56	957,17±1667,95	476,5±498,62	982,75±1112,99	0,096
	0	544,33±532,87ab	734,08±519,89a	757,67±339,58a	211,42±237,66b	258,08±364,26bc	190,25±355,5bd	245,17±150,62ab	0,003
p- value		0,915	0,430	0,403283938	0,461249502	0,20065392	0,064896649	0,064190499	
Platelets	1	293200±81342,62ab	298666,67±130878,38a	303666,67±179279,53a	372250±128986,35abc	373500±133788,5bc	474666,67±258589,3bc	452500±182957,27c	0,001
	0	274500±90252,68ac	242083,33±84410,75a	261916,67±114720,58a	304000±104575,33ab	305666,67±77769,28ab	376250±136247,19b	355833,33±141309,35bc	0,001
p- value		0,284	0,307	0,510582639	0,16815337	0,182777399	0,441512584	0,257063475	

Legend: M.C.V. = mean corpuscular volume; MCHC = Mean corpuscular hemoglobin concentration; RDW = Red Cell Distribution Width;. Differences between groups was considered when the P-value was < 0.05

Supplementary table 2. Mean, standard deviation of Biochemistry exam in BLV+ (12) and BLV- (n=12) dairy heifers during transition period.

		-3	-2	-1	0	+1	+2	+3	
AST	1	90,1±43,83a	79,08±21,15a	72,75±17,93ab	89,92±54,56a	77,58±12,92a	80,33±24,81a	56,42±20,21b	0,043
	0	81,92±61,87	88,42±82,86	52,75±13,18	59,33±24,63	73±19,02	90,42±91,27	64,25±13,86	0,066
p-value		0,153	0,303	0,010	0,045	0,484	0,139	0,480	
GGT	1	18,85±8,08	22,28±3,95	19,61±6,56	26,42±12,21	24,48±7,85	25,54±7,28	25,91±15,15	0,175
	0	21,77±6,19ab	16,72±5,2a	18,91±4,4acd	19,78±5,42ab	23,97±6,95b	22,6±5,96bc	23,25±9,61bd	0,019
p-value		0,461	0,016	0,478	0,159	0,991	0,301	0,759	
BHB	1	0,33±0,12	0,35±0,13	0,32±0,08	0,42±0,18	0,53±0,18	0,54±0,13	0,66±0,23	0,001
	0	0,36±0,1	0,33±0,09	0,34±0,1	0,35±0,13	0,57±0,18	0,58±0,25	0,6±0,14	0,001
p-value		0,540	0,908	0,731	0,184	0,456	0,772	0,545	
Triglycerides	1	29,37±12,89a	26,97±3,65a	25,88±11,69a	22,18±16,15ab	10,63±7,3bc	7,47±3,04c	9,67±3,67bcd	0,001
	0	29,42±8,7a	27,75±9,62a	29,99±9,4a	15,89±15,04b	7,79±3,74b	9,34±6,1b	11,6±8,12b	0,001
p-value		0,806	0,744	0,243	0,008	0,325	0,441	0,685	
Cholesterol	1	79,59±14,45ab	74,23±12,29a	72,31±14,6a	71,39±12,78a	76,01±18,75a	83,98±23,22ab	94,83±23,8b	0,003
	0	69,83±11,72ab	68,23±9,86ab	64,82±12,02a	61,34±14,5a	67,83±14,49ab	78,62±17,93ab	90±25,26b	0,001
p-value		0,175	0,375	0,197	0,081	0,249	0,564	0,483	
T.P.P.	1	6,69±0,52abc	6,23±0,48a	6,28±0,51a	6,41±0,55ab	6,78±0,41abc	7,06±0,62bc	7,29±0,5c	0,001
	0	6,57±0,88ab	6,38±0,96ab	6,04±0,66a	6,28±0,65ab	6,66±0,67ab	6,94±0,46ab	7,09±0,56b	0,001
p-value		0,669	0,579	0,345	0,624	0,624	0,647	0,434	
Haptoglobin	1	3,64±1,69ab			13,96±18,1a			9,3±21,16b	0,005
	0	2,55±1,46ab			6,04±5,64a			2,07±0,99b	0,018
		0,198			0,149			0,243	
Fibrinogen	1	330±133,75ade	316,67±158,59abf	250±183,4ac	350±183,4ade	500±244,95d	308,33±197,52bcef	425±237,89df	0,015
	0	350±131,43	383,33±180,07	358,33±172,99	483,33±220,88	325±186,47	375±142,22	325±165,83	0,689
p-value		0,740	0,314	0,145	0,183	0,036	0,398	0,218	

Legend: AST = Aspartate aminotransferase; GGT = Gamma-glutamyl transferase; BHB = β-Hydroxybutyrylation; T.P.P = Total plasma protein. Differences between groups was considered when the P-value was < 0.05

Supplementary table 3 - Haptoglobin concentration in pairs of BLV+ (12) and BLV- (n=12) dairy heifers during transition period

	Time	Haptoglobin concentration (mg/dL) pairs												Mean SD
BLV+	-3	4,74	1,73	1,52	2,09	3,00	1,54	6,13	4,33	4,33	6,26	4,45	3,52	3,64±1,69
	0	4,27	2,20	*	*	3,23	1,45	32,22	12,64	4,24	6,42	5,01	21,51	13,96±18,1
	1	3,92	1,33	73,02	60,33	3,02	1,49	1,49	3,18	3,28	3,76	4,42	3,42	9,3±21,16
BLV-	-3	1,5	2,0	*	1,7	1,6	1,8	1,7	1,7	1,8	4,5	3,7	5,8	2,55±1,46
	0	*	2,8	1,3	9,1	18,9	1,7	*	9,1	*	3,7	3,0	4,6	6,04±5,64
	1	*	1,7	1,3	1,4	1,6	1,6	1,6	1,4	1,5	3,3	3,8	3,9	2,07±0,99

Legend: * Represents missing results due to hemolysis. SD = Standard deviation.

Supplementary table 4 - Linear Mixed Model to evaluate the effect of Leucosis on the hematological and biochemistry parameters of dairy heifers during the transition period

	Variables	BLV+ (n=6)	BLV- (n=6)	Time	Group	Time x Group
High BLV proviral load	Red blood cell/µL	5.71±0.8	5.85±0.67	0.547	0.669	0.942
	Hemoglobin g/dL	9.47±1.18	9.52±1.28	0.215	0.808	0.467
	Hematocrit (%)	28.54±3.74	28.67±5.79	0.474	0.914	0.529
	MGV.fL	50.19±4.43	50.38±4.14	0.008	0.809	0.767
	MCHC.g/dL	33.3±2.29	32.42±2.01	0.132	0.138	0.216
	R.D.W.(%)	19.31±15.55	16.89±0.92	0.003	0.686	0.328
	Metarrubricytes(%)	0.02±0.16	0.1±0.37	0.828	0.238	0.490
	Leucocytes/µL	22968.29±9546.66	13300±4804.88	0.173	0.018	0.402
	Myelocytes/µL	-	-	-	-	-
	Metamyelocytes/µL	-	-	-	-	-
	Bands/µL	-	2.79±18.05	-	-	-
	Neutrophil/µL	6153.51±3864.86	4755.12±2703.55	0.342	0.428	0.499
	Lymphocytes/µL	15636.2±8445.18	7933.5±3057.33	0.842	0.036	0.184
	Monocytes/µL	197.93±341.71	155.9±199.55	0.011	0.62	0.941
	Eosinophil/µL	980.66±1255.58	452.69±474.61	0.003	0.22	0.414
	Basophil/µL	-	-	-	-	-
	Platelets/µL	292439.02±118562.44	323380.95±118387.32	-	-	-
	AST U/L	76.02±17.66	67.83±20.04	0.129	0.279	0.130
	GGT U/L	23.07±9.89	19.68±5.72	0.517	0.407	0.130
	BHB	0.43±0.18	0.46±0.17	0.001	0.451	0.813
	Triglycerides	20.35±12.69	19.29±12.6	0.001	0.47	0.463
	Cholesterol	83.03±18.47	72.39±15.99	0.001	0.157	0.967
	T.P.P. g/dL	6.82±0.54	6.68±0.74	0.001	0.498	0.671
	Haptoglobin (g/dL)	6.65±7.68	4.08±4.18	0.018	0.21	0.864
	Fibrinogen mg/dL	319.51±197.76	371.43±192.91	0.912	0.395	0.375
Low BLV proviral load	Red blood cell/µL	6.25±1.76	6.06±0.710	0.001	0.857	0.694
	Hemoglobin g/dL	10.01±1.61	9.54±1.520	0.001	0.522	0.449
	Hematocrit (%)	31.08±4.05	29.71±4.02	0.001	0.481	0.948
	MGV.fL	51.21±6.81	49.04±3.52	0.711	0.136	0.408
	MCHC.g/dL	32.14±2.21	32.04±1.97	0.839	0.854	0.272
	R.D.W.(%)	16.76±0.83	16.16±0.79	0.005	0.105	0.722
	Metarrubricytes(%)	0.02±0.16	0.14±0.470	0.485	0.206	0.197
	Leucocytes/µL	12814.63±5369.43	13021.43±2814.64	0.726	0.587	0.678
	Myelocytes/µL	-	-	-	-	-
	Metamyelocytes/µL	-	-	-	-	-
	Bands/µL	3.34±21.4	-	-	-	-
	Neutrophil/µL	4574.05±2254.25	4747.4±2132.28	0.091	0.693	0.778
	Lymphocytes/µL	7477.93±5128.01	7691.26±2071.78	0.692	0.502	0.774
	Monocytes/µL	209.8±324.1	195.17±210.28	0.951	0.79	0.587
	Eosinophil/µL	549.51±653.58	387.6±393.1	0.094	0.257	0.443
	Basophil/µL	-	-	-	-	-
	Platelets/µL	445000±189197.38	282404.76±108878.11	-	-	-
	AST U/L	79.44±41.24	77.9±73.4	0.186	0.045	0.457
	GGT U/L	23.74±9.2	22.32±7.32	0.026	0.667	0.105
	BHB	0.47±0.21	0.43±0.2	0.001	0.127	0.61
	Triglycerides	16.9±12.41	18.36±13.2	0.001	0.924	0.124
	Cholesterol	74.75±18.43	70.66±19.51	0.034	0.586	0.473
	T.P.P. g/dL	6.54±0.67	6.45±0.78	0.001	0.796	0.704
	Haptoglobin (g/dL)	11.24±21.94	2.62±2.57	0.024	0.286	0.963
	Fibrinogen mg/dL	390.24±207.13	371.43±156.62	0.202	0.924	0.092

Legend: M.C.V. = mean corpuscular volume; MCHC = Mean corpuscular hemoglobin concentration; RDW = Red Cell Distribution Width; AST = Aspartate aminotransferase; GGT = Gamma-glutamyl transferase; BHB = β-Hydroxybutyrylation; T.P.P = Total plasma protein. Differences between groups was considered when the P-value was < 0.05

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