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COLIBACILOSE EM AVES DE PRODUÇÃO: CURCUMINA COMO ALTERNATIVA PARA MINIMIZAR OS EFEITOS NEGATIVOS CAUSADOS PELA DOENÇA

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COLIBACILOSE EM AVES DE PRODUÇÃO: CURCUMINA COMO ALTERNATIVA PARA MINIMIZAR OS EFEITOS NEGATIVOS CAUSADOS PELA DOENÇA

Elaborada por
Gilneia da Rosa

como requisito parcial para obtenção do grau de
Mestre em Zootecnia

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"Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível" (Charles Chaplin, 1956).

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Zootecnia
Universidade do Estado de Santa Catarina

COLIBACILOSE EM AVES DE PRODUÇÃO: CURCUMINA COMO ALTERNATIVA PARA MINIMIZAR OS EFEITOS NEGATIVOS CAUSADOS PELA DOENÇA

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Chapecó, 06 de fevereiro de 2020.

A colibacilose aviária é uma doença infecciosa causada pelo agente *Escherichia coli*, que ocasiona elevada morbidade e diminuição de produtividade. Com a proibição da utilização de antibióticos na produção animal, a utilização de extratos vegetais com propriedades antimicrobianas, vem se destacando, em especial, a curcumina, princípio ativo da planta *Curcuma longa*, que já possui efeito antimicrobiano, ação antioxidante e anti-inflamatória comprovados. Desta forma, o objetivo foi determinar os impactos da doença na produção avícola e os efeitos da utilização da curcumina via dieta. Para isso, foram realizados três experimentos distintos. No experimento 1, foram utilizadas galinhas poedeiras com infecção natural por *E. coli*, divididas em grupo controle (sem sinais clínicos) e infectados (com diarréia e apáticas), com 40% de postura diária, sendo o agente infeccioso isolado de amostras fecais e tecido (ovário, fígado e peritônio), submetido ao teste de suscetibilidade antimicrobiana e instaurado o tratamento com norfloxacina por três dias. Antes do tratamento, as aves do grupo infectados apresentavam níveis elevados de lipoperoxidação (LPO), superóxido dismutase (SOD), glutationa peroxidase (GPx), creatina quinase (CK) e piruvato quinase (PK). Após o tratamento, os níveis de LPO permaneceram mais altos em aves com doença clínica, enquanto SOD e GPx não diferiram entre os grupos, já a produção elevou-se para 90%. Experimento 2, utilizamos 36 galinhas poedeiras com colibacilose, divididas em grupo controle (aves alimentadas com ração basal) e grupo curcumina (aves alimentadas com ração basal + 200 mg de curcumina/kg/ração). Fezes e ovos apresentaram menor contagem bacteriana total, coliformes totais e *E. coli* quando administrado a curcumina aos 21 e 42 dias. Em ovos frescos, a luminosidade e a intensidade de amarelo da gema foram maiores para grupo curcumina, bem como para ovos armazenados: maior densidade específica, altura do albúmen, menor pH da gema, menores níveis de peroxidação lipídica e maior capacidade antioxidante total, indicando melhor qualidade dos ovos. Também para o grupo curcumina foi observada menor contagem total de leucócitos, neutrófilos e linfócitos, menores níveis de proteína total, fosfatase alcalina e alanina aminotransferase, garantindo melhor saúde dos animais pelo efeito anti-inflamatório e antioxidante obtido. No experimento 3, utilizamos vinte matrizes de corte juvenis, divididas em dois grupos: grupo infectado intraperitonealmente (1 mL contendo 1,5 x 10^8 UFC de *E. coli*) e grupo controle (aves não infectadas). Após 10 dias da infecção, houve menor crescimento e ganho de peso das aves infectadas, com desenvolvimento de pericardite, congestão hepática e infiltrados inflamatórios periportais moderados com predominância de

neutrófilos, número maior de leucócitos totais, linfócitos, heterófilos e monócitos, bem como níveis significativamente mais altos de proteínas e globulinas enquanto que os valores de albumina diminuíram no mesmo período. Para biomarcadores oxidativos séricos, níveis de peroxidação lipídica (TBARS) e radicais livres (ROS) foram significativamente maiores, assim como a atividade da glutationa S-transferase (GST) durante o mesmo período para grupo infectado. Os níveis de radicais livres e de tióis proteicos a nível hepático foram significativamente maiores nas aves infectadas, bem como atividade de catalase e superóxido dismutase. No baço, apenas a atividade Glutationa S-Transferase foi significativamente maior no grupo infectado, ao contrário do cérebro, onde a atividade de superóxido dismutase, níveis de radicais livres e de tióis não protéicos foram significativamente maiores nas aves infectadas do que no grupo controle. A atividade da acetilcolinesterase (AChE) no córtex cerebral foi menor no grupo infectado do que no controle; houve um aumento na atividade de nucleosídeo trifosfato difosfohidrolase (NTPDase), 5'-nucleotidase e adenosina desaminase (ADA), possivelmente indicando maior hidrólise de adenosina trifosfato (ATP) ($P < 0,001$), adenosina difosfato (ADP) ($P < 0,01$) e adenosina monofosfato (AMP) ($P < 0,01$), seguidos por aumento da desaminação da adenosina ($P < 0,001$). Apesar disso, nenhum animal apresentou sinais clínicos da doença durante todo período experimental. Diante disso, concluímos que a infecção por *E. coli* em aves de produção causa estresse oxidativo nos animais, diminui significativamente produção e afeta as enzimas responsáveis pela neurotransmissão e imunomodulação, como acetilcolina, adenosina trifosfato e adenosina desaminase. A curcumina possui efeito antimicrobiano atuando no controle da infecção, melhora a qualidade microbiológica e físico-química de ovos, bem como a saúde dos aimais pelo seu efeito antioxidante e anti-inflamatório, sendo um aditivo natural com alto potencial para utilização na alimentação de aves.

Palavras-chave: aditivos naturais, avicultura, curcumina, colibacilose, comportamento animal, estresse oxidativo, produção animal.

ABSTRACT
Master's Dissertation
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COLLIBACILLOSIS IN PRODUCTION BIRDS: CURCUMIN AS AN ALTERNATIVE TO MINIMIZE THE NEGATIVE EFFECTS CAUSED BY THE DISEASE

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Chapecó, 06 february 2020

Avian colibacillosis is an infectious disease caused by the agent *Escherichia coli*, which causes high morbidity and decreased productivity. With the ban on the use of antibiotics in animal production, the use of plant extracts with antimicrobial properties stood out, in particular curcumin, the active ingredient of the *Curcuma longa* plant, which already has a proven antimicrobial, antioxidant and anti-inflammatory action. Thus, the objective was to determine the impacts of the disease on poultry production and the effects of using curcumin via diet. For this, three different experiments were carried out. In experiment 1, laying hens with natural infection by *E. coli* were used, divided into a control group (without clinical signs) and infected (with diarrhea and apathy), with 40% of daily posture, with the infectious agent isolated from fecal samples and tissue (ovary, liver and peritoneum), subjected to antimicrobial susceptibility test and treatment with norfloxacin for three days. Before treatment, as the birds in the infected group have high levels of lipoperoxidation (LPO), superoxide dismutase (SOD), glutathione peroxidase (GPx), creatine kinase (CK) and pyruvate kinase (PK). After treatment, the levels of OLP will remain higher in birds with clinical disease, while SOD and GPx did not differ between groups, with an increase in production to 90%. Experiment 2, we used 36 laying hens with colibacillosis, divided into a control group (birds fed basal feed) and a curcumin group (birds fed basal feed + 200 mg curcumin / kg / feed). Feces and eggs showed lower total bacterial count, total coliforms and *E. coli* when curcumin was administered at 21 and 42 days. In fresh eggs, yolk brightness and yellow intensity were higher for curcumin group, as well as for stored eggs: higher specific density, albumen height, lower yolk pH, lower levels of lipid peroxidation and higher total antioxidant capacity, indicating better egg quality. Also for the curcumin group, a lower total count of leukocytes, neutrophils and lymphocytes, lower levels of total protein, alkaline phosphatase and alanine aminotransferase were observed, ensuring better health of the animals due to the anti-inflammatory and antioxidant effect obtained. In experiment 3, we used twenty juvenile broiler breeders, divided into two groups: intraperitoneally infected group (1 mL containing 1.5 x 108 CFU of *E. coli*) and control group (uninfected birds). After 10 days of infection, there was less growth and weight gain in infected birds, with the development of pericarditis, liver congestion and moderate periportal inflammatory infiltrates with a predominance of neutrophils, a greater number of total leukocytes, lymphocytes, heterophils and monocytes, as well as significantly levels higher protein and globulin levels while albumin values decreased in the same period. For serum oxidative biomarkers, levels

of lipid peroxidation (TBARS) and free radicals (ROS) were significantly higher, as was the activity of glutathione S-transferase (GST) during the same period for the infected group. The levels of free radicals and protein thiols at the hepatic level were significantly higher in infected birds, as well as catalase and superoxide dismutase activity. In the spleen, only Glutathione S-Transferase activity was significantly higher in the infected group, unlike in the brain, where superoxide dismutase activity, levels of free radicals and non-protein thiols were significantly higher in infected birds than in the control group. Acetylcholinesterase (AChE) activity in the cerebral cortex was lower in the infected group than in the control group; there was an increase in the activity of nucleoside triphosphate diphosphohydrolase (NTPDase), 5'-nucleotidase and adenosine deaminase (ADA), possibly indicating greater hydrolysis of adenosine triphosphate (ATP) ($P < 0.001$), adenosine diphosphate (ADP) ($P < 0,01$) and adenosine monophosphate (AMP) ($P < 0.01$), followed by increased deamination of adenosine ($P < 0.001$). Despite this, no animal showed clinical signs of the disease during the entire experimental period. Therefore, we conclude that *E. coli* infection in production birds causes oxidative stress in animals, significantly decreases production and affects the enzymes responsible for neurotransmission and immunomodulation, such as acetylcholine, adenosine triphosphate and adenosine deaminase. Curcumin has an antimicrobial effect acting on infection control, improves the microbiological and physical-chemical quality of eggs, as well as the health of animals due to its antioxidant and anti-inflammatory effect, being a natural additive with high potential for use in poultry feed.

Keywords: natural additives, poultry, curcumin, colibacillosis, animal behavior, oxidative stress, animal production.

SUMÁRIO

CAPÍTULO I

1. REVISÃO DE LITERATURA

1.1 INTRODUÇÃO

1.1.1 AVICULTURA

A avicultura, nos últimos anos, é o setor do agronegócio que apresenta maior expansão entre os principais setores produtivos que integram o complexo carne a nível mundial. A integração entre genética, nutrição, sanidade e ambiência torna esta atividade altamente competitiva no mercado de carnes e ovos (PROCÓPIO, 2020).

O Brasil, em 2019, ocupou o primeiro lugar no *ranking* mundial de exportação de carne de frango e a sétima posição na produção de ovos no mesmo ano (ABPA, 2019). Com um amplo plantel de matrizes de corte e postura, a tendência é a expansão contínua tanto do mercado produtor quanto consumidor nas próximas décadas.

A expansão deste mercado deve-se não somente ao alto grau de controle sanitário, mas a conjuntura com o controle, pela indústria, do ciclo produtivo das aves e a melhoria da taxa de conversão de proteína vegetal em proteína animal, possibilitando um aumento na produtividade, redução de custos e consequentemente uma queda absoluta e relativa do preço da carne de frango em relação a outras carnes (SCHMIDT; SILVA, 2018).

Além disso, a avicultura oferece um produto de excelente qualidade, que é o ovo, o qual, possui todos os nutrientes necessários para formar um animal completo bem como contribui consideravelmente com a nutrição humana, sendo fonte de proteína de alta qualidade e pequena porcentagem calórica (ASSIS et al., 2016), além disso, por ser comercializado a baixo custo, muitas vezes é a única fonte de proteína animal para famílias de menor poder aquisitivo.

1.1.2 QUALIDADE E COMPOSIÇÃO NUTRICIONAL DE OVOS

O ovo é um alimento nutricionalmente completo, rico em proteínas de alto valor biológico com fornecimento de 6,25 g de proteína por dia (15% da quantidade diária recomendada), vitaminas do complexo B, A, E, K, minerais como ferro, fosforo selênio e zinco, carotenóides como a luteína e zeaxantina bem como fonte de colina (NOVELLO et al., 2006).

Devido a sua composição nutricional, pode ser utilizado para compor uma dieta variada e equilibrada (ASSIS et al., 2016). Nos últimos anos houve aumento expressivo no consumo de ovos devido ao aumento do poder de compra da população, que passaram a consumir mais ovos e produtos industrializados que contenham ovos (AMARAL et al., 2016). Além disso, aproveitando o fato de que o ovo é um alimento completo e que gera maior saciedade, muitos consumidores adotaram seu consumo na formulação de dietas para emagrecimento (UBABEF, 2014).

No entanto, a qualidade deste produto é motivo de preocupação, não só para as granjas comerciais, mas, também, para comerciantes e consumidores. Como é um produto rico em nutrientes, pode favorecer o desenvolvimento de microrganismos deteriorantes e patogênicos, quando ocorrem trincas, quebras ou manuseio incorreto (ARAGON-ALEGRO et al., 2005). Além dos aspectos econômicos com perdas do produto, defeitos na qualidade podem significar riscos para a saúde pública (PIRES et al., 2015).

A contaminação do conteúdo dos ovos pode ocorrer tanto no trato reprodutor da galinha, resultando na transmissão vertical do microrganismo, que durante a formação da gema ou do albúmen resultará em ovos já contaminados, quanto à transmissão horizontal, aquela pela qual há a passagem do ovo com casca pela cloaca e o seu contato com excretas, ou em equipamentos e embalagens gerando a contaminação (BARCELOS, 2017).

O microrganismo, após penetrar pelos poros da casca, chega à gema e ocasiona inúmeras alterações, como sua desintegração pela oxidação de ácidos graxos, escurecimento da coloração amarela, odor desagradável devido a produção de gás sulfídrico, indol e ureia, e com isso também a liquefação do albúmen, consequentemente alterando o sabor dos ovos, tornando-os impróprios para consumo (FRAZIER; WESTHOFF, 2000).

Além disso, ovos mantidos em temperatura elevada tanto em armazenamento como durante a comercialização, têm maior probabilidade de possuir microrganismos patogênicos como *Salmonella* spp., *Escherichia coli, Staphylococcus aureus, Streptococcus, Aspergillus fumigatus* e organismos esporulados (STRINGHINI, 2008), pois, por ser um produto com alta concentração de nutrientes aliado a manutenção em altas temperaturas, propicia ainda mais a sobrevivência e rápida multiplicação bacteriana. Mesmo assim, a legislação brasileira não exige a refrigeração dos ovos desde sua coleta até a chegada ao consumidor final, aumentando ainda mais o risco de disseminação de patógenos (XAVIER et al., 2008).

Dentre estes microrganismos e que podem determinar alterações significativas na qualidade de ovos, está a *Escherichia coli* (SILVA LIMA et al., 2018), a qual, representa 95% das bactérias que compõem o grupo dos coliformes fecais (LACERDA, 2011), sendo considerado um dos principais indicadores de qualidade microbiológica de alimentos.

1.1.3 *Escherichia coli*: COLIBACILOSE

A *E. coli* é uma bactéria pertencente à família *Enterobacteriaceae*, descrita pela primeira vez em 1884, pelo microbiologista alemão Theodor Escherich, típico bacilo Gram negativo, fermentadora de lactose e produtora de indol, possui membrana externa composta de lipopolissacarídeos (LPS), não formadora de esporos, móvel ou imóvel quando há presença de flagelos peritríquios e metabolismo respiratório facultativo (QUINN et al., 2005).

É a primeira bactéria a colonizar o intestino das aves, através de penetração pela casca dos ovos e pelo contato após o nascimento com a mãe e meio ambiente. Cepas não patogênicas são consideradas desejáveis e benéficas, uma vez que a presença deste agente exerce efeito protetor contra a inserção de outros microrganismos como a *Salmonella* spp., além disso, auxilia nos processos de digestão de alimentos, síntese e absorção de nutrientes (FERREIRA; KNÖBL, 2009).

No entanto, subgrupos de *E. coli* apresentam fatores de virulência que os tornam capazes de causar doenças. Na produção avícola a *Avian Pathogenic E. coli* (APEC) de origem extraintestinal é responsável pela colibacilose, grave doença caracterizada por

quadros respiratórios e septicêmicos em aves de todas as idades (CAMARGO; SUFFREDINI, 2015), sendo a principal causa mundial de perdas econômicas neste setor devido à morbidade e mortalidade, ocasionadas pela doença (HUSSEIN et al., 2013).

Os fatores de virulência de APEC, ainda não estão completamente elucidados, no entanto, os mais frequentemente descritos incluem expressão de adesinas, enterotoxinas, sistemas de captação de ferro no sangue e nos tecidos do hospedeiro e resistência a ação microbicida do soro do hospedeiro com fator citotóxico necrosante e hemolisina (RODRIGUEZ-SIEK et al., 2005; JOHNSON et al., 2008). Todos estes fatores contribuem para a sobrevivência e rápida evolução da doença (FERREIRA; KNOBL, 2009).

A principal porta de entrada utilizada pela *E. coli* é o trato respiratório superior, com a adesão das fímbrias do microrganismo as células ciliadas epiteliais da traqueia e faringe, onde multiplicam-se e posteriormente invadem a corrente sanguínea, se disseminando para fígado, pulmão, coração e sacos aéreos (MALO, 2009).

A progressão da doença pode ser favorecida por inúmeros fatores, como ocorrência de lesões no trato respiratório superior devido à poeira e altas concentrações de amônia aliada a criações com alta densidade e processos de desinfecção ineficientes (FERREIRA; KNOBL, 2009) e principalmente o estado imunológico dos animais que não são capazes de bloquear a multiplicação do patógeno (POURBAKHSH et al., 1997), além disso, as cepas podem permanecer por longos períodos no ambiente, contaminando água, cama e ração que serão fonte de disseminação para as demais aves (FERREIRA; KNOBL, 2009).

A bactéria pode causar lesões em diferentes órgãos, geralmente em aves está associada aos quadros de: colisepticemia, peritonite, pneumonia, pleuropneumonia, aerossaculite, pericardite, celulite, coligranuloma, doença crônica respiratória complicada (DCR), onfalite, salpingite síndrome da cabeça inchada, panoftalmia, osteomielite, sinovite e artrite (FERREIRA; KNOBL, 2009). Há também sinais clínicos inespecíficos, como aumento de mortalidade e conversão alimentar, sonolência ou prostração, baixo consumo de ração e ganho de peso, baixa uniformidade, aumento da refugagem no lote e diarreia (CAMPOS; TRABULSI, 1999).

Apesar da *E. coli* acometer as aves em diferentes idades, a sua susceptibilidade e a severidade dos sinais clínicos são maior em aves mais jovens, porém quando presente em aves adultas é considerado o patógeno de maior preocupação sanitária devido aos prejuízos

produtivos que acarreta, pois pode diminuir em até 70% a produção de ovos em galinhas de postura (AHMED et al., 2013).

Essa bactéria geralmente infecta matrizes e galinhas em pico de postura, quando os animais estão em estresse continuo devido à produção intensa e ocorrência de diversos eventos metabólicos, como o aumento dos níveis de estrógeno no organismo promovendo uma hipertrofia do tecido uterino e maior secreção glandular, favorecendo a ocorrência da infecção via ascendente pela cloaca ao oviduto e sacos aéreos (BERCHIERI JUNIOR; MACARI, 2009), podendo levar a morte dos animais entre 6 a 12 horas (BARNES et al., 2008).

Em matrizes de corte são observados altos índices de mortalidade devido á peritonite em decorrência da infecção. Porém, em alguns plantéis nenhum sinal clínico está presente e, consequentemente, nenhuma galinha doente é observada, fêmeas que morrem estão em boas condições corporais, no entanto, com baixa produção de ovos (CAMPOS; TRABULSI, 1999).

Outra habilidade característica da *E. coli* é de resistir aos fatores séricos inibitórios permitindo seu escape da ação do sistema complemento e da fagocitose nos processos de infecção sistêmica. Esta resistência sérica tem sido atribuída a diversas estruturas destas células incluindo o lipopolissacarídeo (LPS), a cápsula K1 e as proteínas de membrana externa (OMPs), além da produção de endotoxinas pela liberação de complexos lipopolissacarídeos de suas paredes celulares durante a lise, ocasionando febre e morte dos hospedeiros (MOREIRA, 2007).

Com a proibição da utilização de antimicrobianos na forma de aditivo na alimentação animal, a ocorrência da colibacilose aviária vem se acentuando. Com isso, novas alternativas com a utilização de extratos vegetais que atuem como fitoterápicos e possuem efeito antibacteriano e mantenham a eficiência produtiva tornaram-se de extrema relevância e valia (DONO, 2013), com destaque especial para a curcumina, componente herbário obtido a partir da planta *Curcuma longa*, com inúmeros benefícios comprovados e amplo uso na alimentação animal (RAJPUT et al., 2013).

1.1.4 *Curcuma longa*: CURCUMINA

1.1.4.1 Origem

A curcumina é um composto fenólico natural extraído do rizoma do açafrão da Índia também popularmente conhecido como açafrão da terra (*Curcuma longa*) pertencente à família Zingiberaceae, é uma planta herbácea originária do sudeste asiático. Essa planta é amplamente cultivada em todo mundo, com relatos de sua utilização pelo homem nos últimos 6000 anos com fins medicinais, como especiaria, tempero e corante natural (MALA et al., 1995).

A parte do vegetal com maior utilização é o rizoma, onde os curcuminóides são em sua maioria acumulados, que pode ser consumido fresco ou seco. Para fins de conservação e comercialização, esse rizoma é desidratado e moído, gerando um pó de coloração amarelada a vermelho alaranjada (LI et al., 2011). Sendo que na Índia pode-se encontrar este composto na forma de cápsulas, pomadas, unguentos, cremes e curativos para aplicação tópica, misturada ou não com outros componentes.

Na alimentação animal, a curcumina vem apresentando inúmeros benefícios nas mais diferentes espécies animais, como ação hepatoprotetora em frangos de corte submetidos à intoxicação por aflatoxina (YARRU et al., 2009), efeito antibacteriano contra salmonelose (VARMUZOVA et al., 2015), efeito antiparasitário e anti-inflamatório em peixes (MALLO et al., 2017), coccidiostático (GALLI et al., 2018) e ação antioxidante (MARCHIORI et al., 2019).

Em ovinos é descrita como eficaz em tratamento de mastite subclínica (JAGUEZESKI et al., 2018), promovendo maior ganho de peso de cordeiros em crescimento (MOLOSSE et al., 2019), menor oxidação lipídica de produtos farmacêuticos, mantendo qualidade por longos períodos de armazenamento (AK; GÜÇIN, 2008).

Na avicultura, de acordo com pesquisa de Al-Jaleel (2012), dosagens menores que 2% incorporadas à ração propiciam a ação de seus componentes antioxidantes, estimulando a síntese proteica pela ave e apresentando maior conversão alimentar em frangos de corte, enquanto que Abdel-Rahman et al. (2014), observaram maior profundidade de criptas para aves que receberam cúrcuma em pó em associação com feno-grego via dieta.

1.1.4.2 ATIVIDADE ANTIMICROBIANA DA CURCUMINA

O mecanismo de ação antimicrobiano da curcumina ainda não se encontra completamente elucidado, no entanto, tem sido demonstrada sua ampla ação contra diversos microrganismos de importância veterinária. Em sua forma microencapsulada, Wang et al. (2009), obtiveram efeito antimicrobiano para cepas de *Escherichia coli*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Aspergillus níger* e *Penicillium notatum*.

Nascimento et al. (2019), relataram que a cúrcuma na ração de frangos de corte é hábil em impedir a colonização intestinal de bactérias patogênicas, indicando que a curcumina a 1% é capaz de expressar suas atividades antimicrobianas, promovendo um desequilíbrio na população de bactérias componentes da microbiota e principalmente contra as bactérias inoculadas experimentalmente.

Alguns autores supõem que os grupamentos metoxila e hidroxila presentes na curcumina sejam os responsáveis pela sua ação antimicrobiana (KAUR et al., 2010; HAN; YANG, 2005; RAI et al., 2008) e que sua forma de ação esteja relacionada pela interação com a proteína FtsZ do citoesqueleto bacteriano, impedindo sua atuação na citocinese bacteriana.

A FtsZ (Filamentation Temperature-Sensitive protein Z) é uma proteína altamente conservada em bactérias, sendo a primeira a sinalizar o futuro sitio de divisão bacteriana. Suas moléculas se entrelaçam formando filamentos que irão se agrupar na formação de um anel contrátil, chamado de anel Z, que marca o local onde ocorrerá a divisão (HURLEY et al, 2016). Este anel serve como uma estrutura organizadora para montagem do divisoma, recrutando proteínas que agem de forma coordenada promovendo a invaginação da membrana e da parede celular, formando o septo de divisão que divide a célula-mãe em duas células-filhas idênticas (MEIER; GOLEY, 2014).

Em experimento realizado por Kaur et al. (2010) e Rai et al. (2008), utilizaram cepas de *Bacillus subtilis* e *E. coli*, onde comprovaram que os oxigênios ligados aos anéis fenólicos estruturais da curcumina (funções fenol e metoxila) juntamente com suas carbonilas impediram a polimerização da proteína FtsZ e consequentemente a divisão celular bacteriana destas cepas.

Demais pesquisas demonstram sua atividade nas mais diversas formas utilizadas, em associação com cobalto na inibição de *E. coli* (HATAMIE et al., 2012), como nanocristais em água (BHAWANA et al., 2011), em combinação com sulfanamidas impedindo, além da *E. coli*, a replicação de *S. aureus, B. subtilis* e *Salmonella typhimurium* (LAL et al., 2013).

1.1.5 ESTRESSE OXIDATIVO

Frente as lesões ocasionadas por este patógeno e a disseminação da doença via corrente sanguínea, o organismo animal apresenta mecanismos endógenos e exógenos de defesa, que atuam prevenindo, reparando ou inativando as reações moleculares dos elétrons desemparelhados ocasionados pela alta produção de espécies reativas ao oxigênio (EROs), ocasionando estresse oxidativo (MIROŃCZUK-CHODAKOWSKA et al., 2018).

Estresse oxidativo é conhecido pelo desiquilíbrio entre oxidantes e antioxidantes, sendo que o sistema antioxidante pode ser enzimático e não enzimático. Na produção animal, o estresse oxidativo tem sido relacionado com numerosas afecções, como sepse, mastite, enterites, pneumonia, doenças respiratórias e articulares (CELI, 2010). A oxidação é parte fundamental do metabolismo celular, no entanto, a produção de espécies reativas ao oxigênio (EROs) eleva-se em lesões teciduais por traumas, infecções, parasitoses, hipóxia e produção de toxinas, devido a ativação da fagocitose, liberação de ferro e cobre ou interrupção da cadeia transportadora de elétrons (ROCK et al., 1996).

Em processos inflamatórios a presença prolongada e exacerbada de EROs produzidas pelo organismo como forma de defesa, podem ocasionar danos irreversíveis, como a peroxidação lipídica, ruptura de DNA e de proteínas teciduais, inativação de enzimas (MOHANTY et al., 2012) e impedimento de remodelação de tecidos lesionados e cicatrização de ferimentos (THANGAPAZHAM et al., 2013).

No entanto, conjuntamente com a produção exacerbada de EROs, enzimas antioxidantes também são sintetizadas com a função de proteção e neutralização dos radicais livres, através da capacidade de doar elétrons (SAEIDNIA; ABDOLLAHI, 2013) e fornecer proteção celular eficiente. De acordo com a literatura, como principais enzimas antioxidantes importante para minimizar a exacerbação do estresse oxidativo destaca-se a glutationa redutase (GSH), superóxido dismutase (SOD), glutationa peroxidase (GPx),

glutationa S-transferase (GST) e catalase (CAT) (EL-BAHR, 2015; GARCÍA-NIÑO et al., 2015). O sistema antioxidante não-enzimático, também faz parte do grupo endógeno e compõe-se de melatonina, bilirrubina, proteínas de ligação de metal, ácido úrico, poliaminas, entre outros (MIROŃCZUK-CHODAKOWSKA et al., 2018).

A catalase é uma enzima encontrada no sangue, medula óssea, mucosa, rim e fígado e sua atividade é dependente de Nicotinamide Adenosine Dinucleotide Phosphate (NADPH) (FERREIRA; MATSUBARA, 1997). Esta enzima evita o acúmulo da metahemolgobina e reage com o H_2O_2, formando H_2O e O_2. Desempenha um papel importante na resposta adaptativa das células e na tolerância ao estresse oxidativo (MATÉS; SÁNCHES-JIMENEZ, 1999), sendo uma das principais defesas antioxidantes que atuam no organismo frente a infecções, juntamente com a glutationa peroxidase impedindo o acúmulo de peróxido de hidrogênio (HALLIWELL; GUTTERIDGE, 1989).

Já a enzima superóxido dismutase (SOD) tem papel fundamental no sistema imune, pois catalisa a remoção do radical superóxido. Antes, esta enzima já foi descrita como uma peroteína que contém cobre, mas não se sabia de nenhuma atividade catalítica que ela poderia realizar (HALLIWELL; GUTTERIDGE, 1989). A forma que contém cobre e zinco em sua forma estrutural é denominada de superóxido dismutase cobre-zinco dependente (Cu Zn SOD) e está presente no citosol de todas as células eucarióticas, enquanto que a superóxido dismutase dependente de manganês (MnSOD), está presente na mitocôndria, sendo uma das mais importantes defesas antioxidante das células expostas aos radicais livres durante o estresse oxidativo (MATÉS; SÁNCHES-JIMENEZ, 1999).

1.1.6 SISTEMA PURINÉRGICO

A sinalização purinérgica é uma importante via modulatória do sistema nervoso central, estando envolvida em muitos mecanismos neuronais e não neuronais e em eventos de curta e longa duração, incluindo secreção exócrina e endócrina, respostas imunes, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK, 2006).

Fazem parte deste sistema, três componentes principais: nucleotídeos e nucleosídeos extracelulares, adenosina trifosfato (ATP), adenosina difosfato (ADP) e adenosina monofosfato (AMP), seus receptores e as ectoenzimas responsáveis pela

regulação dos níveis dessas moléculas com a finalidade de manutenção da homeostase de todo o organismo (YEGUTKIN, 2008).

O ATP é uma molécula energética, também conhecida como um sinal de dano, ou padrão molecular associado ao dano (DI VIRGILIO, 2005). Quando liberado, contribui para o desencadeamento da resposta inflamatória juntamente com os padrões moleculares associados a patógenos com a finalidade de reparação do processo inflamatório associado a traumas ou processos infecciosos (MARIATHASAN; MONACK, 2007). Sua liberação nos terminais pré e pós-sinápticos pode ocorrer como um mecanismo fisiológico, ou em resposta a danos celulares, como a hipóxia e injúrias (BURNSTOCK, 2006).

No entanto, este nucleotídeo também pode ser armazenado em vesículas sinápticas, sendo liberado por exocitose como um co-transmissor, juntamente com neurotransmissores como a acetilcolina (ACh) e o glutamato (ZIMMERMANN, 2000) ou nas células neuronais e nas células não neuronais através de transportadores que se ligam a esse nucleotídeo ou via canais acoplados à conexina ou panexina (SABIROV; OKADA, 2005).

O ATP, dependendo da sua concentração e local de ação, possui ações pró e anti-inflamatórias, dependendo da sua concentração extracelular e tipo de receptor envolvido (DI VIRGILIO et al., 2009). Em situações pró-inflamatórias, ocorre à estimulação e proliferação de linfócitos, sendo necessário para a secreção de importantes citocinas dependentes das células T, como interferon gama (INF-γ) e interleucina (IL-2), intimamente envolvidas na indução de resposta imune a antígenos estranhos (SNEDDON et al., 1999). Além disso, está envolvido no recrutamento de monócitos para tecidos, na produção de interleucina 1β e fator de necrose tumoral alfa (TNF-α) por macrófagos (ELSSNER et al., 2004) e na migração e diferenciação de células dendríticas (LA SALA et al., 2003).

Porém, contraditoriamente, o ATP extracelular também pode desempenhar um papel imunossupressor, por inibir a proliferação de células T e, consequentemente, bloquear a liberação de citocinas próinflamatórias (DEAGLIO et al., 2007). Esse mecanismo se dá principalmente quando o ATP está em baixas concentrações extracelulares, aumentando a sua afinidade por receptores do tipo P2Y, localizados na superfície dos linfócitos. Estes receptores quando estimulados promovem uma liberação de citocinas pró-inflamatórias,

estimulam uma resposta Th2, e levam a liberação de citocinas anti-inflamatórias, promovendo um efeito protetor contra danos teciduais excessivos (BOURS et al., 2006).

As concentrações dos nucleotídeos e nucleosídeos extracelulares, em muitos tecidos, são reguladas pela ação de enzimas ancoradas à membrana celular, com o seu sítio catalítico voltado para o meio extracelular. Essas enzimas hidrolisam os nucleotídeos extracelulares em seus respectivos nucleosídeos, e dentre elas estão as E-NTPDases, que hidrolisam tanto ATP quanto ADP a AMP, na presença de cátions divalentes como cálcio e magnésio, e a E-5‟-nucleotidase com a hidrólise dos nucleotídeos monofosfatados, resultando em adenosina. Esta por sua vez é hidrolisada pela enzima adenosina deaminase (E-ADA), transformando a adenosina em inosina, seu metabólito inativo (ZIMMERMANN, 1996).

A enzima E-5‟-nucleotidase também desempenha funções não enzimáticas, como a indução da sinalização intracelular e mediação de processos de adesão célula-célula e célula-matriz e de migração (VOGEL et al., 1991). O aumento na atividade dessas enzimas pode surgir como um mecanismo adaptativo com a finalidade de reduzir os níveis de ATP e aumentar os níveis de adenosina no meio, conferindo neuroproteção (KAIZER et al., 2007).

Já o grupo de enzimas das ectonucleotidases é constituído pelas famílias das ectonucleotídeo pirofosfatase/fosfodiesterase (E-NPP), ectonucleosideo trifosfato difosfoidrolases (E-NTPDases), ecto-5‟-nucleotidase e fosfatases alcalinas, que estão situadas na membrana celular, possuindo seu sítio ativo voltado para o meio extracelular, ou na forma solúvel no meio intersticial (ZIMMERMANN, 2011). A E-NTPDase desempenha importante controle da função dos linfócitos, incluindo o reconhecimento do antígeno e ativação de funções efetoras das células T citotóxicas (FILIPPINI et al., 1990), além de gerar e amplificar sinais de interação célula a célula em um organismo parasitado (KACZMAREK et al., 1996).

1.2 OBJETIVOS

1.2.1 OBJETIVO GERAL

Verificar se a adição de curcumina na dieta de aves de produção com colibacilose é uma alternativa eficaz para minimizar os efeitos negativos causados pela doença.

1.2.2 OBJETIVOS ESPECÍFICOS

- Avaliar se a adição de curcumina na dieta de poedeiras infectadas naturalmente por *Escherichia coli*, tem efeitos sobre produção, qualidade físico química de ovos e efeito antimicrobiano.

- Analisar se o consumo de ração contendo curcumina na dieta reduz os níveis oxidativos e aumenta os níveis de antioxidantes a níveis séricos e em ovos frescos e armazenados.

- Avaliar se a infecção experimental por *E. coli* causa estresse oxidativo.

- Avaliar se a infecção experimental por *E. coli* interfere no metabolismo energético.

-Avaliar se a infecção experimental por *E. coli* tem participação da via anti-inflamatória colinérgica.

- Avaliar se a infecção experimental por *E. coli* aumenta a atividade de enzimas relacionadas ao sistema purinérgico a fim de estimular a resposta inflamatória.

- Mensurar os impactos sobre o desempenho de aves jovens e eficiência das galinhas e matrizes.

CAPÍTULO II

2. ARTIGOS E MANUSCRITO

Os resultados desta dissertação são apresentados na forma de três artigos e um manuscrito, com sua formatação de acordo com as orientações das revistas aos quais foram publicados e submetido.

2.1 – ARTIGO I

Impact of colibacillosis on production in laying hens associated with interference of the phosphotransfer network and oxidative stress

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Impact of colibacillosis on production in laying hens associated with interference of the phosphotransfer network and oxidative stress

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ABSTRACT

The objective of this study was to evaluate the productive impact of colibacillosis on laying hens and to investigate whether energetic metabolism and oxidative stress were involved in the pathogenesis of the disease. An experimental shed containing 270 laying hens of the Hy-Line lineage (32 weeks old) presented approximately 40% daily laying, and many birds presented with diarrhea and apathy followed by death. Necropsy revealed macroscopic lesions compatible with colibacillosis and infectious agent *Escherichia coli* was isolated from fecal samples of all birds in the infected group, as well as from tissue (ovary, liver and peritoneum). Sixteen chickens were selected for this study, divided into two groups: Control (animals without clinical alterations) and infected (with diarrhea and apathetic). *E. coli* isolates were subjected to the antimicrobial susceptibility testing according to the methodology approved by CLSI, 2018. This testing showed sensitivity to gentamicin, amoxicillin, norfloxacin and colistin. It was then determined that laying hens would be treated with norfloxacin (15 mg/kg) diluted in water offered at will to the birds for three days. Blood collections were performed via brachial vein after the diagnosis of *E. coli* (before starting treatment) and seven days after treatment. Three debilitated chickens died on the second day after initiating therapy. Before treatment, birds with clinical signs had higher levels of lipoperoxidation (LPO) and activities of antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPx) than in the control group (asymptomatic animals). After treatment, LPO levels remained higher in birds that had clinical disease (infected group), whereas the activity of SOD and GPx enzymes did not differ between groups. Activity levels of creatine kinase (CK) and pyruvate kinase (PK) were higher in the group of chickens with clinical disease before treatment. Post-treatment, no differences were observed between groups in terms of CK; however, PK activity remained high in these animals. In the hens that died, there were lesions characteristic of avian colibacillosis, with ovary involvement, explaining the low laying activity of the birds at their peak of production. For 10 days after starting treatment, the percentage of laying increased to 90%. Therefore, we conclude that colibacillosis interferes with the phosphotransfer network by stimulating ATP production, in addition to causing oxidative stress of the birds during laying, that negatively affects health and productive efficiency.

Keywords: Poultry; Biochemical Disorders; *Escherichia coli*; pathology.

1. Introduction

Avian colibacillosis is an infectious disease caused by *Escherichia coli*, an extra-intestinal pathogen (ExPEC) responsible for respiratory infections and septicemia in birds [1]. Colibacillosis has a great economic impact because of mortality and productivity decreases of up to 70% in laying birds [2]. The main risk factors that contribute to the development of the disease is inadequate environment, respiratory diseases, immunosuppressive factors and animal metabolism [3]. In addition to controlling environmental conditions, including moisture, ventilation and chlorination of drinking water, control of the disease can be achieved by antibiotics or by preventive vaccination [4].

In birds as well as mammals, the immune response to infectious diseases produces free radicals as a way to destroy invading organisms, causing oxidation of lipids, DNA and protein molecules via an imbalance between the production of these compounds and the antioxidant defenses; collectively, this is known as oxidative stress [5]. Oxidative stress directly interferes with the energy metabolism that responsible for providing energy, maintaining the osmotic balance and guaranteeing vital functions [6]. According to the literature, these biochemical alterations negatively affect the health and production of the organism [7, 8, 9].

Considering that Brazil is one of the world's largest producers and exporters of eggs, it is extremely important to identify and control colibacillosis. This is because colibacillosis is one of the major infectious diseases affecting the production and health of laying birds; therefore, knowledge of the mechanisms involved in the pathogenesis of this disease can indicate tools for prevention, diagnosis and control. Therefore, the objective of this study was to report the productive impact of colibacillosis on laying hens and to investigate whether an imbalance in energy metabolism occurs, as well as whether oxidative stress is involved in the pathogenesis of the disease.

2. Materials and Methods

2.1. Animals and history

A group of 270 laying hens of the Hy-Line lineage, 32 weeks of age, were maintained in an experimental coop. The percentage of laying was approximately 40%, well below the expected standard for this age group and lineage. Diarrhea and apathy were seen in approximately 15% of the chickens, associated with the mortality of 12 birds in a three-day interval. Three of these laying hens underwent necropsy, at which time tissue samples were collected for histopathological (preserved in 10% formaldehyde) and microbiological analyses. Macroscopically, pericarditis, necrotic spots on the liver, hepatic congestion, fibrin coating, airsacculitis, mesentery thickening and salpingitis with diffuse caseous material were observed (Figure 1). In the heart, liver, ovaries and bursa of Fabricius, no histopathological lesions were observed. However, in the small intestine, moderate-to-severe multifocal deposition of fibrin was associated with moderate multifocal heterophilic inflammatory infiltrates (Figure 2).

2.2. Microbiological Analysis: Isolation

First, the infectious agent was isolated from feces and tissue samples (ovary, liver and peritoneum) of the dead chickens prior to the experiment, as described below. Stool and tissue were aseptically weighed (1g each) and homogenized in 9 mL of brain-heart infusion (BHI) broth in sterile tubes and later incubated in a bacterial oven 36 ± 1 °C for 24 h growth. Subsequently, using the depletion technique, both samples were duplicated in Petri dishes containing eosin methylene blue (EMB) agar and MacConkey agar, and then incubated at 36 ± 1 °C for 24 hours. Three to five colony forming units (CFU), had characteristics of *E. coli* (metallic green colonies on EMB agar and fermentation of lactose in MacConkey agar) were selected and subjected to biochemical tests: with a sterile platinum needle, the same colony was inoculated for biochemical tests on base-urea agar, triple sugar iron agar, hydrogen sulphite agar, indole and motility, and Simmons citrate agar and later incubated at 36 ± 1 °C for 24 hours, and the results were read

The collected samples were also used for *Salmonella* spp. An aliquot of 1 mL of the culture added to BHI was transferred to 9 mL of selenite cystine broth and 1–9 mL of Rappaport-Vassiliadis broth and incubated in a bacteriological oven at 37 °C for 24 hours. Then, aliquots of the aforementioned broths were plated by the seeding technique in duplicate for selective media: Brilliant green agar and Hektoen enteric agar, and were again incubated at 37

°C for 24 hours. Three to five CFU were harvested in triple-sugar iron agar (TSI), and subjected to the urease test, indole production, methyl red, motility, lysine decarboxylase, malonate test and Simmons citrate, and incubated at 37 °C for 24 hours.

In all stool and tissue samples analyzed, *E. coli* were isolated. There was no isolation of the bacterium *Salmonella* spp. in the samples tested.

2.3. E. coli susceptibility testing

For the antimicrobial susceptibility test, seven isolates from three sacrificed hens (section 2.1) were used, including ovaries (n = 3) and livers (n = 3) (one from each hen), as well as *E. coli* isolates from the peritoneum (n = 1). For antimicrobial susceptibility testing, we used the methodology approved by the Clinical and Laboratory Standards Institute [10] and the National Agency for Sanitary Surveillance [11], included in the IN M-2A-8 Standardization of Antimicrobial Sensitivity Tests by disk-diffusion using Mueller-Hinton agar. Various classes of antimicrobials (ATBs) with different mechanisms of action were tested: beta-lactams (amoxicillin plus clavulanic acid 30 μg - AMC); third-generation cephalosporins (ceftiofur 30 μg - CTF); fluoroquinolones (enrofloxacin 5 μg - ENO); quinolones (ciprofloxacin 5 μg - CIP, norfloxacin 10 μg - NOR, nalidixic acid 30 μg - NAL); aminoglycosides (gentamicin 10 μg - GEM); sulfonamides (trimethoprim/sulfamethoxazole 25 μg - SUT); polymyxins (colistin 10 μg - COL); and tetracyclines (tetracycline 30 μg - TET). After 24 hours of incubation, the plates were subjected to reading, measuring the diameter of the inhibitory halos of each disk and classified according to the tables described by the CLSI (2018), classifying the *E. coli* isolates into sensitive, intermediate or resistant. The samples that presented an intermediate profile was considered resistant [11]. For control, we used a sample of *Escherichia coli* ATCC® 25922.

2.4. Experimental design and treatment

Sixteen hens were selected for biochemical analyses. *E. coli* was isolated from the feces of the 16 chickens, according to methodology previously described. Hens were divided into two groups: infected and with clinical signs of diarrhea and apathy (n = 8) and without clinical signs used as controls (n = 8). All hens used as controls were apparently

healthy with daily laying. Based on the antibiotic sensitivity results, we treated the animals for three consecutive days using norfloxacin diluted in water (200 g in 1000 L), corresponding to a dose of 15 mg/kg. Antimicrobial delivery began 24 hours after diagnosis (day 1). Norfloxacin was chosen for the treatment for laying hens because of its effectiveness in the *in vitro* test, its availability in our laboratory, its wide use in the western region of Santa Catarina, and its easy administration.

2.5. Blood samples

Blood samples were taken via the brachial route at two time points: the day of confirmation of the diagnosis of colibacillosis (day 0 of the experiment) and seven days after the start of treatment. Blood was placed in tubes without anticoagulant, centrifuged (5500 rpm for 10 minutes) and serum was frozen in a microtube (-20 °C) until analysis.

2.6. Protein levels

Serum protein concentrations were determined by the Bradford method using the BG-250 Coomassie brilliant blue dye, following the methodology described by Read and Northcote [12], using bovine serum albumin as the standard.

2.7. Lipid peroxidation

To evaluate lipid peroxidation (LPO), serum samples were diluted (1:1 v/v) in cold methanol and centrifuged at 1000 x *g* for 10 min at 4 °C. This technique is based on the reaction of hydroperoxides present in the sample with Fe^{2+} ($FeSO_4$) in acid medium (H_2SO_4) in the presence of xylenol orange dye, as proposed by Monserrat et al. [13], and described in detail by da Silva Barreto et al. [14]. Serum LPO was measured in the supernatants using a microplate reader at 550 nm using cumene hydroperoxide as the standard. The results were expressed as nmol CHP/mL serum.

2.8. Superoxide dismutase (SOD) and glutathione peroxidase (GPx)

Superoxide dismutase activity was determined according to the auto-oxidation principle of pyrogallol, inhibited in the presence of SOD. The optical density change was determined kinetically for two minutes at 420 nm, at ten-second intervals, according to the methodology described by Beutler [15], and the activity was expressed in U SOD/mL. Glutathione peroxidase (GPx) was measured indirectly through the oxidation rate of NADPH at 340 nm using copper hydroperoxide (CuOOH), according to Wendel [16]; and results were expressed as U GPx/mL.

2.9. Serum creatine kinase and pyruvate kinase activities

Creatine kinase (CK) activity was evaluated based on the colorimetric method established by Hughes [17], that estimates creatine levels at wavelength 540 nm, as reported in detail by Baldissera et al. [18]. The results were expressed as nmol of creatine formed by min per mg of protein. For pyruvate kinase (PK) activity, we used the methodology described by Leong et al. [19], at a wavelength of 340 nm, as also reported in detail by Baldissera et al. [18]. The results were expressed as nmol of pyruvate formed per min per mg of protein.

2.10. Statistical analysis

The data were first subjected to normality testing. All data that did not present normal distribution were transformed into logarithms. Thereafter, a two-way analysis of variance was applied for comparison between groups and over time. Results were presented as mean and standard deviation. Significance was considered when $P < 0.05$.

3. Results

The *E. coli* isolate was sensitive to the following antimicrobials: gentamicin, amoxicillin, norfloxacin and colistin. The bacterium proved to be resistant or intermediately

resistant to the other antibiotics tested (Table 1). The results of the susceptibility tests were the same for all isolates.

Three chickens were infected with *E. coli* and became debilitated despite treatment, and died on the second day of antibiotic therapy by euthanasia. They underwent histopathological analysis. The lesions were similar to those previously described in the methodology section and illustrated in Figures 1 and 2. After this period, there were no deaths, as we observed no further symptoms of diarrhea or apathy. Prior to treatment, the laying percentage was 40%; however, between seven and ten days after administration of norfloxacin, the productive efficiency was again evaluated, and the percentage of laying rose to 90%. To evaluate the pathogenicity of the *E. coli* isolates, they were inoculated in 10 young hens (30 days old), causing severe disease with pericarditis, hepatic congestion, fibrin coating, airsacculitis and salpingitis; these lesions similar to those described in the case of natural infection, section 2.1.

Before treatment, chickens infected with *E. coli* with clinical signs had higher levels of LPO and activity of SOD and GPx enzymes compared to asymptomatic (control) birds ($P < 0.05$, Figure 2). After treatment, LPO levels remained higher in the animals that had clinical disease ($P < 0.05$), whereas SOD and GPx did not differ between groups ($P > 0.05$). Over time (i.e., after treatment), we observed a significant reduction in GPx activity. LPO and SOD were numerically smaller after treatment, but with no differences over time.

CK and PK activity was higher in the group of chickens with clinical disease before treatment (Figure 4). After treatment, no differences were observed between groups for CK ($P> 0.05$), however PK activity remained high in animals that had clinical disease (Figure 4). Over time, no differences were observed for the enzymes CK and PK ($P > 0.05$).

4. Discussion

In this study, animals with colibacillosis displayed alterations in the activity of enzymes involved in energy metabolism and oxidative stress. According to the literature, oxidative stress is a metabolic response induced by infections by microorganisms; it contributes to several aspects of pathogenesis of several infectious diseases, including

apoptosis, loss of immune function, and replication and inflammatory responses [20], because of the action of free radicals originating in large quantity in lesioned tissues [21].

We found high levels for SOD and GPx in order to minimize the negative effects of lipoperoxidation, because SOD acts by a superoxide anion dismutation mechanism, producing hydrogen peroxide (H_2O_2) [22] in an attempt to protect cellular targets against the attack of this anion. Avoiding or minimizing the harmful effects that this reactive species is mediated by this enzyme, along with vitamin E, neutralizing radicals via catalysis [23]. Similarly, GPx is responsible for the immune defense and maintenance of intracellular homeostasis, being detected at high levels when the organism endures considerable periods of oxidative stress [5]. Elevated levels of LPO measured in this study before and after treatment with norfloxacin indicated exacerbated lipid peroxidation. Certainly the lipoperoxidation is related to the presence of lesions observed in the intestine, abdomen and thorax, because the polyunsaturated fatty acids of the cell membranes when being degraded cause their disintegration and destruction, an injury that is demonstrated by increases in formation of MDA that determines the extent of oxidative damage in pathological conditions [24], as well as justified by the high levels of GPx and SOD in this group, as part of the body's attempt to maintain its homeostasis and cellular and tissue defense.

Oxidative stress is an undesirable pathological condition. According to researchers, oxidative stress is an important mechanism of metabolic damage in broiler chickens, and is considered one of the main pathologies that affects their growth and development, as well as the quality of their final product, because this dysfunction is one of the main causes of food spoilage during and after processing [25]. *E. coli* is a frequently observed bacterium in the feces of broiler chickens [26], and thus it may contribute considerably to exacerbation of oxidative stress in broilers, as seen in chickens in the present study.

In poultry, the peak production phase is around 32 to 35 weeks of age, when the greatest predisposition to pathological alterations due to *E. coli* occurs. Infection is associated with sex hormone levels, as reported in experimental infections, where colonization of the oviduct by *E. coli* was greater when estrogen levels were high, and peritonitis followed initial colonization by *E. coli* from the sewer [27]; at peak stage, because of high egg production, the immune system of these animals is weakened, predisposing them to infection. Ferreira and Knobl [26] emphasized that the bacterial

strains remain in the environment for long periods, contaminating the bed, water, air and feed that serve as the route of dissemination to other animals. Systemic cases of *E. coli* infection are usually caused by primary respiratory tract infections that spread to various organs. In these cases, tracheitis, pneumonia, airsacculitis, pericarditis and polyserositis [28] are observed, negatively affecting energy metabolism.

The production and consumption of intracellular ATP are fundamental to the bioenergetics of living organisms, maintaining a broad range of cellular activities. Nevertheless, the interaction of enzymes in energy production appears to be insufficient to satisfy all cellular energetic needs for tissue [18]. The damage to lipids may be related to the impairment of CK and PK activities [18] as observed in the present study, i.e., elevated CK and PK activities were observed in clinically ill chickens. According to the literature, CK is an enzyme active in recent muscle injury; its high levels affirm the occurrence of cell damage by the pathogen in the disease cycle; and PK as well may be moderately elevated when muscle damage occurs [29]. CK is located in muscle, heart and brain tissue. In muscle, it catalyzes the conversion of ATP and creatine to phosphorylated creatine, serving as a high energy phosphate donor for muscle activity [30]. Therefore, increases in enzyme activity in laying hens may be associated with muscle injuries [31]; however, because it is a nonspecific enzyme, the possible causes of elevated levels include trauma, forced exercise, restraint, intramuscular injection of irritant fluids and systemic infections affecting the skeletal or cardiac muscle [32], which in this study was colibacillosis. Increases in CK and PK activities may also be considered attempts to produce more ATP in response to impairment in energetic metabolism caused by disease, such as decreases in serum glucose levels [3]. A study demonstrated that activity of enzymes belonging to the phosphotransfer network were highly susceptible to free radicals and oxidative damage [33]. Therefore, we evaluated some parameters associated with oxidative stress in order to identify a possible pathway involved in the impairment of cardiac energetic metabolism.

According to González et al. [34], when properly interpreted, serum biochemical values provide important information regarding the clinical status of individual animals and populations, nutritional balance, loss situations, treatment and prognostic monitoring, as well as regarding prevention and aggravation of serious illnesses. In this study, we verified that oxidative stress, common in laying hens [35], was aggravated in *E. coli* infections,

interfering with productive efficiency; therefore, the treatment was necessary and effective. As was observed in our study, even 7 days after initiation of treatment levels of lipid peroxidation remained high, however, as a response of the antioxidant system via the enzyme SOD.

Laying hens symptomatically-infected with *E. coli* had higher levels of LPO, as well as activities of SOD, GPx and CK than did asymptomatic hens. These results are a consequence of the high pathogenicity of the *E. coli* isolate used in this study; therefore, it is important to clarify for the readers that the results of these variables may differ in cases of infections by other *E. coli* isolates with higher or lower pathogenicity's.

5. Conclusion

Colibacillosis interferes with the productive efficiency of laying hens, and norfloxacin was effective for treatment. The disease alters the activity of energy metabolism-regulating enzymes associated with the synthesis and use of ATP, suggesting an attempt to maintain energy homeostasis by increasing ATP levels. Finally, the infection leads to clinical disease, associated with exacerbation of oxidative stress, negatively affecting chicken health and contributing to worsening clinical signs and pathological lesions.

Ethics committee

The use of samples collected during the outbreak was approved by the Animal Use Committee in the survey (CEUA), protocol number 7826240718.

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Table 1

Antimicrobial susceptibility test for *Escherichia coli* isolates from laying hens at the State University of Santa Catarina, according to the methodology approved by CLSI (2018) and standardized by ANVISA (2003).

Antimicrobials	**Results**
Gentamicin	Sensitive
Amoxicillin	Sensitive
Norfloxacin	Sensitive
Enrofloxacin	Sensitive
Colistin	Sensitive
Trimethoprim/sulfamethoxazole	Intermediate*
Ceftiofur	Intermediate*
Ciprofloxacin	Intermediate*
Nalidixic acid	Resistant
Tetracycline	Resistant

* clinically considered to be resistant.

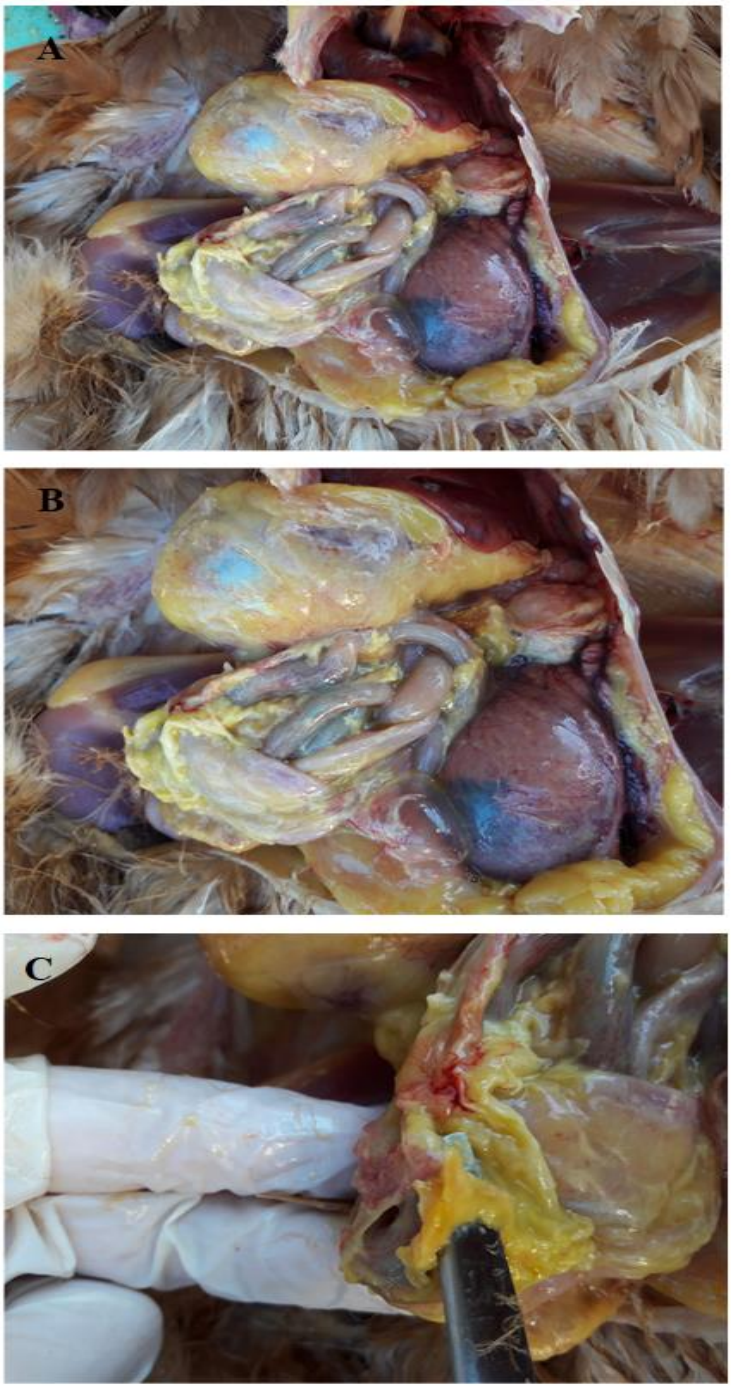

Fig.1. Chickens naturally infected with *Escherichia coli*. Macroscopically, there was evidence of pericarditis, necrotic spots in the liver, hepatic congestion, fibrin coating, airsacculitis, thickening of mesentery and salpingitis with diffuse caseous material.

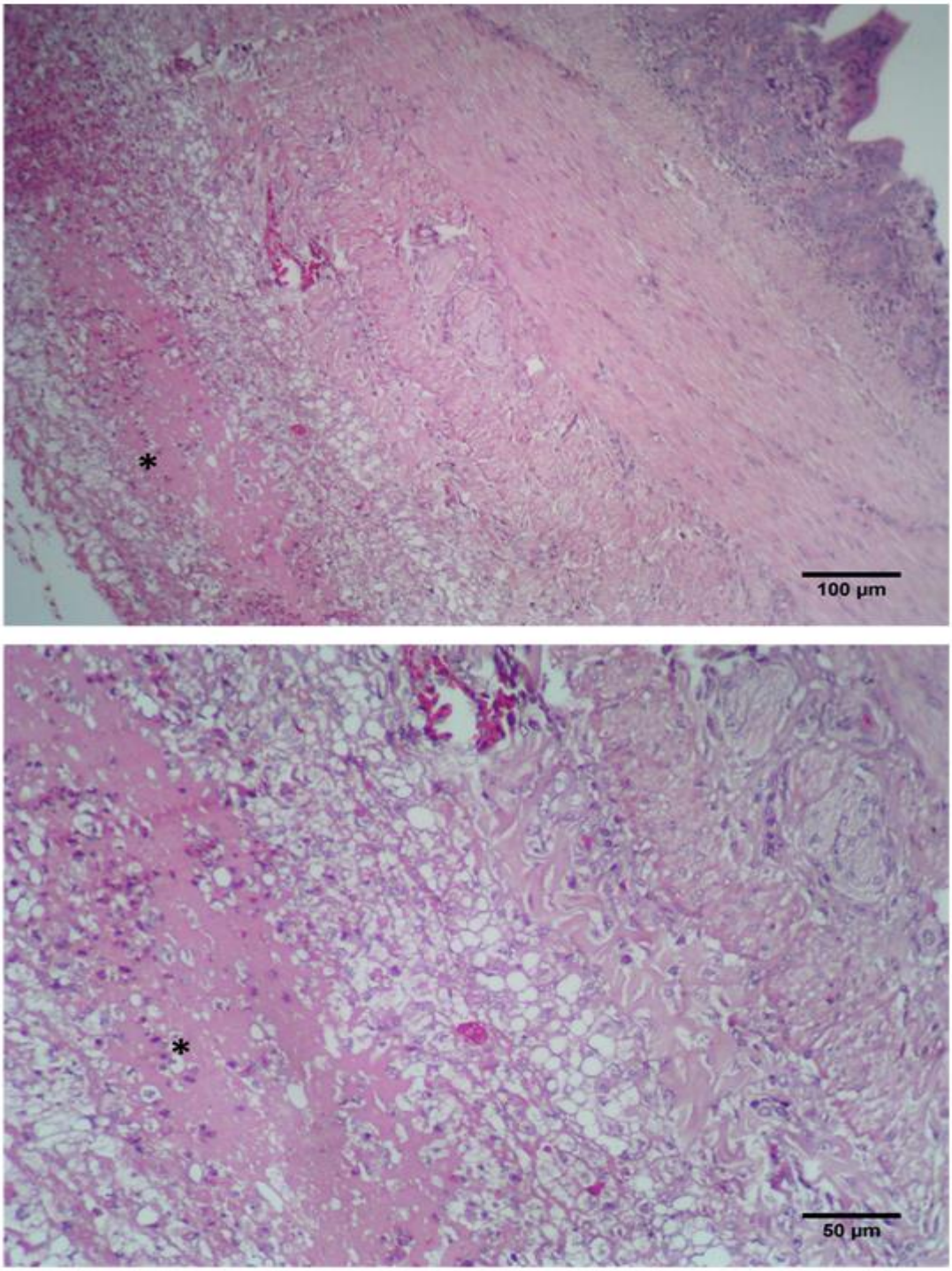

Fig. 2. Chickens naturally infected with *Escherichia coli*. Small intestine: in the serosa we observed moderate-to-severe fibrin deposition associated with moderate heterophilic inflammatory infiltrates (*). Bar: 50 and 100 µm.

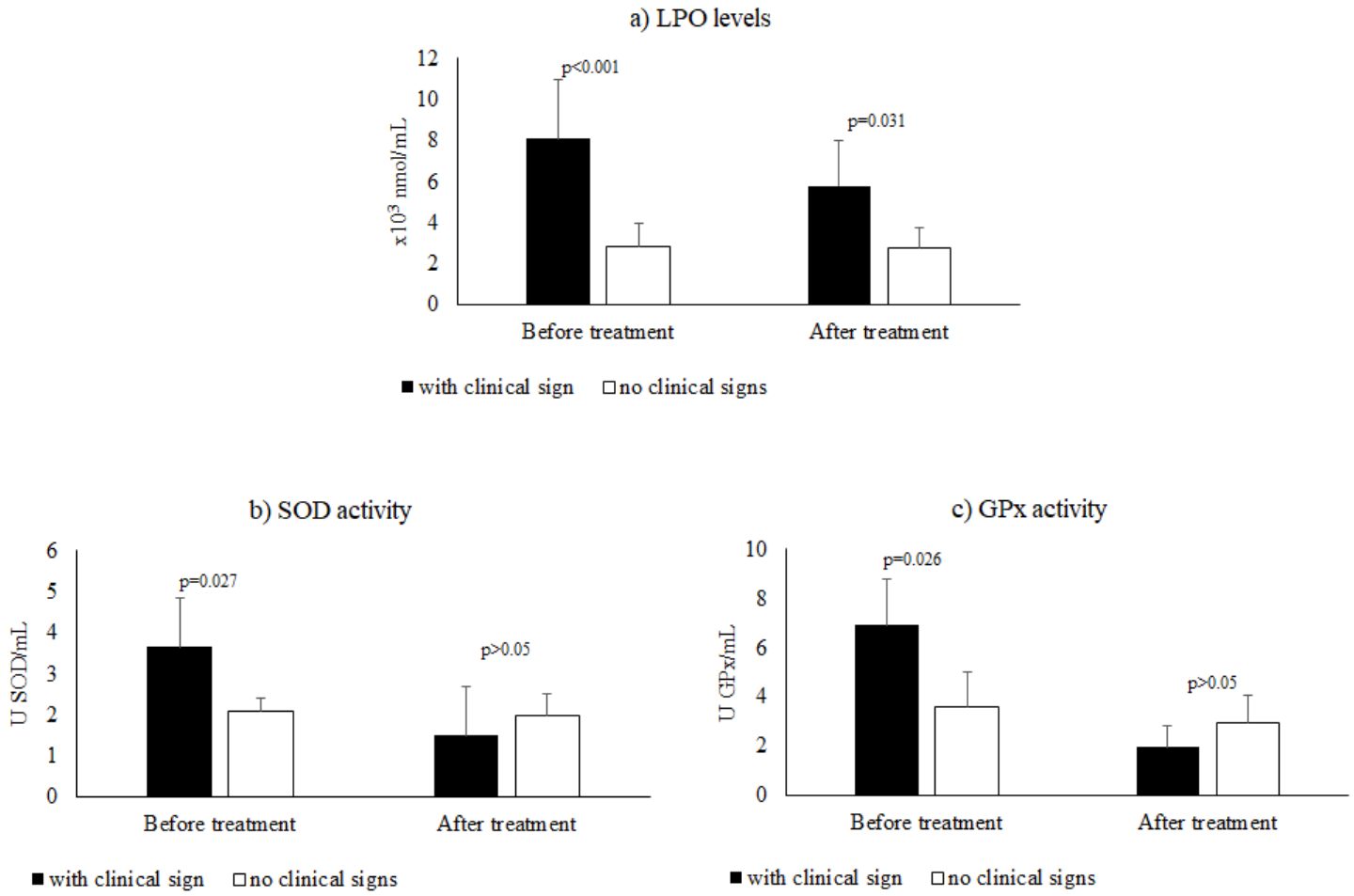

Fig. 3. Lipoperoxidation (LPO) [a] and activities of superoxide dismutase (SOD) [b] and catalase (CAT) [c] in the serum of chickens naturally infected with *Escherichia coli* with clinical signs compared to asymptomatic, before (day 0) and after treatment (day 7) with norfloxacin.

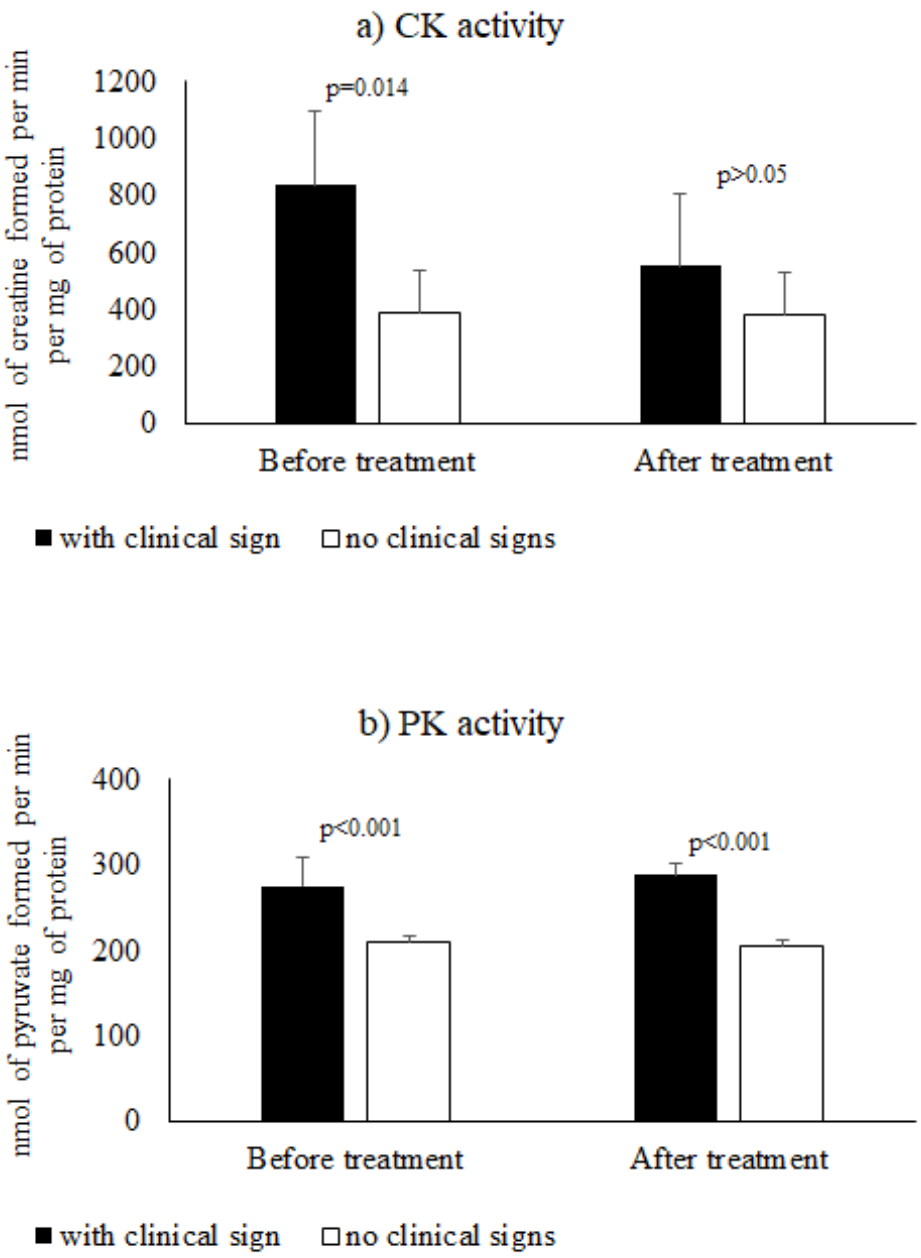

Fig.4. Activity of creatine kinase (CK) [a] and pyruvate kinase (PK) [b] in chickens naturally infected with *Escherichia coli* with clinical signs compared to asymptomatic, before (day 0) and after treatment (day 7) with norfloxacin.

2.2 – MANUSCRITO I

Curcumin supplementation for laying hens reduces *Escherichia coli* infections, modulates metabolism, inflammatory and antioxidant response and improves egg quality

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SUBMETIDO

Curcumin supplementation for laying hens reduces *Escherichia coli* infections, modulates metabolism, inflammatory and antioxidant response and improves egg quality

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Abstract. We determined whether curcumin supplementation in the diets of laying hens that had been naturally infected with *Escherichia coli* would control infection, and would have positive effects on overall health, as well as egg production and quality. We allocated 36 chickens that had been naturally infected with *E. coli* into two groups, with six repetitions and three chickens per repetition: control group (birds fed basal feed) and curcumin group (birds that ate basal feed supplemented with 200 mg curcumin/kg). The experimental period lasted 42 days, and fecal and egg samples were collected at 1, 21 and 42 days to determine total bacterial count, and counts of total coliforms and *E. coli*. Blood samples were collected at the same times for complete blood counts and markers of antioxidant capacity. At 21 and 42 days, we collected fresh eggs to analyze their physicochemical compositions as well as their oxidant/antioxidant status. At 41 days, we collected eggs stored the for 30 days to determine quality and shelf-life. We found that feces and eggs had lower total bacterial counts, *E. coli* counts and total coliform counts in the curcumin group at 21 and 42 days. In fresh eggs, the brightness and yellow intensity (b+) were significantly higher in the curcumin group. In stored eggs, higher specific gravity, albumen height and lower yolk pH were observed in curcumin group. Fresh eggs collected and stored on day 42 showed lower levels of lipid peroxidation in the curcumin group, while the total antioxidant capacity in the stored eggs was significantly higher in the curcumin group. The curcumin group showed lower total leukocyte counts as a result of lower numbers of neutrophils and lymphocytes, as well as lower levels of total protein, alkaline phosphatase and alanine aminotransferase. Lower serum lipoperoxidation at 42 days was observed in the curcumin group, probably because of the higher activity of glutathione peroxidase and glutathione transferase in other words, because of antioxidant stimulation. Despite the positive health effects of curcumin, the laying and feed conversion rates did not differ between groups. Taken together, our findings suggest that curcumin supplementation in laying hens with colibacillosis has positive effects on infection control, animal health and egg quality.

Keywords: *Curcuma longa*, *Escherichia coli*, egg quality, laying hens.

1. Introduction

Avian colibacillosis, caused by *Escherichia coli*, is one of the major causes of losses in the poultry industry, affecting the entire production chain. Control of colibacillosis has become complicated because recent prohibition of antibiotics in animal feed difficulties. In response natural alternatives with antimicrobial properties are being sought to maintain levels of production and quality of products (Yesilbag et al., 2013). In particular, there has been a sharp rise in studies of phytogens, essential oils, herbal components and extracts that are added to poultry feed, particularly those that can improve bird performance and egg quality (Botsoglou et al., 2012). Of particular interest is curcumin that is extracted from *Curcuma longa.* Curcumin has antimicrobial (Sarkar et al., 2016) and antioxidant properties (Akinyemi et al., 2017), as well as activities as a growth promoter and animal performance enhancer (Dono, 2013). Some years ago, curcumin was considered a food additive and was released for wide use in animal feed (Rajput et al., 2013); nevertheless, there remains a lack of studies of the positive and negative effects of curcumin in animals.

Curcumin's ability to prevent intestinal colonization of pathogenic bacteria is the most desirable property in animal production because it promotes an imbalance in the population of microbiota, mainly against experimentally inoculated bacteria (Nascimento, 2019). In addition to its antimicrobial action, researchers found that the inclusion of 0.5% ground *Curcuma longa* in chicken diets improved productive performance and oxidative stability of fresh eggs, as well as reduced yolk lipid oxidation during storage (Radwan et al., 2008). The antioxidant properties of curcumin are also desirable because this enables sequestration of free radicals and chelation of metal ions. Curcumin donates electrons or hydrogen atoms, thereby stabilizing reactive species, preventing chain reactions such as lipid peroxidation (Itokawa et al., 2008).

According to the literature, the inclusion of ingredients or additives with antioxidant properties in the feed is an effective method of minimizing lipid peroxidation in eggs (Eid et al. 2008; Akdemir and Sahin, 2009). A study showed that curcumin supplementation in laying hens provided coccidiostatic activity and prevented egg depreciation (Galli et al. 2018). Another study found an antimicrobial effect of curcumin against *E. coli* (Oliveira et al., 2018). Based on these findings, we determined whether curcumin supplementation

would control *E. coli* in naturally-infected laying hens, and would improve animal health, production and egg quality.

2. Material and methods

2.1 Curcumin

Curcumin powder was acquired from Sigma-Aldrich (St. Louis, MO, EUA) and provided to the hens at concentrations of 200 mg/kg of the by the feed.

2.2 Bird husbandry

A total of 36 commercial Hy-Line Brown laying hens, 84-weeks-old with average body weight of 1680 ± 10 g were used in this study. The birds were housed (three birds/cage) in an experimental poultry house divided into 12 cages for 42 days receiving water *ad libitum* and 16 h of natural and artificial light/8 h of dark.

2.3 Treatments

The birds were randomly distributed in two groups with six repetitions each and three chickens per repetition: control group (T-CON), chickens fed with basal feed; and curcumin group (T-CUR), birds given basal feed supplemented with 200 mg curcumin/kg. The basal diet was formulated with corn and soybean meal (Table 1) according to the composition of the ingredients and nutritional requirements described in the Brazilian poultry and pork tables (Rostagno et al., 2011).

2.4 Performance

We measured evaluated egg production (%) and egg mass (g/bird/day). The eggs were weighed on an electronic balance during the final three days of the cycle, and average weight was determined. Egg mass was calculated the percentage of oviposition in relation to the average egg weight.

The diets provided at each repetition were weighed and offered to the birds. We calculated the amount of food consumed over 21 days. Feed conversion per gram of egg was calculated as the average food intake in the experimental cycle divided by egg mass,

i.e., the amount of food consumed to produce one kilogram of eggs. To calculate the feed conversion per dozen eggs produced, the amount of food consumed in the period was divided by the number of eggs produced multiplied by 12.

2.5 Bacterial examinations: feces and eggs

Fecal samples, (n = 24) and eggs (n = 24), i.e., 12 samples per repetition, were collected on days 1, 21 and 42 to perform total bacterial counts (TBC), using Plate Count Agar according to Parisi et al. (2015). Counts of *E. coli* and total coliforms were quantified using a technique involving 3M Petrifilm EC Plates. Eggs with intact shells were not washed, but were collected and analyzed within one hour after laying. Entire shells were broken and internal contents were discarded. We placed the shells in sterile beakers, ground and homogenized with sterile glass rod. We used 1 gram of shell for the subsequent analyses.

For these analyses, 1 gram of feces and eggshells were weighed in different environments and diluted in 9 mL of buffered peptone water until dilution 10^{-6} for feces and 10^{-1} for eggs, always inoculating 1 mL for subsequent dilutions. Then, 1 mL of the 10^{-6} dilution (feces) and 10^{-1} dilution (eggs) of each sample were inoculated into 3M Petrifilm Plates for TBC and *E. coli* (CE) and 100 μL was inoculated into plate count agar plates. Subsequent incubation was carried out for 24 and 48 hours in a bacteriological greenhouse at 37 °C. Results were expressed as colony forming unit per g (CFU/g).

For both eggs and feces, during the three collection periods tests were performed for *Salmonella* spp., and a 1-mL aliquots of dilutions (10^{-1} and 10^{-6} of eggs and feces, respectively) were transferred to 5 mL Brain-Heart Infusion broth in test tubes. Tubes were incubated in a bacteriological greenhouse for 24 hours at 37 °C and we then transferred 1 mL to 9 mL of Selenite Cystine broth and Rappaport Vassiliadis broth, incubated again at 37 °C for 24 hours. Then, aliquots of these broths were plated using enriched seeding technique in duplicate for selective media: Brilliant Green Agar and Hektoen, again incubated at 37 °C for 24 hours. All samples obtained bacterial growth, and then three to five colonies were chosen fo Triple Sugar Iron Agar (TSI), urease test, indole production, methyl red, motility, lysine decarboxylase, malonate test and Simmons Citrate, incubated at 37 °C for 24 hours. All results were negative for *Salmonella*.

2.6 Egg analysis: physicochemical composition

To assess egg quality, a sample consisting of two eggs per repetition per group (total 12 eggs per group) was collected at 21 and 42 days and evaluated immediately after collection (fresh eggs). At the end of the experiment (day 41), two eggs were stored in commercial trays and stored at a controlled temperature of 27 °C for 30 days for stored egg analysis to determine whether curcumin had an effect on shelf-life.

We measured specific gravity according to the methodology of Freitas et al. (2004). We measured eggshell resistance (kgf) using a Texture Analyzer TA.XT plus texturometer (SMS P75), with pre-test speed of 5.0 mm/second, test speed 1.0 mm/second and post-test speed 20 mm/second, to measure force required to break the shells. Albumen height (HA) was determined at three points near the bud using a digital micrometer. Haugh units (HU) were calculated according to the technique described by Haugh (1937) according to the equation: HU = 100 log (H (mm) + 7.57 - 1.7 W (g) 0.37). Yolk color was measured using a colorimetric matrix (DSM-Yolk Color Fan®) and a colorimeter (Minolta CR-400) that determined the brightness (L∗), red intensity (a∗) and yellow intensity (b∗). Yolk and albumen pH were measured manually using an electronic pH parameter. Yolk weight was measured using a digital scale. The shells were washed and dried at room temperature for 48 hours, then weighed and thickness was measured using digital caliper at three points (apical, basal and equatorial) with an accuracy of 0.01 mm to obtain an average value.

2.7 TBARS levels in egg yolks

Thiobarbituric acid reactive substances (TBARS) in the reaction mixture were analyzed according to Buege and Aust (1978) and expressed as nanomoles of malondialdehyde equivalents per milligram of protein. Briefly, a portion of the reaction mixture (0.5 mL) was added to 0.5 mL of 5% trichloroacetic acid and 2 ml of thiobarbituric acid stock reagent containing 15% trichloroacetic acid, 0.375% thiobarbituric and 0.25 N HCl were added to the mixture. The mixture was boiled for 15 minutes and centrifuged at 1,000 x *g* for 15 min. The supernatant absorbance was measured at 535 nm.

2.8 Antioxidant capacity against peroxyl radicals (ACAP) in egg yolks

This fluorometric method was based on the thermal decomposition (37 ºC) of ABAP (2,2'-azobis(2-methylpropionamidine) dihydrochloride that generates peroxyl radicals, as described by Amado et al. (2009) and reported in detail by da Reis et al. (2019). Yolk was used to detect fluorescence using wavelength 485 nm (excitation) and 520 nm (emission) for 40 min at 37 ºC. The results were expressed as fluorescence units per mg of protein.

2.9 Blood sampling

Blood sampling (approximately 1 mL) was performed on days 1, 21 and 42 of the experiment from the brachial vein of one laying hen per repetition (total of six animals per treatment). Blood samples were collected into tubes without anticoagulant and were centrifuged at 3500 rpm for 10 min to obtain serum. The serum was stored at –20 ºC until use.

2.10 Hemogram

Leukocyte and erythrocyte counts were performed using the Neubauer chamber method, according to the methodology described by Natt and Herrick (1952), and hemoglobin content was determined using a commercial kit according to manufacturer´s recommendations. At sampling, blood smears were created and stained with commercial dye (*Panótipo Rápido*) to perform leukocyte differential counts using a light microscope at 1000 x magnification as described by Lucas and Jamroz (1961). Hematocrit was measured using micro-capillary tubes, centrifuged at 14000 x *g* for 5 min.

2.11 Biochemistry analyses

Serum levels of total proteins, albumin, alkaline phosphatase (AF) and alanine aminotransferase (ALT) were evaluated using a semi-automated BioPlus (Bio-2000) and commercial kits. Serum globulin levels were calculated as the difference between serum levels of total proteins and albumin.

2.12 Oxidant and antioxidant levels in serum

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. This technique is based on the reaction of hydroperoxides with Fe^{2+} ($FeSO_4$) in an acid medium (H_2SO_4) in the presence of the dye xylenol orange, as proposed by Monserrat et al. (2003) and reported in detail by da Silva Barreto et al. (2018). Serum LPO levels were measured in the supernatants using a microplate reader at 550 nm, with cumene hydroperoxide as the standard. Results were expressed as nmol CHP/mL of serum.

GST activity was assayed spectrophotometrically at 340 nm by the method of Giusti and Galanti (1984). The mixture contained serum as test, 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH and 100 mM CDNB, used as substrate. The enzymatic activity was expressed as U/mg of protein. Glutathione peroxidase (GPx) enzyme activity was measured using tert-butyl hydroperoxide as substrate (Wendel, 1984), with NADPH disappearance at 340 nm monitored in potassium phosphate buffer 100 mM + EDTA (1 mM), pH 7.7. Results were calculated and expressed as U mg $protein^{-1}$.

2.13 Statistical analyses

All variables were subjected to normality testing (Shapiro–Wilk). Variables that were not normally distributed were transformed to logarithms to use a parametric test for comparison of means. The data were then subjected to the Student t-test. Significance was considered when $p \leq 0.05$.

3. Results

3.1 Egg Production and Quality

Egg production, egg mass, feed intake and feed conversion (kg/kg) (kg/dozen) of the birds did not differ between treatments (Table 2). No significant differences were observed with respect to Haugh units, albumen pH, yolk weight, egg shell strength, red intensity (a +), shell thickness or weight on days 21 and 42 for fresh and stored eggs at 30 days (Table 3). However, the curcumin group had significantly greater intensity of yellow (b +) and brightness (L +) in fresh eggs at 21 and 42 days; however, there was no difference

between treatments for stored eggs at 30 days. For stored eggs, higher specific gravity, albumen height and lower yolk pH were found in the curcumin group (Table 3).

For fresh egg yolk (day 42) and stored egg yolk, TBARS levels were lower in the egg yolk of the curcumin group (Figure 1a), while total antioxidant capacity (ACAP) was significantly higher in eggs stored for 30 days (Figure 1b).

The bacterial counts are shown in Figure 2. On days 21 and 42, there were lower total bacterial counts, total coliforms and *E. coli* counts in the eggs of the curcumin group than in the control group.

3.2 Serum biochemistries and oxidant/antioxidant status

Serum LPO concentration was lower at 42 days in the curcumin group. Also, at 42 days, there were higher serum GPx and GST activities (Figure 3).

Red cell numbers, hematocrits, hemoglobin concentrations, and eosinophil and monocyte counts did not differ between groups. Total leukocyte counts were lower in birds that consumed curcumin owing to a reduction in lymphocyte and neutrophil counts on days 21 and 42 (Table 4).

Levels of serum total protein, alkaline phosphatase and alanine aminotransferase at 21 and 42 days were lower in the curcumin group (Table 5). Globulin and albumin levels did not differ significantly between groups.

3.3 Fecal microbiology

The total bacterial counts at 21 and 42 days showed significant difference between treatments. On day 21 total bacterial count values were as follow: T-CUR= 180 CFU/mL; T-CON = 389 CFU/mL, total coliforms were as follows: T-CUR = 119 CFU/mL; T-CON = 202 CFU/mL); and *E. coli* counts were as follows: T-CUR = 104 CFU/mL; T-CON = 153 CFU/mL; similarly on day 42: total bacterial counts were T-CUR = 104 CFU/mL; T-CON = 310; total coliform counts were T-CUR = 56 CFU/mL; T-CON = 215 CFU/mL; and *E. coli* counts were T-CUR = 44 CFU/mL; T-CON = 211) (Figure 4).

4. Discussion

Curcumin supplementation did not influence feed intake, feed conversion, egg production or weight gain, suggesting that its use did not affect palatability; rather, it increased the intensity of yellow and egg yolk brightness. Similar results have been described by other researchers, particularly the absence of differences in feed intake, production and body weight gain (Park et al., 2012; Hassan, 2016). However, Park et al. (2012) also reported higher yolk color for laying hens supplemented with 0.5% curcumin. According to the literature, the color of the yolk is one of the main requirements that influence the purchase decision by the consumer, because it is associated (wrongly) with the nutritional value of the egg. The food industry also makes this same association, that is, eggs with a more intense yolk color to yellow are preferred for production of pasta and derivatives (Zahroojian et al., 2013). These results suggest that curcumin is a potential natural alternative, primarily because with the ban on the use of artificial additives in poultry feed with the goal of more intense yolk color (Englmaierová and Skrivan, 2013). In addition, according to Gul et al. (2012), the yellow coloration of the yolk, in addition being more attractive to consumers, is linked to the amount of xanthophylls and carotene present in the diet, acting as antioxidants in the protection of lipid oxidation and free radical elimination. These findings suggest that these effects lead to greater preservation of eggs for long-term storage.

In this study, for stored eggs, higher specific gravity, albumen height and lower yolk pH were observed in the curcumin group. Naturally, with increased egg storage time, the dense albumen decomposes and loses its consistency as a result of decreased water. There is consequent loss of height, spreading easily and changing its acidity and pH elevation (Barbosa et al., 2012), altering the product taste and causing loss of egg weight (Copur et al., 2008). These processes were minimized in the present study, demonstrated by eggs maintaining albumen structure and pH. According to researchers, pH elevations are related to losses of CO_2 and moisture to the environment via pores in the shell. According to Souza et al. (2015), commercial eggs in storage have a pH of 9.7, a level that provides rapid lipid oxidation, significantly altering the taste of eggs and derivatives. Our findings, that therefore, suggest curcumin supplementation gave rise to greater water retention and consequent pH stability (pH 5.61), ensuring higher egg quality within 30 days after laying.

According to the literature, egg quality is related to higher total antioxidant levels and lower lipid peroxidation in egg yolk, biological effects that occur when curcumin is added to poultry diet (Marchiori et al., 2019; Galli et al., 2018), similar to what we observed in our study. According to Al-Jaleel (2012) supplementation with 1.5% *C. longa* provided antioxidant action and stimulated protein synthesis in birds. This author also reported that curcumin supplementation in broilers at levels of 0.5%, 1.0% and 1.5% gave rise to lower feed conversion and improved animal health. In addition to the antioxidant activity in the eggs, it stimulated serum antioxidant responses, manifested as higher levels of GST and GPx in supplemented laying hens, associated with lower LPO at 42 days. These findings suggest that curcumin activated the enzymatic antioxidant pathway, one of the body's most important defenses against infectious and inflammatory processes (Schneider and Oliveira, 2004), because GPx is responsible for the immune defense and maintenance of cellular homeostasis, acting in conjunction with GST in catalyzing and reducing hydrogen peroxide and lipid hydroperoxides (Costantini and Moller, 2009). High antioxidant activity during this period associated with lower lipid peroxidation suggest control of oxidative reactions and greater stability for oxidan/antioxidant status, ensuring better health for laying hens in the presence of *E. coli* infection.

Decreased total white blood cell counts throughout the experimental period and decreased lymphocyte and heterophil counts support reports of antiinflammatory effects reported in numerous studies of curcumin-supplemented diets. Sharma et al. (2007) reported that in vitro curcumin inhibited interleukin-1, which stimulates lymphocyte proliferation and reduced IgG secretion; this is a determining mechanism in the decrease of serum globulins and total protein levels. Total protein reduction was observed in the present study at 21 days, but had no cumulative effect, as it was not observed at day 42 of the experiment.

In addition to the antioxidant and anti-inflammatory effects, curcumin significantly lowered bacterial counts in eggs and feces, with greatest efficacy at 42 days, in addition to controlling *E. coli* during disease. According to Aya et al. (2018), curcumin acts by binding to lipophilic bacterial cell walls (such as *E. coli*). As reported by Kaur et al. (2010), curcumin inhibits the Ftsz cytoskeletal protein polymerization in *E. coli*, which is essential cell division, thereby acting as a potent bacteriostatic. According to Nascimento et al.

(2019), curcumin prevents intestinal colonization of pathogenic bacteria, promoting bacterial microbiota imbalance mainly against inoculated pathogens, and preserving intestinal integrity, favoring weight gain, feed intake and feed conversion, in the face of experimental *Salmonella typhimurium* infection in broiler chickens using curcumin instead of conventional antimicrobials.

Conclusion

Curcumin supplementation in laying hens with colibacillosis improved animal health, mediated by anti-inflammatory, antioxidant and antimicrobial effects. Dietary consumption of curcumin was effective in controlling infection, with emphasis on reducing *E. coli* and consequently on eggs, improving egg quality. Curcumin supplementation increased antioxidant capacity and reduced egg lipid peroxidation, generating internal stability of the stored egg for 30 days, thereby minimizing common deleterious effects and preserving egg quality.

Ethics Committee

This study has been approved by the Ethics Committee in Animal Research of the Universidade do Estado de Santa Catarina (UDESC), under protocol number 8942070519.

Conflict of interest

The authors declare no conflict of interest.

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Table 1

Percentage and calculated composition of the experimental diets.

Ingredients	**Composition (kg)**
Corn	65.7
Soybean meal	21.8
DL- Methionine	0.20
Limestone	8.90
Dicalcium phosphate	1.50
Soy oil	1.10
Sodium chloride	0.50
Vitamin and Mineral Premix*	0.30
Total	100
Calculated values according to the centesimal composition of Rostagno (2011).	
Metabolizable energy (kcal/kg)	2.84
Crude protein	15.7
Calcium	3.87
Available phosphorus	0.37
Sodium	0.23
Digestible lysine	0.68
Digestible methionine	0.42
Digestible methionine + cystine	0.65

* Product composition (kg): vit. A 7,000,000 UI; vit. D3 4,000,000 UI; vit. E 5000 mg; vit. K 1200 mg; vit. B1 360 mg; vit. B2 2000 mg; vit. B6 700 mg; vit. B12 7000 mcg; niacin 7500 mg; biotin 30 mg; pantothenic acid 6000 mg; folic acid 300 mg; iron 1 1000 mg; copper 3000 mg; iodine 204 mg; chloride 360 mg; growth promoter and feed efficiency 20 mg; coccidiostatic 100 g; antifungal 2000 mg; antioxidant 10 mg; magnesium 50 g; sulfur 40 g; energy and protein vehicle (q. s. p.) 1000 g.

Table 2

Mean and coefficient of variation (CV) of the results obtained for oviposition percentage (PP) and egg weight after 21 and 42 days of the addition of curcumin in the diet.

Treatments	Egg production (%)	Egg mass (g/hen/day)	Feed intake (g/hen/day)	Feed conversion (kg/kg)	Feed conversion (kg/dz)
1º cycle					
T-CON	63.2	46.1	120	1.63	2.07
T-CUR	62.9	44.6	126	1.81	2.24
P-value	0.84	0.80	0.69	0.55	0.42
CV (%)	10.7	11.7	8.41	7.21	5.73
2º cycle					
T-CON	57.1	39.5	137	2.40	2.95
T-CUR	58.9	31.0	142	2.88	2.92
P-value	0.86	0.21	0.62	0.36	0.79
CV (%)	12.7	10.3	5.37	4.97	6.53
Mean two cycle					
T-CON	60.1	42.8	128	2.01	2.51
T-CUR	60.9	37.8	134	2.34	2.58
P-value	0.85	0.38	0.55	0.50	0.66
CV (%)	10.6	12.5	6.34	6.37	6.04

Note: There was no difference between groups in terms of performance-related variables.

Table 3

Mean and coefficient of variation (CV) of specific gravity (SG), Haugh units (HU), yolk index (YI), yolk pH (YpH), albumen pH (ApH), yolk weight (YW), eggshell p weight (EW), albumen height (AH), eggshell strength (ES. Kgf), luminosity (L) red intensity (a*), yellow intensity (b^*) and thickness bark (TB, mm) of fresh eggs (days 21 and 42 of the experiment) and eggs stored for 30 days at controlled temperature (27 °C).

Parameters	Time	T-CON	T-CUR	P-value*	CV (%)
SG	Fresh eggs: 21	1.079	1.078	0.94	2.74
	Fresh eggs: 42	1.078	1.076	0.91	1.95
	Stored eggs	0.990	1.029	**0.03**	1.75
HU	Fresh eggs: 21	84.9	76.9	0.54	8.74
	Fresh eggs: 42	72.3	61.2	0.21	13.4
	Stored eggs	16.6	16.5	0.89	9.64
YpH	Fresh eggs: 21	5.85	6.15	0.84	3.60
	Fresh eggs: 42	5.86	5.89	0.91	2.06
	Stored eggs	7.88	5.61	**0.01**	2.25
ApH	Fresh eggs: 21	8.29	8.09	0.89	3.12
	Fresh eggs: 42	8.30	8.40	0.34	2.18
	Stored eggs	9.45	9.54	0.47	3.98
YW	Fresh eggs: 21	18.0	17.5	0.94	2.74
	Fresh eggs: 42	18.0	19.0	0.52	3.94
	Stored eggs	19.3	18.7	0.89	3.01
EW	Fresh eggs: 21	6.49	6.05	0.21	2.87
	Fresh eggs: 42	6.39	6.44	0.19	3.74
	Stored eggs	6.44	6.10	0.26	3.24

AH	Fresh eggs: 21	7.60	7.17	0.45	4.41
	Fresh eggs: 42	6.28	6.23	0.79	5.70
	Stored eggs	1.75	2.25	**0.05**	3.82
ES	Fresh eggs: 21	4508	3932	0.24	15.7
	Fresh eggs: 42	4329	3575	0.06	18.6
	Stored eggs	4221	3903	0.35	11.7
L^+	Fresh eggs: 21	61.1	61.1	0.93	3.79
	Fresh eggs: 42	61.4	63.2	**0.04**	2.06
	Stored eggs	52.9	52.4	0.92	3.57
a^+	Fresh eggs: 21	- 7.74	- 7.73	0.90	5.67
	Fresh eggs: 42	-6.79	- 8.90	0.11	2.46
	Stored eggs	- 4.21	- 4.94	0.88	3.90
b^+	Fresh eggs: 21	45.0	47.2	0.05	2.53
	Fresh eggs: 42	41.2	44.5	0.01	2.04
	Stored eggs	53.0	56.6	0.14	3.97
TB	Fresh eggs: 21	0.33	0.33	0.96	1.03
	Fresh eggs: 42	0.37	0.38	0.94	1.74
	Stored eggs	0.36	0.33	0.41	2.87

Note: *Means with $P \leq 0.05$ in the same line significantly differ from one another (Student's t-test).

Table 4

Mean and coefficient of variation (CV) of hemogram on days 1, 21 and 42 of the experiment using laying hens fed with curcumin (T-CUR).

	Days	T-CON	T-CUR	p-value	CV
Erythrocytes	1	1.72	1.74	0.86	7.21
($x10^6$/µL)	21	1.66	1.70	0.74	6.30
	42	1.78	1.84	0.30	4.57
Hemoglobin	1	8.95	8.50	0.82	5.74
(g/dL)	21	12.3	14.7	0.28	8.04
	42	14.4	12.5	0.31	7.55
Hematocrit (%)	1	28.5	27.0	0.87	4.40
	21	33.5	31.0	0.62	5.74
	42	32.5	31.5	0.79	7.84
Total leukocytes	1	8.27	8.29	0.77	14.4
($x10^3$/µL)	21	12.8	9.63	0.05	8.79
	42	15.2	10.1	0.04	11.3
Heterophil	1	4.16	4.10	0.84	16.7
($x10^3$/µL)	21	5.77	3.73	0.04	10.1
	42	6.87	4.70	0.01	12.8
Lymphocyte	1	3.42	3.55	0.80	12.7
($x10^3$/µL)	21	5.45	3.78	0.02	8.20
	42	6.57	4.38	0.01	9.37
Monocyte	1	0.62	0.57	0.78	18.4
($x10^3$/µL)	21	1.01	0.79	0.60	15.4
	42	1.16	0.72	0.12	14.3

Eosinophil	1	0.07	0.07	0.82	9.70
($x10^3/\mu L$)	21	0.57	0.36	0.62	14.0
	42	0.60	0.32	0.46	15.9

Note: *Means with $P \leq 0.05$ in the same line significantly differ from one another (Student's t-test).

Table 5

Mean and coefficient of variation (CV) of seric levels of total protein, albumin, and globulin, and activities of alkaline phosphatase (AF) and alanine aminotransferase (ALT) on days 1, 21 and 42 of the experiment using laying hens fed with curcumin (T-CUR).

	Days	T-CON	T-CUR	p-value	CV
Total proteins	1	5.30	5.00	0.81	2.94
(g/dL)	21	4.70	3.90	0.05	3.09
	42	4.85	4.40	0.38	3.65
Albumin	1	1.70	1.60	0.86	4.12
(g/dL)	21	1.35	1.20	0.25	3.45
	42	1.30	1.15	0.32	2.84
Globulin	1	3.60	3.40	0.89	3.41
(g/dL)	21	3.35	2.70	0.08	6.03
	42	3.55	3.25	0.58	5.12
AF (U/L)	1	333	313	0.78	15.6
	21	277	198	0.07	10.3
	42	330	170	0.01	11.4
ALT (U/L)	1	13.0	16.0	0.70	8.36
	21	22.5	17.5	0.10	9.74
	42	39.0	20.1	0.01	10.6

Note: *Means with $P \leq 0.05$ in the same line significantly differ from one another (Student's t-test).

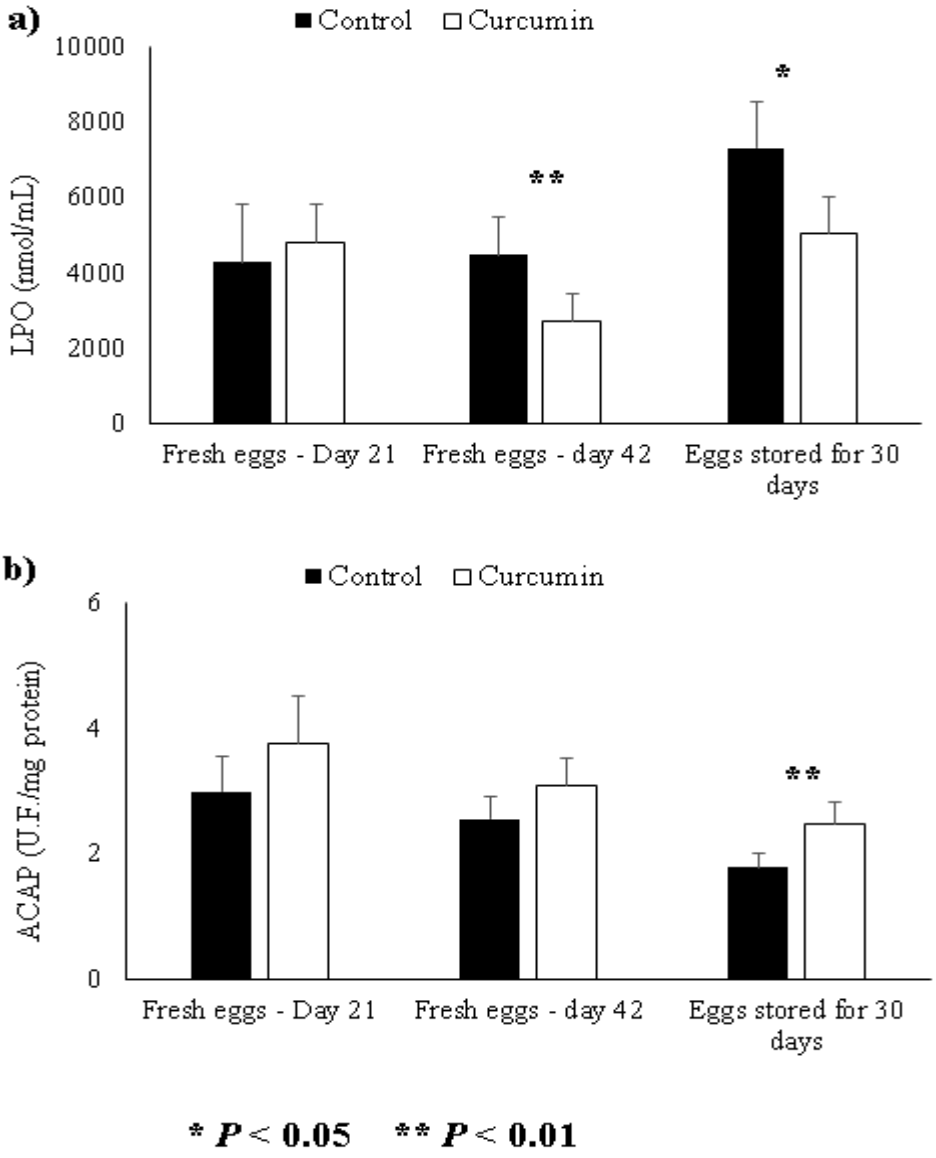

Fig. 1: Thiobarbituric acid reactive substances (TBARS/nmol/mL) and total antioxidant capacity (ACAP – UF/mg protein) in yolk of fresh eggs (days 21 and 42 of the experiment) and eggs stored for 30 days at room temperature (27 ºC) of laying hens supplemented with curcumin.

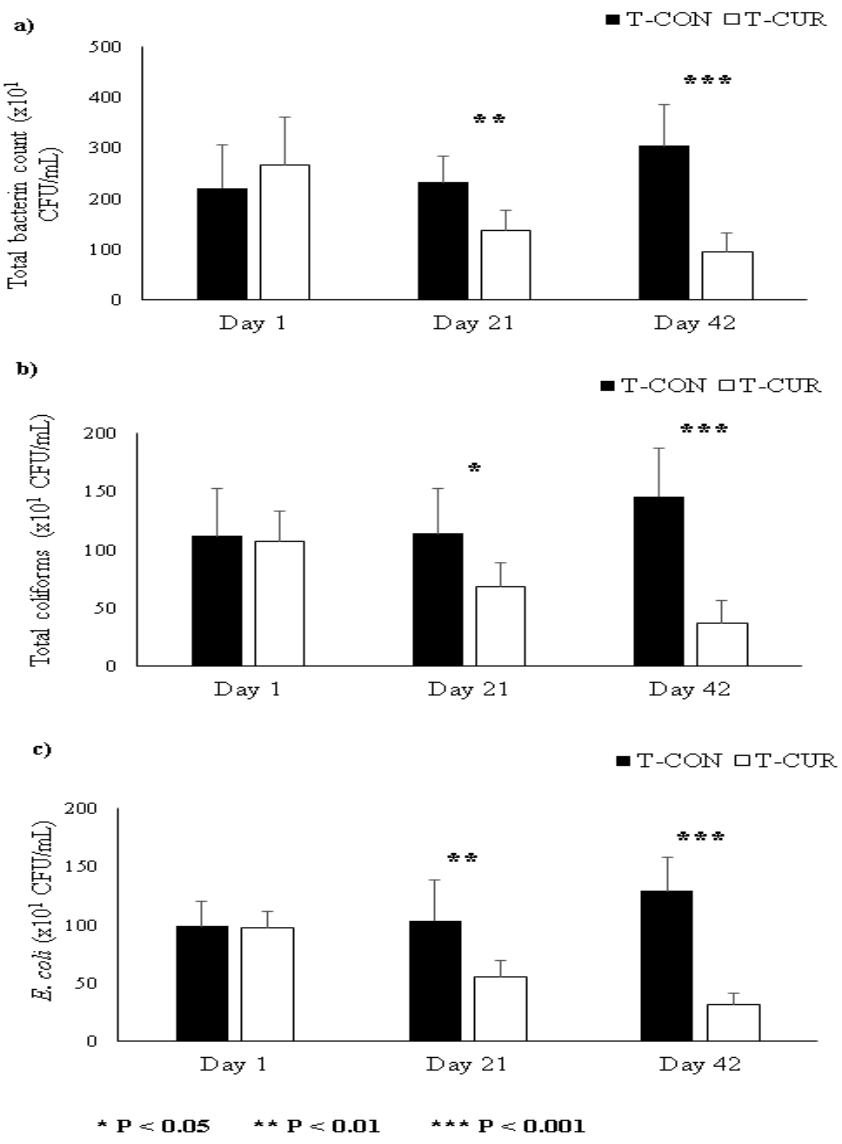

Fig. 2. Microbiological analysis of eggs, total bacterial count, *Escherichia coli* and total coliforms in laying hens naturally infected *E. coli* and supplemented with curcumin. Asterisk (*) indicates difference between groups at various times ($P < 0.05$).

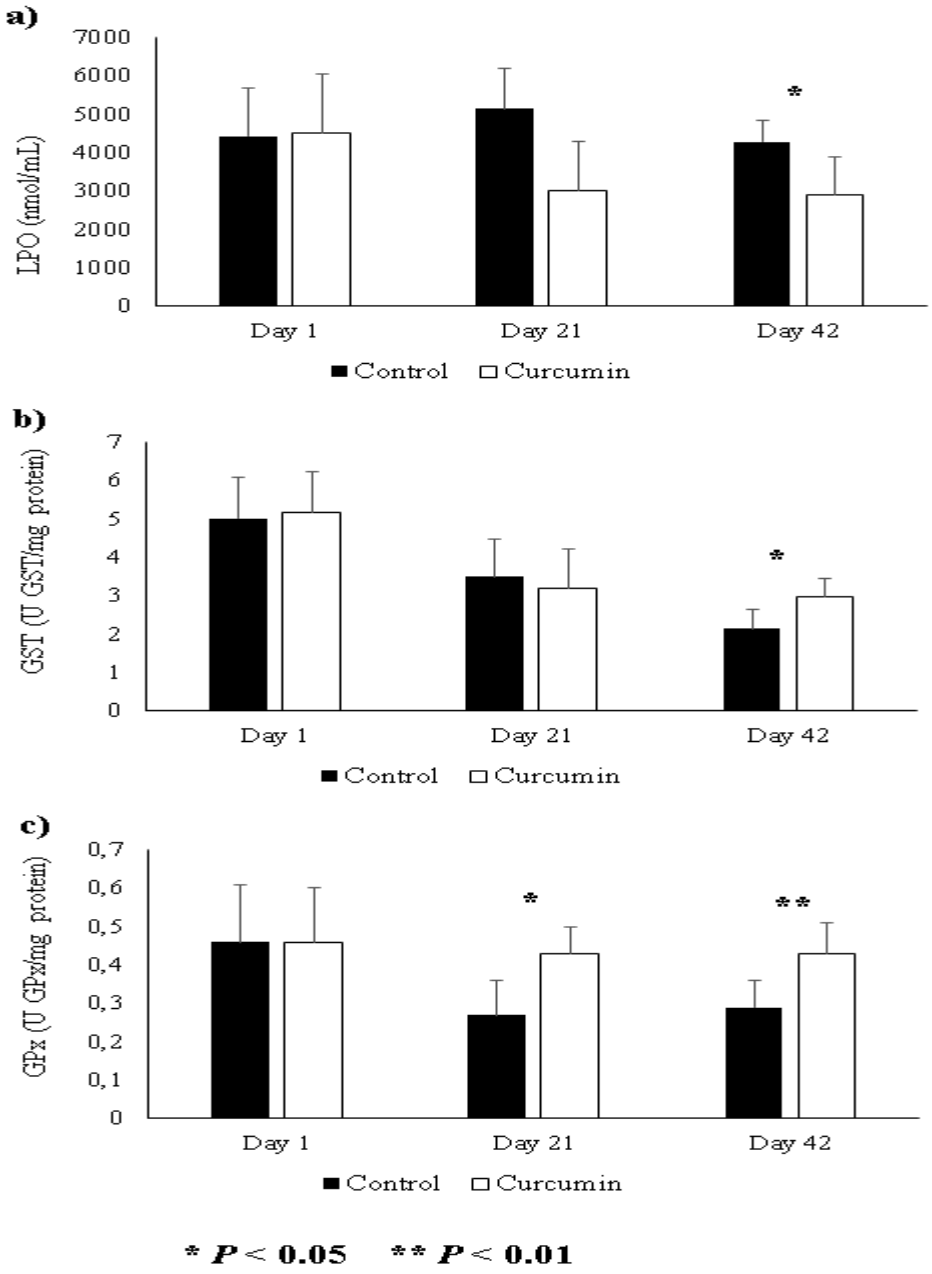

Fig. 3: Biochemical variables LPO (nmol/mL) GPx (U/mg protein) and GST (U/mg protein) in serum from laying hens naturally infected with *Escherichia coli* and supplemented with curcumin compared with the control group on days 1, 21 and 42.

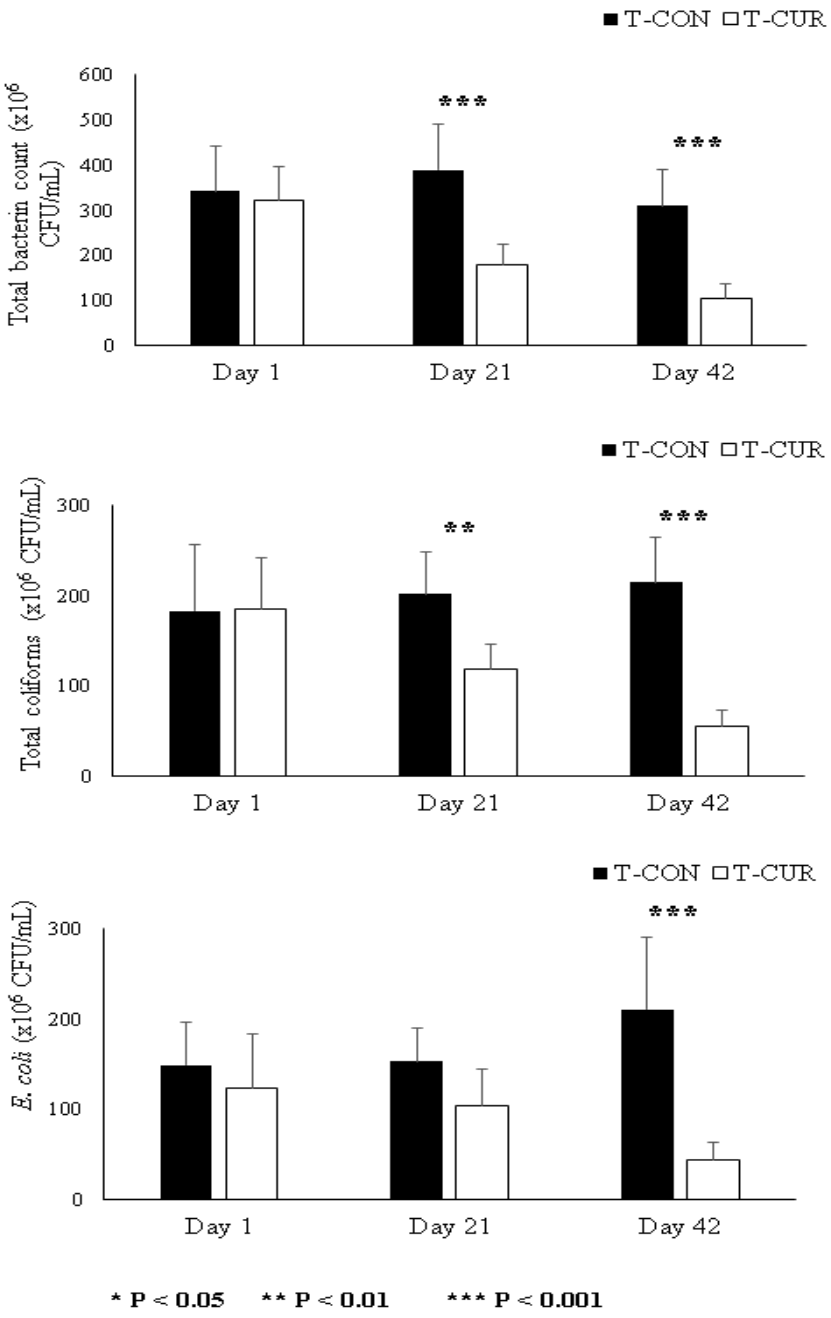

Fig. 4: Microbiological analysis of feces, total bacterial count, *Escherichia coli* and total coliforms from laying hens naturally infected with *E. coli* and supplemented with curcumin. Asterisk (*) indicates differences between groups at various times (P <0.05).

2.3 ARTIGO II

Impact of experimental *Escherichia coli* infection in broiler breeder chicks: the effect of oxidative stress on weight gain

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Impact of experimental *Escherichia coli* infection in broiler breeder chicks: the effect of oxidative stress on weight gain

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ABSTRACT

The aim of this study was to determine whether oxidative stress occurs in *Escherichia coli*-infected broiler breeder chicks, as well as the impact of this infection on bird growth. Twenty birds, 25-day-old female birds were divided into two groups (n = 10 per group): an intraperitoneally-infected group (1 mL containing 1.5 x 10^8 CFU of *E. coli*) and a control group that received 1 mL of culture medium (uninfected birds). Birds were weighed individually at the beginning and at the end of the experiment, and samples were collected on days 0, 5 and 10 post-infection (PI). No clinical signs were observed throughout the experimental period; nevertheless, on day 10 PI, there was lower growth and weight gain in infected birds than in the control group. The infected birds showed pericarditis and liver congestion, as well as moderate periportal inflammatory infiltrates with predominance of neutrophils. Significantly higher numbers of total leukocytes, lymphocytes, heterophils and monocytes were observed in the infected group on days 5 and 10 PI, as well as significantly higher total protein and globulin levels; albumin values significantly decreased over the same period. Levels of serum oxidative biomarkers (lipid peroxidation (TBARS) and free radicals (ROS)) were significantly higher at 10 PI, as was glutathione S-transferase (GST) activity during the same period. Hepatic ROS and protein thiol levels were significantly higher in *E. coli*-infected birds, as well as activities of the antioxidant enzymes catalase, superoxide dismutase. In the spleen, only GST activity was significantly higher for the infected group, unlike the brain, where SOD activity, ROS and non-protein thiol levels were significantly higher in infected birds than in the control group. These data suggested that colibacillosis causes oxidative stress in broiler breeder chicks, negatively affecting their weight gain.

Keywords: Colibacillosis. Oxidative stress. Performance. Poultry farming.

1. Introduction

Pathogenic poultry *Escherichia coli* causes colibacillosis, a disease responsible for substantial economic losses in the poultry industry worldwide due to decreased productivity and significant morbidity and mortality [1]. One of the characteristics that confers high pathogenicity to the bacterium is the production of endotoxins and lipopolysaccharides during its migration through the bloodstream. These bacteria also have a K1 capsule and outer membrane proteins that confer resistance to serum factors and inhibitory drugs, allowing evasion of immune factors, such as the complement system and phagocytosis [2]. This microorganism causes, directly or indirectly, an oxidative imbalance and consequent oxidative stress in the host, drastically affecting health and productive capacity. Oxidative stress may occur in response to increased generation of reactive species or depletion of defense antioxidants [3]. The cytotoxicity associated with oxidative stress derives from the potential of free radicals, among them reactive oxygen species (ROS) that are responsible for causing oxidation of cellular constituents, including proteins, lipids and DNA. These processes interfere with cell structures and functionalities, often evolving to cell death [4].

Reactive species are constantly produced by the body during cellular respiration; in moderate concentrations, they are also involved in physiological functions such as regulation of muscle tone and phagocytosis [5]. However, in excessive concentrations, ROS stimulate oxidation of lipids, proteins and DNA [3], thereby requiring activation of antioxidant defense mechanisms (enzymatic and non-enzymatic) that are important for regulation of free radical levels, thereby protecting cells against the effects of oxidation [5]. This antioxidant defense mechanism acts by providing electrons and neutralizing free radicals, particularly the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) [6].

With increased activity of these enzymes, there is normally a reduction in oxidative reactions, minimizing oxidative stress and cell damage [7]. However, in birds, the immune response to infectious diseases generates free radicals as a means of destroying invasive organisms. Hematological variables are also used as indicators of oxidative damage in biomembranes [8], due to their susceptibility to peroxidation. Numerous studies traced the etiology or progression of diseases to the ability of microorganisms to release toxins, as

well as their ability to resist serum and immune defense factors [2, 8, 9]. Resistance is markedly present in *E. coli* infections [2]. Therefore, in this study, we aimed to determine whether oxidative stress occurs in broiler breeder chicks infected by *E. coli*, as well as the impacts of the disease on animal growth.

2. Material and Methods

2.1. Strain and inoculum

The standard strain used for the preparation of the inoculum was ATCC® 25922 that belongs to Laboratory of Molecular Biology and Microbiology of Santa Catarina State University (UDESC-CEO). The strain was grown in brain heart infusion broth, washed in Milli-Q® water and standardized at a concentration equivalent to 1.5 x 10^8 CFU/mL, according to 0.5 McFarland scale and confirmation by plating on 3M plates ™ Petrifilm EC.

2.2. Animals and accommodation

Twenty 25-day-old female broiler breeder chicks were used, weighing an average of 754 ± 102 g. Birds were placed in an experimental poultry house, with an adaptation period of 15 days and the experiment lasted 10 days (total of 25 days). The diet used throughout the experimental period was the same for all birds, where the main ingredients were corn and soybean, according to the nutritional requirements for the birds described by Brazilian Poultry Nutrition Tables [10]. Feed and water was provided *ad libitum*.

Prior to the beginning of the experiment (adaptation period) all birds were tested for *E. coli* and *Salmonella* spp, as previously described by Rosa et al. [41]. Results show absence of *Salmonella*; and *E. coli* at the dilutions tested.

2.3. Experimental design

The birds were divided into two groups (n = 10 each) as follows: intraperitoneal injection with 1 ml inoculum containing 1.5 x 10^8 CFU of *E. coli* (infected group); and birds that received 1 mL of culture medium without the bacteria (control group).

2.4. Clinical examinations and body weights

Birds were observed daily for clinical signs of colibacillosis. At the beginning and end of the experiment, the birds were weighed individually on a digital scale.

2.5. Sample collection and tissue preparation

Blood samples were collected from the ulnar vein at three time points (days 0, 5 and 10 post-infection), allocated in two tubes; one with anticoagulant (EDTA) for hemogram and the other without anticoagulant to biochemistry. Whole blood without anticoagulant was centrifuged at 3500 *g* for 10 min, and serum was collected and stored (-20 ° C) until analysis. On the 10^{th} day of experiment (end of study), after blood collection, all animals were euthanized (cervical dislocation). Necropsy was performed, and macroscopic lesions were recorded. Liver, spleen and brain were collected for histopathological and biochemical analyses. To evaluate oxidative stress, tissue structures were gently homogenized in a glass Potter homogenizer in specific buffer. The homogenates (liver and spleen) were centrifuged at 10,000 *g* at 4 ºC for 10 min to yield a supernatant (S1) that was used for analyses. Brain homogenates were centrifuged at 3,000 *g* at 4º C for 10 min to yield an S1. Aliquots of tissue homogenates were stored at −80 °C until assay.

2.6. Microbiological isolation

Liver samples were aseptically collected and separated. Each sample was inoculated and homogenized in 9 mL of brain-heart infusion broth and incubated for 24 hours at 36 ± 1 °C. They were then seeded in petri dishes containing methylene blue eosin agar and MacConkey agar and incubated at 36 ± 1 °C for 24 hours. Colonies with metallic green characteristics on EMB agar and pink rose color on MacConkey agar were subjected to biochemical tests (urea base agar, TSI agar, SIM medium agar and Simmons citrate agar) and subsequently incubated at 36 ± 1 ° C for 24 hours. Brain and spleen were not microbiologically evaluated because of the small size of the organs after collection for biochemical analysis and histopathology.

2.7. Hemogram

Leukocyte and erythrocyte counts were performed using the Neubauer chamber method, according to the methodology described by Natt and Herrick [11], while hemoglobin content was determined using a commercial kit according to manufacturer´s recommendations. At sampling, blood smears were created and stained with commercial dye (*Panótipo Rápido*) to perform leukocyte differential counts using a light microscope at 1000 x magnification [12]. Hematocrit was measured using micro-capillary tubes, centrifuged at 14000 *g* for 5 min.

2.8. Plasma biochemistry

Plasma total protein and albumin levels were measured using a semi-automated analyzer BioPlus 2000 with commercial kits (Analisa Gold®) following the manufacturer's recommendations. Globulin levels were calculated using the formula total protein – albumin.

2.9. Protein content

Protein contents in serum and tissue homogenates were determined using the method of Lowry et al. [13] with serum bovine albumin as the standard. These results were used in the analyses described below.

2.10. Oxidants: TBARS and ROS

Lipid peroxidation was measured as TBARS and expressed in terms of malondialdehyde (MDA) content. In this method, MDA, an end-product of fatty acid peroxidation, reacts with TBA to form a colored complex. TBARS levels were measured in serum according to Jentzsch et al. [14]. Brain, liver and spleen tissues (200 μL of S1) were incubated at 95 ºC for 60 min in acid medium containing 8.1% sodium dodecyl sulfate, 0.5 mL of acetic acid buffer (500 mM, pH 3.4) and 0.6% TBA. TBARS levels were measured at 532 nm, and the absorbance was compared with the standard curve using malondialdehyde according to the method of Ohkawa et al. [15].

Determination of 2´-7´-dichlorofluorescein (DCFH) levels was performed as an index of the peroxide production by cellular components. This experimental method of analysis is based on the deacetylation of the probe DCFH-DA, and its subsequent oxidation by reactive species to DCFH, a highly fluorescent compound [16]. Serum (10 μL), and liver or spleen homogenates (0.8 μg of protein) were added to a medium containing Tris–HCl buffer (10 mM, pH 7.4) and DCFH-DA (1 mM). After DCFH-DA addition, the medium was incubated in the dark for 1 h until the start of fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm, with both slit widths 1.5 nm).

2.11. Protein and non-protein thiols

NPSH (non-protein thiols) was measured spectrophotometrically using Ellman's reagent [17]. An aliquot of 100 μL for serum and tissue homogenates in a final volume of 900 μL of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 mL). A standard curve using cysteine was added to calculate the content of thiol groups in samples.

For the measurement of protein thiols (PSH), we used DTNB (5,5-dithiobis-(2-nitrobenzoic acid; Sigma) based on Sedlak and Lindsay [18]. The pellet formed by the precipitated protein was re-suspended in homogenization buffer for determination of PSH content. The absorbance readings (405 nm) were performed using a spectrofluorimeter (Biotek, Synergy HT).

2.12. Antioxidant enzymes: CAT, SOD and GST

SOD activity in brain, liver and spleen was assayed by measuring the inhibition of 1 mM adrenaline auto-oxidation by absorbance at 480 nm using a glycine buffer (50 mM, pH 10.2) as described by Bannister and Calabrese [19]. CAT activity was determined using the method of Aebi by measuring the rate of catalysis of 30 mM H_2O_2 at 240 nm in 50 mM of potassium phosphate buffer at pH 7.0. GST activity (tissue and serum) was assayed spectrophotometrically at 340 nm according to Giusti and Galanti [20]. The mixture contained serum and tissue homogenates as the test, and 0.1 M potassium phosphate buffer (pH 7.4), 100 mM GSH with 100 mM CDNB used as substrate.

2.13. Histopathology

After euthanasia, samples of brain, spleen, and liver were fixed in buffered formalin solution (10%), processed routinely, and stained with hematoxylin and eosin (H&E) for histopathological analyses.

2.14. Statistical analysis

Hemogram data did not show normality and were transformed to logarithms. Data regarding enzymatic activities showed normal distribution using the Shapiro-Wilk test. Subsequently, the data were subjected to the Student *t* test for independent samples considering $P<0.05$. All results were expressed as mean and standard deviation.

3. Results

3.1. Clinical signs, histopathology and microbiological findings

No clinical signs were observed during the experimental period. However, after 10 days of experiment, we observed a delay on body growth of *E. coli*-infected birds; body weights were 944 g in infected birds and 1182 g in uninfected controls. At necropsy, macroscopically, pericarditis with the presence of caseous lumps, as well as hydropericardium and hepatic congestion were observed (Figure 1). The liver had a soft consistency. Histopathological lesions were not observed in the brain and spleen. In liver, we observed a moderate periportal inflammatory reaction, primarily heterophilic infiltrates in birds infected with *E. coli* (Figure 1). *E. coli* was not isolated from the livers of birds in the control group, whereas the *E. coli* counts in the livers of experimentally infected birds were 159.1×10^4 CFU/g.

3.2. Hemogram and serum biochemistry

Erythrocyte counts, hematocrit, hemoglobin concentration and number of eosinophils did not differ significantly between groups. The number of total leukocytes, lymphocytes, heterophils and monocytes were significantly higher in the infected group on days 5 and 10 PI (Table 1).

Total protein and globulin levels were higher in birds in the infected group at 10 days PI, while albumin values decreased significantly over the same period. Over time, total protein and globulin levels increased in the infected group (Table 2).

3.3. Oxidants and antioxidant levels in serum

TBARS levels were higher in the infected group at 10 days PI than in the control group; this was also the case for ROS concentrations during the same period. This difference was observed because over time there was an increase in the levels of TBARS and ROS in the *E. coli*-infected birds (Table 3).

GST activity was significantly higher at day 10 PI in the infected group. PSH and NPSH levels did not differ between groups (Table 3).

3.4. Antioxidant and oxidants levels in cerebral, hepatic and splenic tissues

Details of the results of oxidant and antioxidant status of brain, liver and spleen are described in Table 4. TBARS levels did not differ significantly between groups in liver, spleen and brain, unlike the concentration of ROS in liver and brain that were higher in the *E. coli*-infected group. Activities of the antioxidant enzymes CAT, SOD and GST were higher in the livers of the infected group. A similar result was found for PSH levels in the livers of infected birds. In the spleen, GST activity was higher in birds infected with *E. coli*. In the brain, SOD activity and NPSH levels were higher in the infected group than in the control group (Table 4).

4. Discussion

Leukocytosis was one of the findings of colibacillosis in young broiler breeders, indicating a cellular immune response; this included a higher predominance of neutrophils, as expected in bacterial infections. In acute inflammatory responses to bacteria, fungi and viruses, leukocytosis may be evident as early as 6 h PI, with a peak at 3 days and may persist for 7 to 14 days PI even when the agent is no longer present [21]. Other factors that

favor leukocytosis are dehydration (causing hemoconcentration), body cavity bleeding and stress. Nevertheless, in the present study, no clinical signs were observed in animals during the entire experimental period. During necropsy, no cavitary hemorrhages were observed, only pericarditis. During the inflammatory process, the increase in circulating leukocytes resulted in higher oxidant production (EROS) to combat invading agents [22]. The increased production of these oxidants combined with endotoxin production by the microorganism, as in *E. coli* infections, generates excessive production of free radicals that exceeds the rate of removal, establishing a picture of oxidative stress, inhibiting tissue remodeling and wound healing, and severely weakening the host [23]. Mild monocytosis in infected birds suggests that there was a greater demand for phagocyte mononuclear system cells and antigen stimulation during the inflammatory response [24], as well as moderate heterophilia, indicating severe cell damage and possible tissue necrosis due to *E. coli* infection. Heterophils are the main phagocytic cells involved in the inflammatory response, as well as being involved in attacking bacteria through chemotaxis, opsonization, phagocytosis and lysis [25].

Studies have shown that neutrophils/ heterophils are capable of producing oxygen free radicals, presenting oxidative activity as a way of protecting cell membranes against infections [26]. The increase in monocytes was possibly due to stimulation of the hematopoietic organs in response to severe inflammation caused by *E. coli*, as they serve important phagocytic functions, and can migrate from blood to damaged organs. Importantly, moderate lymphocytosis in birds infected with *E. coli* was consistent with the evolution of the disease. Latimer and Bienzle [27] reported reactive lymphocytes in blood smears, as observed in our study, suggesting antigenic stimulation associated with chronic bacterial infections, acting directly on cellular immunity through the production of antibodies. Feng et al. [28] reported that an increase in leukocyte and lymphocyte numbers improved short-term serum activity with respect to the immune response; however, in the long term, such reactions may be detrimental to animals and may cause significant production losses. It is important to mention that blood counts in this study were complementary data, used to confirm the infection, as well as to monitor the pathogenesis of the disease at the inflammatory level.

We observed an increase in total protein levels as a result of increased globulin, as well as a decrease in albumin levels on day 10 PI. In poultry, the largest protein fraction is albumin (40–60%), 100% of which is synthesized in the liver. Its measurement complements the clinical diagnosis of liver disease and the occurrence of lesions in this organ, capable of interfering with synthesis. In this group, macroscopically, liver congestion and inflammatory infiltrates were observed, a common finding during systemic *E. coli* infection, attributable to high endotoxin production, overloading the liver, which is unable to detoxify circulating blood quickly and efficiently [29], thereby contributing to the death of the animals within hours [9]. Nevertheless, the decrease in albumin levels is worrying, because it acts on the transport of anions, cations, fatty acids and hormones. Changing their rate of production affects the concentrations of these compounds, as well as all physiological reactions important for survival and immune defenses in birds [30]. The increase in globulins is consistent with the increase in lymphocytes, a cell type responsible for the production of immunoglobulins, reflected in the gamma-globulin fraction of the proteinogram.

In response to injuries caused by high toxin production as well as exacerbated free radical production from bacterial infection, the antioxidant system of birds infected by *E. coli* in this study was activated. We found high activity of the enzymes SOD, GST and CAT. Superoxide anion dismutation produces hydrogen peroxide [31] in an attempt to protect cellular targets against, protecting cells from the harmful effects that this free radical can cause [32]. Together with SOD, CAT acts by dismutation and antioxidant defense, because it decomposes hydrogen peroxide that highly toxic to water and molecular oxygen [33]. In our study, *E. coli* infection stimulated hepatic CAT activity, together with SOD and GST, and increased PSH levels in response to elevated ROS levels in the liver.

Similarly, the increase in GST activity observed in infected birds reinforces the high oxidation of cell membranes by the constant damage suffered by *E. coli*, as this enzyme stands out for its detoxification potential and cellular protection, and has high activity in cases of oxidative stress, as an attempt to reduce free radical production [34], especially in infectious diseases [35]. Therefore, it is plausible that liver, brain and spleen present high levels of GST in an attempt to maintain homeostasis against injury and high endotoxin production by *E. coli*.

Cellular inflammatory response and oxidative stress were observed in young chicks infected with *E. coli*, associated with 20% lower body weight than the control group. Ahmed et al. [36] found that colibacillosis in laying birds can reduce egg production by up to 70%, while Boratto et al. [37], in a study of *E. coli* infection in broiler chickens, found a reduction in weight gain by 16% and reductions in feed intake of 7%, as well as feed conversion increasing by 9% when compared to uninfected birds. These authors also observed higher relative weights for heart, liver and intestine and decreased villus height; the liver remained injured for a long period after infection. According to the authors, this was related to the neutralization of toxic substances produced from the bacterial metabolic activity of *E. coli*, requiring higher energy expenditure for detoxification by liver, inducing hepatocyte hypertrophy and aggravation of the disease.

Similar results of high production loss were also reported by Moraes et al. [38], who found that, at 22 and 42 days after infection with *E. coli* in commercial turkeys, 48% of birds died in the challenged group, and 42.4% of this total died within the first 48 hours after challenge. At necropsy, lesions of perihepatitis, pericarditis and airsaculitis were observed, similar to those observed in the present study.

In research on oxidative stress in birds that corroborated the findings of the present study, researchers [39] induced biochemical disturbances in broiler chickens using lipopolysaccharide from *E. coli*. They observed lower average weight gain, and higher relative weights of the spleen and bursa of Fabricius, decreased enzymatic activity of CAT, SOD and GPx in serum and liver, and increased MDA levels. Similar results were also described by Rosa et al. [40]. The authors detected a 60% reduction in laying associated with higher levels of lipoperoxidation (LPO) and activity of antioxidant enzymes SOD and GPx, as well as higher activity levels for creatine kinase (CK) and pyruvate kinase (PK) in laying hens naturally infected by *E. coli*. Taken together, the data suggest that, under natural or experimental conditions, *E. coli* infection induces oxidative stress and interferes with animal performance.

5. Conclusion

Colibacillosis causes oxidative stress in broiler breeder chicks, negatively affecting health and growth. During *E. coli* infection, there is an increase in serum and tissue free

radicals that consequently activate enzymatic and non-enzymatic antioxidant responses. It is likely that the strong response of the antioxidant system was decisive in minimization or avoidance of negative effects of the disease, thereby avoiding serious injuries. This may explain the absence of severe tissue damage.

Ethics committee

The use of the birds and samples collected during the experiment was approved by the animal use committee (CEUA), protocol number 7826240718.

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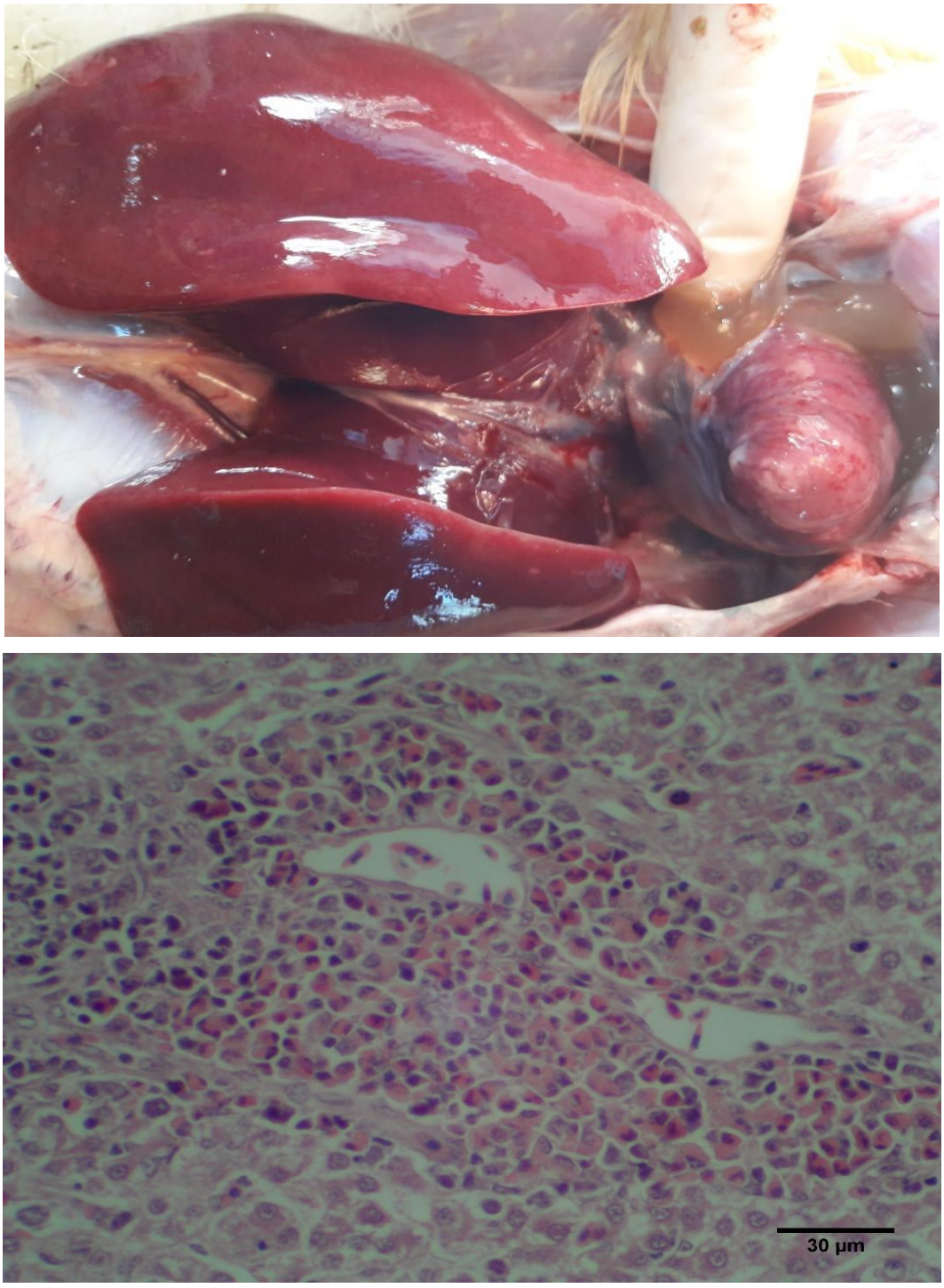

Fig. 1: Pericarditis with the presence of caseous lumps, as well as hydropericardium and liver congestion in birds infected experimentally by *Escherichia coli* 10 days post infection (PI). Liver with moderate periportal inflammatory reaction primarily by heterophilic infiltrates.

Table 1
Mean and standard deviation of hemogram (hematocrit, erythrocytes, hemoglobin, total leucocytes, lymphocytes, neutrophils, monocytes and eosinophils) of birds experimentally infected by *Escherichia coli* compared with the negative control group on days 0, 5 and 10 post infection (PI).

Variables	**Days (PI)**	**Control**	**Infected**	**P value**
Total erythrocytes	0	10.0 (0.44)	8.74 (1.46)	**>0.05**
($x10^6$ µL)	5	8.62 (0.75)	10.1 (3.45)	**>0.05**
	10	9.87 (1.09)	9.08 (2.05)	**>0.05**
	P-value	**>0.05**	**>0.05**	
Hematocrit (%)	0	27.0 (2.92)	27.4 (3.84)	**>0.05**
	5	32.2 (3.11)	30.8 (3.76)	**>0.05**
	10	29.8 (2.86)	31.4 (3.2)	**>0.05**
	P-value	**>0.05**	**>0.05**	
Hemoglobin (mg/dL)	0	8.44 (2.71)	9.06 (1.11) [b]	**>0.05**
	5	12.0 (3.2)	10.0 (4.4) [ab]	**>0.05**
	10	11.6 (3.32)	15.3 (3.6) [a]	**>0.05**
	P-value	**>0.05**	**<0.01**	
Total leucocytes	0	11.1 (1.44)	12.7 (1.49) [b]	**>0.05**
($x10^3$ µL)	5	16.0 (4.40)	47.4 (15.6) [a]	**<0.001**
	10	14.7 (4.5)	47.6 (19.3) [a]	**<0.001**
	P-value	**>0.05**	**<0.001**	
Lymphocytes	0	5.74 (1.56)	6.12 (1.44) [b]	**>0.05**
($x10^3$ µL)	5	6.18 (1.03)	7.27 (3.68) [b]	**>0.05**
	10	5.63 (1.27)	14.0 (3.0) [a]	**<0.001**
	P-value	**>0.05**	**<0.001**	
Heterophils	0	4.90 (1.58)	5.66 (2.79) [b]	**>0.05**
($x10^3$ µL)	5	8.30 (4.84)	29.4 (10.5) [a]	**<0.001**
	10	7.08 (3.08)	26.8 (16.3) [a]	**<0.001**
	P-value	**>0.05**	**<0.001**	
Monocytes (µL)	0	0.43 (0.19)	0.85 (0.31) [b]	**>0.05**
($x10^3$ µL)	5	0.95 (0.74)	10.3 (6.3) [a]	**<0.001**
	10	1.61 (1.42)	5.44 (2.03) [a]	**<0.001**
	P-value	**>0.05**	**<0.001**	
Eosinophils (µL)	0	0.02 (0.04)	0.14 (0.20)	**>0.05**
($x10^3$ µL)	5	0.55 (0.65)	0.34 (0.52)	**>0.05**
	10	0.39 (0.44)	1.24 (1.20)	**>0.05**
	P-value	**>0.05**	**>0.05**	

Note: Values of P <0.05 were considered statistically significant, on the same line. Over time, P <0.05 and different letters (a, b) in the same column show differences between moments of infection.

Table 2

Mean and standard deviation of biochemical variables (total protein, albumin and globulin) of birds experimentally infected with *Escherichia coli* compared with the negative control group at days 0, 5 and 10 post infection (PI).

Variables	**Days (PI)**	**Control**	**Infected**	**P value**
Total protein (g/dL)	0	6.41 (1.62)	5.98 (2.01)	**>0.05**
	5	6.49 (2.07)	6.95 (1.14)	**>0.05**
	10	6.21 (1.36)	8.92 (1.85)	**<0.01**
	P-value	**>0.05**	**<0.001**	
Albumin (g/dL)	0	2.23 (0.74)	2.28 (0.36)	**>0.05**
	5	2.09 (0.57)	2.14 (0.65)	**>0.05**
	10	2.26 (0.24)	1.95 (0.13)	**<0.05**
	P-value	**>0.05**	**>0.05**	
Globulin (g/dL)	0	4.18 (1.16)	3.70 (1.47) [b]	**>0.05**
	5	4.40 (1.27)	4.81 (1.05) [ab]	**>0.05**
	10	3.95 (0.95)	6.97 (1.63) [a]	**<0.001**
	P-value	**>0.05**	**<0.001**	

Note: Values of P <0.05 were considered statistically significant, in the same line. Over time, P <0.05 and different letters (a, b) in the same column show differences between moments of infection.

Table 3

Mean and standard deviation of biochemical variables as TBARS (ηmol MDA/mL), ROS (UDCF/mL), GST (nmol/h/mL), protein thiols (PSH) (nmol/mL) and non-protein thiols (NPSH) (nmol SH/mg protein) in serum of birds experimentally infected with *Escherichia coli* compared with the negative control group at days 0, 5 and 10 post infection (PI).

Variables	**Days (PI)**	**Control**	**Infected**	**P value**
TBARS	0	1.63 (0.65)	1.06 (0.50) [b]	**>0.05**
	5	1.30 (0.25)	1.39 (0.60) [ab]	**>0.05**
	10	1.19 (0.44)	2.08 (0.64) [a]	**<0.01**
	P-value	**>0.05**	**<0.05**	
ROS	0	20.1 (4.1)	19.3 (2.3) [b]	**>0.05**
	5	23.3 (6.8)	22.9 (8.8) [b]	**>0.05**
	10	23.6 (5.5)	42.0 (11.9) [a]	**<0.01**
	P-value	**>0.05**	**<0.001**	
GST	0	10.1 (2.53)	10.6 (2.08) [b]	**>0.05**
	5	10.2 (2.67)	9.53 (2.8) [b]	**>0.05**
	10	7.30 (2.1)	21.1 (6.5) [a]	**<0.001**
	P-value	**>0.05**	**<0.001**	
PSH	0	0.08 (0.02)	0.10 (0.03)	**>0.05**
	5	0.08 (0.03)	0.12 (0.03)	**>0.05**
	10	0.07 (0.01)	0.09 (0.03)	**>0.05**
	P-value	**>0.05**	**>0.05**	
NPSH	0	0.50 (0.05)	0.61 (0.38)	**>0.05**
	5	0.41 (0.01)	0.36 (0.08)	**>0.05**
	10	0.27 (0.05)	0.27 (0.01)	**>0.05**
	P-value	**>0.05**	**>0.05**	

Note: Values of P <0.05 were considered statistically significant, in the same line. Over time, P <0.05 and different letters (a,b) in the same column show differences between moments of infection.

Table 4

Mean and standard deviation of biochemical variables CAT, SOD, TBARS, ROS, GST, protein thiols (PSH) and non-protein thiols (NPSH) in hepatic tissues of birds experimentally infected with *Escherichia coli* compared with the negative control group at day 10 post infection (PI).

Variable	**Control**	**Infected**	**P-value**
Liver			
TBARS	0.60 (0.02)	0.61 (0.01)	>0.05
ROS	65.1 (5.3)	77.3 (6.7)	<0.05*
CAT	90.3 (5.8)	78.6 (5.7)	<0.01*
SOD	36.7 (3.2)	51.3 (2.3)	<0.001*
GST	40.6 (3.5)	50.1 (4.7)	<0.05*
PSH	0.14 (0.01)	0.18 (0.01)	<0.05*
NPSH	0.43 (0.02)	0.46 (0.02)	>0.05
Spleen			
TBARS	0.42 (0.08)	0.40 (0.008)	>0.05
ROS	21.1 (4.9)	19.7 (6.5)	>0.05
SOD	35.9 (5.0)	30.3 (8.5)	>0.05
GST	10.9 (3.1)	17.0 (3.9)	<0.05*
PSH	0.25 (0.04)	0.21 (0.01)	>0.05
NPSH	0.41 (0.007)	0.42 (0.008)	>0.05
Brain			
TBARS	1.31 (0.5)	1.15 (0.4)	>0.05
ROS	50.3 (14.8)	76.8 (13.6)	<0.001*
SOD	33.6 (1.45)	37.0 (2.5)	<0.001*
GST	19.8 (7.3)	15.1 (3.2)	>0.05
PSH	0.11 (0.03)	0.14 (0.07)	>0.05
NPSH	0.42 (0.01)	0.47 (0.04)	<0.05*

Note: ROS **(**U DCF/mg of protein); GST (U GST/mg of protein); SOD (U SOD/mg of protein); TBARS (nmol MDA/mg protein); CAT (nmol CAT/mg of protein); PSH (nmol SH/mg protein) NPSH (nmol/mL).

2.4 ARTIGO III

Impacts of *Escherichia coli* infection in young breeder chicks on the behavior and cerebral activity of purinergic and cholinergic enzymes involved in the regulation of molecules with neurotransmitter and neuromodulator function

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ABSTRACT

The objective of this study was to evaluate if infection by *Escherichia coli* in juvenile breeder chicks alters the activity of enzymes involved in neurotransmission and cerebral immunomodulation, including acetylcholinesterase (AChE), nucleoside triphosphate diphosphohydrolase (NTPDase), 5'-nucleotidase (5'NT) and adenosine deaminase (ADA), as well as their effects on the pathogenesis of the disease. We divided 20 growing breeder chicks into two groups (n = 10 per group). One group was experimentally infected with 1 mL of culture medium containing $1x10^8$ CFU of *E. coli* intraperitoneally. The other was the negative control. On the tenth day after infection, the animals were euthanized and brain samples were collected. Macroscopically, pericarditis and hepatic congestion were observed in the birds, but without histopathological lesions in the encephalon although the bacterium was present in the cerebral cortex of all animals in the infected group (i.e., they were PCR-positive). The activity of AChE, NTPDase, 5'-NT and ADA were evaluated in the cerebral homogenates of the birds after 10 days of infection. AChE activity in the cerebral cortex was lower in the infected group than in the control; there was an increase in the activity of NTPDase, 5'-nucleotidase and ADA, possibly indicating greater hydrolysis of ATP ($P<0.001$), ADP ($P<0.01$) and AMP ($P<0.01$), followed by increased adenosine deamination ($P<0.001$). Despite these changes, no apparently diseased animals were observed throughout the experimental period. Therefore, such changes in enzymatic activity may affect the functioning of the central nervous system because these enzymes are responsible for extracellular regulation of molecules that act on neurotransmission and immunomodulation such as acetylcholine, ATP and adenosine.

Keywords: *Escherichia coli*; breeding chicks; purinergic signaling.

1. Introduction

Avian colibacillosis is an infectious disease with great economic impact on the poultry industry. Bacteria primarily infect the upper respiratory system; however, more worrying is the rapid dissemination through the bloodstream [1] owing to the characteristic ability of this microorganism to resist inhibitory serum factors, allowing escape from complement and phagocytosis, as well as the production of endotoxins during migration. This resistance is due to the cellular structure containing bacterial lipopolysaccharides (LPS), outer membrane proteins (OMPs) and especially the K1 capsule which has anti-phagocytic properties. According to the literature the capsule is essential for the penetration of the blood-brain barrier with consequent septicemia and death [2].

According to researchers, some neuro-metabolites produced by *Escherichia coli* can act as neurotransmitters or modulators of neuronal enzymes, including γ-amino butyric acid, noradrenaline, serotonin and acetylcholine [3]. This facilitates the interference of this microorganism directly with the purinergic signaling pathway, one of the most important modulatory pathways of the central nervous system and of the inflammatory immune response [4]. One of the mechanisms described for these events involves the nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP), as well as their derivative (adenosine), which at high levels in the extracellular environment indicates severe cell damage on the part of pathogens, with potential risk to host health [5]. The increase in the activity of these enzymes, especially in nucleoside triphosphate diphosphohydrolase (NTPDase), reduce levels of extracellular ATP and consequently increases levels of adenosine in a purinergic cascade, as an adaptive and neuroprotective mechanism [6]. NTPDase and 5'-nucleotidase participate in the control of ATP levels in synapses to modulate neuromodulation and purinergic neurotransmission. This enzyme hydrolyzes di- and triphosphate extracellular nucleotides in the presence of Ca^{2+} or Mg^{2+} in the central nervous system, in platelets and other tissues [7]. Bacterial infections such as colibacillosis at the neurological level can cause changes in the activity of these enzymes and thereby cause damage to the entire organism. Adenosine deaminase (ADA) regulates levels of adenosine, a nucleotide considered a marker of cellular and neuroprotective damage [8]. It is a molecule with anti-inflammatory and

immunosuppressive actions capable of inhibiting cytokine production by inflammatory and proliferating lymphocytes [9]. In pathological conditions, ADA also modulates the release of neurotransmitters, to defend against excessive tissue injury [10]. It possesses cholinergic signaling capabilities that are involved in anti-inflammatory actions through ecto-enzymes [11]. Acetylcholinesterase (AChE) is found primarily in cholinergic synapses and neuromuscular junctions, where it serves a regulatory role in neurotransmission, being responsible for the rapid hydrolysis of the neurotransmitter acetylcholine (ACh), regulating the levels of serotonin, dopamine and other neuropeptides, modulating both immune responses and neurotransmission.

Because systemic colibacillosis causes a number of metabolic disorders, as well as neurological disorders, it is important to understand the mechanisms involved. Our hypothesis is that the purinergic and cholinergic systems act to regulate the inflammatory process at the cerebral level, activating alternative anti-inflammatory pathways that are extremely important to minimize the negative effects of infection, as well as resulting tissue damage because the bacteria can reach the brain. Therefore, the objective of this study was to evaluate whether *E. coli* infection in young breeder chicks alters the activity of enzymes involved in neurotransmission and cerebral immunomodulation such as AChE, NTPDase, 5'NT and ADA, as well as to study the pathogenesis of the disease.

2. Material and Methods

2.1 Animals and accommodations

We used 25 female breeder chick's (apparently healthy) in the growth phase at 25 days of age, weighing 754 ± 102 g, in an experimental poultry house. The animals went through an adaptation period of 15 days.

At the end of the adaptation period, feces were collected from all birds for microbiological testing. One gram of each stool sample was aseptically weighed and diluted in 9 mL of buffered peptone water in a sterile test tube and homogenized on a vortex shaker, giving a 10^{-1} dilution. Dilutions were then made up to 10^{-6}, always inoculating 1 mL of the previous dilution in 9 mL of buffered peptone water. Then 1 mL of

the 10^{-6} dilution of each sample was inoculated into a 3M ™ Petrifilm ™ *Escherichia coli* plate and incubated in a bacteriological oven at 37 °C for 24 and 48 hours. The collected samples were also used for *Salmonella* spp. A 1 mL aliquot of the 10^{-1} dilution from each sample was transferred to 9 mL of cystine selenite broth and 1 to 9 mL of Rappaport-Vassiliadis broth and incubated in a bacteriological oven at 37° C for 24 hours. Then, aliquots of these broths were seeded using the duplicate sowing technique for the selective media (bright green agar and Hektoen enteric agar) and incubated again at 37 °C for 24 hours. Three to five CFU were collected on triple iron agar (TSI) and subjected to the urease test, indole production, methyl red, motility, lysine decarboxylase, malonate and Simmons citrate test, and were incubated at 37 °C for 24 hours. In all stool samples analyzed prior to the start of the experiment, no *E. coli* or *Salmonella* were isolated. Therefore, the birds were free of these two specific pathogens.

The diets were formulated according to the nutritional requirements of laying hens and breeders, described in the Brazilian tables for birds [12]. Ground corn and soybean meal were the two main ingredients used in the diet formulation, which fed all the birds in this experiment. The supply of food and water was ad libitum throughout the experimental period.

2.2 Inoculum

For inoculum preparation, the bacteria were placed in culture medium of brain-heart infusion (BHI) broth for growth (bacteriological oven; 37 °C for 24h). Subsequently, this culture medium was diluted in milli-Q® water and the concentration standardized according to the McFarland scale at 0.5; what characterized the inoculum used in this study.

2.3 Experimental design

The birds were divided into two groups (n = 10 each), as follows: those infected intraperitoneally with 1 mL of inoculum containing $1x10^8$ CFU of *E. coli* reference strain ATCC® 25922, belonging to the culture collection at the Molecular Biology and Microbiology Laboratory of the State University of Santa Catarina (UDESC-CEO)

(positive control); and birds that received 1 mL of the culture medium + milli-Q® water without the bacterium (negative control). The experimental period lasted 10 days.

2.4 Sample collection and tissue preparation

At 10 days post-infection (PI), brain and liver samples were collected after euthanasia for histopathological and biochemical analyses. Brain fragments were gently homogenized in a glass Potter homogenizer in specific buffer.

For measurement of NTPDase, 5'nucleotidase and AChE, a fragment of cerebral cortex was removed, weighed and homogenized (1v/10v) with Tris-HCl 50 mM with 4 mM EDTA (to exclude possible interference of endogenous divalent cations). Each homogenate was centrifuged at 2200 g for 10 min and the supernatants were collected and frozen at –20 ºC until analyses.

For ADA analyses in brain, a cerebral cortex fragment was weighed, homogenized in 10 volumes of 50 mmol/L per mM phosphate buffer (pH 7.0) and centrifuged for 30 min at 14,000 *g* at 4 ºC.

Protein content was determined using the Coomassie blue method according to Bradford [13] using bovine sera albumin as standard. The protein supernatant 1 (S1) of tissue were maintained as 0.6–0.8 mg/mL of protein until analysis. These results were used in the analyses described below.

2.5 AChE enzymatic assay in cerebral cortex

The AChE enzymatic assay in cerebral cortex was determined using a modification of the spectrophotometric method of Ellman et al. [14]. The reaction mixture (330 μL final volume) contained 100 mL buffer. The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25 °C. The enzyme was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were processed in triplicate; enzymatic activity was expressed in μmol AcSCh/h/mg of protein.

2.6 Enzymatic assays: NTPDase, 5'-nucleotidase and adenosine deaminase

NTPDase and E-5'-NT enzymatic activities of cerebral cortex were determined using the methods of Schetinger et al. [15] and Heymann et al. [16], respectively. The enzyme preparation (20 μL; 8–12 μg of protein) was added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of substrate (ATP, ADP, or AMP), and enzyme activities are reported as nmol Pi released/min/mg of protein. All samples were processed in triplicate.

ADA activity in the cerebral cortex was determined according to Giusti and Galanti [17]. Brain samples (50 μL) were incubated with 21 mmol/L adenosine pH 6.5 and incubated at 37 °C for 60 min; all samples were processed in triplicate, and the results were expressed as U ADO/mg protein.

2.7 E. coli count in liver

We aseptically weighed 1 g of each liver sample, then homogenized and diluted them in 9 ml of buffered peptone water in sterile test tubes. We then inoculated 1 mL of the 10^{-4} dilution of each sample into 3M ™ Petrifilm ™ EC plates and incubated in a bacteriological oven at 37 °C for 48 hours. Characteristic colony counts were performed using a colony counter and the log number CFU/g was calculated.

2.8 PCR in brain tissue

We performed DNA extraction using phenol, with fragments of cerebral cortex as described in detail by Machado et al. [18]. We used a pair of primers specific for the Fel A gene from *E. coli* that produce amplicons of 270 base pairs, according to Rocha et al. [19]. After the amplification reaction, the sample were run on 1.5% agarose gel and finally subjected to electrophoresis. Purified *E. coli* samples were used as controls.

2.9 Histopathology

After euthanasia, fragments of brain was fixed in buffered formulation solution (10%), processed routinely, and stained with hematoxylin and eosin (H&E) for histopathological analyses.

2.10 Statistical analysis

Data showed normal distribution using the Shapiro-Wilk test. Therefore, all data were subjected to the Student *t* test for independent samples, considering $P<0.05$. All results were expressed as mean and standard deviation.

3. Results and discussion

No clinical signs of disease were observed in the animals during the entire experimental period; but bird behavior changed, i.e., there was higher water consumption and lower feed intake. *E. coli* was not isolated from the livers of birds in the control group, whereas the *E. coli* counts in the livers of experimentally infected birds were $1,59 \times 10^6$ UFC/g. Brain PCR was negative in all animals in the uninfected group; however, brain PCR was positive for *E. coli* in all infected animals. Macroscopically, pericarditis and hepatic congestion were observed in the infected animals (Figure 1a), while no histopathological lesions were observed in brain tissue (Figure 1b). In the livers of infected birds, we observed moderate periportal inflammatory, primarily heterophilic infiltrates (Figure 1c). Hepatic congestion is a common pathological finding in systemic colibacillosis, characterized by overload of the organ such that it is unable to effectively detoxify the bloodstream [20]. Many liver lesions are not specific to the etiology, but rather provide important information on the occurrence of systemic diseases. This is especially true for diseases caused by pathogenic bacteria with a high potential for invasion and cell adhesion, as well as high endotoxin production during their migration through the bloodstream such as *E. coli* [21]. Pericarditis is a pathological change that has attracted attention, because it occurs about six hours after infection, depending on the host's immunity and pathogenicity of the infecting strain [22]. With the evolution of the disease,

pneumonia, salpingitis, swollen head syndrome (SCI) and synovitis can be observed, causing animal death in a few hours [1].

All these systemic and pathological changes caused by colibacillosis, either directly or indirectly, were responsible for changes in the activities of NTPDase, 5'-nucleotidase, ADA and AChE. AChE activity in the cerebral cortex was significant lower in infected birds than in uninfected birds 10 days post-infection ($P<0.001$) (Figure 2). Brain NTPDase, 5'-nucleotidase and ADA activities were significant higher in infected birds than the control group on day 10 post-infection (Figure 3).

ATP is an important source of energy that is responsible for regulating several metabolic processes when present intracellularly, including neurotransmission, cardiac function and bone metabolism [4]. It is regulated by several enzymes, including NTPDase. Nevertheless, when present at high levels in the extracellular environment, ATP acts as a damage-associated molecular pattern (DAMP) and a proinflammatory molecule [10]. In this study, we can interpret the increase in the activity of NTPDase as an anti-inflammatory effect; that is, it increased the hydrolysis of ATP in the extracellular space, lowering ATP levels. ATP is responsible for stimulation and proliferation of lymphocytes as well as the release of mast cell histamines, prostaglandins production and cytokines [23]. Therefore, greater hydrolysis of ATP and ADP up to 10 days PI in infected birds suggests an increase of these nucleotides in the extracellular space, because the bacteria are present in the chickens' brains, and ATP acts as a pro-inflammatory agent by stimulating lymphocyte proliferation and potentiating the release of cytokines such as interleukin 1 (IL-1) and interferon-γ (IFN-γ) [8]. We believe that the increase in ATP hydrolysis mediated by NTPDase may have been a form of defense of the organism, reducing the inflammatory response, which, when exacerbated, causes tissue damage and aggravates clinical symptoms.

ADP levels were altered because of events related to platelet aggregation, primarily due to lesions. It is released to recruit new platelets from the circulation in order to amplify the aggregate signal and to reestablish equilibrium [24]; nevertheless, in this study, because no hemorrhagic regions were observed in the encephalon, we attributed the increase of ADP hydrolysis to a direct effect on the purinergic cascade, that is, as ATP hydrolysis increased, and more ADP was available in the extracellular space. Similarly, we observed

elevation of AMP levels, because this compound is the result of ATP/AMP hydrolysis by the action of NTPDase and subsequent hydrolysis by 5'-nucleotidase, released as adenosine [7]. AMP is a low-energy compound that is usually detected in large concentrations in situations of extreme low energy, as well as in situations where ATP and ADP have been dephosphorylated to obtain energy for fundamental cellular functions [25].

Based on the increase in the hydrolysis of ATP, ADP and AMP, as observed in the group infected with *E. coli*, we infer a possible elevation in circulating levels of adenosine. This molecule has anti-aggregating effects mediated by adenosine metabotropic receptors, specifically the A2A and A2B receptors [25]. These receptors regulate the production of cAMP, inhibit platelet aggregation, and inhibit protein kinase A (PKA), reducing intracellular release of Ca^{2+} reserves and damaging the stability of the cell structure [25]. Because adenosine is associated with anti-inflammatory autocrine signaling via P1-type receptors [26], we speculate that an increase in circulating levels of this molecule may represent a dynamic physiological mechanism that modulates vascular responses to endothelial damage to avoid exacerbated effects of exposure to pathogens. According to the literature, adenosine also acts on synaptic transmission and neuroprotection, as well as on inflammatory processes; therefore, the increased activity of ADA may be a mechanism to reduce extracellular adenosine levels, with anti-inflammatory effects.

Another important molecule involved in inflammatory processes is acetylcholine (ACh). This is rapidly hydrolyzed by AChE and butyrylcholinesterase (BChE), and an increase in the activity of these enzymes directly reflects the reduction of ACh levels that in turn reinforces local and systemic inflammatory events due to the absence of control negative feedback from ACh [11]. In the present study, AChE activity was inhibited in the brains of infected chickens, which should have increased levels of ACh, characteristic of an action in the cholinergic anti-inflammatory pathway to minimize brain damage. Corroborating these results, Jaguezeski et al. [27] evaluated acute infection by *Listeria monocytogenes* in cattle. They also observed lower AChE activity and higher ACh activity in the cerebral cortex of infected animals, even in animals showing no signs of disease.

This preliminary study showed that colibacillosis alters enzyme activity of the cholinergic and purinergic system in the brains of birds. Nevertheless, we did not evaluate the expression of enzymes and receptors, nor did we quantify ACh, ATP, ADP, AMP or

adenosine levels in such a way that could clarify the pathogenetic mechanisms involving the purinergic and cholinergic system in cerebral colibacillosis. Importantly, we used the standard *E. coli* strain ATCC25922; being necessary in isolated future studies identified as responsible for causing miningitis.

4. Conclusion

Bird infected with *E. coli* have increased hydrolysis of ATP, ADP and AMP as well as increased adenosine deamination in the cerebral cortex; however, the birds showed no neurological clinical signs. We also found a reduction in AChE activity in the brains of birds infected by *E. coli*. These changes suggest new pathways of colibacillosis pathogenesis that need to be investigated and elucidated.

Ethics committee

The use of the birds and samples collected during the experiment was approved by the animal use committee in the survey (CEUA), protocol number 7826240718.

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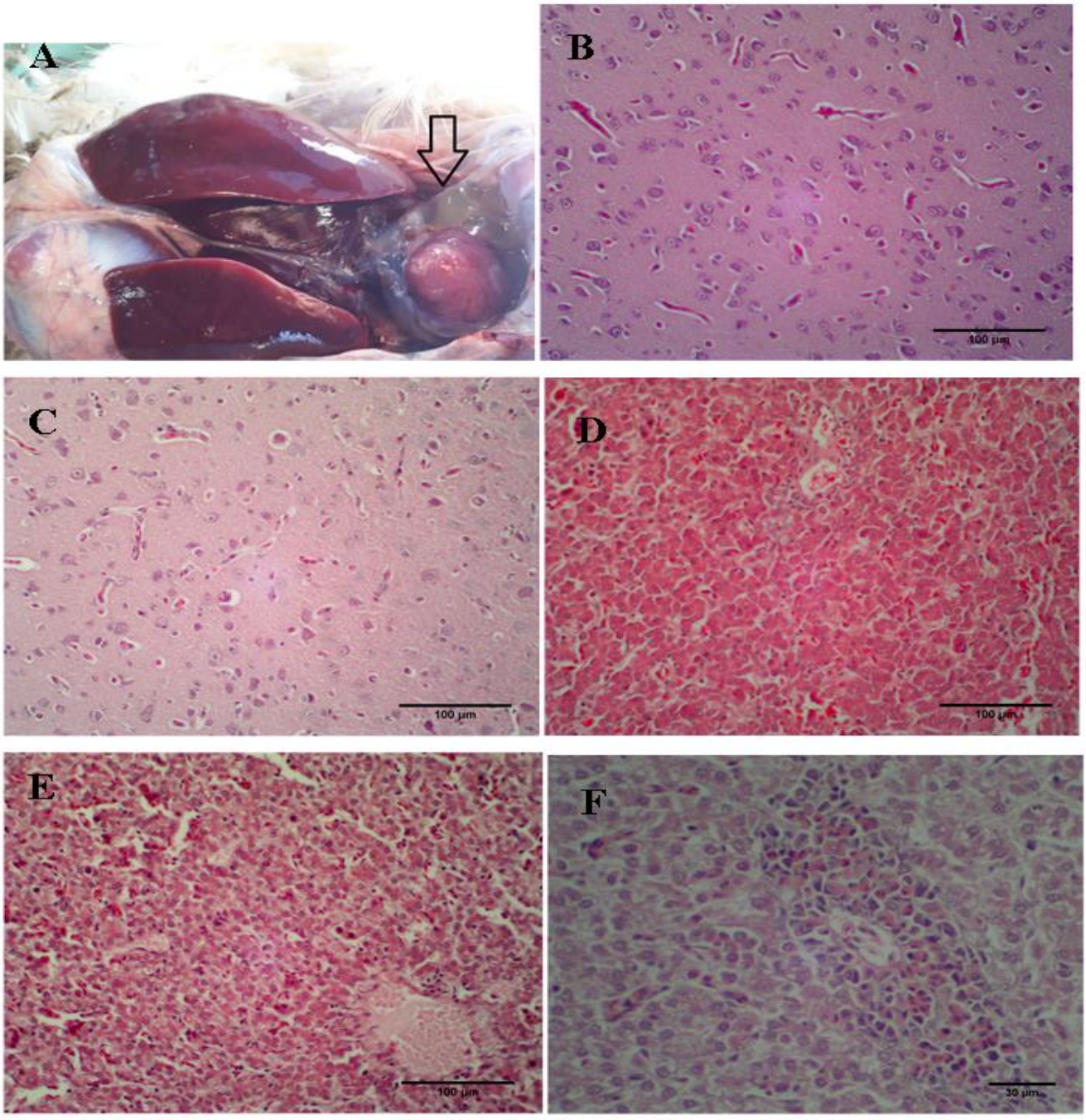

Fig. 1. A) Pericarditis in birds infected experimentally by *Escherichia coli* 10 days after infection. B and C) brain histological images, no change, of uninfected and infected birds by *E. coli*, respectively). D) Uninfected bird liver, unchanged. E and F) Liver with moderate periportal inflammatory, primarily heterophilic infiltrates in infected birds by *E. coli* at 20x and 40x (optical microscope), respectively.

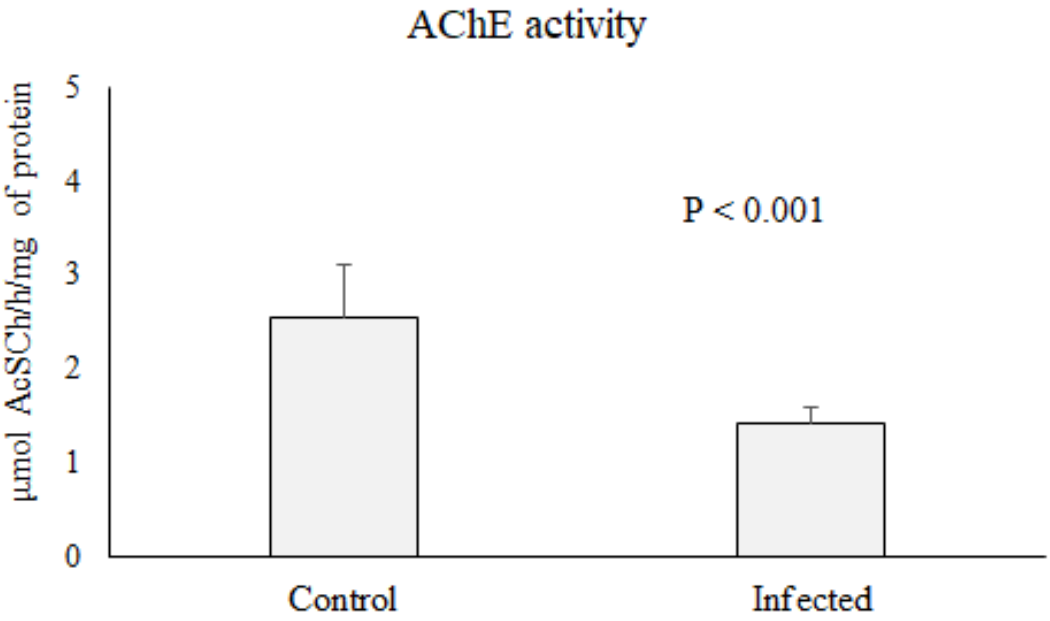

Fig.2. Brain acetylcholinesterase (AChE) activity in birds experimentally infected with *Escherichia coli* 10 days post-infection (PI).

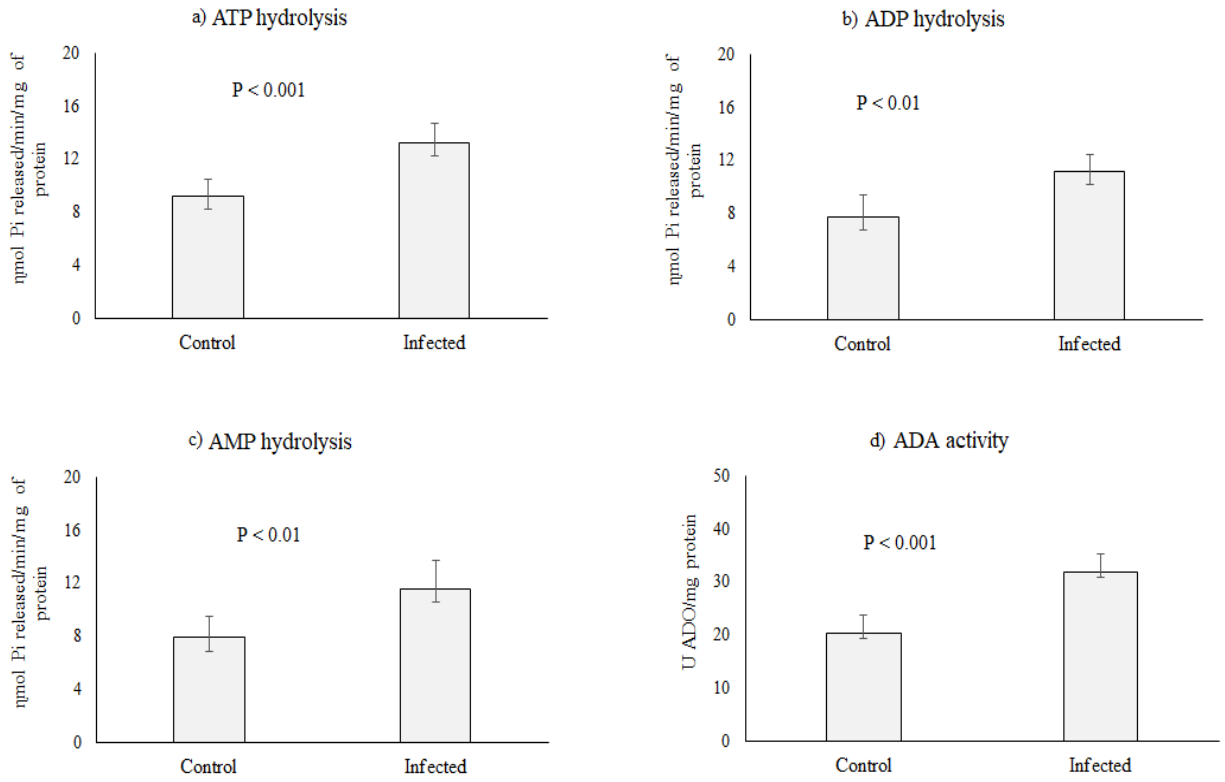

Fig. 3. Brain nucleoside triphosphate diphosphohydrolase (NTPDase) [a,b], 5'-nucleotidase [c] and adenosine deaminase (ADA) [d] activities in birds experimentally infected with *Escherichia coli* on day 10 post-infection (PI).

3 – CONSIDERAÇOES FINAIS

A utilização de curcumina via dieta proporcionou efeitos benéficos na saúde dos animais com potente efeito antimicrobiano frente à infecção por *Escherichia coli*, podendo ser utilizada como aditivo na prevenção e controle desta infecção em granjas comerciais. Além de apresentar atividade antibacteriana, proporcionou efeito anti-inflamatório e antioxidante a níveis séricos e nos ovos. Destaque para menor peroxidação lipídica e maior capacidade antioxidante para ovos frescos e armazenados, garantindo maior tempo de vida útil do produto, fornecendo aos consumidores ovos com melhor qualidade microbiológica e segurança alimentar, pois reduziu consideravelmente a contagem bacteriana, em especial da *E. coli*.

O uso deste fitoterápico deve de ser considerado, pois com a proibição da utilização de antibióticos na produção animal, se faz necessário à substituição por produtos naturais com ação semelhante, pois a colibacilose é uma das mais preocupantes doenças infecciosas da cadeia produtiva avícola e como observado no decorrer destes experimentos, interfere diretamente na eficiência produtiva tanto de poedeiras como nas matrizes de corte, com redução de até 70% na produção de ovos, menor crescimento das aves e perda de peso dos animais em produção que reflete em perdas econômicas consideráveis.

Além disso, a doença causa estresse oxidativo nos animais, mesmo sem sinais clínicos, com alta produção de radicais livres no soro e tecidos e ativação das vias de defesas antioxidantes na tentativa de minimizar lesões sofridas ocasionadas pelo microrganismo e manter homeostase energética e funcional, pois no decorrer da doença, teve-se a alteração da atividade das enzimas reguladoras do metabolismo energético, com maior hidrólise de ATP, ADP e AMP, aumento da desaminação da adenosina no córtex cerebral ao tempo que reduziu a atividade da AChE sugerindo novas vias a serem consideradas na patogênese da colibacilose ainda não elucidadas.

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UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Adição de curcumina na dieta de poedeiras infectadas experimentalmente com Escherichia coli: efeitos sobre produção e qualidade do ovo ", protocolada sob o CEUA nº 8942070519 (ID 000910), sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 10/05/2019.

We certify that the proposal "Addition of curcumin in the diet of laying hens experimentally infected with Escherichia coli: effects on egg production and quality", utilizing 80 Birds (80 females), protocol number CEUA 8942070519 (ID 000910), under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 05/10/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 05/2019 a 07/2019 Área: Zootecnia

Origem:	Aviário do setor de avicultura CAV/UDESC						
Espécie:	Aves	sexo:	Fêmeas	idade:	95 a 100 semanas	N:	80
Linhagem:	Hy-line Brown			Peso:	2 a 3 kg		

Local do experimento: O projeto ocorrerá no município de Chapecó, Santa Catarina, em galpão experimental da Universidade do Estado de Santa Catarina, Centro de Educação Superior do Oeste (UDESC\CEO).

Lages, 12 de janeiro de 2020

Ubirajara Maciel da Costa
Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

em aberto
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

UDESC UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Infecção experimental por Escherichia coli em galinhas poedeiras e matizes de corte: distúrbios bioquímicos e suas consequência na patogenia da doença", protocolada sob o CEUA nº 7826240718 (ID 000689), sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 08/08/2018.

We certify that the proposal "Experimental infection by Escherichia coli in laying hens and cutting hues: biochemical disorders and their consequences in the pathogenesis of the disease", utilizing 80 Birds (80 females), protocol number CEUA 7826240718 (ID 000689), under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 08/08/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 08/2018 a 07/2019 Área: Zootecnia

Origem:	Aviário do setor de avicultura CAV/UDESC						
Espécie:	Aves	sexo:	Fêmeas	idade:	4 a 35 semanas	N:	80
Linhagem:	a definir - poedeiras e matirzes			Peso:	1 a 3 kg		

Local do experimento: Setor de avicultura da udesc oeste

Lages, 12 de janeiro de 2020

Ubirajara Maciel da Costa
Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

em aberto
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina