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Hematological parameters and frequency of salmonella spp. in swabs of caiman yacare after of probiotics

The C. yacare placed in breeding conditions have a preserved genetic pool, which requires the enhancement of its zootechnical characteristics to solve some obstacles. Among them is the natural occurrence of Salmonella spp. in wild C. yacare. To address this problem, some breeders have adopted the use of probiotics to reduce Salmonella spp. in the digestive tube of crocodilians. The purpose of this study was to verify whether the addition of probiotics in animal feed reduces the isolation frequency of Salmonella spp. in cloacal swabs and to assess whether the additive interferes with the hematology and the complement system. During an 18-month period, four groups of caimans received 0% (control group), 0.25%, 0.5% and 1% of probiotic supplement. The probiotics did not reduce the isolation frequency of Salmonella spp. We have identified directly proportional relation between probiotic concentration and Hemoglobin concentration (Hb) and Mean Corpuscular Hemoglobin Concentration (MCHC) and inversely proportional relation to the total concentration of Eosinophils and Heterophils. Finally, we verify that the prolonged use of probiotics at 0.25% concentration is safe and increase the activity of the complement system.

Keywords: Probiotic; Hemoglobin; Salmonella; Complement; Erythrocytes.

Parâmetros hematológicos e frequência de salmonella spp. em suabes de caiman yacare após uso de probióticos

O pool genético dos C. yacare colocados em condições de cultivo é preservado, o que requer o aprimoramento de suas características zootécnicas para transpor alguns obstáculos. Entre eles está a ocorrência de Salmonella spp. em C. yacare em animais selvagens trazidos para o ambiente de cultivo. Para resolver esse problema, alguns criadores adotaram o uso de probióticos para reduzir a presença de Salmonella spp. no tubo digestivo desses crocodilianos. O objetivo deste estudo foi verificar se a adição de probióticos na alimentação animal reduz a frequência de isolamento de Salmonella spp. em suabes cloacais e para avaliar se o aditivo interfere na hematologia e no sistema complemento. Durante um período de 18 meses, quatro grupos de jacarés receberam 0% (grupo controle), 0,25%, 0,5% e 1% de suplementação de probiótico. Os probióticos não reduziram a frequência de isolamento de Salmonella spp. Foi verificada uma relação diretamente proporcional entre a concentração de probiótico com a concentração de hemoglobina (Hb), bem como com a Concentração de Hemoglobina Corpuscular Média, e relação inversamente proporcional à concentração total de Eosinófilos e Heterófilos. Finalmente, verificamos a segurança e o aumento da atividade do sistema do complemento no uso prolongado de probióticos a uma concentração de 0,25%.

Palavras-chave: Probióticos; Hemoglobina; Salmonella; Complemento; Eritrócitos.

Topic: **Uso Sustentável da Biodiversidade**

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INTRODUCTION

The planet's largest density of crocodilians is found in the flooded regions of Argentina, Bolivia, Brazil and Paraguay. This and the resilience of the *Caiman yacare* population allows the sustainable management of the species (FARIAS et al., 2013), presenting as an alternative to environmental preservation and income for the Pantanal population (HARRIS et al., 2005; NOGUEIRA et al., 2011). Aiming to optimize the breeding system for Pantanal caimans, some work fronts have sought to enhance the zootechnical characteristics of caimans, optimizing weight gain, improving health conditions and reducing mortality rates (ALEIXO et al., 2002; MACIEL et al., 2003). As a result, it is possible to point out some obstacles to breeding, such as feeding and sanitation in the zootechnical systems. Even though *C. yacare* present high tolerance to infections, poor hygiene conditions may increase the risks of epizootic outbreaks due to foster of potential pathogenic/zoonotic bacteria (PRESSINOTTI et al., 2013; SCOTT; FOSTER, 1997). In Brazil, federal law regulates *C. yacare* breeding in the ranching system, where an annual quota of wild egg harvest and hatchlings capture are determined by the population size of the species (BAMPI et al., 2002; VERDADE, 2004). The negative side of this model is the introduction of microorganisms found in wild populations into the breeding system, which may maximize sanitary risks and increase the need of controls even further. Among other bacteria, different serovars of *Salmonella* spp. make up the natural gut microbiota of wild and farmed caimans (AUSTRALIA, 2000; HUCHZERMEYER, 2002; MITCHELL et al., 2001; UHART et al., 2011), with some serovars considered potentially zoonotic (MADSEN et al., 1998).

The ingestion of contaminated caiman meat, the contact with live caimans or with the environment where caimans evacuate are potential contamination routes to humans by these bacteria (ENG et al., 2015). Crocodilian meat may be contaminated by *Salmonella* spp. during slaughter and processing, but these crossed contamination risks may be mitigated by hygiene, monitory and treatment strategies (AUSTRALIA, 2000). However, the presence of *Salmonella* spp may debilitate *C. yacare* (HUCHZERMEYER, 2002; MITCHELL et al., 2001), making necessary to control the presence of this bacteria in the microbiota. As a prophylactic measure, caiman feeding can be supplemented with probiotics to promote competitive exclusion and/or produce bactericide substances capable of reducing the frequency of *Salmonella* spp. in the digestive tube. The probiotics are used to improve the health condition of its consumers and may vary depending on the supplement doses, frequency, and treatment length. Moreover, the microorganisms acting as probiotics are species-specific, so that generalizations regarding the actions of a certain microorganism combination in distinct zootechnical species may be misleading (GATESOUPE, 1999; HAI, 2015). The literature highlights the probiotic effect of the bacteria *Enterococcus faecium* (OLIVEIRA et al., 2014), *Lactococcus lactis* (DOYLE; ERICKSON, 2012), *Bacillus subtilis* (SADEGHI et al., 2015), *Bifidobacterium* spp., (IÑIGUEZ PALOMARES et al., 2006), *Lactobacilus acidophilus* (PRISCILLA et al., 2008; VANDEPLAS et al., 2010) and *Lactobacilus casei* (HUDAULT et al., 1997). There is no published data on use of probiotics in crocodilians. Because of the occurrence of *Salmonella* spp. in the digestive tube of *C. yacare*, we tested a commercial probiotic composed by combination of *Bifidobacterium bifidum*, *Bacillus subtilis, Enterococcus faecium, Lactobacillus acidophilus,*

Lactobacillus casei and *Lactobacillus lactis* as feed additive (BERGE et al., 2012). The systemic effects of the use of probiotics were verified by evaluating the activity of the alternative pathway of the complement system and by hematologic parameters (GATESOUPE, 1999; HAI, 2015). Once use of probiotics represents an alternative to diminishing the prevalence of *Salmonella* spp in the gut and improvement of general health conditions, it is necessary to study the hematological parameters and frequency of *Salmonella* spp in cloacal swabs in *C. yacare* after the prolonged use of probiotics.

MATERIALS AND METHODS

The *C. yacare* used herein (120 sub-adults of 3-year and 2-month-old; see table 2 for Snout-Vent Length) were made available by a legal ranch located in the State of Mato Grosso (Brazil), where they are farmed in a barn with 4.0 m^2 brick stalls on uneven floor and with water available in one third of this area (ALEIXO et al., 2002). This study was carried out in accordance with federal laws on use and handling of wild animals and the protocol was approved by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA), the Authorization and Biodiversity Information System (SISBio), Brazil (number 42831)

Experimental Design

Feeding was carried out three times a week and provided at 5% of live weight of the animals, with a diet based on ground bovine viscera, soymeal, limestone, vitamins A, D3, E, K3, B1, B2, B6, B12, Biotine, Niacine, Calcium Panthotenate, Folic Acid, Manganese, Zinc, Iron, Copper, Iodine, Selenium (ALEIXO et al., 2002; MARCÓ; PIÑA; LARRIERA, 2009), and supplemented with commercial probiotic Nutrimix Aves® (Base Fértil, São Paulo, Brazil, MAPA EPSP07471). The probiotic used contains vitamin A, butylated hydroxytoluene (BHT), methionine, lysine, flavoring agent, *Bifidobacterium bifidum*, *Bacillus subtilis, Enterococcus faecium, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus lactis.*

Four concentrations of probiotics supplementation were used. The 120 *C. yacare* were randomly distributed into control group that did not receive addition of probiotics in the feed or three remaining groups that received additions of 0.25%, 0.5% or 1% (w/w) of probiotics in the feed for a period of 18 months. We assayed 29, 31, 30 and 30 caimans in the control, 0.25%, 0.5% and 1% groups respectively (Table 1).

Table 1: Final concentration of probiotics at 0.25%, 0.50% and 1% in the feed offered to *C. yacare.*

Probiotic Concentration	0,25%	0,50%	1%
vitamin A	6.5 UI/g	13 UI/g	26 UI/g
BHT	0.0025 mg/g	0.005 mg/g	0.01 mg/g
Methionine	0.0075% in mass	0.015% in mass	0.03% in mass
Lysine	0.0075% in mass	0.015% in mass	0.03% in mass
Flavoring agent	0.175% in mass	0.35% in mass	0.70% in mass
Bifidobacterium bifidum	595 UFC/g	1190 UFC/g	2380 UFC/g
Bacillus subtilis	875 UFC/g	1750 UFC/g	3500 UFC/g
Enterococcus faecium	595 UFC/g	1190 UFC/g	2380 UFC/g
Lactobacillus acidophilus	595 UFC/g	1190 UFC/g	2380 UFC/g
Lactobacillus casei	560 UFC/g,	1120 UFC/g,	2240 UFC/g,
Lactobacillus lactis	300 UFC/g.	600 UFC/g.	1200 UFC/g.

BHT = Butylated hydroxytoluene

Hematology

The number of caimans sampled were 23, 23, 24 and 25 in the control, 0.25%, 0.5% and 1% groups respectively. The maximum blood volume collected was 0.3% of live weight of the caimans, not detrimental to the viability of the organisms after sampling (NARDINI; LEOPARDI; BIELLI, 2013; SYKES; KLAPHAKE, 2008). Blood was harvested from the spinal venous sinus located continuously with the tail to the occipital condyle (MYBURGH et al., 2014; SYKES et al., 2008) with aid of a needle and syringe, previously treated with heparin solution (1 mL of 5.000UI heparin for 50 mL of 0.9% saline solution) after asepsis with iodine 2% (AREE et al., 2011; MYBURGH et al., 2014). The hematology evaluation was adapted from fish to crocodilians. Blood smeared on glass slides were stained by May Grünwald and Giemsa (ROSENFELD, 1947) and total cell counting was determined using Neubauer chamber (NARDINI et al., 2013). The hematocrit percentage (Ht) was determined by the microcapillary method (RANZANI-PAIVA et al., 2013), whereas total hemoglobin was determined by the cyanometahemoglobin method (Hb) (COLLIER, 1944) using laboratory kit for human hemoglobin (Labtest, Brazil). Total leukocyte counting was determined by the relative count (RANZANI-PAIVA et al., 2013). A total of 2,000 cells were counted in blood smears, distinguishing between erythrocytes (Er), thrombocytes (Tr) and leukocytes (Lc). Subsequently, the numbers were used in the indirect calculation of these cell types concentration (RANZANI-PAIVA et al., 2013). With the aforesaid hematimetric parameters, we calculated Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC):

$$\mathrm{MCV} = \frac{\mathrm{Ht\ (L/L)\ x\ 1000}}{\mathrm{Er\ (pL^{-1})}} = \mathrm{fL} \qquad \mathrm{MCH} = \frac{\mathrm{Hb}\ \left(\frac{\mathrm{g}}{\mathrm{dL}}\right) \mathrm{x}\ 10}{\mathrm{Er\ (pL^{-1})}} = \mathrm{pg} \qquad \mathrm{MCHC} = \frac{\mathrm{Hb}\ \left(\frac{\mathrm{g}}{\mathrm{dL}}\right)}{\mathrm{Ht}\ \left[\frac{\mathrm{L}}{\mathrm{L}}\right]} = \%$$

The leukogram was performed by active search in glass slides blood smears of monocytes, lymphocytes, eosinophils, heterophils and basophils until counting 200 cells (GREER et al., 2014).

Activity of the alternative pathway of the Complement System

The efficiency of the Complement System was assessed by the method of sheep (*Ovis aries*) Er hemolysis. An Er suspension was prepared in PBS pH 7,4 at 2% concentration (v/v) (SRBC – Suspension of Red Blood Cells) (MERCHANT, 2012; MERCHANT et al., 2006; SIROSKI et al., 2010). A total of 20 mL of blood was harvested from each of the 30 *C. yacare,* distributed as 8, 7, 9 and 6 *C. yacare* in the control, 0.25%, 0.5% and 1% probiotic groups respectively, with aid of non-heparinized syringes. Blood was transferred into 15 mL test tubes, allowed to coagulate for 4 hours at 25º C and centrifuged at 2,000 g allowing blood serum to be harvested. Absorbance was blanked with a 2 mL mixture of PBS and 2 mL of serum. Serum and SRBC were incubated for 5 minutes at 30º C. All incubations were centrifuged for sedimentation of non-hemolyzed Er and the hemoglobin released on the supernatant was estimated by spectrophotometry at the 540 nm wavelength (AREE et al., 2011; MERCHANT, 2012). Negative control (0% hemolysis) consisted of heat inactivation of 2 mL serum (56º C for 30 min) and subsequently tested by incubation with 2 mL SRBCs for 5 minutes (ISBERG, 2007; FINGER et al., 2012; MACHHA et al., 2011). Positive control (100% hemolysis)

consisted of incubating 2 mL SRBCs with 2 mL of 2% Triton-X, aggressively homogenized with an insulin syringe. Total hemolysis was assessed in microscope before absorbance measuring.

Frequency of *Salmonella* spp.

Cloacal swabs were sampled once at the end of the 18 months period from all the 120 *C. yacare* of the experimental design. The swabs were inoculated in Stuart medium and kept refrigerated until being selectively enriched in sodium tetrathionate broth at 37° C for 48h, followed by plating in XLT4 selective medium and reincubation at 37° C for 24-48h (MEAD et al., 1989). The identification of isolates was carried out through a biochemical series medium (Enterokit – Probac®, São Paulo, Brazil) and was confirmed serologically with polyvalent somatic commercial antigen (Probac®, São Paulo, Brazil). Isolates were kept in storage mediums and sent to the strains collection.

Bacterial genomic DNA was extracted as previous described (BOOM et al., 1990) to confirm genus *Salmonella* spp. by Polymerase Chain Reaction (PCR) (ALVAREZ et al., 2004). Amplification was performed using 50 µL of the mixture containing 20 ng of DNA, 1.5 mM/L of MgCl2, 0.2 mM/L of each dNTP, 0.4 µM of each oligonucleotide, 1 U of Taq enzyme DNA polymerase (LGC Biotecnologia, São Paulo, Brazil), 1 X PCR buffer and ultrapure water. The amplified fragments were visualized after 1.5% agarose gel electrophoresis, stained by BlueGreen® (LGC Biotecnologia, São Paulo, Brazil), and amplicon sizes identified with the assistance of a 100 base pair molecular marker.

Data analysis

Data are presented as descriptive statistics and Kruskal-Wallis non-parametric test was used for testing the hypothesis of hematological differences between treatments and control. The Mann-Whitney U non-parametric test was used for testing the differences in the hemolysis test. Differences in *Salmonella* spp. frequency between treatments and control was tested by chi-square. Linear regressions were traced in dispersion graphs correlating probiotic concentration and the measured parameters, and tested by ANOVA F Test.

RESULTS

Hematology

SVL, Er, Lc, Ht, Hb, MCV, MCH results and total concentration of monocytes, lymphocytes, basophils and heterophils were not significantly different among treatments. MCHC values were significantly higher for caimans receiving 1% probiotics compared to the control group. Total eosinophils concentration presented lower levels in caimans receiving 0.5% probiotics compared to the control group (Table 2).

Table 2: Hematological analysis of *C. yacare* at probiotic concentration of 0%, 0.25%, 0.5% and 1 %.

PbC	**0%**	**0.25%**	**0.5%**	**1%**
SVL	57.73 ± 3.50	60.73 ±4.32	58.83 ± 4.12	59.40 ± 2.51
Er /uL	53.75 10^4 ± 10.52 10^4	55.77 10^4± 8.96 10^4	56.92 10^4± 9.47 10^4	58.07 10^4± 8.39 10^4

Lc /uL	11.23 10^3 ± 4.46 10^3	12.83 10^3± 7.48 10^3	11.94 10^3± 6.02 10^3	11.97 10^3± 5.27 10^3
Ht%	24.74 ± 2.78	24.78 ± 3.46	24.46 ± 3.02	25.28 ± 3.38
Hb (g/dL)	9.24 ± 1.83	9.80 ± 1.93	10.05 ± 2.25	10.63 ± 2.61
MCV (fL)	474.13 ± 88.13	452.71 ± 82.69	437.27 ± 69.57	438.26 ± 46.61
MCH (pg)	176.41 ± 42.09	180.03 ± 46.12	178.75 ± 40.57	184.00 ± 40.87
MCHC (g/dL)	37.43 ^{a} ± 6.50	39.58^{ab} ± 5.86	41.02^{ab} ± 7.20	41.99^{b*} ± 7.80
Total Monocytes	766.07 ± 463.36	819.77 ± 487.58	679.90 ± 299.94	709.10 ± 406.92
Total Lymphocytes	6996.98 ± 2738.58	9479.313 ± 5259.58	9529.21 ± 5361.10	9178.96 ± 4109.67
Total Eosinophils	806.14 ^{a} ± 639.62	655.15 ^{ab} ± 624.82	348.25^{b**} ± 376.84	442.30^{ab} ± 325.12
Total Basophils	251.06 ± 225.62	242.08 ± 307.74	196.98 ± 167.37	158.30 ± 132.26
Total Heterophils	2410.38 ± 1879.74	1634.33 ± 1685.90	1190.01 ± 738.45	1486.91± 1097.61

PbC, Probiotic Concentration; SVL, Snout-Vent Length; Er, Erythrocytes; Ht, Hematocrit; Hb, Hemoglobin; Lc, Leukocytes; MCH, Mean Corpuscular Hemoglobin; MCV, Mean Corpuscular Volume; MCHC, Mean Corpuscular Hemoglobin Concentration. Different letters refer to significant differences between the columns. The *p* value was calculated using the Kruskal-Wallis non-parametric test. * p=0,014, ** p=0,004.

Linear regressions of Hb and MCHC were significantly and directly proportional to the probiotic concentration (Figure 1). Concentrations of eosinophils and heterophils were significantly and inversely proportional to the PbC as shown by the linear regression (Figure 2).

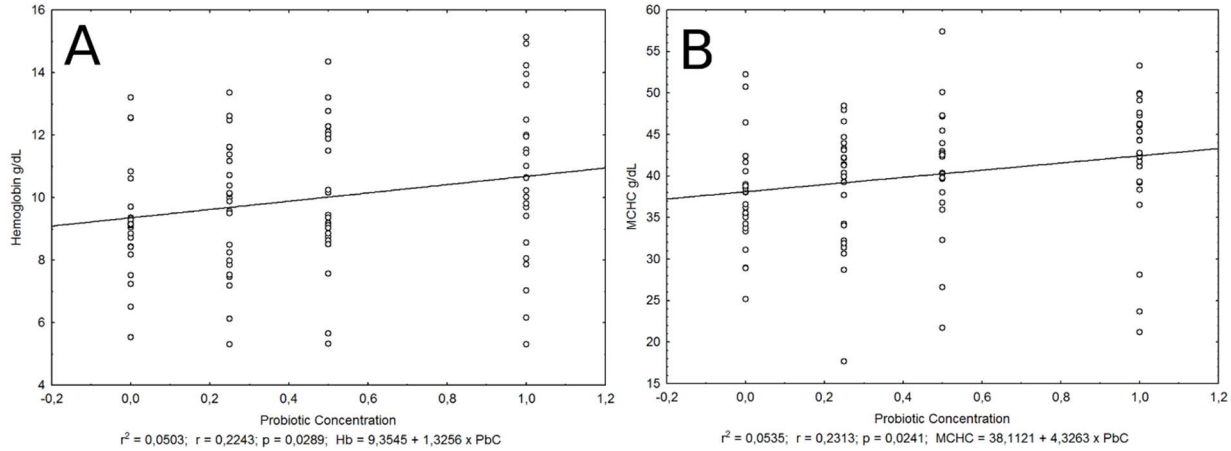

Figure 1: Linear regression graphs of parameters directly proportional to probiotic concentration**.** (A) Probiotic Concentration vs Hemoglobin. (B) Probiotic Concentration vs Mean Corpuscular Hemoglobin Concentration. p < 0.05. Correlation levels and the linear equations are shown. **Footnote:** PbC, Probiotic Concentration; Hb, hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration

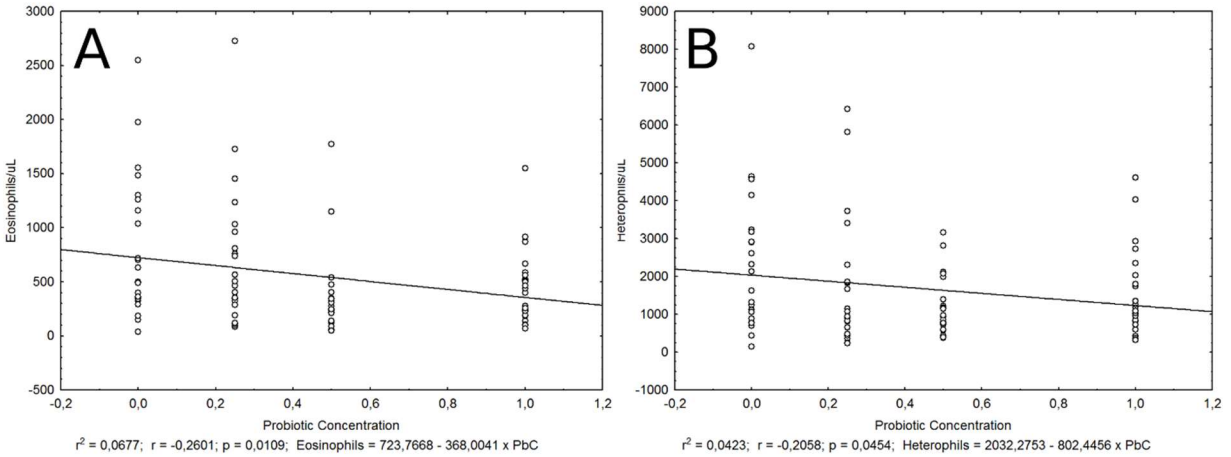

Figure 2: Linear regression graphs of parameters inversely proportional to probiotic concentration. (A) Probiotic Concentration vs Eosinophils / µL. (B) Probiotic Concentration vs Heterophils / µL. p < 0.05. Correlation levels and the linear equations are shown. **Footnote:** PbC, Probiotic Concentration.

Activity of the alternative pathway of the Complement System

Hemolysis values of caimans receiving probiotics at 0.25% concentration were significantly higher when compared to the control group (Table 3).

Table 3: Hemolysis of serum from *C. yacare* treated with different concentrations of probiotics.

PbC	SVL	Hemolysis % Mean	-95%	+95%
0%	58.81	12.83	3.64	22.02
0.25%	64.00	23.75*	14.76	32.74
0.5%	60.50	20.44	8.25	32.63
1%	60.10	15.40	5.11	25.70

PbC, Probiotic Concentration; SVL, Snout-Vent Length. * Significant difference according to Mann-Whitney U in comparison to control. $p < 0.01$. Inactivation by EDTA or serum heated at 56 ºC fully inhibited hemolysis.

Frequency of *Salmonella* spp.

Salmonella spp. was present in 13.79% of cloacal swabs from the control group, in 22.58%, 40% and 43.33% of cloacal swabs, respectively, from 0.25%, 0.5% and 1% probiotic groups. There were no significant differences in the occurrences reported among treatments.

DISCUSSION AND CONCLUSIONS

To our knowledge, there are no prior reports on the use of probiotics in crocodilian feeding, consequently, our starting point were the already established protocols used for studies in other animal groups, such as birds and fish. In such studies, the use of probiotics allows colonization of the gut by a different microbiota, improving the immune system and antimicrobial activity (GATESOUPE, 1999; HAI, 2015; SMITH, 2014). Improvements in weight gain (RAMOS et al., 2015), immunomodulation (BALCÁZAR et al., 2006) and hematological profile changes (SADEGHI et al., 2015) are correlated to the use of probiotics; however, the biological processes explaining the cause and consequence relations are still being investigated (HOU et al., 2015).

The prolonged use of probiotics did not change Ht values, which are within the expected range for *C. yacare* (BARBOZA et al., 2004; 2007; 2012; KOZA et al., 2012; VIEIRA et al., 2002). *C. yacare* are ectothermic, and the temperature of the environment interferes with sex determination (PINHEIRO et al., 2001), development (MIRANDA et al., 2002) and hematimetric indexes (BARBOZA et al., 2004), such as the Ht. Other conditions also provoke variations in reptiles Ht besides the temperature, such as blood viscosity (BARBOZA et al., 2004) and age group, but there is no significant difference when other parameters are taken into account, such as sexes and species, or if farmed versus wild caimans (BARBOZA et al., 2010; PADILLA et al., 2011). Caimans of the same age were used herein in order to minimize Ht physiological variations correlated to weight gain and aging (BARBOZA et al., 2007). Ht increase may result of stress caused by the confinement of caimans, which basal levels resume to normal levels after 8 hours (BARBOZA et al., 2007). The lowest Ht averages are found in ranching system animals (BARBOZA et al., 2007; 2011), that can be explained by the fact that these animals are used to constant handling, reducing confinement stress. We highlight that no *C. yacare* deaths were registered throughout the experiment. In addition, visual body score indicated the

maintenance of a healthy status in all caimans. This corroborate the hypothesis that there are healthy *C. yacare* with Ht lower than 20%, considering the minimum reference level for maintenance of the healthy status in reptiles (STACY et al., 2011). Hb results of the control and supplemented treatments are considered higher than the reference for *C. yacare* (KOZA et al., 2012), potentially correlated to feeding regularity, constant management and/or lower thermal fluctuation (VIEIRA et al., 2002) when compared to values for caimans described in the literature. This can be explained by the fact that the *C. yacare* sampled in this experiment are kept in ranching conditions at latitudes near the Equator, lower altitude and near the geodesic point of the continent, free of ocean air masses and, therefore, under more constant weather conditions. The higher Hb values increased MCH and MCHC values. MCH values described in the literature vary between warm and cold seasons (BARBOZA et al., 2010), and the values obtained in this study correspond to the values described for the warm season, corroborating our hypothesis regarding the latitude of the animals in the experiment. MCHC of *C. yacare* presented higher values compared to the values obtained in the Northeast region of Argentina (BARBOZA et al., 2004). The MCHC values obtained in this study were higher, as registered for *C. yacare* maintained in the ranching system (BARBOZA et al., 2007). Thus, the constant management may contributed to the increase of Hb and, as a consequence, of MCHC. The use of probiotics did not change the MCH, as observed in the *O. mykiss* fish (CRETU et al., 2013). The MCV measures the volume of red blood cells, indicating and classifying different types of anemia. The MCVs of farmed caimans were higher than those found in the literature (BARBOZA et al., 2010, 2011), which can be explained by the geographical location of the *C. yacare* in this experiment.

Lyophilized probiotics use the antioxidant butylated hydroxytoluene (BHT) as preserver (CASTRO, 2017). In mice, BHT at 3 g/kg may originate hepatocellular carcinomas, increases platelet count, Er, Ht and Hb and decreases leukocytes (COTTRELL et al., 1994). In *Rhesus* monkeys, 50 mg/kg concentrations during 2 years did not significantly influence Hb, Ht, and white and red blood cells (EFSA, 2012). In this study, the highest BHT concentration was of 10 mg/kg. Vitamin A is frequently added to probiotics as compensation for hepatic losses of vitamin caused by BHT (CASTRO, 2017). Experiments with methionine supplementation at 1.20% concentration of feed increased leukogram in mice (WEBB et al., 2003); in early-stage broiler chickens, methionine supplementation at concentrations from 0.19% to 0.64% increased white blood cells (ADEYEMO et al., 2010) and significantly increased hematimetric parameters (AL-MAYAH, 2006). In fish, lysine supplementation at concentrations from 0.69% to 3.08% of dry diet incremented weight, growth and hematology (WANG et al., 2005), stimulated growth and nutrient retention (BICUDO et al., 2009).

In this study, supplementation was lower than the aforesaid concentrations, thus, it is unlikely that methionine, lysine or the BHT led to the highlighted hematological results, such as Hb increase and granulocyte count decrease. When compared to mammal leukocytes, atypical leukocytes, such as azurophils and heterophils, stand out. Azurophils are small cells with unspecific azurophilic granules and, in crocodilians, they are considered monocyte morphological variations. Heterophils present acidophil, elliptical granulation, with similar role to neutrophils (NARDINI et al., 2013; STACY et al., 2011; SYKES et al., 2008). The percentage

of monocytes obtained corresponds to the values established for *C. yacare* (BARBOZA et al., 2010). Probiotics may increase the number of *Oreochromis niloticus* monocytes (NAKANDAKARE et al., 2013), which has not been investigated in this study. The percentage of lymphocytes is in accordance with the literature available, as for *C. yacare* in Argentina. Lymphocyte concentrations vary greatly, reaching up to 33% in wild caimans (FUENTES et al., 2011). The year's season and age increase may reduce the concentration of lymphocytes in *C. yacare* (BARBOZA et al., 2010; 2012). The use of probiotics may act as immunostimulant, increasing the number of lymphocytes (GATESOUPE, 1999), which may take place in response to the colonization of the digestive tube, increasing the number of lymphocytes and other leukocytes (TEVES et al., 2012). However, lymphocyte concentration did not differ significantly between treatments.

Eosinophils in mammals are responsible for the defense against parasites, but information on this matter in reptiles is scarce (ZIMMERMAN et al., 2010). A diet based on bone meal, crude protein, ethereal extract, calcium and phosphorus may increase the number of eosinophils (BARBOZA et al., 2011); however, it did not significantly influence the percentage of eosinophils in *R. catesbeiana* frogs (FRANÇA et al., 2008). In reptiles we find eosinophil count in the range between 7 and 20% active against *Staphylococcus aureus* and protozoans (STACY et al., 2011). The eosinophil concentration found was higher than the values reported in the literature for C. yacare from Northeast of Argentina kept in ranching conditions (BARBOZA et al., 2010). Higher results of eosinophils in the control group may be explained by the positive correlation with temperature or the persistence of some preexistent challenge as parasitoses. The reduction of eosinophils in the peripheral blood of the treated caimans may be related to decrease or preexistent gut parasitoses by probiotics, thus, presenting less haematopoiesis. Maintaining the concentration of basophils in all treatments weakens the hypothesis of an allergic condition or chronic inflammation. It has been reported that the number of basophils in *R. catesbeiana* did not change with the use of probiotics (FRANÇA et al., 2008).

In the literature, the percentage of heterophils is not consensual, and there is great discrepancy between the values presented for crocodilians (BARBOZA et al., 2010). Some species of crocodilians present higher frequency of heterophils instead of lymphocytes (FUENTES et al., 2011) and vice versa (BARBOZA et al., 2010). Heterophils are the first cells to reach the infection (LATORRE et al., 2012). The concentration of heterophils observed is not indicative of the existence of acute infection, since our values are low compared to the literature (HARIKRISHNAN et al., 2011). The decrease of eosinophils and heterophils concentration inversely proportional to probiotic concentration may be related to problems in haematopoiesis of granulocytes caused by exhaustion of physiological condition after probiotic overstimulation.

In *O. mykiss*, immunostimulation was observed with feed supplementation with 0.22% of probiotics (CRETU et al., 2013). In caimans, the greatest activity of the alternative pathway of the complement system happened at concentration of 0.25% of probiotics, concomitantly to the maintenance of the values of the other parameters compared to the control, corroborating the hypothesis that the 0.25% concentration is the most appropriate. Doses higher than 0.25% may have caused physiological disturbances such as reduction in weight gain in caimans (data to be published) and disrupting the haematopoiesis of granulocytes.

There was an attempt to minimize the frequency of *Salmonella* spp in the digestive tube of *C. yacare* with the use of probiotics, but the extended use was no effective to reduce the number of animals colonized by *Salmonella* spp, under these study conditions. Further studies are required to verify whether there are quantitative differences in bacterial cell counts in the digestive tract of probiotic-treated and untreated animals, establishing more effective and safe protocols for *C. yacare*.

In this study, we addressed a prolonged use of probiotics for a period of 18 months. This constant use may have different effects compared to the use for short periods, that is, part of the effects in the hematological and immunological parameters may be due to the energy depletion and saturation of the physiological phenomena, such as innate immunity. Considering the values presented in this study in 0.25% supplementation with probiotics, it is possible to assert that this dosage is safe even after an 18-month chronic treatment. At this concentration, we verified the maintenance of the hematological parameter with an increase of activity in the complement system. Finally, the use of probiotics at 0.25% concentration may constitute an interesting management strategy to be tested on the basis of its immunostimulation, even though the mixture is not sufficient to prevent shedding of *Salmonella* spp at the cloaca.

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