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Inflammatory responses, energy metabolism enzymes, oxidative status in *Clostridium perfringens* infection in broilers

Respostas inflamatórias, enzimas do metabolismo energético, status oxidativo na infecção por *Clostridium perfringens* em frangos de corte

Respuestas inflamatorias, enzimas del metabolismo energético, estado oxidativo en la infección por *Clostridium perfringens* en pollos de engorde

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Abstract

The aim of this study was to determine whether infection by *Clostridium perfringens* negatively interferes with the oxidant/antioxidant status and activity of energy metabolism enzymes (creatine kinase (CK), adenylate kinase (AK) and pyruvate kinase (PK)), as well as the zootechnical performance of broilers. A completely randomized design with three treatments, with five replications by treatment, and 10 birds by repetitions was used: T1: non-infected group; T2: group infected with *C. perfringens*; T3: group infected with *C. perfringens*, and basal diet with performance enhancers (antibiotic and coccidiostatic). At 21 days of age, the birds were experimentally infected orally with 4.0 x 10^8 CFU/mL *C. perfringens*. At strategic moments during the experimental period, zootechnical data and blood samples were collected. There were no significant differences among treatments in terms of weight gain, feed intake, or feed conversion. At 20 days, heterophils counts were significantly lower in the T3 group birds than in the others. A significant reduction in the number of total leukocytes, because of the lower number of lymphocytes, was observed in groups T2 and T3 on day 34. Neutrophil and monocyte counts were significantly lower in group T3 than in group T1 on day 34. On day 42, there were significantly lower levels of total protein and globulin in birds in T3 than in T1. On day 27, PK activity was significantly higher in groups T2 and T3; similar behavior observed on day 34 when CK and PK activities in T2 and T3 were significantly higher than those of T1. AK activity was significantly higher in the serum of T3 birds than in those of the other groups at 34 days. At 41 days, CK and PK activities were significantly higher only in birds in group T3 compared to other groups. The levels of reactive oxygen species and lipoperoxidation, as well as the activity of glutathione S-transferase, were significantly lower in birds from group T3 compared to other groups at age 20 days. After a challenge with *C. perfringens*, there were fluctuations in the behavior of oxidative stress biomarkers; in particular, at 41 days of age, birds in T2 had elevated levels of lipoperoxidation, different from what was seen in birds in T3 that consumed feed with a performance enhancer. Preliminary results suggest that clostridiosis affects serum activity of enzymes in the phosphotransfer network, requiring compensatory enzymatic changes in an attempt to maintain energy homeostasis. In addition, the infection reduces the number of inflammatory cells and causes oxidant/antioxidant imbalance that may contribute to the pathophysiology of the disease. Además, la infección reduce el número de células inflamatorias y provoca un desequilibrio en el estado oxidante / antioxidante que puede contribuir a la fisiopatología de la enfermedad.

Keywords: Antioxidant; ATP; Birds; Clostridiosis; Pathogenesis.

Resumo

O objetivo deste estudo foi determinar se a infecção por *Clostridium perfringens* interfere negativamente no status oxidante / antioxidante e na atividade das enzimas do metabolismo energético (creatina quinase (CK), adenilato quinase (AK) e piruvato quinase (PK)), bem como no desempenho zootécnico de frangos de corte. O delineamento experimental foi inteiramente casualizado com três tratamentos, com cinco repetições por tratamento e 10 aves por repetições: T1: grupo não infectado; T2: grupo infectado com *C. perfringens*; T3: grupo infectado com *C. perfringens* e dieta basal com promotores de crescimento (antibiótico e coccidiostático). Aos 21 dias de idade, as aves foram infectadas experimentalmente por via oral com 4,0 x 10^8 UFC/mL de *C. perfringens*. Em momentos estratégicos do período experimental, foram coletados dados zootécnicos e amostras de sangue. Não houve diferenças significativas entre os tratamentos em termos de ganho de peso, consumo de ração e conversão alimentar. Aos 20 dias, as contagens de heterófilos foram significativamente menores nas aves do grupo T3 do que nas outras. Foi observada uma redução significativa no número de leucócitos totais, devido ao menor número de linfócitos nos grupos T2 e T3 no dia 34. As contagens de neutrófilos e monócitos foram significativamente menores no grupo T3 do que no grupo T1 no dia 34. No dia 42, houve níveis significativamente menores de proteína total e globulina nas aves do T3 em relação ao T1. No dia 27, a atividade da PK foi significativamente maior nos grupos T2 e T3; comportamento semelhante observado no dia 34 quando as atividades de CK e PK em T2 e T3 foram significativamente maiores em relação ao T1. A atividade de AK foi significativamente maior no soro das aves do T3 em comparação aos outros grupos aos 34 dias. Aos 41 dias, as atividades de CK e PK foram significativamente maiores apenas nas aves do grupo T3 em comparação com os outros grupos. Os níveis de espécies reativas ao oxigênio e lipoperoxidação, bem como a atividade da glutationa S-transferase, foram significativamente menores nas aves do grupo T3 em relação com os outros grupos aos 20 dias de idade. Após um desafio com *C. perfringens*, houve flutuações no comportamento dos biomarcadores de estresse oxidativo; em particular, aos 41 dias de idade, as aves do T2 apresentaram níveis elevados de lipoperoxidação, diferente do que foi observado em aves do T3 que consumiram ração com promotores de crescimento. Estes resultados preliminares sugerem que a clostridiose afeta a atividade sérica de enzimas da rede de fosfotransferência, o que exige alterações enzimáticas compensatórias na tentativa de manter a homeostase energética. Além disso, a infecção reduz o número de células inflamatórias e causa desequilíbrio no status oxidante/antioxidante que pode contribuir para a fisiopatologia da doença.

Palavras-chave: Antioxidante; ATP; Aves; Clostridiose; Patogênese.

Resumen

El objetivo de este estudio fue determinar si la infección por *Clostridium perfringens* interfiere negativamente con el estado oxidante / antioxidante y la actividad de las enzimas del metabolismo energético (creatina quinasa (CK), adenilato quinasa (AK) y piruvato quinasa (PK)), así como comportamiento zootécnico de pollos de engorde. O delineamento experimental foi inteiramente casualizado com três tratamentos, com cinco repetições por tratamento e 10 pollos por repetições: T1: grupo no infectado; T2: grupo infectado por *C. perfringens*; T3: grupo infectado por *C. perfringens* y dieta basal con promotores del crecimiento (antibiótico y coccidiostático). A los 21 días de edad, las aves fueron infectadas experimentalmente por vía oral con 4.0 x 10^8 UFC/mL de *C. perfringens*. En momentos estratégicos del período experimental, fueron recolectaron datos zootécnicos y muestras de sangre. No hubo diferencias significativas entre los tratamientos en términos de aumento de peso, consumo de alimento y conversión alimenticia. A los 20 días, los recuentos de heterófilos fueron significativamente más bajos en las pollos del grupo T3 que en las otras pollos. Se observó una reducción significativa en el número de leucocitos totales, debido al menor número de linfocitos, en los grupos T2 y T3 el día 34. Los recuentos de neutrófilos y monocitos fueron significativamente más bajos en el grupo T3 que en el grupo T1 el día 34. El día 42, hubo niveles significativamente más bajos de proteína total y globulina en pollos de T3 en comparación con T1. El día 27, la actividad PK fue significativamente mayor en los grupos T2 y T3; comportamiento similar observado en el día 34 cuando las actividades de CK y PK en T2 y T3 fueron significativamente mayores en relación con T1. La actividad de AK fue significativamente mayor en el suero de pollos de engorde T3 en comparación con los otros grupos a los 34 días. A los 41 días, las actividades de CK y PK fueron significativamente más altas sólo en las aves del grupo T3 en comparación con los otros grupos. Los niveles de especies reactivas al oxígeno y la lipoperoxidación, así como la actividad de la glutatión S-transferasa, fueron significativamente menores en los pollos del grupo T3 en comparación con otros grupos a los 20 días de edad. Después de un desafío con *C. perfringens*, hubo fluctuaciones en el comportamiento de los biomarcadores de estrés oxidativo; en particular, a los 41 días de edad, los pollos de T2 mostraron altos niveles de lipoperoxidación, diferente a lo observado en pollos de T3 que consumieron alimento con promotores de crecimiento. Estos resultados preliminares sugieren que la clostridiosis afecta

la actividad sérica de las enzimas en la red de fosfotransferencia, lo que requiere cambios enzimáticos compensatorios en un intento por mantener la homeostasis energética.

Palabras clave: Antioxidante; ATP; Clostridiosis; Patogénesis; Pollos.

1. Introduction

Clostridium perfringens is a gram-positive anaerobic spore-forming bacterium (Alnoman et al., 2017) that produces toxins and enzymes. It is one of the causative agents of necrotic enteritis. Clostridiosis has been more frequent in countries where there are bans in place regarding the use of antibiotics as growth promoters (Khalique et al., 2020). Necrotic enteritis is the subclinical form of infection caused by *C. perfringens* "type A", which is responsible for producing an alpha toxin, as well as "type C", which produces the beta toxin (Van Immerseel et al., 2004). Other diseases such as coccidiosis and ingredients present in the feed that contain large amounts of non-starch polysaccharides are predisposing factors for infection in birds (Khalique et al., 2020). Clostridiosis causes productivity losses, in addition to significantly increasing treatment costs that can reach $6 billion per year (Yin et al., 2017). Researchers Lacey et al. (2018) observed that the pathogenic strains of *C. perfringens* interact with the intestinal microbiota, causing imbalances and affecting the zootechnical performance of birds. This interaction influences the pathogenicity and severity of the disease, contributing significantly to the death of the affected animals.

Little is known about the effects of clostridiosis on adenosine triphosphate (ATP) metabolism. Nevertheless, it is important to understand the mechanisms involved in this pathophysiology and its relationship with the enzymes involved in the phosphotransfer network, including creatine kinase (CK), adenylate kinase (AK) and pyruvate kinase (PK) (Galli et al., 2019). This is because CK controls energy metabolism by reversibly transferring the phosphoryl group from ATP to adenosine diphosphate (ADP), and creatine to produce phosphocreatine. In so doing, it acts as a buffer source and energy transporter in which it transports energy from mitochondria to the cytosol, playing an important role in regulating energy balance during bioenergetic deregulations (Schlattner et al., 2006). AK catalyzes the conversion of two molecules of ADP into one of ATP and an adenosine monophosphate (AMP), causing a doubling of the energetic potential, an effect that is fundamental for intracellular energy communication and for tissues with high energy demands (Morselli et al., 2019). Finally, PK is an irreversible enzyme of the glycolysis pathway that originates pyruvate and ATP, thereby participating in the production of energy for fabrics with high

energy demands (Wang et al., 2002). Recently, a study conducted by researchers Da Rosa et al. (2019) reported damage to the phosphotransfer network by reducing the serum activity of CK and PK directly contributes to the pathophysiology and mortality of laying hens experimentally infected with *Escherichia coli*. This finding led us to hypothesize that the phosphotransfer network may be involved in the pathogenesis of clostridiosis.

The activity of these enzymes help regulate energy regulation (ATP), as well as being variables that are susceptible to increases in free radical levels. The latter impairs the antioxidant system (Glaser et al., 2010). Oxidative stress is characterized by an imbalance between pro-oxidant and antioxidant systems, when there is an excess of free radicals, and depletion of the antioxidant system (Xing et al., 2012). This biochemical condition, often subclinical, contributes to tissue damage, which favors the pathogenesis of various disease. The hypothesis of this study is that clostridiosis causes oxidative stress that alters the activity of the phosphotransfer network enzymes in broilers. If this hypothesis is borne out, it will become very important to understand the pathophysiology of clostridiosis and how it affects the health and performance of birds. Therefore, the aim of the present study was to determine whether *C. perfringens* infection affects the oxidant/antioxidant status and enzyme activity mediating energy metabolism, as well as the zootechnical performance of broilers.

2. Materials and Methods

2.1. Setting and animals

A total of 180 1-day-old male Cobb chicks were reared over a period of 42 days, divided into three phases: initial (1–21 days), growth (22–35 days) and finishing (36–42 days). The birds were housed in an experimental house divided into 1.80-m2 boxes, with 20 birds allocated per box based on the average weight of the repetition, aiming at homogeneity among the birds in each box. We followed management guidelines indicated by the pedigree manual. Water and feed were provided ad libitum throughout the experiment. The basal diet was formulated based on the requirements presented in the Brazilian Tables for Poultry and Swine (Rostagno et al., 2017).

2.2. Experimental design

A completely randomized design with three treatments, with five replications by treatment, and 10 birds by repetitions was used (total 180 chicks). All groups received the same basal diet (BD). The treatments were divided into T1 – negative control; T2: positive control (infected with *C. perfringens*); T3: treatment group (infected with *C. perfringens*). The challenge with *C. perfringens* was performed at 21 days of experiment (groups T2 and T3), when each bird received a 1.0-mL inoculum orally corresponding to dose of 4.0 x 108 CFU/mL. BD (without growth promoter) was consumed by birds in T1 and T2; T3 birds consumed BD containing an antimicrobial (4.4 mg/kg lincomycin).

To confirm the infection, five samples of fresh chicken feces by repetition were collected on day 27 of the experiment, mixed to produce a pool used for isolation of C. perfringens. Stools were inoculated in cooked meat medium and incubated anaerobically at 37 ° C for 48 hrs in an anaerobic jar containing GasPakTM. Enriched samples were streaked on Sulphite Polymixin Sulphadiazine agar plates and were incubated anaerobically as above. Colonies that grew with characteristics of *C. perfringens* were stained with Gram's stain and sub-cultured on brain heart infusion agar plates until they were free from contaminating bacteria. The pure colonies were further streaked on the 5% sheep blood agar and egg yolk agar plates and incubated anaerobically for 24 h; the colonies producing characteristic double zone of hemolysis around them on blood agar and producing zone of opalescence around the colonies on egg yolk agar were tentatively identified as *C. perfringens*. The bacteria were identified in the feces of groups infected with *C. perfringens* (T2 and T3); but it was not isolated from the control group (T1).

2.3 Bird performance parameters

To evaluate the performance parameters of the birds, the study was divided into two experimental periods, 1 to 35 days and 1 to 42 days of growth, where the birds were weighed at the beginning and end of each period, resulting in obtaining the average weight gain (g), average daily bird weight gain (g) and the average bird weight at the end of each period (g). The feeds were also weighed at the beginning and end of each experimental period, and the average feed intake per bird (g) was calculated, obtained from the feed intake of each batch, in each period, divided by the number of birds in each batch. Feed consumption in each period also served as the basis for calculating feed conversion, calculated by dividing feed

consumption in the period by the average bird weight during the same period. The number of birds that died in each period (mortality) was also calculated and expressed as percentages.

2.4 Sample collection

Blood was collected from seven birds per treatment, at 20, 27, 34, and 41 of the experiment. The birds were restrained manually; blood was collected from the ulnar vein into insulin syringes. Blood was allocated in two Eppendorf tubes, one containing EDTA to obtain whole blood for analysis of antioxidant enzymes and blood counts, and the other without anticoagulant to obtain the serum. Subsequently, the material was centrifuged at 3500 rpm for 10 minutes and the serum was separated, collected and frozen (–20 °C) for biochemical analysis.

At the end of the experiment (day 42), all birds were sent to a municipal slaughterhouse. We took the opportunity to collect the intestine of two chickens by repetition (total of 10 birds per treatment) for histopathological analysis. All slaughter procedures followed current regulations; and were the responsibility of the slaughterhouse.

2.5 Hemogram

After blood collection, blood smears stained with a commercial dye (Panótico Rápido) for leukocyte differential and reading under a light microscope (100 ×). Hematocrit was measured using microcapillary tubes, centrifuged (10.000 rpm for 5 min) and read according to the manufacturer's guidelines. The counting of leukocytes and erythrocytes was performed in a Neubauer chamber, according to the method described in literature (Thrall et al., 2015).

2.6 Serum clinical biochemistry

We measured liver enzymes levels (alanine amino transferase [ALT] and aspartate amino transferase [AST]), as well as protein metabolism variables (total protein, albumin, and uric acid) using commercial kits (Analisa®) and a semi-biochemical analyzer automatic (BioPlus 2000®). Globulin levels were determined by subtracting total protein and albumin levels.

2.7 Oxidative/antioxidant profile

Lipid peroxidation (LPO) levels were measured using serum samples diluted in cold methanol (1:1 v/v) and centrifuged at 1000 x g for 10 min at 4 ºC [15, 16]. Serum LPO levels were measured using a microplate reader at 550 nm, with cumene hydroperoxide as the standard.

Serum reactive oxygen species (ROS) levels were determined using the DCFH oxidation method described by researchers Ali et al. (1992), recently published in detail by Biazus et al. (2017). 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.

2.8 Antioxidant profile

Serum superoxide dismutase (SOD) activity was determined by inhibiting the O2 reaction with adrenaline as described by researchers Mccord and Fridovich (1969). One unit of SOD enzyme is defined as the amount of enzyme that inhibits the adrenaline oxidation rate by 50%.

This leads to the formation of a red-colored product, adrenochrome, that is detected using a spectrophotometer. Serum SOD activity was determined by measuring the rate of adrenochrome formation observed at 480 nm in a reaction medium containing 50 mM glycine-NaOH, pH 10 and 1 mM adrenaline. Results were expressed as IU SOD/mg protein.
Serum glutathione S-transferase (GST) activity was measured using spectrophotometry at 340 nm according to literature Habig et al. (1974). The mixture contained muscle homogenate supernatant as a test, 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH and 100 mM CDNB, used as substrate. Enzyme activity was expressed as µmol/CDNB/mg protein.

Glutathione peroxidase (GPx) activity was quantified using tert-butyl hydroperoxide as a substrate (Wendel, 1981). Enzyme activity was quantified by monitoring the disappearance of NADPH at 340 nm in a medium containing 100 mM potassium phosphate buffer, 1 mM EDTA, pH 7.7, 2 mM GSH, 0.1 U/mL GR, 0.4 mM azide, 0.5 mM tert-butyl hydro peroxide, 0.1 mM NADPH and tissue supernatants. The results were expressed as U GPx/mg protein.

2.9 Enzyme of metabolism energetic

Serum 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 MgSO4 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. Creatine levels were measured according to the colorimetric method Hughes (1962). 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.

Serum AK activity was measured with a coupled enzymatic assay using hexokinase (HK) and glucose 6-phosphate dehydrogenase (G6PD) (Dzeja et al., 1998). The reaction mixture contained 100 mM KCl, 20 mM HEPES, 20 mM glucose, 4 mM MgCl2, 2 mM NADP+, 1 mM 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/min/mg of protein.

Serum PK activity was assayed essentially as described by researchers Leong et al. (1981). The incubation medium consisted of 0.1 M Tris/HCl buffer, pH 7.5, 10 mM MgCl2, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7 U L-lactate dehydrogenase, 0.1 % (v/v) Triton X-100 and 20 µL of 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/min/mg of protein.

2.10 Histopathology

Fragments of the intestine (duodenum and jejunum) were collected and stored in 10% formaldehyde. The tissues were embedded in paraffin for transverse sections of 4 µm thickness and stained with hematoxylin and eosin.

2.11 Statistical analysis

Data were subjected to normality testing. Data showing no normal distribution were transformed and the analysis test was applied in sequence analysis of variance. In cases of

significant differences, the means were subjected to the Tukey test accepting 5% as significant.

3. Results

3.1 Zootechnical performance and histology

There were no significant differences among treatments in terms of zootechnical performance (Table 1). No histological lesions were observed in the duodenum and jejunum of chickens from all treatments at the end of the experiment.

3.2 Hemogram

At 20 days of the experiment, heterophils counts were significantly lower in birds in group T3 compared to those of the other groups (P <0.05 – Table 2).

The numbers of leukocytes and lymphocytes were significantly lower in groups T2 and T3 compared to T1 (P <0.01) on day 34.

There were significantly lower heterophils and monocyte counts in T3 compared to T1 (P <0.05) on the same day. On days 27 and 41, no significant differences were observed between groups (P >0.05) (Table 2).

Table 1 - Mean values obtained for feed intake (FI, kg/bird), average weight (AW, kg/bird), average weight gain (AWG, kg/bird), daily weight gain (DWG, g/bird/day), feed conversion (FC) and mortality (MORT, %) of birds subjected to different treatments in the periods from 1 to 35 days and from 1 to 42 days.

Period: 1 to 35 days						
Treatment	**FI**	**AW**	**AWG**	**DWG**	**FC**	**MORT**
T1	3.80	2.43	2.38	68.23	1.59	4.00
T2	3.81	2.45	2.40	68.74	1.58	3.75
T3	3.80	2.44	2.39	68.44	1.59	1.25
P-value	0.181	0.081	0.074	0.080	0.885	0.534
CV (%)	3.52	2.42	2.37	2.78	3.61	12.8
Period: 1 to 42 days						
T1	5.27	3.16	3.11	74.22	1.69	4.5
T2	5.19	3.14	3.09	73.74	1.67	4.0
T3	5.02	3.06	3.02	71.92	1.72	3.75
P-value	0.175	0.349	0.349	0.349	0.127	0.621

CV (%)	4.25	2.78	2.84	2.84	3.61	10.03

Note 1: CV = coefficient of variation. T1 = control; T2 = positive control for *Clostridium perfringens*; T3 = commercial growth enhancer (oral *C. perfringens* challenge + 4.4 mg/kg lincomycin). Source: Authors.

3.3 Clinical biochemistry

On day 41, levels of total protein and globulin variables were significantly lower in T3 group than in T1 ($P < 0.05$). For the variables ALT, AST, albumin, and uric acid, no significant differences were found between groups ($P > 0.05$) (Table 3).

Table 2. Hemogram of broilers experimentally infected with *Clostridium perfringens*.

	Hematocrit (%)	Erythrocytes (x 10^6 µL)	Leukocytes (x 10^3 µL)	Heterophil (x 10^3 µL)	Lymphocyte (x 10^3 µL)	Monocyte (x 10^3 µL)	Eosinophil (x10^3 µL)
Day 20							
T1	26.0	1.58	7.85	1.77 ^{A}	5.08	0.64	0.33
T2	27.5	1.48	7.40	1.44 ^{A}	5.22	0.51	0.23
T3	27.7	1.42	6.75	0.85 ^{B}	5.06	0.47	0.35
P-value	**0.91**	**0.74**	**0.61**	**0.02***	**0.78**	**0.56**	**0.64**
CV (%)	**5.74**	**3.96**	**17.4**	**15.7**	**20.1**	**33.4**	**37.9**
Day 27							
T1	23.4	1.86	11.6	2.88	7.61	0.52	0.24
T2	24.0	1.66	9.91	2.08	6.47	0.67	0.68
T3	25.1	1.59	8.97	2.50	6.12	0.82	0.51
P-value	**0.61**	**0.32**	**0.09**	**0.19**	**0.23**	**0.44**	**0.50**
CV (%)	**4.14**	**5.79**	**20.7**	**15.0**	**18.4**	**30.7**	**40.9**
Day 34							
T1	24.8	1.64	13.9 ^{A}	3.57 ^{A}	8.36 ^{A}	1.24 ^{A}	0.76
T2	25.4	1.83	9.69 ^{B}	2.71 ^{AB}	5.37 ^{B}	0.98 ^{AB}	0.62
T3	24.1	1.49	8.37 ^{B}	2.03 ^{B}	5.01 ^{B}	0.73 ^{B}	0.59
P-value	**0.80**	**0.27**	**0.01***	**0.04***	**0.01***	**0.05***	**0.58**
CV (%)	**6.17**	**5.01**	**18.6**	**21.3**	**25.7**	**28.0**	**0.31**
Day 41							
T1	24.4	1.46	11.2	3.70	7.29	0.84	0.34
T2	24.8	1.51	10.0	2.76	6.09	0.73	0.43
T3	25.4	1.55	11.0	3.45	6.51	0.50	0.58
P-value	**0.82**	**0.45**	**0.51**	**0.09**	**0.11**	**0.39**	**0.61**
CV (%)	**5.74**	**3.96**	**13.7**	**24.2**	**20.2**	**26.8**	**25.0**

$^{A, B}$ – Mean values followed by different letters overwritten in the same column indicate significant difference for $P < 0.05$. CV = coefficient of variation. T1 = control; T2 = positive control for *Clostridium perfringens*; T3 commercial growth enhancer. Source: Authors.

Table 3. Serum biochemistry of broilers experimentally infected with *Clostridium perfringens.*

Treatments	ALT (U/L)	AST (U/L)	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Uric acid (mg/dL)
Day 20						
T1	7.28	160	2.62	1.15	1.47	10.5
T2	3.57	164	2.25	1.05	1.20	10.0
T3	2.57	166	2.31	0.98	1.32	7.90
P-value	**0.24**	**0.84**	**0.70**	**0.67**	**0.65**	**0.39**
CV (%)	**41.5**	**19.2**	**11.9**	**13.2**	**14.6**	**22.0**
Day 27						
T1	7.40	184	2.51	1.40	1.11	6.08
T2	6.42	182	2.71	1.34	1.42	4.51
T3	3.71	211	2.65	1.40	1.26	5.94
P-value	**0.54**	**0.20**	**0.53**	**0.85**	**0.12**	**0.42**
CV (%)	**39.4**	**21.3**	**6.51**	**8.10**	**7.86**	**17.6**
Day 34						
T1	2.85	261	2.80	1.15	1.64	7.01
T2	3.00	235	2.58	1.14	1.44	6.74
T3	2.42	264	2.71	1.11	1.60	6.71
P-value	**0.74**	**0.59**	**0.55**	**0.82**	**0.74**	**0.78**
CV (%)	**36.7**	**28.3**	**10.2**	**11.0**	**14.1**	**20.2**
Day 41						
T1	4.00	318	3.21 ^{A}	1.04	2.17 ^{A}	5.08
T2	2.10	268	2.67 ^{B}	1.05	1.62 ^{B}	4.21
T3	3.00	414	2.94 ^{AB}	1.15	1.78 ^{AB}	3.77
P-value	**0.60**	**0.09**	**0.05***	**0.80**	**0.05***	**0.12**
CV (%)	**37.9**	**33.1**	**6.47**	**5.12**	**7.85**	**15.4**

$^{A, B}$ – Mean values followed by different letters overwritten in the same column indicate significant difference for P < 0.05. CV = coefficient of variation. T1 = control; T2 = positive control for *Clostridium perfringens*; T3 = commercial growth enhancer. Source: Authors.

3.4 Oxidant and antioxidant status

Serum levels of ROS and LPO, as well as serum GST activity, were significantly lower in the T1 and T3 group compared to T2 (P <0.05) on day 20 of the experiment. Serum LPO levels were significantly lower in group T1 and T3 compared to the other (P <0.05) on day 34 of the experiment. The enzymatic activity of GST was significantly lower in groups T1 and T3 compared to T2 (P <0.05), while the serum activity of GPx was significantly lower in group T2 compared to T1 (P <0.05). On day 41, a significant decrease in serum ROS levels was observed in groups T2 and T3 compared to T1 (P <0.05). For the variable LPO, the highest concentration was observed at T2 in relation to T1 and T3 (P <0.05). The activity of the GPx enzyme was significantly higher in birds from T2 and T3 compared to T1 (P <0.05). On day 27, there was no significant difference between the variables studied (P >0.05) (Table 4).

3.5 Energetic metabolism

On day 27, serum PK activity was significantly higher in groups T2 and T3 than in T1 (P <0.05). On day 34, a significant increase in serum CK and PK activity was observed in groups T2 and T3 compared to T1 (P <0.05). AK serum activity was significantly higher in T3 than in T1 (P <0.05).

At 41 days of experiment, greater activity of the enzymes CK and PK was observed in birds in group T3 than those in T1. At 20 days of experiment there was no significant difference in the activity of the enzymes CK, PK, or AK between the groups (P> 0.05) (Table 5).

4. Discussion

Clostridiosis did not alter zootechnical performance as expected; just as we did not observe histopathological lesions in the intestines of birds at 42 days of life (21 days' post-infection).

The higher count of 10^6 CFU/g of *C. perfringens* digestion in the small intestine can cause necrotic enteritis in birds (Kiu and Hall, 2018). However, there needs to be other predisposing factors for this to occur, including the following: digestion viscosity, presence of non-starch polysaccharides, intestinal transit time, and intestinal pH (Yang et al., 2019).

It is important to note that the dose, strain, and form of infection used in this study may have been insufficient to cause changes in animal performance. Nevertheless, in this context, the predisposing factors mentioned above may also have influenced our results.

Table 4. Oxidant and antioxidant variables in the serum (Concentrate: per mg of protein or per mL in serum) of broilers experimentally infected with *Clostridium perfringens*.

Treatments	ROS (U DCF/mg)	LPO (nmol/mL)	GST (U GST/mg)	GPx (U GPx/mg)	SOD (U SOD/mg)
Day 20					
T1	3.66 ^{A}	510 ^{B}	3.52 ^{B}	0.35	2.78
T2	3.58 ^{A}	2378 ^{A}	6.12 ^{A}	0.33	2.92
T3	2.38 ^{B}	465.0 ^{B}	3.96 ^{B}	0.32	2.72
P-value	**0.02***	**0.01***	**0.01***	**0.89**	**0.79**
CV (%)	**24.7**	**40.3**	**19.4**	**21.6**	**18.6**
Day 27					
T1	1.43	764.7	3.47	0.21	1.89
T2	1.57	1241	4.73	0.39	2.02
T3	1.63	970.5	3.93	0.20	2.10
P-value	**0.71**	**0.07**	**0.64**	**0.06**	**0.74**

CV (%)	**32.0**	**49.7**	**27.9**	**26.3**	**26.2**
Day 34					
T1	0.77	1209 ^{B}	5.47 ^{B}	0.40 ^{A}	1.89
T2	1.30	2208 ^{A}	3.53 ^{A}	0.20 ^{B}	2.16
T3	1.18	1101 ^{B}	5.17 ^{B}	0.37 ^{AB}	2.14
P-value	**0.23**	**0.05***	**0.05***	**0.04***	**0.09**
CV (%)	**30.7**	**36.4**	**30.8**	**23.7**	**14.3**
Day 41					
T1	1.49 ^{A}	2081 ^{B}	4.36	0.21 ^{B}	2.05
T2	0.60 ^{B}	4168 ^{A}	4.33	0.38 ^{A}	2.08
T3	0.80 ^{B}	2159 ^{B}	3.43	0.44 ^{A}	2.10
P-value	**0.04***	**0.03***	**0.28**	**0.02***	**0.81**
CV (%)	**28.3**	**36.7**	**28.32**	**19.9**	**18.5**

$^{A, B}$ – Mean values followed by different letters overwritten in the same column indicate significant difference for P < 0.05. CV = coefficient of variation. T1 = control; T2 = positive control for *Clostridium perfringens*; T3 = commercial growth enhancer. Source: Authors.

Table 5. Serum creatine kinase (CK), pyruvate kinase (PK) and adenylate kinase (AK) activities of experimentally infected by *Clostridium perfringens.*

Treatments	**CK (U/L)**	**PK (**nmol of pyruvate formed/min/mg of protein**)**	**AK (**nmol of ATP formed/min/mg of protein)
Day 20			
T1	0.17	1.05	1.07
T2	0.21	1.89	1.46
T3	0.19	1.65	1.40
P-value	**0.87**	**0.56**	**0.36**
CV (%)	**16.3**	**33.2**	**38.0**
Day 27			
T1	0.17	0.91 ^{b}	1.20
T2	0.18	1.79 ^{A}	1.22
T3	0.17	1.76 ^{A}	1.36
P-value	**0.93**	**0.01***	**0.07**
CV (%)	**13.8**	**27.3**	**36.2**
Day 34			
T1	0.16 ^{B}	0.92 ^{B}	1.06 ^{B}
T2	0.26 ^{A}	1.55 ^{A}	1.72 ^{A}
T3	0.28 ^{A}	1.59 ^{A}	1.86 ^{A}
P-value	**0.05***	**0.04***	**0.05***
CV (%)	**14.6**	**19.3**	**21.8**
Day 41			
T1	0.15 ^{B}	0.93 ^{B}	1.35
T2	0.27 ^{A}	1.78 ^{A}	1.60
T3	0.21 ^{AB}	1.65 ^{AB}	1.63
P-value	**0.01***	**0.01***	**0.59**
CV (%)	**20.3**	**24.0**	**33.3**

$^{A, B}$ – Mean values followed by different letters overwritten in the same column indicate significant difference for P < 0.05. CV = coefficient of variation. T1 = control; T2 = positive control for *Clostridium perfringens*; T3 = alternative growth enhancer; T4 = commercial growth enhancer. Source: Authors.

Researchers Bortoluzzi et al. (2019) verified that infected broilers with *C. perfringens* at 1 x 10^{8} CFU/mL in 200 mL of water have lower zootechnical performance compared to the uninfected group. These authors observed that necrotic enteritis is a multifactorial disease. In

this sense, perhaps the predisposing factors did not occur in this study to develop severe disease that could have a negative effect on growth performance. In addition, the bedding material can also contribute to the establishment of the intestinal microbiota, and can mitigate the negative impacts of the enteric challenge (Bortoluzzi et al., 2019). Finally, there may have been decreased numbers of pathogenic bacteria and increased numbers of beneficial bacteria, as observed by researchers Bortoluzzi et al. (2019). The main damage in the field described on clostridiosis is associated with coccidiosis; therefore, we are planning a future study to assess the impacts of these concomitant diseases.

Lower number of leukocytes and lymphocytes in birds may be due to the antimicrobial activity of the additives (Voemesse et al., 2019) lincomycin in the present study. Lower lymphocyte counts may occur due because of clostridiosis affecting lymphatic organs, which is common in some diseases such as eimeriosis (Ademola et al., 2019). Lower monocyte counts may be related to immune system stress Duskaev et al. (2018), while lower heterophils counts may indicate that these birds did not need to mount cellular immune responses, which may have occurred with the use of antibiotics. The smaller amount of blood leukocytes could be related to the migration of these cells to the intestine in order to act in the direct fight against bacteria; however, on days 27 and 34 when this change was seen, we did not perform a bowel histological analysis.

The levels of total protein and globulin are related to protein and immune metabolism (Zhu et al., 2014). The decrease in total protein and globulin levels in birds experimentally infected with *C. perfringens* may be related to lower levels of stimulation of the immune system and inflammatory responses. ROS are produced mainly in mitochondria, and in cases of bacterial diseases, the amount of ROS produced can increase in an attempt to eliminate the infectious agent; however, this can cause both mitochondrial and cellular injuries, in addition to being responsible for oxidative stress (Dmitry et al., 2014). There was a reduction in the serum levels of ROS and LPO in the group infected with *C. perfringens* and who received the antibiotic. This is beneficial, because it shows that the antibiotic reduced the harmful effects caused by ROS and LPO. Organisms possess several non-enzymatic and enzymatic antioxidant defense mechanisms (Winterbourn, 2015). In cases of oxidative stress, enzymes can act in different ways. At 20 days, there was a decrease in the GST activity in T3. This reduction decreases the expression of pro-inflammatory cytokines, blocking transcription factors sensitive to redox conditions (Gius et al., 1999). This effect may also be related to the lower production of free radicals, such that enzymes do not need to increase their activity levels. Further studies are needed to understand the role of oxidative stress and inflammatory

response in clostridiosis in chickens, since in our study there were non-logical effects; that would need other complementary analyzes to clarify the mechanisms involved in the changes.

The enzymes CK, AK, and PK are responsible for phosphotransfer, reactions that are associated with communication between the cytosol and the nucleus, and are therefore essential for cellular homeostasis (Dzeja and Terzic, 2003). These enzymes catalyze the exchange of nucleotides, facilitating communication between the places of generation and use of ATP (Saupe et al., 1998). The enzymes participating in the phosphotransfer network are fundamental to bioenergetic regulation in tissues (Stanojevic et al., 2008). For this reason, it is important to understand how the phosphotransfer network works in the context of clostridiosis. In this study, infection by *C. perfringens* in broilers altered the serum activities of AK, CK and PK, so as to avoid compromising this energy network. Increased serum CK activity was observed in the infected groups, possibly as an attempt to store and distribute energy as a form of energy compensation to try to maintain bioenergetic homeostasis by producing phosphocreatine and avoiding a drop in ATP concentration, as noted by researchers Perin et al. (2019) in mice experimentally infected by *Staphylococcus aureus.*

Increased serum PK activity, by contrast, may be a mechanism designed to increase glucose production, with the purpose of maintaining energy metabolism, especially in tissues that require high energy such as the liver (Perin et al., 2019). Researchers Galli et al. (2019) observed increased AK activity in birds challenged with *Eimeria spp*. These findings suggest that increased AK activity can be seen as an attempt to maintain the bioenergetic balance as well. In this way, the phosphotransfer network enzymes are upregulated in an attempt to maintain bioenergetics during infection, or to reduce the negative effects of bioenergetic dysregulation during clostridiosis.

Among the limitations of our studies, we would like to highlight the absence of histoptological analysis shortly after infection (days 27 and 34), when the main hematological and biochemical changes were observed. It would also be useful in this study to have isolated *C. perfringens* on days 34 and 41, as this information could assist in the discussions of biochemical data. It is important to make it clear to readers that infections by *C. perfringens* are generally opportunistic, therefore, as already mentioned, the pathogenesis and pathogenicity of the infection is dependent on many factors. Another limitation of our research was the absence of another group, that is, similar to T3, but that was not as challenged as *C. perfringens*. This group could allow for stronger conclusions, given this, it is important to make it clear that we have preliminary results in this manuscript.

5. Final Considerations

The results allow to conclude that the consumption of antimicrobial by the birds (control group - T3) avoids fluctuations in the hematological and biochemical variables when the birds are challenged by *C. perfringens*.

This study did not allow a conclusion on the relationship between cellular inflammatory response and oxidative stress, because there was a fluctuation between these variables during the experimental period. Phosphotransfer network enzymes compensate for infection-related disruptions in energetic homeostasis via a feedback mechanism.

Clostridiosis affects energy metabolism, although it did not alter animal performance in our study; this may be the case because of the dose used for the infection and the absence of predisposing factors. For future work it is suggested to consider a more challenge for *C. clostridium* that provides changes in animal performance, in addition to the prediponent factors mentioned in this article. Therefore, in order to simulate a field situation and, therefore, evaluate the relationship between cellular inflammatory response and oxidative stress, together with the enzymes of the phosphotransfer network, in order to understand more clearly the mechanisms of metabolism energy in cases of challenge by clostridiosis.

The main contribution is related to the understanding of how the phosphotransfer network enzymes work in cases of clostridiosis, and there are not many studies in this area. Para trabalhos futuros sugere-se considerar um maior desafio por *C. clostridium* que proporcione mudanças no desempenho animal, além dos fatores prediponentes citados neste artigo. Portanto, com objetivo de simular uma situação de campo e, com isso, avaliar a relação entre a resposta inflamatória celular e o estresse oxidativo, juntamente com as enzimas da rede de fosfotransferência, com o intuito de entender de forma mais clara os mecanismos do metabolismo energético em casos de desafio por clostridiose. A principal contribuição está relacionada com o entendimento de como as enzimas da rede de fosfotransferência atuam em casos de clostridiose, sendo que não possui muitos estudos nesta área.

Ethics committee

This study was approved by the Animal Use Ethics Committee (CEUA) of the State University of Santa Catarina (UDESC), protocol number 3369060819, under the rules of the National Council for Animal Experimentation Control (CONCEA).

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