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Gingivitis in calves: longitudinal hematological and metabolic profiles- and salivary buffering capacity in animals treated with virginiamycin

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ABSTRACT: Gingivitis is an infectious inflammatory process that generates local and systemic conditions, compromising the masticatory capacity of animals. Virginiamycin controls and prevents gingivitis and necrotizing gingivitis in cattle. However, the interaction and effect with different sera and salivary elements remain unknown. The present 6-month longitudinal study evaluated the hematological, metabolic, and salivary buffering capacity profiles of calves with gingivitis treated with virginiamycin. Ten calves were divided into two groups: control and virginiamycin (n = 5 each). Calves in the virginiamycin group had a lower occurrence of gingivitis ($P < 0.01$, Student's *t*-test). The animals that developed gingivitis in both experimental groups had higher salivary levels of alkaline phosphatase ($P = 0.01915$) and lower serum levels of albumin ($P = 0.0028$). Additionally, animals receiving virginiamycin had higher serum levels of magnesium ($P = 0.008$), albumin ($P = 0.0008$), urea ($P = 0.008$), alkaline phosphatase ($P = 0.008$), total proteins ($P = 0.008$), and plasma protein ($P = 0.018$). Salivary buffering capacity was negatively correlated with salivary variables such as calcium, magnesium, albumin, total protein, and aspartate aminotransferase and serum variables such as albumin and aspartate aminotransferase. Results of the present study suggested that the occurrence of periodontopathies in episodes is reflected in the local and systemic alterations in animals. In this context, clinical periodontal monitoring also showed the benefits of virginiamycin supplementation on gingival conditions and systemic health markers, in addition to controlling the two precursor forms of periodontitis.
Key words: salivary biochemistry, serum biochemistry, cattle, gingivitis, necrotizing gingivitis, virginiamycin.

Gengivites em bezerros: perfis longitudinais hematológicos, metabólicos e capacidade tamponante salivar em animais tratados com virginiamicina

RESUMO: Gengivites são processos infecto-inflamatórios que geram quadros locais e sistêmicos, com comprometimento da capacidade mastigatória dos animais. A virginiamicina controla e previne a gengivite e a gengivite necrosante em bovinos; no entanto, a interação e efeito com diferentes elementos séricos e salivares permanecem desconhecidos. O presente estudo teve como objetivo avaliar, em um estudo longitudinal com duração de seis meses, os perfis hematológicos, metabólicos e de capacidade tamponante salivar de bezerros com gengivite e tratados com virginiamicina. Foram utilizados 10 bezerros divididos em dois grupos: grupo controle (n = 5) e grupo virginiamicina (n = 5). Os bezerros do grupo virginiamicina apresentaram menor ocorrência de gengivite ($P < 0,01$, teste T – Student). Os animais que desenvolveram gengivite, de ambos os grupos experimentais, apresentaram níveis salivares mais elevados de fosfatase alcalina ($P = 0,01915$) e níveis séricos menores de albumina ($P = 0,0028$). Observou-se também que os animais que receberam virginiamicina apresentaram níveis séricos mais elevados de magnésio ($P = 0,008$), albumina ($P = 0,0008$), ureia ($P = 0,008$), fosfatase alcalina ($P = 0,008$), proteínas totais ($P = 0,008$) e proteína plasmática ($P = 0,018$). As análises de capacidade tamponante salivar foram correlacionadas negativamente às variáveis salivares como cálcio, magnésio, albumina, proteína total e aspartato aminotransferase e às variáveis séricas como a albumina e aspartato aminotransferase. Os resultados do presente estudo sugerem que a ocorrência em episódios das periodontopatias refletem-se em alterações locais e sistêmicas nos animais. Neste contexto, o monitoramento clínico periodontal também evidenciou os benefícios do emprego de suplementação pela virginiamicina sobre as condições gengivais e marcadores sistêmicos de saúde, além do controle das duas formas precursoras de periodontites.
Palavras-chave: bioquímica salivar, bioquímica sérica, bovino, gengivite, gengivite necrosante, virginiamicina.

INTRODUCTION

The imbalance of the oral microbiota, particularly the supra- and sub-gingival microbial biofilm, a phenomenon known as dysbiosis, is a precursor of gingivitis and necrotizing gingivitis, and diseases of infectious-inflammatory origin that affect the periodontium lining the teeth (HAJISHENGALLIS, 2014; COLOMBO &TANNER, 2019; RAMOS et al., 2019). These

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changes are precursors of periodontitis in ruminants, with severe repercussions on animal health and production (SILVA et al., 2016; BORSANELLI et al., 2017; BORSANELLI et al., 2018; BORSANELLI et al., 2021; CAMPELLO et al., 2019).

The development and selection of biomarkers capable of determining the intensity and evolution of these diseases is a challenge in ruminants due to the limited knowledge of the particularities of these multifactorial processes in different animal species. Several periodontal biomarkers are used in humans, highlighting enzymes derived from host cells released during the degradation of connective tissue (MANDEL, 1990). The fate of cells and cellular remains affected by periodontal infectious conditions, such as alkaline phosphatase (AP), gamma glutamyl transferase (GGT), and aspartate amino transferase (AAT), are measured to verify the progression of these diseases as well as the effectiveness of periodontal therapies (LOBÃO et al., 2019; ROMANO et al., 2020).

Minerals, such as calcium, phosphorus, and magnesium, can also exemplify molecular phenomena in periodontal tissues, as they can be rapidly mobilized, changing their concentrations in saliva and/or blood serum, in addition to acting in the remineralization process of dental support tissues, physiological reactions associated with hemostasis, enzymatic metabolism, and tissue repair (JAWED et al., 2011; ROMANO et al., 2020).

Other markers, such as urea, total protein, and albumin, act on protein/energy metabolism and are used as indicators of inflammatory processes in liver and kidney diseases as well as periodontitis in humans (OGAWA et al., 2006; IWASAKI et al., 2008; KAUR et al., 2015). Hematological changes can also contribute to the understanding of the impact of periodontal and oral infectious and inflammatory processes on systemic health (BEYDOUN et al., 2020).

As a control and prophylaxis of periodontitis, the use of virginiamycin, an antibiotic of the streptogramin class, is effective for the clinical recovery of calves with aggressive periodontitis (TIMS et al., 1992), as well as for the control and prevention of gingivitis and necrotizing gingivitis (RAMOS et al., 2019), which are precursors of periodontitis and are known to be silent in humans. However, the mechanism of action by which this occurs remains unknown, as do possible correlations with different serum and salivary elements.

The pharmacological characteristics that allow the use of this drug are based on the fact that virginiamycin presents limited absorption by the body and rapid metabolism, with more than 94% excreted in feces. However, in the environment, it is rapidly degraded (ARAÚJO et al., 2016), which is a very interesting feature to be explored in conscious animal production.

In horses treated with virginiamycin, the absence of antimicrobial resistance has been verified in the microbiota (MENZIES-GOW; YOUNG, 2011). In cattle that ingested virginiamycin mixed with mineral salt at 0.94 mg/Kg/day, the risk to public health in terms of bacterial resistance, until the time of slaughter of the animals, was not observed (BESSEGATTO et al., 2017).

In this context, considering the use of virginiamycin to control periodontal diseases and the possible elucidative role of multiple biochemical and hematological indicators in infectious-inflammatory processes, the present study evaluated the longitudinal hematological and metabolic profiles and salivary buffering capacity in calves treated with virginiamycin, and clinically monitored the occurrence of gingivitis and necrotizing gingivitis.

MATERIALS AND METHODS

Experimental groups and sample collection.

The present study evaluated eight incisor teeth of ten male Jersey calves, aged between four and six months, weaned, clinically healthy in terms of general physical and oral parameters, and divided into two groups: control and virginiamycin (n = 5 each). The study lasted 6 months, during which, the virginiamycin group received 340 mg of virginiamycin daily via oral *"pour dressing"* for a period of 5 months. The administration of virginiamycin was then suspended for 1 month, and the animals were monitored. Blood and saliva samples were collected monthly to assess biochemical indicators, totaling five blood and six saliva samples. Collections were always performed simultaneously and at the same pattern (in the morning).

Feed management in rotational grazing system.

The animals in both groups were maintained under the same feeding management in rotational grazing systems of Massai (*Panicum maximum*) and Mombaça (*Panicum maximum* cv. Mombaça) grass in a recently renovated and fertilized area, as described by RAMOS et al. (2019). The diet remained the same, but small changes due to the seasonality of the pastures due to the season of the year had an effect on all the animals, not interfering with the objectives of the study, which simulated conditions similar to those found in animal production.

Clinical examination.

The periodontal clinical condition monitoring records included 6 clinical examinations, in which 40 incisor teeth per group were evaluated (8 incisor teeth per animal), totaling 480 dental units evaluated throughout the entire experimental period. Periodontal clinical examinations were also performed as described by RAMOS et al. (2019), based on visual inspection and probing of the incisors (labial and lingual surfaces; Figure 1), which were adapted from the gingival probing process as classically performed in humans (CHAMBRONE et al., 2010; NEWBRUN, 1996). In the initial clinical examination of the oral cavity, the animals presented with deciduous dentition, normal dental units (tooth and periodontium), and no apparent evidence of gingival alteration. In periodontal clinical examinations, probing (Williams periodontal probe) is a safe procedure for both qualified professionals and animals. After physically restraining the animal, the

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CLINICAL EXAMINATION FORM FOR BOVINE INCISOR TEETH

DATE: _____

ANIMAL IDENTIFICATION:	
SEX:	
BREED:	
AGE:	

Affections	Faces	Triadan Teeth							
		4I(404)	3I (403)	2I(402)	1I (401)	1I (301)	2I (302	3I(303)	4I (304)
Bleeding	Lip								
	Lingual								
Suppuration	Lip								
	Lingual								
Gingivitis	Lip								
	Lingual								
NUG	Lip								
	Lingual								

Subtitle:
- Bleeding: S (Spontaneous); P (probing).
- NUG: Necrotizing Ulcerative Gingivitis.
- Gingivitis: Edema and/or discoloration and/or bleeding.
- 1I: first incisor; 2I: second incisor; 3I: third incisor; 4I: fouth incisor.

Other observations (example: stomatitis, periodontitis, gingival recession, angular cheilitis, etc.):

GENERAL INFORMATION:

-Up to 1.5 years: Deciduous teeth - 3.5 to 4 years: Subst. third incisors

-1.5 to 2 years: Subst. first incisors - 4.5 to 5 years: Replace. fourth incisors

- 2.5 to 3 years: Subst. second incisors

Figure 1 - Clinical examination form for bovine incisor teeth.

periodontal probe was inserted into the subgingival sulcus and gently moved around the gingival margin of the tooth in which the pocket/sulcus depth measurement was obtained, in addition to the clinical aspects related to gingivitis and necrotizing gingivitis. Gingivitis is characterized by the presence of edema at the gingival margin, appearance, color, spontaneous bleeding, or bleeding associated with probing. In necrotizing gingivitis, an attempt was made to visualize the presence of ulcerations at the gingival margin, with or without the presence of a grayish white/yellowish pseudomembrane and pain during handling (RAMOS et al., 2019).

After randomizing the groups and verifying the homogeneity of the batches, all calves started the experimental period in full general and oral health. After the 30-day adaptation period, oral changes in the incisor teeth, such as gingivitis and necrotizing gingivitis, began to be observed in different numbers of episodes and intensities, which were recorded in individual odontograms (RAMOS et al., 2019).

Sample collection.

1. *Blood collection.* Five collections of total blood (10 ml) were performed by puncturing the jugular vein with the aid of BD Vacutainer needles (25x7) ®. A set quantity was placed in microtube (1 mL), with 10% ethylene diamictetraceticdodic acid, to perform the hemogram, and the remainder was placed in tubes without anticoagulants for later biochemical analysis and stored at -80 ºC.

2. *Saliva collection.* For the six saliva collections, samples were obtained directly from the oral cavity using Salivette® devices (CD Genomics, NY, USA) By removing the top cap of the device tube, a cotton cone was inserted into the calf' buccal cavity such that it came into contact with the saliva on the buccal surface of the incisor teeth. The cotton cone was the then returned to the container that remained refrigerated until centrifugation to obtain 2 mL of saliva. After collection, the samples were stored at -80 °C until biochemical analyses, except for the analysis of salivary buffering capacity, which was performed immediately after collection.

Determinations of serum and salivary biochemical parameters.

Biochemical analyses were performed using commercial kits (Bioclin®, Belo Horizonte, MG, Brazil) following the manufacturer's recommendations. The analyzed enzymes were aspartate aminotransferase (AAT), gamma glutamyl transferase (GGT), alkaline phosphatase (AP), as well as calcium, phosphorus, magnesium, urea, total protein, and albumin levels.

Hematological profile analysis.

For the hemogram, the samples were homogenized and read using an automated hematological counter (CELM, DA-500, and CC-530®), followed by differential counting using blood smears stained using the rapid panoptic method (WEISS&TVEDTEN, 2004) and later analyzed by optical microscopy (1000x).

Measurement of pH and salivary buffering capacity.

Immediately after saliva collection, pH and buffering capacity were analyzed using a portable pH-meter (METTER TOLEDO, Seven Multi®) monthly, totaling six collections. The buffering capacity was determined by titration with aqueous hydrochloric acid solution (HCl 0.01 N). In an aliquot of 0.5 mL of saliva, certain volumes (0.1 mL) of hydrochloric acid (0.01 N) were added and the pH recorded with each addition of the acid until reaching the analyzed pH ranges (initial pH - pH 8, 0; pH 7.9–7.0; pH 6.9–6.0; pH 5.9–5.0; pH 4.9–4.0), according to BASSOUKOU et al. (2009).

Statistical analysis.

Oral clinical, hematological, and biochemical data were tabulated and analyzed using Statística software (Stat Soft Ltd.), version 7, and Graph Pad Prism (version 6.0) spreadsheets. For the clinical signs, the graphical representation in percentage was used, and the Student's *t*-test was used to compare the quantitative parameters referring to the biochemical, hematological, and salivary buffering capacity and clinical signs. The clinical dichotomous variables (presence/absence) were subjected to the chi-square test, whereas the correlations between all hematological, serum, salivary, and clinical parameters between the animals of each group were analyzed using the Spearman correlation test. For the analysis of salivary buffering capacity, the Student's *t*-test and – the Wald-Wolfowitz run test were used. The correlations observed or suggested by the tests were also analyzed using the Mann-Whitney test. In all tests, $P \leq 0.05$ was adopted as significant, except for clinical analyses, where $P < 0.01$, Student's *t*-test.

RESULTS

The results of the present study allowed the characterization of the evaluated animals in terms of clinical periodontal, hematological, serum, and

salivary biochemical parameters, and analysis of salivary buffering capacity.

Periodontal clinical analyses did not show loss of conjunctival attachment in the animals. Regardless of the experimental group, the animals showed episodes of involvement of the lining periodontium, with spontaneous bleeding or on probing and gingival edema, characteristic of gingivitis (Figure 2A), or even formation of pseudomembrane or ulcer characteristic of necrotizing gingivitis (Figure 2B).

The percentage of sites affected by gingivitis and necrotizing gingivitis in the 480 evaluations of the dental units of the 10 calves is shown in figure 3. In figure 3A, it is also possible to observe that the animals in the virginiamycin group had a lower percentage of gingival alterations, characterized as gingivitis, on clinical periodontal examination, using $P < 0.01$, Student's *t*-test.

In addition, a seasonal pattern was observed in the occurrence of gingivitis. In both groups, the presence of gingival necrosis characterized by necrotizing gingivitis was frequent but restricted to a few periodontal sites (Figure 3B).

For serum biochemical analyses, a few variables changed along the longitudinal axis. The ANOVA test of repeated measures for categorical data showed that the highest concentrations of serum albumin occurred in calves that received virginiamycin. However, at the end of the evaluations, the levels of this indicator were similar in both groups (Figure 4).

Animals with gingivitis in both experimental groups showed lower serum levels of albumin ($P = 0.0028$) by Student's *t*-test; animals with episodes of necrotizing gingivitis had higher levels of serum phosphorus ($P = 0.015$).

Using Student's *t*-test, it was observed that in addition to albumin ($P = 0.0008$), animals in the virginiamycin group had higher serum levels of urea ($P = 0.008$), alkaline phosphatase ($P = 0.008$), and total proteins ($P = 0.008$) throughout the entire experimental period. Serum magnesium values ($P = 0.008$) also showed significant differences between the groups, with concentrations that increased exponentially over the observed period, but with higher values in the virginiamycin group (Figure 5). In salivary biochemical analyses, animals with gingivitis in both experimental groups had higher salivary levels of alkaline phosphatase ($P = 0.01915$, Student's *t*-test).

In the evaluation of the results of the hematological analysis conducted using the Student's*t*-test, it was possible to show that animals that presented episodes of gingival necrosis also showed leukocytosis ($P = 0.034$, Student's *t*-test) when compared with the animals evaluated as clinically healthy. In addition to higher levels of eosinophils in relation to animals with gingivitis but without tissue necrosis ($P = 0.0149$, Student's *t*-test).

Animals in the virginiamycin group had higher levels of plasma proteins ($P = 0.018$), and this difference was maintained throughout the experimental period ($P = 0.028$). The other hematological variables showed few fluctuations over time and were not significant.

Spearman's correlation test showed a positive correlation between fibrinogen levels and

Figure 2 - A. Calf incisors with episodes of involvement of the lining periodontium, bleeding on probing and gingival edema, characteristic of gingivitis. Figure 1B. Calf incisors with episodes of pseudomembrane formation or ulceration on the buccal surface (left pinch) characteristic of necrotizing gingivitis (RAMOS et al., 2019).

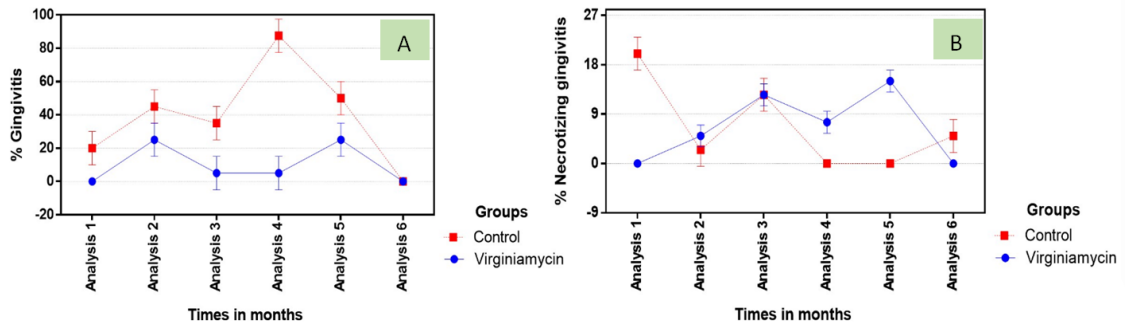

Figure 3 - A. Clinical analysis expressed as a percentage of periodontal sites with gingivitis in incisors of calves in the control and virginiamycin groups (Analysis 1 to 5), and after drug withdrawal (Analysis 6). Figure 2B. Clinical analysis expressed as a percentage of periodontal sites with necrotizing gingivitis in incisors of calves in the control and virginiamycin groups (Analysis 1 to 5), and after drug withdrawal (Analysis 6).

the highest levels of serum GGT (CI = 0.76), and the monocyte population also showed a positive correlation with serum AAT levels (CI = 0.60).

In the analyses of salivary buffering capacity, the control and virginiamycin groups did not show significant differences (P = 0.94, Student's t-test), but in the control group that had more episodes of gingivitis, a significant difference was observed between diseased and clinically healthy animals (P = 0.010, Wald-Wolfowitz Runs test). This difference between healthy and diseased animals was not observed within the virginiamycin group (P > 0.05).

When correlated with salivary variables, salivary buffering capacity showed negative values for total salivary protein in the virginiamycin group. In the control group, salivary calcium, salivary magnesium, salivary total protein, salivary albumin, and salivary AAT showed negative correlations (Table 1).

When salivary buffering capacity was compared with serum biochemical analyses, a negative correlation was evident in the virginiamycin group for serum albumin; in the control group the correlation was positive for serum AAT levels (Table 2).

DISCUSSION

Virginiamycin, an antimicrobial from the streptogramin class, is used as a growth promoter, and its mechanism of action involves inhibition of bacterial protein synthesis and control of the microbiota (COCITO, 1979). Its use in the promotion of animal health refers to the control of acidosis (ARAÚJO et al., 2016) and in, recovery of calves

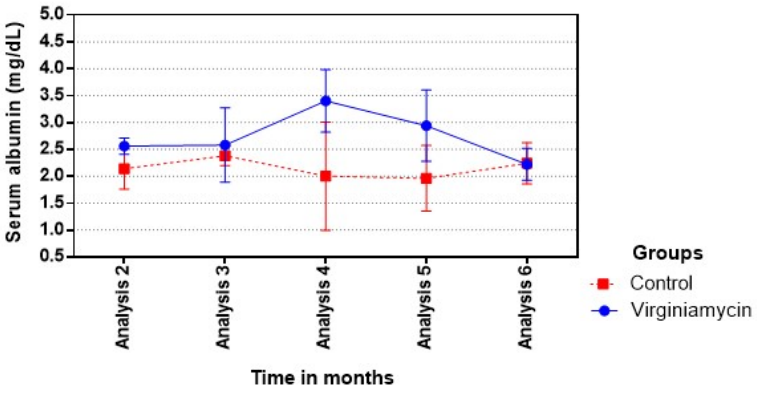

Figure 4 - Clinical analysis of serum albumin expressed as mean and standard deviation of calves in control and virginiamycin groups (Analysis 1 to 5), and after drug withdrawal (Analysis 6).

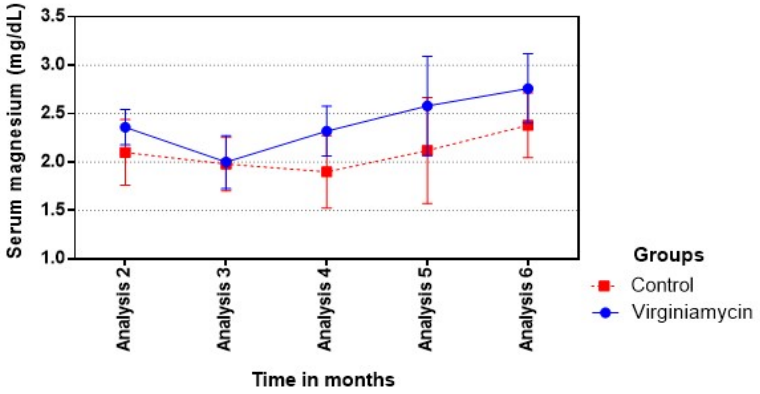

Figure 5 - Clinical analysis of serum magnesium expressed as mean and standard deviation of calves in the control and virginiamycin groups (Analysis 2 to 5), and after drug withdrawal (Analysis 6).

with aggressive periodontitis (TIMS et al., 1992) and control and prevention of gingivitis and necrotizing gingivitis (RAMOS et al., 2019). In the context of periodontal diseases in ruminants, virginiamycin is the only alternative for the control and prevention of this group of silent and often neglected diseases affecting animal health and production.

Regarding the serum parameters of calves that ingested virginiamycin, the results of the present longitudinal study showed higher levels of magnesium (Figure 5), which could be linked to the lower occurrence of gingivitis in this group of animals. Lower serum levels of this element indicate inflammatory conditions, disseminated intravascular coagulation, and oxidative and immunological stress, which may affect metabolism (SPASOV et al., 2012; HASTURK & KANTARCI, 2015; PELCZYŃSKA et al., 2022). Likewise, the increase in serum levels of magnesium, albumin, and urea may also indicate a change in the pattern of nitrogen metabolism and an increase in resistance to infections, whereas magnesium has been implicated in the reduction of inflammatory responses (PELCZYŃSKA et al., 2022) and as a regulatory element of tissue repair and cellular metabolism (CASTAGNINO et al., 2018).

The serum profile of calves that received virginiamycin may also suggested or indicated a possible superior capacity for absorbing and

Table 1 - Spearman correlation of salivary buffering capacity with the salivary variables of the virginiamycin and control groups.

Salivary variables	Virginiamycin Group	Control Group
Calcium (mg/dL)	-0.26	-0.47*
Phosphorus (mg/dL)	0.24	-0.21
Magnesium (mg/dL)	-0.27	-0.53*
Total Protein (mg/dL)	-0.42*	-0.51*
Albumin (mg/dL)	-0.24	-0.54*
Urea (mg/dL)	-0.20	-0.32
AAT (U/L)	-0.18	-0.54*
GGT (U/L)	-0.01	-0.21
Alkaline phosphatase (U/L)	-0.19	-0.15

*variables with significant correlation.

Table 2 - Spearman correlation of salivary buffering capacity with serum variables in virginiamycin and control groups.

Serum variables	Virginiamycin Group	Control Group
Calcium (mg/dL)	-0.11	0.20
Phosphorus (mg/dL)	0.07	-0.04
Magnesium (mg/dL)	0.12	0.24
Total Protein (mg/dL)	-0.05	0.00
Albumin (mg/dL)	-0.60*	0.24
Urea (mg/dL)	0.00	-0.04
AAT (U/L)	0.01	0.40*
GGT (U/L)	0.11	0.21
Alkaline phosphatase (U/L)	0.07	0.24

transporting ions compared to animals in the control group. Specifically, virginiamycin alters ruminal microbiota, modifies feed processing patterns, and indirectly affects the availability of macro- and micro-nutrients (BRETSCHNEIDER et al., 2008). Therefore, magnesium and other ions could present variations in their concentrations as a result of selective alterations in the microbiota by the action of virginiamycin, which suggested the need for additional studies to evaluate this hypothesis.

Magnesium also acts synergistically with calcium in the production and action of cellular secondary messengers, in addition to acting on vascular integrity and angiogenesis, which may affect tissue repair, although the mechanisms involved are unclear (ROWE, 2012), affecting the longevity of individuals who receive this supplementation (ROWE, 2012). The effects of magnesium on repair may have reduced the severity of tissue damage in the virginiamycin-treated group, which showed levels of magnesium and proteins indicative of systemic health, such as albumin and total serum proteins.

This hypothesis is also supported by the fact that virginiamycin can affect the protein metabolism of ruminal bacteria, increasing the availability of propionic acid and other organic acids that participate in numerous anabolic and catabolic pathways, as well as reducing the deamination of amino acids in the rumen, exacerbating their availability for protein synthesis (NAGARAJA & TAYLOR, 1987), which may have contributed to higher levels of serum and plasma proteins, in addition to serum albumin in the virginiamycin group.

Serum albumin levels have also been correlated as indicative of the presence and severity of inflammatory processes, malnutrition, nephropathies and liver diseases, as well as the presence of periodontitis in humans (OGAWA et al., 2006; IWASAKI et al., 2008; KAUR et al., 2015), though presenting low specificity, as it reflects different changes in liver functions and renal excretion (BROTTO et al., 2011). Serum albumin levels were higher in the virginiamycin group ($P = 0.00086$). However, within this group, no significant differences were observed between clinically healthy animals and those with gingivitis (Figure 3), as was also observed in human patients with different periodontal conditions (BROTTO et al., 2011). In contrast, in human patients, lower serum albumin levels have been associated with severely compromised systemic conditions (OGAWA et al. 2006; IWASAKI et al., 2008).

In the present study, the higher levels of albumin observed in animals in the virginiamycin group may reflect their systemic health condition and not just gingival inflammation. The reduction in albumin levels is linked to periodontal bone loss in humans (OGAWA et al., 2006; IWASAKI et al., 2008; LALKOTA et al., 2021), though periodontal bone resorption was not observed in animals from both experimental groups. Thus, a possible correlation between serum albumin levels and host immunocompetence and the relative risk of infection cannot be disregarded, which could impact the development of periodontal infectious-inflammatory conditions (INCHINGOLO et al., 2020; WU et al., 2020).

Some enzymes, such as alkaline phosphatase, aspartate aminotransferase, and gamma glutamyl transferase, are considered efficient indicators of cell damage and are linked to cytostructures and metabolism. Therefore, their release may reflect the existence of tissue damage (LOBÃO et al., 2019; ROMANO et al., 2020). Parameters such as bone metabolism and tissue inflammation have alkaline phosphatase as the main biomarker in humans, where its elevation prior to the establishment of periodontitis may reflect the effects of the inflammatory process on the lining periodontium, the possibility of future involvement of the periodontium of support, and the establishment of loss of conjunctival attachment (PATEL et al., 2016).

Although, it may be related to periodontal bone resorption and remodeling, alkaline phosphatase has been considered an indicator of cell damage or injury (SANIKOP et al., 2012). The present study reinforces the relationship between salivary alkaline phosphatase levels ($P = 0.019$) and gingival inflammation, but not serum alkaline phosphatase ($P = 0.019$), reflecting local conditions. It is possible that part of the salivary alkaline phosphatase originates in the serum of the animals, externalizing itself due to gingival inflammation, which could contribute to exacerbating the salivary levels of this biomarker. However, other factors, such as the age of the animals and follow-up time, may not have allowed the establishment of periodontitis in the animals in both groups.

In general, the results of the present study indicated a substantial increase in protein metabolism. First, this increase could be interpreted as an exacerbation of proteolytic activity, which is normally associated with tissue damage, but the release of enzymes linked to membrane structures (GIGON et al., 2021), such as gammaglutamyl transferase (GGT), would be indirectly responsible for the increase in protein synthesis and may be linked to tissue repair in a subsequent phase of the

inflammatory process, mainly in the virginiamycin group, in which the animals showed less occurrence of gingivitis and higher levels of albumin and total proteins in serum and plasma.

While the GGT enzyme is associated with an increase in the number of neutrophils in the peripheral blood in an initial acute inflammatory condition, the AAT enzymes is released by polymorphonuclear leukocytes and/or cell lesions, indicating not only the existence of an acute inflammatory process but also the extent of periodontal damage. In the following stages of the inflammatory condition, the increase in bone metabolism by the action of the alkaline phosphatase enzyme, as well as the mobilization of monocytes, indicates the establishment of a chronic inflammatory condition, together with the mobilization of calcium and magnesium (LOBÃO et al., 2019; ROMANO et al., 2020), like the data presented in the present study.

The results revealed that salivary levels of AAT, GGT, and AP showed low correlation with serum concentrations, reinforcing the hypothesis that such salivary markers are mainly reflecting gingival conditions and not systemic alterations, such as typical bone remodeling in young animals. NAGLER et al. (2002) identified associations between serum and salivary biochemical parameters and concluded that when the correlation between serum and salivary parameters is low, the process is local and restricted to the mouth.

Regardless of whether virginiamycin was used or not, animals that developed necrotizing gingivitis had higher levels of eosinophils in peripheral blood. Little data on the role of these cells in periodontal tissues are available in ruminants and other animal models (BEYDOUN et al., 2020; GESTAL et al., 2020).

Periodontal infection is associated with a reduction in peripheral blood platelet levels, in which eosinophils play an important role in eliminating, in the medium term, some of the preformed inflammatory mediators released in allergic conditions, antiparasitic activity, as well as antibacterial and phagocytic activity, similar to those observed in neutrophils, in addition to controlling the innate response to infections (GESTAL et al., 2020).

Although, the role of neutrophils and leukocytes in necrotizing gingivitis (DAHLEN et al., 2019) and other infectious conditions (GIGON et al., 2021) is known, the participation of eosinophils in infections and inflammation of periodontal tissues in cattle is not known. In general, the presence of eosinophils in the periodontal tissues of humans represents a response to the release of large amounts of interleukin 1β (Il-1β) that exacerbates the inflammatory condition (ARAL et al., 2020).

In the present study, a high population of eosinophils was observed in the peripheral blood of animals with necrotizing gingivitis. However, the objective of the present study was to evaluate whether this population of inflammatory cells was elevated. However, one of the protective mechanisms involved in tissue invasion by obligate anaerobic microorganisms, which is characteristic of necrotizing gingivitis (DAHLEN et al., 2019), is the production of highly reactive and oxidizing radicals, such as superoxide anions, with a large amount generated by the cell lysis process involving eosinophils (UEKI et al., 2013), which would need to be evaluated in the periodontium of ruminants.

In cattle, few studies have focused on the role of saliva, pH, and salivary buffering capacity in oral health, and other research has focused on the use of buffers and changes in salivary pH, acting in the neutralization of acids from diets to increase production and gain in weight (KHORASANI &KENNELLY, 2001), as well as in the enamel remineralization process (BARDOW et al., 2000). Thus, its role in the diagnosis and elucidation of the etiopathological factors of periodontitis in ruminants is neglected; although, the production of different basic compounds such as ammonia and acids such as butyric and propionic acids is a characteristic phenomenon in these periodontal conditions.

In specific analyses carried out in humans, SHAILA et al. (2013) did not observe a significant relationship between pH and buffering capacity with periodontal conditions. GALGUT (2001) observed significant correlations between salivary pH and periodontal pockets in humans. However, these correlations were not detected in patients with gingivitis. In the present study, it was observed that the group of cattle that presented with more episodes of gingivitis (control group) showed significant differences in relation to salivary buffering capacity when compared to the virginiamycin group ($P = 0.010$), which suggested that the control group presented a lower buffering capacity than the virginiamycin group.

CONCLUSION

Results of the present study revealed that several serum, salivary, and hematological markers and salivary buffering capacity are altered in gingivitis and necrotizing gingivitis, highlighting that the occurrence of episodes of periodontopathy

is a reflection of alterations that occur locally and systemically. Periodontal clinical monitoring also showed the benefits o virginiamycin supplementation on gingival conditions and systemic health markers, reinforcing the benefit of its use in risk situations such as gingivitis or necrotizing gingivitis in cattle.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the design and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

ETHICS AND BIOSAFETY COMMITTEE

All procedures performed on animals were approved by the animal use Ethics Committee of the Faculty of Agricultural and Veterinary Sciences - UNESP Campus of Jaboticabal/SP (Process FCAV-Unesp nº 013967/2017).

REFERENCES

ARAÚJO, D. B., et al. Use of virginiamycin in cattle feeding. In: MILLEN, D. D.; ARRIGONI, M. B.; PACHECO, R. D. L. **Ruminology**, Springer International Publishing Switzerland, 2016. p.189- 2012.

ARAL, K. et al. Inflammasomes and their regulation in periodontal disease: A review. **Journal of Periodontal Research**, v.55, n.4, p.473-487, 2020. Available from: <https://doi.org/10.1111/jre.12733>. Accessed: Aug. 22, 2022. doi: 10.1111/jre.12733.

BARDOW, A. et al. The buffer capacity and buffer systems of human whole saliva measured without loss of CO2. **Archives of Oral Biology**, v.45, n.1, p.1-12, 2000. Available from: <https://doi.org/10.1016/s0003-9969(99)00119>. Accessed: Aug. 22, 2022. doi: 10.1016/s0003-9969(99)00119-3.

BASSOUKOU, I. H. et al. Saliva flow rate, buffer capacity, and pH of autistic individuals. **Clinical Oral Investigations**, v.13, p.23-27, 2009. Available from: <https://doi.org/10.1007/s00784-008-0209-5>. Accessed: Aug. 22, 2022. doi: 10.1007/s00784-008-0209-5.

BESSEGATTO, J. A. et al. Changes in the fecal microbiota of beef cattle caused by change in management and the use of virginiamycin as a growth promoter. **Research in Veterinary Science**, v.114, p.355-362, 2017. Available from: <https://doi.org/10.1016/j.rvsc.2017.06.011>. Accessed: Dec. 03, 2022. doi: 10.1016/j.rvsc.2017.06.011.

BEYDOUN, H. A. et al. Periodontal disease, sleep duration, and white blood cell markers in the 2009 to 2014 National Health and Nutrition Examination Surveys. **Journal of Periodontolology**, v.91, n.5, p.582–595, 2020. Available from: <https://doi.org/10.1002/JPER.19-0055>. Accessed: Aug. 22, 2022. doi: 10.1002/JPER.19-0055.

BORSANELLI, A. C. et al. Black-pigmented anaerobic bacteria associated with ovine periodontitis. **Veterinary Microbiology**, v.203, p.271-274, 2017. Available from: <https://doi.org/10.1016/j.vetmic.2017.03.032>. Accessed: Aug. 22, 2022. doi: 10.1016/j.vetmic.2017.03.032.

BORSANELLI, A. C. et al. Microbiomes associated with bovine periodontitis and oral health. **Veterinary Microbiology**, v.218, p.1-6, 2018. Available from: <https://doi.org/10.1016/j.vetmic.2018.03.016>. Accessed: Aug. 22, 2022. doi: 10.1016/j.vetmic.2018.03.016.

BORSANELLI, A. C. et al. Risk factors for bovine periodontal disease – a preliminary study. **Animal**, v.15, n.2, p.100121, 2021. Available from: <https://doi.org/10.1016/j.animal.2020.100121>. Accessed: Aug. 22, 2022. doi: 10.1016/j.animal.2020.100121.

BRETSCHNEIDER, G. et al. The effect of feeding antibiotic growth promoters on the performance of beef cattle consuming forage-based diets: A review. **Livestock Science**, v.114, n.2-3, p.135–149, 2008. Available from: <https://doi.org/10.1016/j.livsci.2007.12.017>. Accessed: Aug. 22, 2022. doi: 10.1016/j.livsci.2007.12.017.

BROTTO, R. S. et al. Lack ofcorrelation between periodontitis and renal dysfunction in systemically healthy patients. **European Journal of Dentistry**, v.5, n.1, p.8-18, 2011. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3019747/>. Accessed: Aug. 22, 2022.

CAMPELLO, P. L. et al. Occurrence of periodontitis and dental wear in dairy goats. **Small Ruminant Research**, v.175, p.133-141, 2019. Available from: <https://doi.org/10.1016/j.smallrumres.2019.05.004>. Accessed: Aug. 22, 2022. doi: 10.1016/j.smallrumres.2019.05.004.

CASTAGNINO, P. S. et al. Changes in ruminal fermentation and microbial population of feedlot Nellore cattle fed crude glycerin and virginiamycin. **Animal Feed Science and Technology**, v.242, p.69-76, 2018. Available from: <https://doi.org/10.1016/j.anifeedsci.2018.05.007>. Accessed: Aug. 22, 2022. doi: 10.1016/j.anifeedsci.2018.05.007.

CHAMBRONE, et al. Prevalência e severidade de gengivite em escolares de 7 a 14 anos: condições locais associadas ao sangramento à sondagem. **Ciência & Saúde Coletiva**, v.15, n.2, p.337-343, 2010. Available from: <https://doi.org/10.1590/S1413-81232010000200008>. Accessed: Dec. 03, 2022. doi: 10.1590/S1413-81232010000200008.

COCITO, C. Antibiotics of the virginiamycin family, inhibitors, which contain synergistic components. **Microbiological Reviews**, v.43, n.2, p.145–192, 1979. Available from: <https://doi.org/10.1128/mr.43.2.145-192.1979>. Accessed: Aug. 22, 2022. doi: 10.1128/mr.43.2.145-192.1979.

COLOMBO, A. P. V.; TANNER, A. C. R. The role of bacterial biofilms in dental caries and periodontal and peri-implant

diseases: a historical perspective. **Journal of Dental Research**, v.98, n.4, p.373–385, 2019. Available from: <https://doi.org/10.1177/0022034519830686>. Accessed: Aug. 22, 2022. doi: 10.1177/0022034519830686.

DAHLEN, G. et al. Importance of virulence factors for the persistence of oral bacteria in the inflamed gingival crevice and in the pathogenesis of periodontal disease. **Journal of Clinical Medicine**, v.8, n.9, p.1339, 2019. Available from: <https://doi.org/10.3390/jcm809133>. Accessed: Aug. 22, 2022. doi: 10.3390/jcm8091339.

GALGUT, P. N. The relevance of pH to gingivitis and periodontitis. **Journal of the International Academy of Periodontolology**, v.3, n.3, p.61-67, 2001. PMID: 12666943.

GESTAL, M. C. et al. Disrupting *Bordetella* immunosuppression reveals a role for eosinophils in coordinating the adaptive immune response in the respiratory tract. **Microorganisms**, v.8, n.11, p.1808, 2020. Available from: <https://doi.org/10.3390/microorganisms8111808>. Accessed: Aug. 22, 2022. doi: 10.3390/microorganisms8111808.

GIGON, L. et al. Mechanisms of toxicity mediated by neutrophil and eosinophil granule proteins. **Allergology international**, v.70, n.1, p.30-38, 2021. Available from: <https://doi.org/10.1016/j.alit.2020.11.003>. Accessed: Aug. 22, 2022. doi: 10.1016/j.alit.2020.11.003.

HAJISHENGALLIS, G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. **Trends in Immunology**, v.35, n.1, p.3-11, 2014. Available from: <https://doi.org/10.1016/j.it.2013.09.001>. Accessed: Aug. 22, 2022. doi: 10.1016/j.it.2013.09.001.

HASTURK, H.; KANTARCI, A. Activation and resolution of periodontal inflammation and its systemic impact. **Periodontol 2000**, v.69, n.1, p.255-73, 2015. Available from: <https://doi.org/10.1111/prd.12105>. Accessed: Aug. 22, 2022. doi: 10.1111/prd.12105.

INCHINGOLO, F. et al. Chronic periodontitis and immunity, towards the implementation of a personalized medicine: a translational research on gene single nucleotide polymorphisms (snps) linked to chronic oral dysbiosis in 96 caucasian patients. **Biomedicines**, v.8, n.5, p.115, 2020. Available from: <https://doi.org/10.3390/biomedicines8050115>. Accessed: Aug. 22, 2022. doi: 10.3390/biomedicines8050115.

IWASAKI, M. et al. Longitudinal study on the relationship between serum albumin and periodontal disease. **Journal of Clinical Periodontology**, v.35, n.4, p.291–296, 2008. Available from: <https://doi.org/10.1111/j.1600-051X.2008.01215.x>. Accessed: Aug. 22, 202. doi: 10.1111/j.1600-051X.2008.01215.x.

JAWED, M. et al. Dental caries in diabetes mellitus: role of salivary flow rate and minerals. **Journal of Diabetes and its Complications**, v.25, n.3, p.183–186, 2011. Available from: <https://doi.org/10.1016/j.jdiacomp.2010.07.001>. Accessed: Aug. 22, 2022. doi: 10.1016/j.jdiacomp.2010.07.001.

KAUR, N. et al. A study to evaluate the correlation of serum albumin levels with chronic periodontitis. **Indian Journal of Dental Research**, v.26, n.1, p.11-14, 2015. Available from: <https://doi.org/10.4103/0970-9290.156788>. Accessed: Aug. 22, 2022. doi: 10.4103/0970-9290.156788.

KHORASANI, G. R.; KENNELLY, J. J. Influence of carbohydrate source and buffer on rumen fermentation characteristics, milk yield, and milk composition in late-lactation Holstein cows. **Journal of Dairy Science**, v.84, p.1707-1716, 2001. Available from: <https://doi.org/10.3168/jds.S0022-0302(01)74606-1>. Accessed: Aug. 22, 2022. doi: 10.3168/jds.S0022-0302(01)74606-1.

LALKOTA, H. et al. Estimation and correlation of serum albumin and serum alkaline phosphatase levels between smokers and non-smokers with generalized chronic periodontitis. **Cureus**, v.13, n.8, p.17474. 2021. Available from: <https://doi.org/10.7759/cureus.17474>. Accessed: Aug. 22, 2022.doi: 10.7759/cureus.17474.

LOBÃO, W. J. M. et al. Relationship between periodontal outcomes and serum biomarkers changes after non-surgical periodontal therapy. **Anais of the Brazilian Academy of Sciences**, v.91, n.2, p.e20170652, 2019. Available from: <https://doi.org/10.1590/0001-3765201920170652>. Accessed: Aug. 22, 2022. doi: 10.1590/0001-3765201920170652.

MANDEL, I. D. The diagnostic uses of saliva. **Journal Oral Pathology e Medicine**, v.19, n.3, p.119-125, 1990. Available from: <https://doi.org/10.1111/j.1600-0714.1990.tb00809.x>. Accessed: Aug. 22, 2022. doi: 10.1111/j.1600-0714.1990.tb00809.x.

MENZIES-GOW, N. J.; YOUNG, N. J. Antibiotic resistance in faecal bacteria isolated from horses receiving virginiamycin for the prevention of pasture- associated laminitis. **Veterinary Microbiology**, v.152, p.424-428, 2011. Available from: <https://doi.org/10.1016/j.vetmic.2011.05.026>. Accessed: Dec. 03, 2022. doi: 10.1016/j.vetmic.2011.05.026.

NAGARAJA, T. G.; TAYLOR, M. B. Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives. **Applied and Environmental Microbiology**, v.53, n.7, p.1620-1625, 1987. Available from: <https://doi.org/10.1128/aem.53.7.1620-1625.1987>. Accessed: Aug. 22, 2022. doi: 10.1128/aem.53.7.1620-1625.1987.

NAGLER, R. M. et al. Saliva analysis in the clinical setting: revisiting an underused diagnostic tool. **Journal of Investigative Medicine**, v.50, n.3, p.214-225, 2002. Available from: <https://doi.org/10.2310/6650.2002.33436>. Accessed: Aug. 22, 2022. doi: 10.2310/6650.2002.33436.

NEWBRUN, E. Indices to measure gingival bleeding. **Journal of Periodontology**. 1996 Jun. 67(6):555-61.. PMID: 8794964 Available from: <https://doi.org/10.1902/jop.1996.67.6.555>. Accessed: Dec. 03, 2022. doi: 10.1902/jop.1996.67.6.555.

OGAWA, H. et al. Association between serum albumin and periodontal disease in community-dwelling elderly. **Journal of Clinical Periodontology**, v.33, n.5, p.312–316, 2006. Available from: <https://doi.org/10.1111/j.1600-051X.2005.00901.x>. Accessed: Aug. 22, 2022. doi: 10.1111/j.1600-051X.2005.00901.x.

PATEL, R. M. et al. Estimation and comparison of salivary calcium, phosphorous, alkaline phosphatase and pH levels in periodontal health and disease: a cross- sectional biochemical stud. **Journal of Clinical and diagnostic research**, v.10, n.7, p.58-65, 2016. Available from: <https://doi.org/10.7860/JCDR/2016/20973.8182>. Accessed: Aug. 22, 2022. doi: 10.7860/JCDR/2016/20973.8182.

PELCZYŃSKA, M. et al. The role of magnesium in the pathogenesis of metabolic disorders. **Nutrients**, v.14, n.9, p.1714,

2002. Available from: <https://doi.org/10.3390/nu14091714>. Accessed: Aug. 22, 2022. doi: 10.3390/nu14091714.

RAMOS, T. N. M. et al. Efficacy of virginiamycin for the control of periodontal disease in calves. **Pesquisa Veterinária Brasileira**, v. 39, n. 2, p. 112-122, 2019. Availablefrom: https://doi.org/10.1590/1678-5150-PVB-5922. Accessed: Aug. 22, 2022. doi: 10.1590/1678-5150-PVB-5922.

ROMANO, F. et al. ICP-mass-spectrometry ionic profile of whole saliva in patients with untreated and treated periodontitis. **Biomedicines**, v.8, n.9, p.354, 2020. Available from: <https://doi.org/10.3390/biomedicines8090354>. Accessed: Aug. 22, 2022. doi: 10.3390/biomedicines8090354.

ROWE, W., J. Correcting magnesium deficiencies may prolong life. **Clinical Interventions in Aging**, v.7, p.51–54, 2012. Available from: <https://doi.org/10.2147/CIA.S28768>. Accessed: Aug. 22, 2022. doi: 10.2147/CIA.S28768.

SANIKOP, S. et al. Gingival crevicular fluid alkaline phosphatase as a potential diagnostic marker of periodontal disease. **Journal Indian Society of Periodontology**, v.16, n.4, p.513-518, 2012. Available from: <https://doi.org/10.4103/0972-124X.106889>. Accessed: Aug. 22, 2022. doi: 10.4103/0972-124X.106889.

SHAILA, M. et al. Salivary protein concentration, flow rate, buffer capacity and pH estimation: A comparative study among young and elderly subjects, both normal and with gingivitis and periodontitis. **Journal Indian Society of Periodontology**, v.17, n.1, p.42-46, 2013. Available from: <https://doi.org/10.4103/0972-124X.107473>. Accessed: Aug. 22, 2022. doi: 10.4103/0972-124X.107473.

SILVA, N. S. et al. Epidemiological, clinical and pathological aspects of an outbreak of periodontitis in sheep an outbreak of periodontitis in sheep. **Pesquisa Veterinária Brasileira**, v.36, n.11, p.1075–1080, 2016. Available from: <https://doi.org/10.1590/S0100-736X2016001100003>. Accessed: Aug. 22, 2022. doi: 10.1590/S0100-736X2016001100003.

SPASOV, A. A. et al. Magnesium and the oxidative stress. **Ross FiziolZhIm I M Sechenova**, v.98, n.7, p.915-923, 2012.

TIMS, F.M. et al. Eficiência da virginiamicina na recuperação de bezerros com a doença peridentária "cara inchada". **Pesquisa Veterinária Brasileira**, v.12, n.7, p.77-80, 1992. Available from: <https://doi.org/10.1590/S0100-736X2004000100011>. Accessed: Aug. 22, 2022. doi: 10.1590/S0100-736X2004000100011.

UEKI, S. et al. Eosinophil extracellular DNA trap cell death mediates lytic release of free secretion competent eosinophil granules in humans. **Blood**, v.121, n.11, p. 2074- 2083, 2013. Available from: <https://doi.org/10.1182/blood-2012-05-432088>. Accessed: Aug. 22, 2022. doi: 10.1182/blood-2012-05-432088.

WEISS, D.; TVEDTEN, H. The complete blood count and bone marrow examination: general comments and selective techniques. In: _____. **Small animal clinical diagnosis by laboratory methods**. Louis, Missouri: Saunders, 2004. p.14-37.

WU, D. et al. Expression profiling and cell type classification analysis in periodontitis reveal dysregulation of multiple incrnas in plasma cells. **Fontiers in Genetics**, v.11, p.382, 2020. Available from: <https://doi.org/10.3389/fgene.2020.00382>. Accessed: Aug. 22, 2022. doi: 10.3389/fgene.2020.00382.