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Impairment of the phosphotransfer network and performance in broiler chickens experimentally infected by *Eimeria* spp.: The role of the oxidative stress

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ABSTRACT

The aim of this study was to evaluate whether infection *Eimeria* spp. in broiler chickens could negatively affect seric enzymes linked to adenosine triphosphate (ATP) metabolism and its relationship to oxidative stress. For this, 30 broiler chickens, 27 days-old, were divided into two groups (n = 15): the control group (C) and the group infected by *Eimeria* spp. (I). On days 1, 7 and 15 of the experiment, the animals were weighed, and fecal and blood samples were collected to evaluate the presence of oocysts and for serum biochemistry and enzymatic parameters, respectively. On day 15, one animal per repetition was submitted to euthanasia and intestinal fragments were collected for histopathological analyses. The body weight was lower in infected animals on day 15 of experiment, while oocyst counts were higher in infected animals on days 7 and 15 of the experiment. Serum levels of globulins were lower in infected animals on days 7 and 15 of experiment, while uric acid levels were higher in the same days, which represent changes on the immune system. Compared to the uninfected animals, on days 7 and 15, levels of serum globulins, triglycerides, creatine kinase and cholesterol were lower. Levels of adenylate kinase and reactive oxygen species (ROS) were higher on both days in infected animals, while levels of thiobarbituric acid-reactive substances (TBARS) were elevated on day 15. Lesions and immature forms of the parasite were observed in the intestines of infected birds. The phosphotransfer network elicited by an oxidative stress negatively affected the performance of broiler chickens with coccidiosis.

1. Introduction

Coccidiosis is caused by protozoan parasites belonging to the genus *Eimeria*, and it is considered one of the most important disease of poultry [1] associated with enteritis, severe diarrhea and consequently, inhibition of intestinal nutrient absorption [2]. The reproductive cycle of this parasite is fast, i.e., between 4 and 7 days post-infection the parasite modifies the structure and appearance of the intestinal villi causing high cell death, contributing to disease pathogenesis [3]. Moreover, infections caused by this coccidian are related to the occurrence of other infections, since the destruction of the intestinal wall is an entry for other opportunistic infectious agents [4].

Pathogenesis of eimeriosis in broiler chickens is associated with impaired animal performance, but the pathways involved in this effect remain poorly understood. In this sense, the measurement of phosphoryltransfer network, which is catalyzed by creatine kinase (CK), adenylate kinase (AK) and pyruvate kinase (PK), provides new perspectives for understanding the alterations in energy metabolism due to diseases [5]. This occurs because these enzymes maintain the homeostasis between release and uptake of energy by transfering phosphoryl groups between the sites of synthesis and utilization of adenosine triphosphate (ATP), contributing to bioenergetic homeostasis [6]. CK is considered a central controller of the energy homeostasis through reversible catalysis of a phosphoryl group from ATP to adenosine diphosphate (ADP) and creatine to produce phosphocreatine (PCr), exerting a putative role on cell division and cell motility [7]. PK is a key

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enzyme of the glycolysis pathway, the main route that provides energy for proper tissue functioning, that catalyzes the irreversible transphosphorylation of phosphoenolpyruvate (PEP) to ADP to form pyruvate and ATP [8]. Finally, AK catalyzes the reversible transfer of the γ-phosphate group from a phosphate donor (normally ATP) to AMP, releasing two molecules of ADP, i.e., molecules involved in the processing of metabolic signals associated with cellular energy utilization [9]. Recently, a study conducted by Baldissera et al. [10] demonstrated that parasitic infections, such as those caused by the ciliate protozoan *Ichthyophthirius multifiliis*, impair the enzymes of the phosphotransfer network of naturally infected freshwater silver catfish *Rhamdia quelen*, causing a severe impairment of bioenergetics and contributing directly to disease pathogenesis. Also, infections caused by *Trypanosoma evansi* were already linked to cerebral, hepatic and cardiac phosphotransfer network impairment, which are contributing factors for disease pathogenesis and the appearance of clinical signs of the disease [11].

It is important to emphasize that the impairment of enzymes belonging to the phosphotransfer network can be directly linked to the occurrence of oxidative damage, mainly by reaction oxygen species (ROS) effect [12]. According to Venkataraman et al. [13], CK activity is highly susceptible to inactivation by oxidative reactions and oxidative damage, being a pathway involved in the impairment of bioenergetics dysfunction. Thus, our hypothesis is that losses on the chicken production chain caused by coccidia can be linked to oxidative stress, as well as by interferences in ATP synthesis. Therefore, the aim of this study was to evaluate whether infections caused by *Eimeria* spp. in broiler chicken could negatively affect seric enzymes linked to ATP metabolism and its relationship with oxidative stress.

2. Material and methods

2.1. Animals and experimental design

Thirty male broilers, 27 days-old, were maintained in cages in order to avoid coccidia infections, since it is a common infection in animals raised using litter. The animals were randomly divided into two groups (control (C) and infected (I)) with five repetitions each and 3 animals per repetition in a completely randomized design, i.e., each cage corresponded to one repetition. The group I was formed by animals experimentally infected by *Eimeria* spp. (BIOVET strains; moderate virulence) at the dose of 35.000 sporulated oocysts per animal (*E. mitis* = 10.000; *E. acervulina* = 8.000, *E. praecox* = 8.000; *E. tenella* = 5.000; and *E. maxima* = 4.000), while the group C was used as negative control group (uninfected animals). The experiment lasted 15 days, and body weight was verified on days 0, 7 and 15 of the experiment using a digital balance.

The diet was formulated based on corn and soybean meal, providing all nutritional requirements for broiler chickens, as described in the Brazilian tables for poultry and pigs [14], being formulated equally for both groups. Food and water was provided ad libitum during the experiment. It is important to emphasize that anticoccidial or antimicrobial drugs were not added in the diet.

2.2. Sampling

Collection of blood and feces was performed on days 0, 7 and 15 of the experiment. Fecal samples were collected together (one sample per cage), allocated in plastic tubes and refrigerated until analysed. Total blood was collected by venipuncture of the ulnar vein from one animal per repetition (randomly selected) in tubes without anticoagulant to obtain serum after centrifugation (3500 rpm, 10 min). The serum samples were stored at −20 °C until analysis. On day 15 of the experiment, one animal per repetition was submitted to euthanasia for collection of intestine (duodenum, jejune and cecum) to perform histopathological analyses.

2.3. Parasitological analyses

Fecal samples were collected from each cage on days 0, 7 and 15 to count the number of *Eimeria* spp. oocysts per gram of feces (OOPG) using the centrifugal-flotation technique [15]. The feces (1 g) was dissolved in 15 mL of sucrose solution and centrifuged during 5 min at 2000 rpm. For fecal examination, a light microscope was used (100×).

2.4. Serum biochemistry

Serum levels of triglycerides, cholesterol, uric acid, total protein, glucose and albumin were evaluated using a semi-automated analyzer BioPlus (Bio-2000) and commercial kits (Analisa®). Serum globulin levels were calculated using the mathematic formula: total protein – albumin.

2.5. Serum CK, AK and PK activities

CK activity was assayed in the reaction mixture containing the following final concentrations: 65 mM Tris-HCl buffer, pH 7.5, 7 mM PCr, 9 mM $MgSO_4$ and 20 μL of sample. After 10 min of pre-incubation at 37 °C, the reaction was started by the addition of 0.3 μmol of ADP, and stopped after 10 min by the addition of 1 μmol of ρ-hydroxymercuribenzoic acid. The creatine level was estimated according to the colorimetric method of Hughes [16]. The color was developed by the addition of 0.1 mL of 2% α-naphthol and 0.1 mL of 0.05% diacetyl in a final volume of 1 mL, and read at 540 nm after 20 min. Results were expressed as U/L.

AK activity was measured with a coupled enzymatic assay using hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD), according to Dzeja et al. [17]. The reaction mixture contained 100 mM of KCl, 20 mM of HEPES, 20 mM of glucose, 4 mM of $MgCl_2$, 2 mM of $NADP^+$, 1 mM of EDTA, 4.5 U mL^{-1} of HK, 2 U mL^{-1} of G6PD, and 20 μL of sample. The reaction was initiated by the addition of 2 mM ADP, and the reduction of $NADP^+$ was evaluated at 340 nm for 3 min in a spectrophotometer. The results were expressed as nmol of ATP formed per min per mg of protein.

PK activity was assayed as described by Leong et al. [18]. The incubation medium consisted of 0.1 M of Tris/HCl buffer, pH 7.5, 10 mM of $MgCl_2$, 0.16 mM of NADH, 75 mM of KCl, 5.0 mM of ADP, 7 U of L-lactate dehydrogenase, 0.1% (v/v) of Triton X-100 and 20 μL of the sample in a final volume of 500 μL. After 10 min of pre-incubation at 37 °C, the reaction was started with the addition of 1 mM PEP. Results were expressed as nmol of pyruvate formed per min per mg of protein.

2.6. Free radicals and lipid peroxidation

Reactive oxygen species (ROS) levels were determined by the DCFH oxidation method as described by Ali et al. [19], recently published in detail by Biazus et al. [20]. Fluorescence was measured using excitation and emission wavelengths of 485 and 538 nm, respectively. A calibration curve was established with standards of 2′,7′-dichlorofluorescein (DCF) (0.1 nm to 1 μm), and results were expressed as U DCF/mg of protein.

As an index of lipid peroxidation, thiobarbituric acid reactive substances (TBARS) formation during an acid-heating reaction was determined, as described by Ohkawa et al. [21]. Malondialdehyde (MDA) solution was used as a reference standard. TBARS levels were determined by the absorbance at 532 nm and were expressed as MDA equivalent (nmol MDA/mg of protein).

2.7. Histopathology

Fragments of the intestines (duodenum, jejune and cecum) of five animals per group (one per repetition) were collected and stored in 10% formaldehyde solution. Intestinal fragments (2 to 5 cm long and 0.5 to

1 cm of thick) were placed in paraffin blocks followed by hematoxylin-eosin (HE) staining and microscopic examination by pathologists.

2.8. Statistical analysis

The data set was tested for normality using the Shapiro-Wilk test, and when it did not show normal distribution (number of oocysts, cholesterol, triglycerides and glucose), it was transformed to logarithm, in order to obtain a normal distribution. Thereafter, all data were submitted to two-way analysis of variance (ANOVA), followed by Tukey post-hoc test considering $P < .05$.

3. Results

3.1. Body weight and parasite counts

Body weight of chickens at days 0, 7 and 15 post-infection was 1630, 2000 and 2590 g (control animals) and 1590, 1730 and 1910 g (infected group animals), respectively. As expected, it was possible to identify a significant impact on body weight in both groups over time, since we used broiler chickens with 27 days of age. Infected animals showed lower body weight when compared to the control group on day 15 of the experiment ($P < .05$).

Animals of the control group did not have oocysts in their fecal samples on days 1, 7 and 15 of the experiment. On the other hand, infected animals showed higher number of oocysts on days 0 (0 ± 0), 7 (499.2 ± 241) and 15 (2188 ± 987), respectively.

3.2. Serum biochemistry

Serum levels of total protein, albumin and glucose did not differ between groups at all evaluated time points. Serum levels of globulin were lower in infected animals on days 7 and 15 of the experiment compared to the control group, while serum levels of uric acid were higher at the same moments (Fig. 1). Serum triglycerides on day 7 and cholesterol on days 7 and 15 were lower in infected animals compared to the control group (Fig. 2).

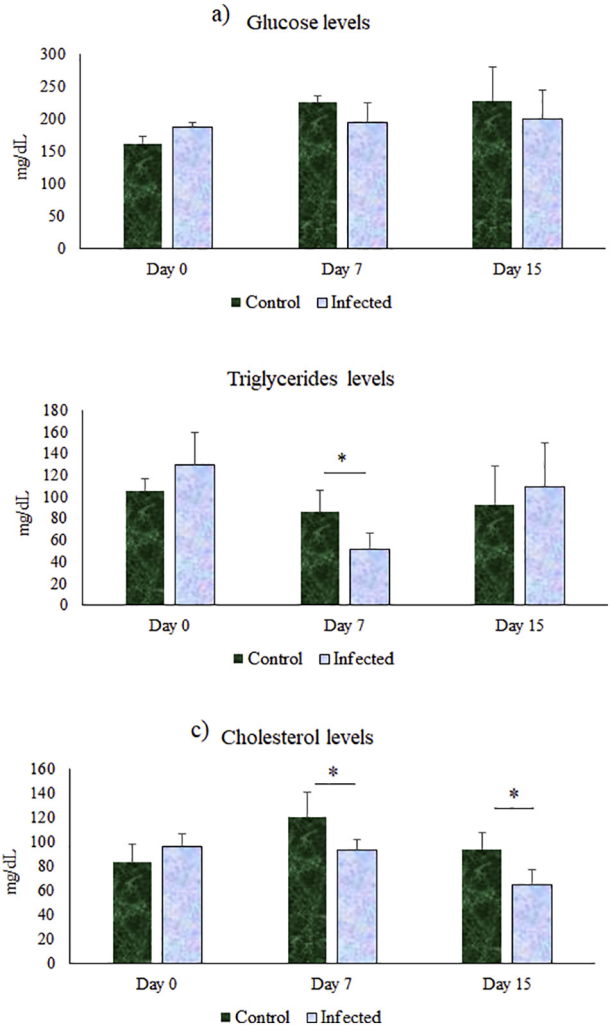

Fig. 2. Serum levels of glucose, triglycerides and cholesterol on days 0, 7 and 15 of the experiment. *Indicate significant difference between groups for $P < .05$.

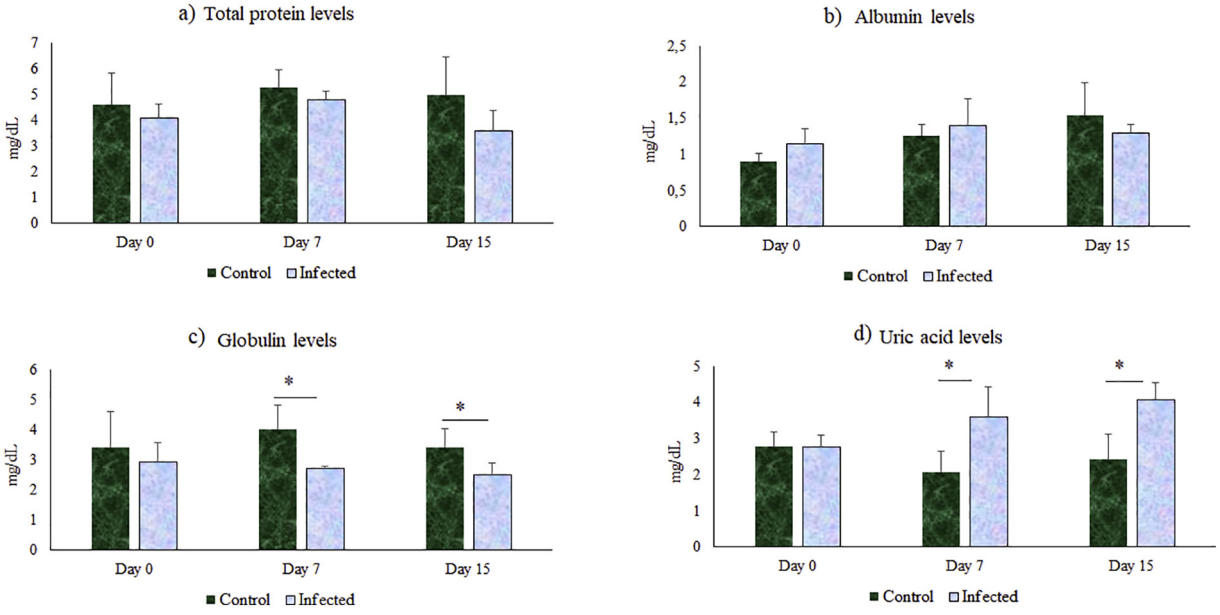

Fig. 1. Serum levels of total protein, albumin, globulin and uric acid on days 0, 7 and 15 of the experiment. *Indicate significant difference between groups for $P < .05$.

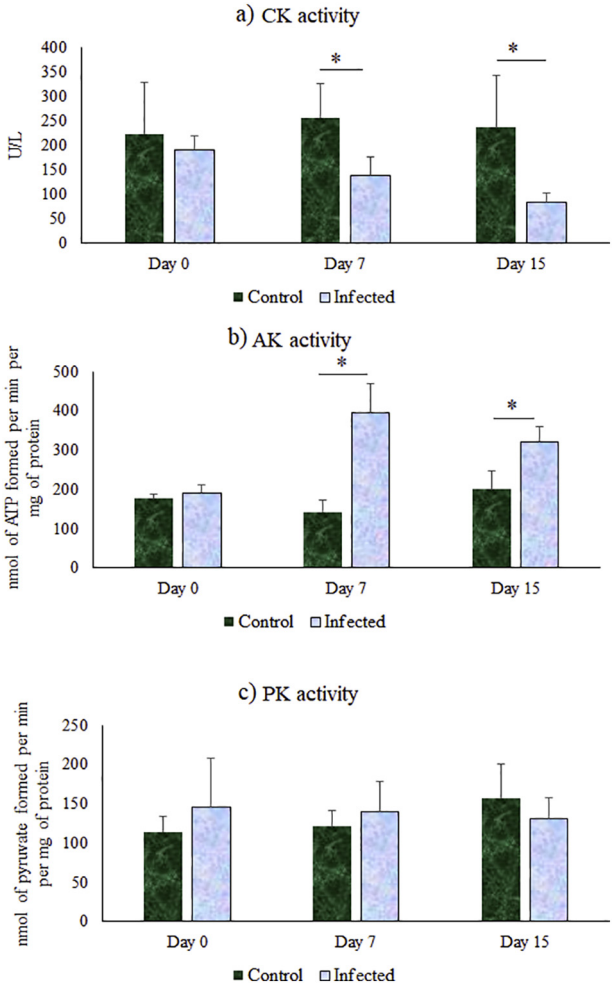

Fig. 3. Serum creatine kinase (CK), adenylate kinase (AK) and pyruvate kinase (PK) activities on days 0, 7 and 15 of the experiment. *Indicate significant difference between groups, considering $P < .05$.

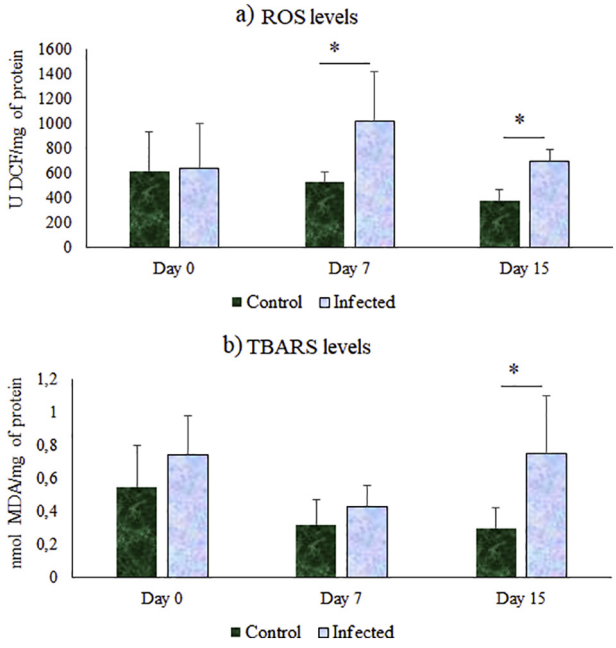

Fig. 4. Serum levels of reactive oxygen species (ROS) and thiobarbituric reactive acid substances (TBARS) on days 0, 7 and 15 of the experiment. *Indicate significant difference between groups for $P < .05$.

3.3. Serum enzymes of phosphotransfer network

Serum CK activity was lower in infected animals on days 7 and 15 of the experiment compared to the control group, while AK activity was higher the same moment of the experiment. No difference was observed between groups regarding PK activity (Fig. 3).

3.4. Serum ROS and TBARS levels

Serum levels of ROS (days 7 and 15 of the experiment) and TBARS (only on day 15) were higher in infected animals compared to the control group (Fig. 4).

3.5. Histopathology

No intestinal lesions were observed in animals of the control group. On the other hand, microscopic lesions were observed in the intestinal wall of infected animals such as micro and macrogametes, schizonts and oocysts comparable to those of *Eimeria* spp. (Figs. 5, 6 and 7).

4. Discussion

As expected, the infection of broiler chickens caused by coccidia led to significantly lower body weight, in agreement to Wang et al. [22] while studying broilers chickens 9 days post-infection (PI) by *E. tenella*. According to Morris et al. [23] and Pirali et al. [24], coccidiosis damages the intestinal tissue, reduces the absorption of nutrients, and consequently, it reduces animal performance, feed intake, mean body weight and mean body weight gain. Intestinal histopathology (duodenum, jejunum and cecum) revealed the presence of micro and macrogametes, schizonts and oocysts consistent with those of *Eimeria* spp., in agreement with Wang et al. [22] while observing damage in the integrity of the cecal mucosa and thickening of the tunica muscular, that directly contributed to the impairment of broiler chicken's performance. In this sense, we highlight that intestinal alterations caused by the presence of *Eimeria* spp. structures contributed to reduce body weight gain of broiler chickens.

In order to obtain more details regarding the effects of eimeriosis in broiler chickens, we evaluated some serum biochemical parameters linked to immunity and metabolism. A significant reduction in serum globulin levels can be considered an impairment of the immune and inflammatory responses, since globulins exert an important role in the recognition of a broad spectrum of specific antigens, playing a major role in the humoral immune response [25]. In agreement with our observation, Dar et al. [26] demonstrated that *E. tenella* infection reduces serum globulin levels in broiler chickens after 7, 14, 21 and 28 days of infection, contributing directly to the impairment of the immune response against infection. On the other hand, increased seric levels of uric acid also can be an indicative of impaired immune responses, in agreement with Koynarski et al. [27] while studying the plasma of broiler chickens naturally infected by *Eimeria* spp. 4 days PI., inducing a high release of pro-inflammatory and pro-oxidative mediators, such as ROS and nitric oxide (NO), as illustrated by Baldissera et al. [28]. In summary, these alterations can be an indicative of impairment of immune response during eimeriosis, which may contribute to disease pathogenesis and mortality. Regarding effects on parameters linked to metabolism, no difference was observed regarding serum glucose levels, different to the observations of Mondal et al. [29] who observed an increase in plasma glucose levels of broiler chickens experimentally infected with *E. tenella* (250,000 sporulated oocysts) 5, 7 and 9 days PI. According to these authors, augmentation of plasma glucose levels may occur due to increased glycogenolysis caused by stress induced release of adreno-corticoid, leading to hyperglycemia or disturbed carbohydrate metabolism. Mondal et al. [29] used highly

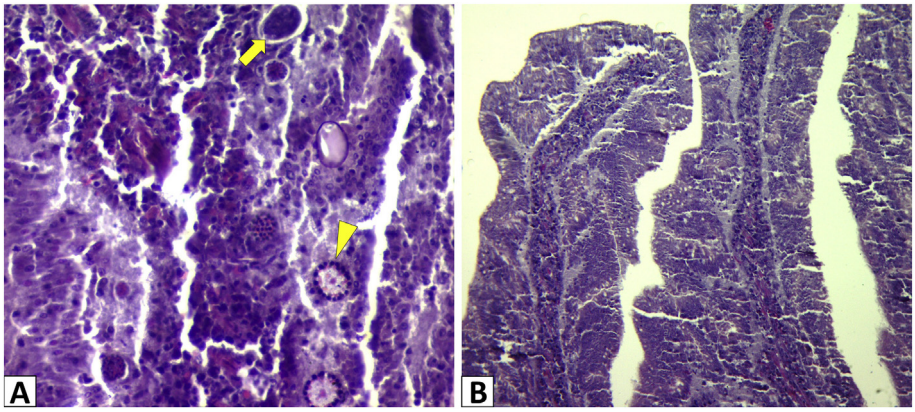

Fig. 5. Duodenum of broiler chickens with coccidiosis. A: Micro (arrow) and macrogametes (arrowhead) of *Eimeria* spp. (morphology compatible with *E. acervulina*) in the enterocytes of the duodenal villi (HE 40×). B: Duodenum without changes of an uninfected chicken (HE 20×).

pathogenic species responsible for hemorrhagic diarrhea, as well as a dose seven times higher than our study (35,000 oocysts), which also used multi-species infection, of which only 17,000 oocysts of pathogenic species, i.e., *E. tenella*, *E. acervulina* and *E. maxima*. Alternately, *E. acervulina* infections caused a reduction in serum glucose levels of broiler chickens, which occurs due to the inhibition of liver glycogenolysis [30]. Thus, effects on the glucose metabolism can be associated with species of coccidian as well as the infecting dose.

Coccidia infection alters lipid metabolism as illustrated by the decrease of serum triglycerides and cholesterol levels, in agreement with what was observed in broiler chickens infected by *E. tenella* and *E. acervulina* [29,30]. According to Mondal et al. [29], anorexia and malnutrition are considered major reasons for reduced triglyceride levels in birds, while liver and intestinal damage explain the reduction of serum cholesterol levels, since these tissues exert important roles in cholesterol synthesis [31]. Summary, our findings corroborate the data already reported in the literature linked to alterations in carbohydrate and lipid metabolism elicited by eimeriosis.

In order to elucidate the pathways involved in the impairment of animal performance and alterations in carbohydrate and lipid metabolism, we decided to evaluate the enzymes of the phosphotransfer network, a system linked to ATP synthesis and utilization. The phosphotransfer network was evaluated since a study conducted by Freitas et al. [32] demonstrated that *E. acervulina* infection causes mild and severe hypoxia, resulting in a reduction in the ATP content. A significant reduction in serum CK activity was observed in infected animals, as observed by Fukata et al. [33] in the plasma of broiler chickens infected by *E. tenella* 7 and 11 days PI. The inhibition of CK activity indicates an impairment in the synthesis of creatine phosphate (PCr) for rapid temporal and spatial buffering of ATP levels, resulting in impaired communication between sites of energy production and energy consumption, as observed by Baldissera et al. [10] in fish naturally infected by the ciliate protozoan *I. multifiliis*. It is worth mentioning that the inhibition of CK activity also indicates an impairment of the bioenergetic homeostasis between ATP use and synthesis, i.e., indicates disequilibrium in ATP/ADP and PCr/Cr ratios, which may result in decreased availability of ATP and impairment of energy supply, in agreement with Freitas et al. [32] which observed a reduction in serum ATP content. On the contrary, serum AK activity was augmented by eimeriosis infection, which may be considered an attempt to improve or prevent the imbalance of energy homeostasis. This fact corroborates with the interaction between AK and CK enzymes, as proposed by Dzeja and Terzic [34]. In this sense, the existence of a reciprocal compensatory relationship between AK and CK phosphotransfer safeguards cellular energy economy and contributes to improved energy metabolism. Thus, a reduction in CK activity promotes high-energy phosphoryl transfer through the AK system [35], as recently observed in the brains of rats experimentally infected by *Trypanosoma evansi* [11]. In summary, the augmentation of serum AK activity may be considered a response to the inhibition of CK activity, aiming to maintain the homeostasis between cellular ATP utilization and synthesis. In order to explain some possible mechanisms involved on impairment of phosphotransfer network, we decided to evaluate parameters linked to oxidative stress, since these enzymes are highly susceptible to inhibition by ROS [12]. As expected, infected experimentally animals with *Eimeria* spp. showed significant augmentation of serum ROS levels and lipid peroxidation, in concordance to Galli et al. [36] using birds naturally infected by *E. necatrix*, *E. acervulina* and *E. maxima*. In this sense, Glaser

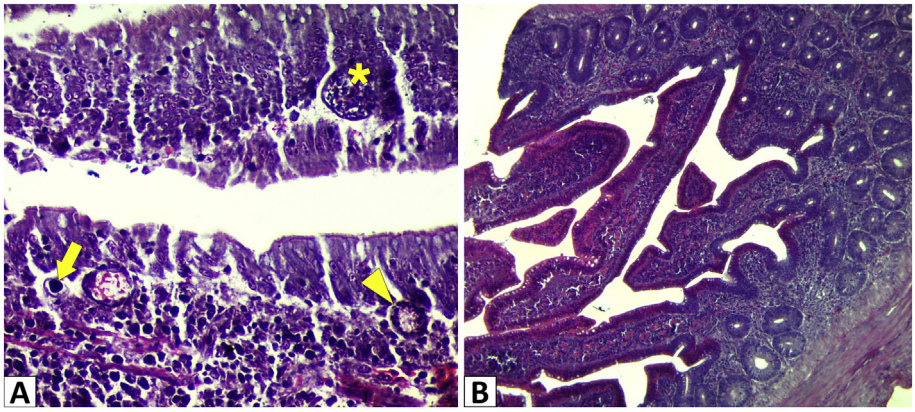

Fig. 6. Jejunum of broiler chickens with coccidiosis. A: Schizonts (arrow), microgametes (asterisks) and macrogametes (arrowhead) of *Eimeria* spp. (morphology compatible with *E. maxima*) in the jejunal villous enterocytes (HE 40×). B: Jejunum without changes of an uninfected chicken (HE 20×).

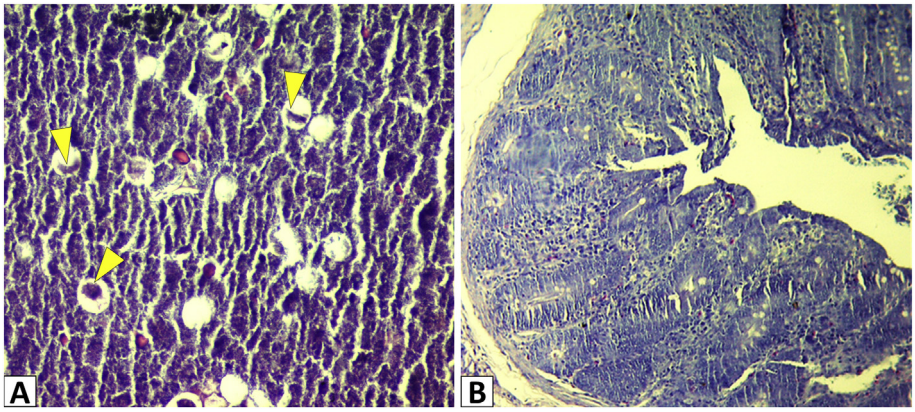

Fig. 7. Cecum of broiler chickens with coccidiosis. A: Large amount of oocysts (arrow heads) of *Eimeria* spp. (morphology compatible with *E. tenella*) in the cecal lumen (HE 40×). B: Cecum without changes of an uninfected chicken (HE 20×).

et al. [12] demonstrated that oxidative damage of lipids and excessive ROS formation are directly associated with the downregulation of CK activity during exposure to methylmercury, as observed in this present study. In summary, lipid oxidative damage elicited by excessive formation of free radicals can be a pathway involved in the inhibition of serum CK activity during eimeriosis.

Based on this evidence, eimeriosis caused alterations in carbohydrate and lipid metabolism which can contribute to the impairment of animal performance. Moreover, alterations in serum enzymes linked to ATP synthesis and utilization, mainly CK, can be a pathway involved in the reduction of body weight. In summary, the impairment of the phosphotransfer network elicited by oxidative stress negatively affects the performance of broiler chickens with eimeriosis.

Ethical note

The methodology used in this experiment was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria under protocol number 3096260917.

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