RAYANNE ANDRADE NUNES

PURIFIED LIGNIN AND CAPSAICINOIDS SUPPLEMENTATION FOR BROILER CHICKENS

Thesis presented to the Federal University of Viçosa, as part of the requirements of the Graduate Program in Animal Science, for the degree of Doctor Scientiae.

Advisor: Arele Arlindo Calderano

Co-advisors: Luiz Fernando Teixeira Albino

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Author

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Arele Arlindo Calderano
Advisor

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BIOGRAPHY

Rayanne Andrade Nunes, daughter of Ana Amélia Tavares Andrade and Raimundo de Jesus Nunes, was born on February 23, 1990, in Aracaju, Sergipe, Brazil. She enrolled in the Bachelor's course in Animal Science at the Federal University of Sergipe in March 2013, graduating in July 2018. In August 2018, she started the graduate program in Animal Science at the Federal University of Viçosa, working in Nutrition and Production of monogastric animals, with a research focus on poultry, under the supervision of Professor Arele Arlindo Calderano. In July 2020, she obtained her master's degree in animal science. In Augustof the same year, she began her Ph.D. in Animal Science with a specialization in Nutrition and Production of Poultry and Swine, also under the supervision of Professor Arele Arlindo Calderano. In Outober 2024, she defended her thesis to obtain the degree of Doctor Scientiae in Animal Science.

ABSTRAT

Nunes, Rayanne Andrade, D.Sc., Federal University of Viçosa, October 2024. **Purified lignin and capsaicinoids supplementation for broiler chickens**. Advisor: Arele Arlindo Calderano. Co-supervisors: Luis Fernando Texeira Albino

Two experiments were conducted to evaluate the effects of lignin and capsaicinoids (CAP) supplementation on the performance and physiological responses of broiler chickens. In the first experiment, the effects of dietary supplementation with purified lignin on performance,relative organ weights, serum metabolites, and gene expression profiles of broilers subjected to cyclic heat stress (HS) were evaluated. At 22 days of age, 280 broilers were allocated in a completely randomized design with four treatments, ten replicates, and seven birds per experimental unit. The birds were subjected to daily cyclic HS, with a temperature of 32 °C (±1) for 10 h/day (08:00–18:00 h) and a temperature of 22 °C (±1) for the rest of the time. The treatments consisted of a basal diet or a basal diet with the addition of 5, 10, or 15 g of purified lignin/kg of diet. There was no effect ($P > 0.05$) of lignin supplementation on performance, carcass yield, relative weights of the bursa, spleen, and liver, or serum levels of glucose, triglycerides, uric acid, malondialdehyde, triiodothyronine, or tetraiodothyronine. The abundance of mRNA for heat shock protein 70, nuclear factor-κB, glutathione peroxidase, and Cu,Zn superoxide dismutase in the liver was also not affected ($P > 0.05$) by the treatments. In the second experiment, the effects of dietary CAP supplementation on performance, intestinal morphometry, and gene expression of LPS-challenged broilers were evaluated. At 8 days of age, 144 male broilers (Cobb 500) were allocated in a completely randomized design with three treatments, eight replicates, and six birds per experimental unit. The treatments consisted of a control diet (CON), a control diet with LPS administration (CON+LPS), and a control diet supplemented with 1 mg CAP/kg of diet and LPS administration (CAP+LPS). The LPS challenge consisted of intraperitoneal injections at 14, 16, 18, and 20 days of age. Four hours after the LPS injection at 20 days, one bird per experimental unit was sacrificed for serum and jejunum sample collection, which were used formRNA analysis. The performance of the birds was evaluated at 21 days of age. The CON birdsshowed higher feed intake ($P = 0.011$) and better feed conversion ratio ($P = 0.022$) compared to CON+LPS birds. The CAP+LPS birds showed higher body weight gain compared to CON+LPS birds, but lower than CON birds ($P <0.001$). The CON+LPS birds had greater crypt depth than CON and CAP+LPS birds ($P = 0.002$).

There was no significant treatment effect on villus height ($P > 0.05$). Higher mRNA expression of superoxide dismutase ($P = 0.046$) and catalase ($P = 0.011$) was observed in the jejunum of CON birds compared to CON+LPS birds. It was concluded that supplementation with 1 mg CAP/kg of diet improves growth performance and intestinal morphometry of LPS-challenged broilers, while purified lignin supplementation does not improve performance, or the antioxidant response of broilers subjected to HS.

Keywords: Additive; Antioxidants; Immune Response

Resumo

Nunes, Rayanne Andrade, D.Sc., Universidade Federal de Viçosa, outubro de 2024. **Suplementação de lignina purificada e capsaicinóides para frangos de corte**. Orientador: Arele Arlindo Calderano. Coorientadores

Dois experimentos foram conduzidos para avaliar os efeitos da suplementação com lignina purificada e capsaicinoides (CAP) na performance e nas respostas fisiológicas de frangos de corte. No primeiro experimento, foram avaliados os efeitos da suplementação dietética com lignina purificada sobre a performance, pesos relativos dos órgãos, metabolitos séricos e perfis de expressão gênica de frangos de corte submetidos a estresse térmico cíclico (HS). Com 22 dias de idade, 280 frangos foram alocados em um delineamento inteiramente casualizado com quatro tratamentos, dez repetições e sete aves por unidade experimental. As aves foram submetidas a HS cíclico diário, com uma temperatura de 32 °C (±1) durante 10 h/dia(08:00–18:00 h) e uma temperatura de 22 °C (±1) para o restante do tempo. Os tratamentos consistiram em uma dieta basal ou uma dieta basal com a adição de 5, 10 ou 15 g de lignina purificada/kg de dieta. Não houve efeito (P > 0,05) da suplementação com lignina sobre a performance, rendimento de carcaça, pesos relativos da bursa, baço e fígado, ou níveis séricosde glicose, triglicerídeos, ácido úrico, malondialdeído, triiodotironina ou tetraiodotironina. A abundância de mRNA para proteína de choque térmico 70, fator nuclear-κB, glutationa peroxidase e superóxido dismutase Cu,Zn no fígado também não foi afetada (P > 0,05) pelos tratamentos. No segundo experimento, foram avaliados os efeitos da suplementação dietética com CAP sobre a performance, a morfometria intestinal e a expressão gênica de frangos de cortedesafiados com LPS. Com 8 dias de idade, 144 frangos de corte machos (Cobb 500) foram alocados em um delineamento inteiramente casualizado com três tratamentos, oito repetições eseis aves por unidade experimental. Os tratamentos consistiram em uma dieta controle (CON),uma dieta controle com administração de LPS (CON+LPS) e uma dieta controle suplementadacom 1 mg de CAP/kg de dieta e administração de LPS (CAP+LPS). O desafio com LPS consistiu em injeções intraperitoneais aos 14, 16, 18 e 20 dias de idade. Quatro horas após a injeção de LPS aos 20 dias, uma ave por unidade experimental foi sacrificada para coleta de amostras de soro e jejuno, que foram usadas para análise de mRNA. A performance das aves foi avaliada aos 21 dias de idade. As aves CON apresentaram maior consumo de ração (P = 0,011) e melhor índice de conversão alimentar (P = 0,022) em comparação com as aves CON+LPS. As aves CAP+LPS mostraram maior ganho de peso corporal em comparação com as aves CON+LPS, mas menor do que as aves CON (P < 0,001). As aves CON+LPS tiveram

maior profundidade de críptas do que as aves CON e CAP+LPS (P = 0,002). Não houve efeito significativo do tratamento na altura das vilosidades (P > 0,05). Foi observada maior expressão de mRNA de superóxido dismutase (P = 0,046) e catalase (P = 0,011) no jejuno das aves CON em comparação com as aves CON+LPS. Concluiu-se que a suplementação com 1 mg de CAP/kg de dieta melhora a performance de crescimento e a morfometria intestinal de frangos de corte desafiados com LPS, enquanto a suplementação com lignina purificada não melhora a performance ou a resposta antioxidante de frangos de corte submetidos a HS.

Palavras-chave: Aditivos; Antioxidantes; Resposta Imune

Summary

General Introduction

The search for feed efficiency has been a critical point in commercial broiler chicken production. Heat stress (HS) poses a significant challenge in hot-climate areas, leading to considerable economic losses. This condition results from an imbalance between the net energy dissipated from the animal's body to the surrounding environment and the heat energy produced by the animal (Lara and Rostagno, 2013). Various factors related to the thermal environment and animal characteristics contribute to this imbalance. Additionally, broilers in production systems are often exposed to inflammatory challenges that further compromise their performance. Pro-inflammatory mediators can suppress appetite, resulting in reduced nutrient intake. This shift in nutrient allocation from growth to the immune response can decrease nutrient availability for growth and promote tissue catabolism (Takahashi et al., 2008; Broom and Kogut, 2018).

Different classes of active principles confer beneficial effects to these substances, such as antimicrobial, antioxidant, antiviral, anti-inflammatory, antifungal, and antiseptic activities (Noleto et al., 2018). Other effects include insecticidal, antihistaminic, expectorant, antispasmodic, analgesic, anesthetic, calming, digestive, antitumor, and mucolytic actions (Azambuja, 2017b). Therefore, the main challenge in poultry production today is to find additives capable of balancing the intestinal health of both the microbiota and the host (Pickler et al., 2011).

Lignin is a group of phenolic polymers that confer strength and rigidity to the woody cell walls of plants. After cellulose, lignin is the most abundant natural compound (Boudet and Grima-Pettenati, 1996) and can make up to one-third of plant cell walls (Pan et al., 2006). In its purified form, it is commercially produced as a byproduct of the paper industry, separated from wood by chemical pulping processes. Different chemical treatments during wood pulping processes produce various types of purified lignin, such as Alcell lignin and Kraft lignin

(Baurhoo et al., 2008). One of the main sources of purified lignin is extracted from Eucalyptus urograndis wood. This product is rich in phenolic compounds such as guaiacol and syringol (Leite et al., 2024).

Purified lignins are low molecular weight phenolic compounds with biological characteristics that differ from those of native lignin, possessing antioxidants, antibacterial, anti-inflammatory, and various other benefits (Tungmunnithum et al., 2018). The main metabolic pathway for the formation of these compounds in plants is the shikimic acid pathway. In this route, a molecule of phosphoenolpyruvate, derived from the glucose cycle, combines with erythrose-4-phosphate, from the pentose phosphate cycle, forming 3-deoxy-D-arabino-heptulosonate phosphate (DAHP). This compound is cycled and reduced to produce shikimic acid, which subsequently participates in various other metabolic routes (Dias et al., 2016).

Although these phenolic fragments could potentially have important applications in animal agriculture, research on purified lignin has not received much attention and there are few published results. In contrast to native lignin, purified lignin does not present a barrier to digestion in monogastric or ruminant animals (Baurhoo et al., 2008).

Literature data suggest the possible positive effect of lignin on the nutrition of monogastrics. Promising results have been observed in the performance of broiler chickens (Makivic et al., 2019; Radulovic et al., 2020). On the other hand, Bogusławska-Tryk et al. (2015), investigating the effects of different levels of lignocellulose in broilers up to 42 days of age, found no significant effect on weight gain and feed conversion.

Supplementation with 1% purified lignin demonstrated improvements in bird performance, as observed by Sozcu (2019) and Leite et al. (2024). These positive results may be related to the antimicrobial effect of lignin (Sozcu, 2019). Although the mechanism of action of lignin is not completely elucidated, it is known that the phenolic compounds present can cause damage to the cell membrane of pathogenic bacteria, resulting in the release of cellular

contents and membrane disintegration (Ayyachamy et al., 2013). This effect can help eliminate intestinal pathogens, improving the health and development of the mucosa, protecting intestinal cells, and modulating the intestinal microbiota (Leite et al., 2024). Studies have investigated the benefits of these phenolic monomers on broiler production and health (Bosetti et al., 2020;Galli et al., 2020), but fewer studies have used purified lignin.

Thus, it is possible to observe that the responses of animals to purified lignin vary according to dosage, animal species, and the type and source of the lignin product. Therefore, more research is needed to conclusively establish the benefits of purified lignin on animal performance and health (Baurhoo et al., 2008), in addition to its effects on animals subjected to thermal challenges.

Red pepper (Capsicum annum L.), a natural additive used in feed, belongs to the capsicum family and is chemically known as 8-methyl-N-vanillyl-6-nonenamide ($C_{18}H_{27}NO_3$), the main component of capsaicinoids found in pepper (Barbero et al., 2016). Rich in ascorbic acid, beta-carotene, potassium, magnesium, and iron (Meghwal and Goswami 2012), red pepper supplementation has been shown to increase the productivity of broiler chickens, serving as an alternative to antibiotics to improve performance (Ndelekwute et al., 2015; Adegoke et al., 2018). Previous research indicates that red pepper contains capsaicin and capsinoids, active substances responsible for its spicy flavor (Boyunaga and Celik 1995; El Husseiny et al. 2002). Piperine, an active compound in black pepper, also improves nutrient digestibility in broiler chickens (Ferreira et al., 1999; Moorthy et al., 2009).

Studies show that red pepper has various biological functions by acting through the TRPV1 pathway, including antimicrobial, antidiabetic, antioxidant, and anti-inflammatory activities (Wang et al., 2021). Additionally, pepper increases the height of intestinal villi (Sirinivasan 2007) and stimulates the release of digestive enzymes, saliva, hydrochloric acid,

and bile acid in the gastrointestinal tract of animals (Platel and Sirinivasan 2004; Lee et al., 2004a; Lee et al., 2004b).

Asli and Rashti (2017) investigated the use of extracts containing cinnamaldehyde or capsaicin in Ross 308 broilers, noting that these extracts protected the microvilli, structures responsible for nutrient absorption. The use of essential oil predominantly composed of thymol resulted in longer intestinal villi than enramycin. Extracts containing carvacrol, cinnamaldehyde, and capsaicin were effective in reducing the amount of *Escherichia coli*, *Clostridium perfringens*, and fungi in the intestines of these birds.

Orndorff et al. (2005) observed that capsaicin, both prophylactically and therapeutically, led to a reduction in the thickness of the cecal lamina propria in broiler chickens. Fan et al. (2023), comparing the effects of capsaicin with antibiotics in broilers, found that animals supplemented with capsaicin showed better performance. Capsaicin significantly increased the total plasma protein content, thyroxine, and reduced glutathione (GSH), while decreasing levels of glucose, uric acid, and malondialdehyde (MDA). Bona et al. (2012) also evaluated the action of plant compounds based on rosemary essential oil (cineole), cinnamon (cinnamaldehyde), red pepper extract (capsaicin), and oregano (carvacrol) on Salmonella Enteritidis, observing a reduction in the colony count of Salmonella after swab collection from the cloaca of 21-day-old birds inoculated with 10^5 CFU/mL of Salmonella Enteritidis (SE).

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Chapter 1:

Purified lignin supplementation on the performance and antioxidant status of broilers submitted to cyclic heat stress

Rayanne Andrade Nunes[1], Luiz Fernando Teixeira Albino[1], Paulo Henrique Reis Furtado Campos[1,2], Hallef Rieger Salgado[1], Samuel Oliveira Borges[1], Rafael de Sousa Ferreira[1], Karine Assis Costa[1], Arele Arlindo Calderano[1,2,].

[1]Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, 36570-900, Brazil

[2]Muscle Biology and Nutrigenomics Laboratory, Universidade Federal de Viçosa, Viçosa, MG, 36570-900, Brazil

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Abstract

The aim of this study was to evaluate the effects of dietary supplementation of purified lignin on the performance, relative organ weights, serum metabolites, and gene expression profiles of broiler chickens submitted to cyclic heat stress (HS). At 22 days old, a total of 280 broilers were distributed in a completely randomized design with four treatments, ten repetitions, and seven birds per experimental unit. The birds were submitted to daily cyclic HS. A high temperature of 32° C (±1) was maintained for 10 h/day (08:00–18:00 h), while a temperature of 22° C (±1) was maintained for the remaining time. The treatments were a basal diet or basal diet with the addition of 5, 10, or 15 g of purified lignin/kg of diet. Data were analyzed using one-way ANOVA and were means compared by the Tukey test at 0.05 significance. There was no effect ($P > 0.05$) of lignin supplementation on performance, carcass yield, relative weights of the bursa, spleen, and liver, or serum levels of glucose, triglycerides, uric acid, malondialdehyde, triiodothyronine, or tetraiodothyronine. The abundance of mRNA of heat shock protein 70, nuclear factor-κB, glutathione peroxidase, and Cu,Zn-superoxide dismutase in the liver was similarly unaffected ($P > 0.05$) by the treatments. In conclusion, purified lignin supplementation not improve performance or the antioxidant response of broiler chickens submitted to HS.

Keywords: performance, phenolic compounds, poultry

1. Introducion

Heat stress (HS) is a critical problem in broiler production in hot-climate areas, triggering significative economic losses. This condition results from a negative balance between the net energy flowing from the animal's body to its surrounding environment and the amount of heat energy produced by the animal (Lara and Rostagno, 2013). In general, various combinations of factors related to the thermal environment and animal characteristics can trigger this imbalance.

HS can influence the performance (Hamidi et al., 2021), immune responses (Hirakawa et al., 2020), and cellular antioxidant system (Habashy et al., 2018; Surai et al., 2019) of broiler chickens. In addition to the utilization of ventilation and cooling systems, nutritional manipulations have been suggested as an alternative to decrease the detrimental impacts of HS on poultry performance and the antioxidant system. Dietary supplementation with polyphenol curcumin improved final body weight, decreased mitochondrial malondialdehyde (MDA) concentration, and enhanced mitochondrial gene expression of superoxide dismutase in broiler chickens submitted to HS (Zhang et al., 2018).

Lignin is a polyphenolic polymer naturally occurring in the cell walls of plants (Vance et al., 1980). In animal nutrition, lignin is mostly regarded as a barrier to nutrient digestibility. Incidentally, in the paper-making industry, purified lignin is recovered as a byproduct of cellulose production after various process (sulfite, kraft, or alcell). In its purified form, lignin contains several low-molecular-weight phenolic monomers, such as carvacrol and cinnamaldehyde, that possess biological effects not characteristic of native lignin (Bozin et al., 2006; Baurhoo et al., 2007a). Studies have investigated the benefits of these phenolic monomers on the production and health of broilers (Bosetti et al., 2020; Galli et al., 2020), but less research has utilized purified lignin.

In this study, it was hypothesized that dietary supplementation of purified lignin can improve performance and the antioxidant responses of broiler chickens submitted to HS.

Therefore, were evaluated the effects of dietary supplementation of purified lignin on the performance, relative organ weights, serum metabolites, and gene expression profiles of broiler chickens submitted to cyclic HS.

2. Material and Methods

2.1. Ethical matters

The Institutional Animal Care and Use Committee approved all animal handling procedures (case number 038/2020), and the experiment was conducted according to the experimental protocol for the use of live birds from the Brazilian College of Animal Experimentation.

2.2. Birds, experimental design, and diets

The experiment was conducted in Viçosa, MG, Brazil (20°45'57.19" S, 42°51'35.42" W, and 682 m altitude). The male broiler chickens (Cobb 500) used in the experiment were obtained from a commercial hatchery (Rivelli Alimentos SA, Matheus Leme, MG, Brazil). Thechicks were vaccinated against bursal disease and Marek's disease (Serotype 3, Live Marek's Disease Vector, Merial Inc., Athens, GA). From one day old until the beginning of the experiment, the birds were reared in a masonry house divided into protected circular pens containing tube feeders, manual drinkers, and a litter of wood shavings. They had free access to water and were fed *ad libitum* with a corn/soybean meal-based mash diet formulated to meettheir nutritional requirements according to Rostagno et al. (2017).

At 22 days old, 280 broiler chickens (983 ± 38 g) were distributed based on body weight in a completely randomized design with four treatments, ten repetitions, and seven birds per

experimental unit. The birds were housed in 40 wire floor cages (1,008 cm^2/bird) in a four-level battery equipped with a trough feeder and a nipple drinker.

The birds were submitted to daily cyclic HS in controlled chambers. A high temperature of 32° C (±1) was maintained for 10 h/day (08:00–18:00 h), while the temperature was set at 22° C (±1) for the remaining time. The relative humidity of the air inside the chambers was maintained at 65.0% (± 5%).

The treatments were a basal diet or basal diet with the addition of 5, 10, or 15 g of purified lignin/kg of diet. The purified lignin used in this research was extracted from *Eucalyptus urograndis* through the kraft process, used in pulp and paper production. The corn/soybean meal basal diet was formulated to meet the nutritional recommendations given by Rostagno et al. (2017; Table 1). Purified lignin in the basal diet was used instead of the inert. Diets were prepared in mash form. Free access to water and feed was provided throughout the experimental period (22 to 42 days old). The light program adopted for the entire experimental period was 18 h of light (4:00 to 22:00) and 6 h of dark.

Table 1. Ingredients and nutrient composition of basal diet (as fed basis).

Ingredients (g/kg)	22-42 days of age
Corn, 78.6 g/kg	568.2
Soybean meal, 450 g/kg	322.5
Soybean oil	60.95
Dicalcium phosphate	13.28
Limestone	6.86
Salt	4.81
Dl- Methionine, 999 g/kg	2.71
L-Lysine HCl, 780 g/kg	1.92
Vitamin premix[1]	1.20
Trace mineral premix[2]	1.00
Choline chloride, 600 g/kg	0.80
L-Threonine, 985 g/kg	0.54
L-Valine, 990 g/kg	0.24
Inert	15.00
Calculated composition (g/kg, unless shown)	
Metabolizable energy, (Kcal/Kg)	3.200
Crude protein	195.0
Calcium	7.05
Available phosphorus	3.41
Sodium	2.03
Digestible glycine + serine	15.52
Digestible lysine	10.77
Digestible Methionine + Cysteine	7.97
Digestible valine	8.29
Digestible threonine	7.11
Digestible thyptophan	2.18

[1]Vitamin premix provided per kg of diet: vitamin A, 11,566 IU; vitamin D_3, 2,892 IU; vitamin E, 43.3 IU; vitamin K_3, 2.32 mg; vitamin B1, 3.12 mg; vitamin B12, 0.019 mg; vitamin B6, 4.33 mg;vitamin B5, 15.54 mg; vitamin B3, 47.0 mg; vitamin B9, 1.08 mg; biotin, 0.11 mg. [2] Trace mineral premix provided per kg of diet: Mn, 58.36 mg; Zn, 54.21 mg; Fe, 41.68mg; Cu, 8.31mg; I, 0.843 mg; Se, 0.250 mg.

2.3. Performance and sample collection

The birds, as well as the feed leftovers, were weighed at 42 days of age to calculate feed intake (FI), weight gain (WG), and the feed conversion ratio (FCR). Mortalities were recorded throughout the experimental period, and the necessary corrections of performance data were carried out.

At 42 days old, three birds with weights closest to the average weight for their respective experimental unit were selected. One bird was used for blood collection. After blood collection, the bird was euthanized by cervical displacement and slaughtered. Liver samples were collected, stored individually in cryogenic tubes, and placed in liquid nitrogen. These samples were transferred to freezer storage at -80°C until the RNA extraction process.

The two remaining birds, after 8 hours of fasting, were euthanized by cervical displacement and slaughtered to measure the yield of carcass, breast, and thigh with a drumstick, as well as the relative weight of the lymphoid organs (bursa and spleen), liver, intestine, and abdominal fat. Carcass yield (CY) was calculated in relation to living weight before slaughter [%CY = (carcass weight x 100)/live weight] and breast and thigh yield with drumstick, and as a function of carcass weight [%Part = (part weight x 100)/carcass weight]. The relative weights of the bursa, spleen, liver, intestine, and abdominal fat were calculated in relation to the live weight of the birds before slaughter.

2.4. Serum parameter measurement

The collected blood was used to analyze serum levels of glucose, uric acid, triglycerides (Cobas c 311; Roche Diagnostics GmbH, Basel, Switzerland), and the hormones triiodothyronine (T3) and tetraiodothyronine (T4; Atellica IM, Siemens Healthcare Diagnostics Inc, New York, USA), following the manufacturer's instructions. To measure MDA, 2.5 ml of 20% trichloroacetic acid and 1.0 ml of 0.67% thiobarbituric acid were added to 0.5 ml of serum;

the mixture was then heated for 30 minutes in boiling water. The resulting chromogen was extracted with 4.0 ml of n-butyl alcohol. The absorbance of the organic phase was determined at a wavelength of 530 nm.

2.5. Total RNA extraction, cDNA synthesis, and RT-qPCR analysis

Total RNA was extracted from 50 mg of powdered liver samples, using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting precipitate was rehydrated with 25 μL of UltraPure DNase/RNase-Free water. The RNA concentration was estimated using a NanoDropTM Lite Spectrophotometer (ThermoFisher Scientific, Beverly, MA, USA). RNA integrity was determined in 1.0% agarose gel. The first cDNA strand was synthesized using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Thermo Fisher Scientific, Beverly, MA, USA). The primer sets used are shown in Table 2; β-actin (β-ACT) was used as the reference gene for data normalization. The following target genes were assessed: heat shock protein 70 (HSP70), nuclear factor-κB (NF-κB), glutathione peroxidase (GPX), and Cu,Zn-superoxide dismutase (SOD1).

The RT-qPCR analyses were performed in duplicate with an Applied Biosystems™ QuantStudio Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Beverly, MA, USA), using the Relative Quantification method and applying the SYBR® Green system (Applied Biosystems, Foster City, CA, USA) and GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, WI, USA). PCR reactions were submitted to the cycles protocol according to the program: 95° C for 2 minutes, 40 cycles of 95° C for 15 seconds, and 60° C for 1 minute. The threshold cycle (Ct) values obtained were later normalized (ΔCt) based on the Ct values of the endogenous control gene β-ACT. The calculation of the gene expression

levels was performed according to the 2-ΔCt method, as described by Livak & Schmittgen (2001).

Table 2. Primer sequences.

Gene	Forward sequence	Reverse sequence
HSP70	CACCATCACTGGCCTTAACGT	TTATCCAAGCCATAGGCAATAGC
NF-κB	GTGTGAAGAAACGGGAACTG	GGCACGGTTGTCATAGATGG
GPX	GACCAACCCGCAGTACATCA	GAGGTGCGGGCTTTCCTTTA
SOD1	AGGGGGTCATCCACTTCC	CCCATTTGTGTTGTCTCCAA
β-actin	TGCTGTGTTCCCATCTATCG	TTGGTGACAATACCGTGTTCA

HSP70: heat shock protein 70; *NF-κB*: nuclear factor-κB; *GPX*: glutathione peroxidase; *SOD1*: Cu, Zn-superoxide dismutase.

2.6. Statistical analysis

Cage averages were considered as an experimental unit for statistical analysis of the growth performance parameters. For analyses of the yield of carcass, breast, and thigh with drumstick—as well as the relative weights of the lymphoid organs, liver, intestine, and abdominal fat—the average of two birds per replicate was considered as the experimental unit. For serum and gene expression analyses, one bird per replicate was considered as the experimental unit. Data were analyzed via one-way ANOVA, according to the following general model:

$$Y_{ij}=\mu + \alpha_i + \varepsilon_{ij},$$

where: Y_{ij} is the measured dependent variable, μ is the overall mean, α_i is the effect of treatments, and ε_{ij} is the random error.

Analyses were carried out using the GLM procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Comparison between treatment averages was performed using the Tukey test. A significant level of 0.05 was applied.

3. Results

3.1. Performance and carcass yield

There was no treatment effect (P > 0.05) on performance the broiler chickens at 42 days of age subjected to cyclical heat stress (Tables 3).

Table 3. Growth performance of broiler chickens from 22 to 42 days of age.

	Purified lignin (g/kg of diet)				SEM[1]	*P*-value
	0	5	10	15		
BWG (kg/bird)	1.986	1.949	1.944	1.895	0.015	0.184
FI (kg/bird)	3.598	3.669	3.645	3.626	0.012	0.176
FCR	1.81	1.89	1.88	1.92	0.01	0.094

[1]SEM: standard error of mean (n = 10 for treatment); BWG: Body weight gain; FI: feed intake; FCR: feed conversion ratio.

3.2. Relative weights of organs

There was no significant treatment effect (P > 0.05) on the relative weights of the bursa, spleen, liver (Table 4). There was no significant treatment effect (P > 0.05) on the carcass yield, intestine and abdominal fat (% of live weight) and breast and thighswith drumstick (% of carcass) broiler chickens at 42 days of age (Table 5).

Table 4. Relative weights of bursa, spleen and liver (% of live weight) of broiler chickens at 42 days of age subjected to cyclical heat stress

	Purified lignin (g/kg of diet)				SEM[1]	*P*-value
	0	5	10	15		
Bursa (%)	0.090	0.088	0.102	0.097	0.006	0.851
Spleen (%)	0.107	0.104	0.113	0.105	0.003	0.789
Liver (%)	1.528	1.586	1.529	1.600	0.021	0.515

[1]SEM: standard error of mean (n = 10 for treatment).

Table 5. Carcass yield, intestine and abdominal fat (% of live weight) and breast and thighs with drumstick (% of carcass) of broiler chickens at 42 days of age subjected to cyclical heat stress

	Purified lignin (g/kg of diet)				SEM[1]	*P*-value
	0	5	10	15		
Carcass yield (%)	78.03	78.29	77.62	77.59	0.25	0.733
Breast (%)	37.39	37.92	37.86	37.57	0.19	0.747
Thighs with drumstick (%)	26.72	26.58	26.82	27.02	0.17	0.854
Intestine (%)	3.12	3.11	3.15	3.09	0.04	0.946
Abdominal fat (%)	0.79	0.80	0.71	0.69	0.03	0.481

[1]SEM: standard error of mean (n = 10 for treatment).

3.3. Serum metabolites

Purified lignin supplementation did not influence (P > 0.05) serum levels of glucose, triglycerides, uric acid, MDA, or the T3 and T4 hormones of broiler chickens at 42 days of age subjected to cyclical heat stress (Table 6)

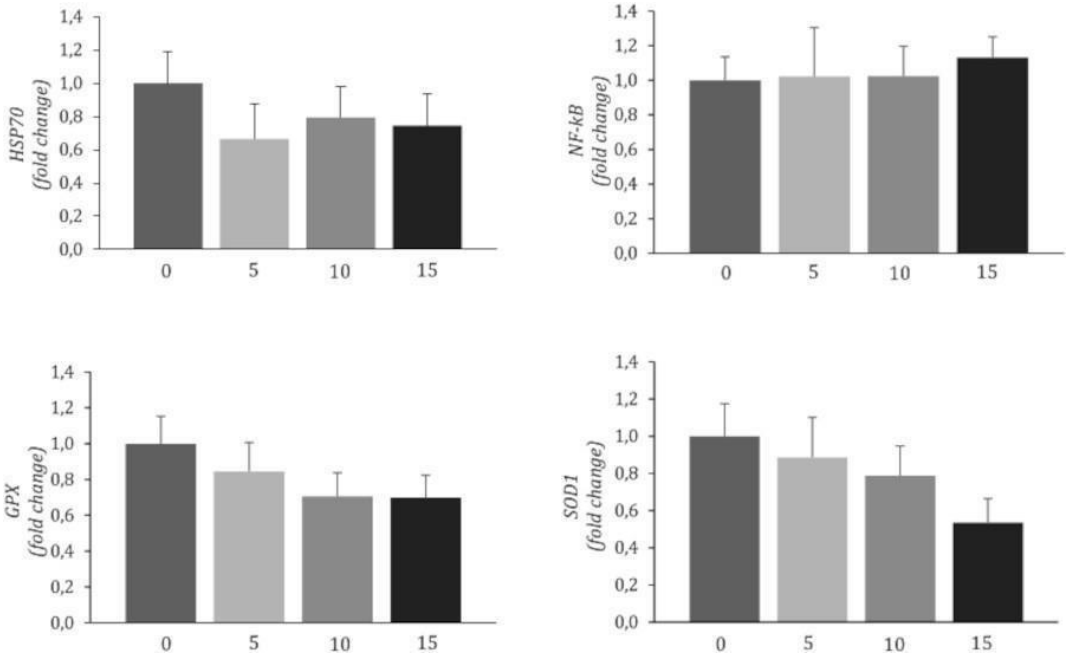
Table 6. Serum metabolites in broiler chickens at 42 days of age

	Purified lignin (g/kg of diet)				SEM[1]	*P*-value
	0	5	10	15		
Glucose (mg/dL)	207.6	205.3	210.7	209.3	3.7	0.965
Triglycerides (mg/dL)	46.5	48.4	37.9	37.8	2.6	0.338
Uric acid (mg/dL)	3.76	4.11	4.33	3.46	0.30	0.768
Malondialdehyde (nmol/mL)	2.89	2.93	2.98	2.80	0.10	0.944
T3 (ng/mL)	0.39	0.30	0.35	0.42	0.04	0.726
T4 (mcg/mL)	0.75	0.96	0.68	0.80	0.06	0.469

[1]SEM: standard error of mean (n = 10 for treatment); T3: triiodothyronine; T4: thyroxine.

3.4. mRNA content

The abundance of mRNA of NF-kB, HSP70, GPX, and SOD1 in the liver was not influenced by the treatments (P > 0.05; Figure 1).



4. Discussion

HS is known to impair the performance of broilers (Hamidi et al., 2021) and cause mitochondrial damage by destabilizing the antioxidant system with an increase in reactive oxygen species (Lu et al., 2017). Furthermore, HS can reduce carcass yield (Baxter et al., 2020). In this study, it was expected that lignin supplementation in its purified form would improve the performance of broiler chickens submitted to HS. However, this hypothesis has not been confirmed. No effects of lignin supplementation were observed on broiler performance, carcass yield, or the relative weights of carcass parts. A previous study reports that broilers supplemented with 12.5 g/kg of purified lignin presented increased villi height and a greater

number of goblet cells in the jejunum, along with a lower population of E. coli in the litter; however, there was no positive effect on performance (Baurhoo et al., 2007b).

Performance is affected when birds are submitted to HS, because their metabolic rates are altered, and their consumption is reduced. This is justified by the diversion of nutrients to meet homeostatic activities, in addition to the impairment of lipid and carbohydrate absorption (Montgomery et. al., 2015) and changes in serum glucose levels due to changes in the gene expression of nutrient transporters, such as the family of glucose transporters (Sun et al., 2015). However, in the present study, according to the performance results, lignin supplementation did not influence the serum levels of glucose, uric acid, or triglycerides.

Atrophy of lymphoid tissues and liver may be associated with a series of HS-induced disorders (e.g., malnutrition, inflammation, and oxidative stress; Quinteiro-Filho et al., 2010; Hirakawa et al., 2020). In the present study, were evaluated the relative weights of the lymphoid organs (bursa and spleen) and liver, finding that lignin supplementation did not influence these variables either.

Metabolic alterations caused by HS are evidenced by changes in the concentrations of hormones responsible for basal metabolism, such as thyroid hormones. HS normally induces reductions in T3 and T4 plasma concentrations. This response is considered an adaptive mechanism to avoid extra heat load by reducing metabolic heat production, thereby reducing maintenance energy requirements (Gonzalez-Rivas et al., 2020). However, no effects were observed on the serum levels of the T3 and T4 hormones with lignin supplementation.

Several researchers have reported that thermal stress increases the expression of the HSP70 gene, which plays an essential protective role against tissue injuries (Yu et al., 2008; Varasteh et al., 2015). In the present study, to assess the ability of lignin to reduce the impact

of HS, was measured the mRNA expression of HSP70 in the livers of broilers; no effect was observed.

HS increases the production of reactive oxygen species and may decrease natural antioxidant capabilities; both of these factors can induce oxidative stress (Gonzalez-Rivas at al. 2020). NF-κB plays an active role in the inflammatory response of chickens (Lan et al., 2017), and studies have shown an association between increased NF-κB expression levels and HS (Sahin and Smith, 2016). Previous studies with broilers indicate that exposure to HS downregulates the mRNA expression of NF-κB in the bursa of Fabricius (Liu et al., 2021), while it upregulates the mRNA expression of SOD (Roushdy et al., 2018). Another consequence of HS is an increase in lipid peroxidation, which generates greater production of MDA (Pamok et al., 2009). In a study with the Isa Brown laying strain, lignin supplementation in a diet contaminated with zearalenone prevented an increase in glutathione peroxidase activityin the duodenal mucosa (Gresakava et al., 2012). Thus, it was hypothesized that dietary supplementation of purified lignin could improve the antioxidant response of broiler chickens submitted to HS. To assess the ability of lignin to influence antioxidant response, wereevaluated the mRNA abundance of NF-kB, GPX, and SOD1 in the liver. However, in accordance with other results observed in this study, these variables were not influenced.

5. Conclusion

Supplementation of 5, 10, or 15 g of purified lignin/kg of diet does not improve performance or the antioxidant response of broiler chickens submitted to HS.

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Chapter 2:

Capsaicinoids supplementation improves the performance and intestinal morphometry of LPS-challenged broiler chickens

Rayanne Andrade Nunes[1], Luiz Fernando Teixeira Albino[1], Marcio de Souza Duarte[2], Claudson Oliveira Brito[3], Ricardo Vianna Nunes[4], Tiago Goulart Petrolli[5], Samuel Oliveira Borges[1], Hallef Rieger Salgado[1], Romário Duarte Bernardes[1], Larissa Pereira Castro[1]; Beatriz Garcia do Vale[1], Arele Arlindo Calderano[1,*]

[1]Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, 36570-900, Brazil

Abstract

This study evaluates the effects of dietary supplementation of capsaicinoids (CAP) on performance, intestinal morphometry, and gene expression of broiler chickens challenged with lipopolysaccharides (LPS). At 8 days of age, 144 male broilers (Cobb 500) were distributed in a completely randomized design with three treatments, eight replications, and six birds per experimental unit. The treatments consisted of a control diet (CON), the control diet and LPS administration (CON+LPS), and the control diet supplemented with 1 mg CAP/kg diet and LPS administration (CAP+LPS). The LPS challenge consisted of intraperitoneal applications at 14, 16, 18, and 20 days of age. Four hours after LPS application at 20 days of age, one bird per experimental unit was sacrificed to collect serum and jejunum samples. Jejunum samples were used to analyze mRNA. The performance of broiler chickens was evaluated at 21 days of age. Data were subjected to ANOVA, and means were compared with the Tukey test at 0.05 significance. The CON broilers exhibited the highest feed intake ($P = 0.011$) and better feed conversion rate ($P = 0.022$) compared to the CON+LPS. The CAP+LPS broilers showed higher body weight gain than the CON+LPS; however, it was lower than the CON broilers ($P < 0.001$). The CON+LPS broilers showed higher crypt depth than the CON and CON+LPS broilers ($P = 0.002$). The treatment had no significant effect on villus height ($P > 0.05$). Higher mRNA expression of superoxide dismutase ($P = 0.046$) and catalase ($P = 0.011$) was observed in the jejunum of CON broilers compared to CON+LPS. However, treatment did not affect the mRNA expression of interleukin 10, interleukin 1β, glutathione peroxidase, and nuclear factor-κB ($P > 0.05$). In conclusion, supplementation with 1 mg CAP/kg diet improves the growth performance and intestinal morphometry of LPS-challenged broiler chickens.

Keywords: feed additive; inflammation; poultry

Abbreviations: CAP, capsaicinoids; FI, feed intake; FCR, feed conversion rate; NF-κB, nuclear factor-κB; LPS, lipopolysaccharides; VH, villus height; CD, crypt depth; BWG, body

weight gain; MDA, malondialdehyde; NF-κB, nuclear factor-κB; IL-1β, interleukin 1β; IL-10, interleukin 10; GPX, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase.

1. Introduction

In practical production systems, broilers are frequently subjected to inflammatory challenges, which harm their overall performance. Pro-inflammatory mediators are known to suppress appetite, resulting in diminished nutrient intake. The balance between the allocation of nutrients for the immune response and the potential decrease in feed intake (FI) contributes to reducing the availability of nutrients intended for growth and can induce tissue catabolism (Takahashi et al., 2008; Broom and Kogut, 2018).

Capsaicinoids (CAP) are compounds naturally present in some species of peppers, and they are receiving increasing amounts of attention due to their bioactive properties and the potential effects they can have on the immune response of broilers (El-Hack et al. 2022; Kreuz et al. 2022). Capsaicin and dihydrocapsaicin are the primary molecular components, representing approximately 90% of the total CAP in peppers (Giuffrida et al., 2013). CAP are characterized by their antioxidant and antimicrobial activities (Hayman and Kam, 2008; Kreuz et al., 2022). Studies have also indicated that these molecules can decrease lipid peroxidation and increase nutrient digestibility, possibly because the production of digestive enzymes is stimulated (Conforti et al., 2007; Oboh et al., 2007; Herrero-Encinas et al., 2023). Furthermore, CAP has been shown to play an important role in mitigating heat stress (Oboh et al., 2007) and improving FI, feed conversion rate (FCR), and carcass characteristics (Li et al., 2022; Herrero-Encinas et al., 2023; Zanotto et al., 2023).

Investigating the effects of CAP is of special relevance in situations where birds are subjected to stress and inflammatory challenges, factors that can induce excessive activation of

nuclear factor-κB (NF-κB) and trigger adverse consequences, compromised health, and reduced productive performance (Nunes et al., 2020; Surai et al., 2021).

Thus, in this study, we hypothesize that CAP has anti-inflammatory properties and that its use as a dietary supplement can mitigate the decrease in broiler performance caused by inflammatory challenges. Thus, we aim to evaluate the effect of dietary supplementation with CAP on performance, intestinal morphometry, and gene expression of broiler chickens challenged with lipopolysaccharides (LPS).

2. Material and methods

2.1. Ethical issues

The Animal Care and Use Committee of the Universidade Federal de Viçosa, Brazil, approved all animal handling procedures (protocol nº 39/2021). The experiment was conducted in accordance with the experimental protocols for the use of live birds from the Brazilian College of Animal Experimentation.

2.2. Birds, experimental design, and diets

For this study, 144 male broiler chickens (Cobb 500) were obtained from a commercial hatchery at 1 day of age (Rivelli Alimentos SA, Matheus Leme, MG, Brazil). Birds were vaccinated against Gumboro and Marek (serotype 3, live Marek's disease vector, Merial Inc. Athens, GA). From 1 to 7 days of age, the birds were raised in protective circles according to lineage management recommendations.

At 8 days of age, the birds were weighed (192.1 ± 66 g) and distributed in a completely randomized experimental design with three treatments, eight replications, and six birds per experimental unit. The birds were housed in wire cages (500 cm^2/bird) equipped with trough-

type feeders and nipple-type drinkers, totaling 24 experimental units. The treatments were a control diet (CON), the control diet and LPS administration (CON+LPS), and a control diet supplemented with 1 mg CAP/kg diet and LPS administration (CAP+LPS). The source of CAP used was Capcin® (ID4Feed, France) at a concentration of 5 g of CAP/kg; this product was used at a concentration of 200 mg/kg diet. The corn/soybean meal basal diet was formulated to meet the nutritional recommendations of Rostagno et al. (2017; Table 1). The diets were prepared in mashed form. The birds had free access to water and feed throughout the experimental period (8 to 21 days of age).

Table 1. Ingredients and nutritional composition of basal diets (g/kg).

Ingredients	8-21 days of age
Corn, 786 g/kg	503.7
Soybean meal, 450 g/kg	411.2
Soybean oil	45.8
Dicalcium phosphate	16.8
Limestone	8.4
Salt	5.2
DL-Methionine, 999 g/kg	3.2
L-Lysine HCl, 780 g/kg	1.5
Vitamin premix[a]	1.3
Trace mineral premix[b]	1.2
Choline chloride, 600 g/kg	1.0
L-Threonine, 985 g/kg	0.6
L-Valine, 990 g/kg	0.1
Calculated composition	
Metabolizable Energy, MJ/kg	12.76
Crude Protein	230.0
Calcium	8.78
Available Phosphorus	4.19
Sodium	2.18
Arginine	14.50
Digestible lysine	12.56
Digestible methionine + cysteine	9.29
Digestible threonine.	8.29
Digestible tryptophan	2.65
Digestible valine	9.67

[a]Vitamin premix provided per kg of diet: vitamin A, 12,528 UI; vitamin D3, 3,132 UI; vitamin E, 46.9 UI; vitamin K3, 2.51 mg; vitamin B1, 3.37 mg; vitamin B12, 0.021 mg; vitamin B6, 4.69 mg; vitamin B5, 16.8 mg; vitamin B3, 51.0 mg; vitamin B9, 1.17 mg; biotin, 0.12 mg.
[b]Trace mineral premix provided per kg of diet: Mn, 70.03 mg; Zn, 65.05 mg; Fe, 50.01 mg; Cu, 9.97 mg; I, 1.012 mg; Se, 0.3 mg.
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Birds in the CON+LPS and CAP+LPS treatment groups received intraperitoneal applications of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO; diluted in saline solution at a concentration of 1.0 mg/mL) at 14, 16, 18, and 20 days of age. The initial dose was 1 mg/kg body weight; this was increased by 12% in subsequent applications (Rakhshandeh and Lange, 2012). Birds in the CON treatment received a similar amount of saline solution.

The environmental temperature was maintained at 22 °C throughout the experimental period, and the birds were exposed to 18 hours of continuous light daily.

2.3. Performance and sample collection

At 20 days, the bird with the weight closest to the average weight of the experimental unit was chosen for sample collection. Four hours after the LPS application, blood was collected from this bird through the wing vein. The blood was centrifuged at 3,600× g at 4 °C for 10 min for separation; serum samples were stored at −20 °C until analysis. After blood collection, the bird was euthanized by cervical displacement. A 2-cm sample of the jejunum was collected, stored individually in a cryogenic tube, and then placed in liquid nitrogen. Subsequently, the jejunum samples were transferred to freezer storage at −80 °C until the RNA extraction process. Another part of the jejunum was also collected to determine the villus height (VH), crypt depth (CD), and their ratio (VH:CD). The lymphoid organs (bursa of Fabricius and spleen) were

removed and weighed separately on a digital scale (0.0001 g) to determine their weight relative to the live weight of the animal.

At 21 days of age, the birds and the leftover feed were weighed to determine body weight gain (BWG), FI, and FCR.

2.4. Serum parameter measurements

The blood samples were used to analyze the serum levels of glucose and triglycerides (Cobas c 311; Roche Diagnostics GmbH, Basel, Switzerland), following the manufacturer's instructions. To measure the serum malondialdehyde (MDA) level, 2.5 ml of 20% trichloroacetic acid and 1.0 ml of 0.67% thiobarbituric acid were added to 0.5 ml of serum, and the mixture was heated for 30 min in boiling water. The resulting chromogen was extracted with 4.0 ml of n-butyl alcohol. The absorbance of the organic phase was determined at a wavelength of 530 nm.

2.5. Determination of mRNA content

The total RNA was extracted from 50-mg samples of jejunum using TRIzol® (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The resulting precipitate was rehydrated with 25 μL of DNase/RNase-free UltraPure water. The RNA concentration was determined using a NanoDrop™ Lite Spectrophotometer (Thermo Fisher Scientific, Beverly, MA, USA), and the RNA integrity was assessed on a 1.0% agarose gel. First-strand cDNA synthesis was performed using high-capacity cDNA reverse transcription kits (Thermo Fisher Scientific, Beverly, MA, USA). The primer sets used are listed in Table 2. Due to its higher expression and stability, *β-actin* was selected as the reference gene for data normalization. The target genes evaluated were nuclear factor-κB (*NF-κB*), interleukin 1β (*IL-1β*), interleukin 10 (*IL-10*), glutathione peroxidase (*GPX*), catalase (*CAT*), and superoxide dismutase (*SOD*). RT-

qPCR analyses were performed in duplicate using Applied Biosystems™ QuantStudio Real-Time PCR Systems (Thermo Fisher Scientific, Beverly, MA, USA) with the relative quantification method. The SYBR® Green system (Applied Biosystems, Foster City, CA, USA) and a GoTaq® qPCR Master Mix kit (Promega Corporation, Madison, USA) were applied. The polymerase chain reactions followed this program: 95 °C for 2 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. The threshold cycle (Ct) values obtained were later normalized (ΔCt) based on the Ct values of the endogenous control gene β-actin. The relative gene expression levels were calculated using the $2^{-\Delta Ct}$ method, as described by Livak and Schmittgen (2001).

Table 2 Primer sequences for quantitative reverse transcription-PCR.

Gene	Forward sequence	Reverse sequences
NF-κB	GTGTGAAGAAACGGGAACTG	GGCACGGTTGTCATAGATGG
IL-1β	GCTCTACATGTCGTGTGTGATGAG	TGTCGATGTCCCGCATGA
IL-10	CATGCTGCTGGGCCTGAA	CGTCTCCTTGATCTGCTTGATG
GPX	GACCAACCCGCAGTACATCA	GAGGTGCGGGCTTTCCTTTA
SOD	AGGGGGTCATCCACTTCC	CCCATTTGTGTTGTCTCCAA
CAT	ACTGCAAGGCGAAAGTGTTT	GGCTATGGATGAAGGATGGA
β-actin	TGCTGTGTTCCCATCTATCG	TTGGTGACAATACCGTGTTCA

2.6. Intestinal morphometry

The 2-cm jejunum samples were washed in saline and kept in 10% formaldehyde phosphate buffer for 48 hours. Cross-sections were then prepared, and the segments were dehydrated in a graded ethanol series, diaphanized with xylene, and embedded in liquid paraffin at 60 °C. Paraffin blocks were fixed in a rotating microtome (Spencer® model 19459, USA), and the transverse sections were sliced to a thickness of 5 μm (the sections were made semi-serially, 1 in each of the 10 sections to avoid repeating the analyses in the same histological area). Six sections were placed on each glass slide and stained with hematoxylin–eosin. Five slides were prepared from the jejunal segment of each bird: 10 well-oriented villi were

measured per slide (50 villi per bird). The sections were examined under an optical microscope (EVOS® XL Core Imaging System, Thermo Fisher Scientific Inc., Bothell, WA) at 10× magnification. Morphometric analysis was performed using ImageJ software (National Institutes of Health, USA). The VH was measured from the top of the villi to the junction of the villus with the crypt, and the CD was measured from the base of the villus to the submucosa.The VH:CD ratio was calculated.

2.7. Statistical analysis

Data were subjected to one-way ANOVA using the GLM procedure of SAS (Statistical Analysis System, 9.4), with subsequent comparison between means using the Tukey test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Performance

The broilers subjected to the CAP+LPS treatment showed higher BWG than those subjected to the CON+LPS treatment; however, it was lower than the CON broilers ($P < 0.001$; Table 3). The broilers in the CON treatment exhibited the highest FI ($P = 0.011$) and better FCR than those in the CON+LPS group ($P = 0.022$).

Table 3 Growth performance of broiler chickens from 8 to 21 days of age.

	CON	CON+LPS	CAP+LPS	SEM	*P-value*
FI (kg/bird)	1.031a	0.974b	1.005ab	0.008	0.011
BWG (kg/bird)	0.679a	0.611c	0.647b	0.006	<0.001
FCR	1.52b	1.59a	1.55ab	0.01	0.022

FI: feed intake; BWG: body weight gain; FCR: feed conversion rate. CON: control diet without LPS administration; CON+LPS: control diet with LPS administration; CAP+LPS: control diet supplemented with 1 mg CAP/kg diet and LPS administration.SEM: standard error of means (n = 8 for each treatment). Means on the same line, followed by different letters, differ from each other by the Tukey test ($P < 0.05$).

3.2. Relative weight of organs

The LPS challenge, independent of the supplementation of CAP, increased the relative spleen weight of the broilers (P < 0.001; Table 4). Treatment had no significant effect on the relative weight of the bursa of Fabricius (P > 0.05).

Table 4. Relative weight of lymphoid organs of broiler chickens at 20 days of age.

	CON	CON+LPS	CAP+LPS	SEM	*P-value*
Bursa (%)	0.180	0.180	0.184	0.007	0.070
Spleen (%)	0.090b	0.165a	0.174a	0.009	<0.001

CON: control diet without LPS administration; CON+LPS: control diet with LPS administration; CAP+LPS: control diet supplemented with 1 mg CAP/kg diet and LPS administration. SEM: standard error of means (n = 8 for each treatment). Means on the same line, followed by different letters, differ from each other by the Tukey test ($P < 0.05$).

3.3. Serum metabolites

The treatment had no significant effect on the serum levels of glucose, triglycerides, or cholesterol ($P > 0.05$; Table 5).

Table 5. Serum metabolites of broiler chickens at 20 days of age.

	CON	CON+LPS	CAP+LPS	SEM	*P-value*
MDA (nmol/Ml)	2.43	2.57	2.53	0.08	0.793
Glucose (mg/dL)	231.2	235.6	230.8	5.1	0.922
Triglycerides (mg/dL)	43.75	47.00	41.62	2.92	0.767

CON: control diet without LPS administration; CON+LPS: control diet with LPS administration; CAP+LPS: control diet supplemented with 1 mg CAP/kg diet and LPS administration. MDA: malondialdehyde. SEM: standard error of means (n = 8 for each treatment). Means on the same line, followed by different letters, differ from each other by the Tukey test ($P < 0.05$).

3.4. Intestinal morphometry

The broilers that received the CON+LPS treatment showed higher CD than those that received the CON and CON+LPS treatments (P = 0.002; Table 6). The VH:CD ratio was higher

in the CON broilers (P = 0.022) than in those that received the CON+LPS treatment. However, the treatment did not significantly affect the VH (P > 0.05).

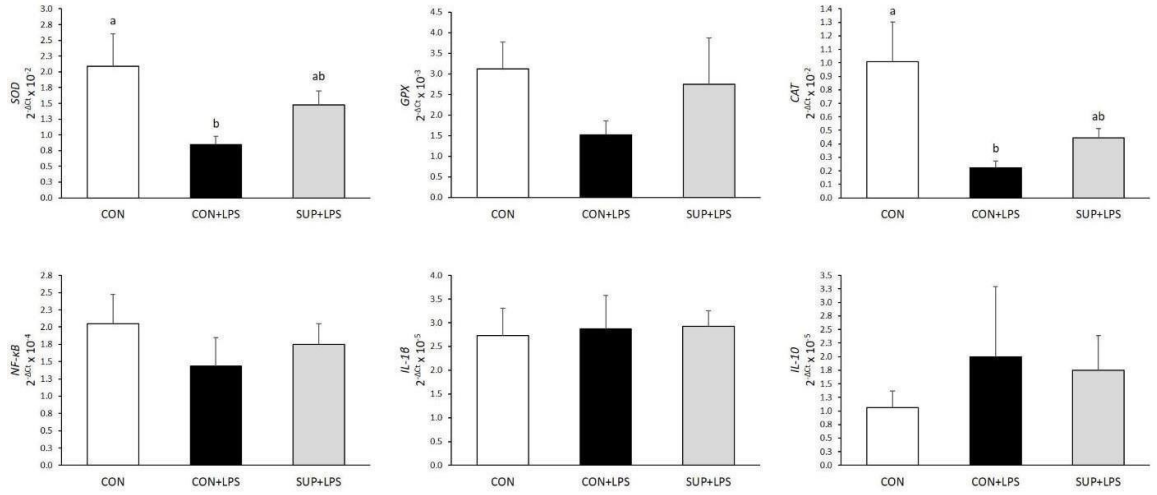
Table 6. Villus height (VH), crypt depth (CD), and VH:CD ratio in the jejunum of broiler chickens at 20 days of age.

	CON	CON+LPS	CAP+LPS	SEM	*P-value*
VH (μm)	935.9	887.9	914.9	21,0	0.664
CD (μm)	195.2b	236.9a	201.2b	5.8	0.002
VH:CD (μm)	4.90a	3.77b	4.56ab	0.17	0.022

CON: control diet without LPS administration; CON+LPS: control diet with LPS administration; CAP+LPS: control diet supplemented with 1 mg CAP/kg diet and LPS administration. SEM: standard error of means (n = 8 for each treatment). Means on the same line, followed by different letters, differ from each other by the Tukey test ($P < 0.05$).

3.5. mRNA content

A higher mRNA expression of SOD ($P = 0.046$) and CAT ($P = 0.011$; Figure 1) was observed in the jejunum of the CON broilers than in broilers given the CON+LPS treatment. However, treatment did not affect the mRNA expression of IL-10, IL-1, GPX, and NF-κB ($P > 0.05$).



4. Discussion

The hypothesis of this study is that CAP have anti-inflammatory properties, and their use as a dietary supplement can mitigate the decrease in broiler performance caused by inflammatory challenges. To test this hypothesis, we used bacterial LPS administration. This method is widely used to induce inflammatory responses in poultry studies (Li et al., 2018; Chen et al., 2020; Kreus et al., 2020). The inflammatory process can negatively affect broiler performance due to the allocation of nutrients to immune response (Nunes et al., 2020; Elazab et al., 2022). Furthermore, inflammatory stress induced by LPS injection causes a reduction of FI in broilers, which might be due to the anorexia response through the control of the hypothalamic–pituitary–adrenal axis (Klasing et al., 1987; Zhang et al., 2010).

In the present study, administering LPS reduced FI and BWG, and worsened the FCR of the broilers. The LPS challenge also affected intestinal morphometry. The challenged broilers presented higher CD and lower VH:CD. This may partly explain the reduction in performance. However, when the broilers were LPS challenged and supplemented with CAP, the BWG partially recovered. In addition, CD was reduced. This was the only finding that could explain the improvement in the BWG of the birds on diets supplemented with CAP. VH and CD are important indicators of the structural integrity of the intestinal mucosal and intestinal digestion and absorption function. Crypts are the site of new enterocyte multiplication (Swatson et al., 2002), and a larger CD is associated with worse intestinal quality and a high intestinal renewal rate (Souza et al., 2020). Li et al. (2022) have identified improvements in the FCR of broilers supplemented with 2 and 4 mg capsaicin/kg and attributed part of this result to the enhanced VH, villus width, and villous surface area in the jejunum of the broilers, suggesting that capsaicin improved the utilization of nutrients.

The assessment of lymphoid organ weights is a crucial indicator of the immune response of birds, providing valuable insights into their overall health (Tong et al., 2012). The spleen is

a lymphoid organ and produces antibodies when subjected to antigenic stimulation (Pozo et al., 2009). In the present study, the LPS challenge caused hypertrophy in the spleen of the broilers. This result is similar to the observations of Chen et al. (2020), highlighting the importance of this organ and the increase in the metabolism of the chickens during the acute-phase immune response. However, supplementation with CAP at the dose studied did not reduce this effect.

Regarding the serum metabolites of the broilers, the LPS injections were expected to induce hypolipidemia and hypoglycemia (Elazab et al., 2022). In addition, an increase in MDA was expected in response to the LPS challenge (Zheng et al., 2016). MDA is an important marker of lipid peroxidation, and higher levels indicate oxidative stress in the body (Ghasemi-Sadabadi et al., 2020). However, in the present study, the LPS challenge did not influence the serum levels of glucose, triglycerides, or MDA. Furthermore, CAP supplementation did not influence the levels of these metabolites in the serum of the broilers. This result is similar to those reported by Kreuz et al. (2022) with supplementation of 1 and 2 mg of CAP/kg feed. However, in a recent study, Zanotto et al. (2023) have observed a reduction of thiobarbituric acid levels in the serum and breast meat of turkeys fed diets supplemented with 4 mg CAP/kg feed.

Oxidative stress in cells can lead to lipid peroxidation, protein nitration, DNA damage, and apoptosis (Mishra and Jha, 2019). However, the burden of reactive oxygen species (ROS) production can be counteracted by an antioxidant defense system, including the enzymes SOD and CAT. The LPS challenge increases the production of ROS in broilers (Wang et al., 2023). In addition, the LPS challenge suppresses the activity of SOD and CAT in the serum, liver, and total antioxidant capacity in broilers (Zheng et al., 2016; Wang et al., 2023). In the present study, the LPS challenge reduced the mRNA expression of SOD and CAT in the jejunum of broilers; however, the CAP supplementation did not mitigate this effect. This result does not

agree with the previous study of Kreuz et al. (2022), which reported that supplementation with 1 or 2 mg CAP/kg diet improved the expression of SOD in the jejunum of broilers.

The inflammatory response of broilers can be measured from the increase in the production of NF-κB and by increased serum concentrations of cytokines, such as TNF-α, IL-1β, IL-6, and IL-10 (Zhang et al., 2019). However, in the present study, neither the LPS challenge nor CAP supplementation changed the mRNA expression of NF-κB, IL-1β, or IL-10 in the jejunum of the broilers. This result also does not agree with Kreuz et al. (2022), which reported a reduction in the expression of NF-κB mRNA in the jejunum of broiler chickens receiving 1 and 2 mg of CAP/kg diet. Liu et al. (2021) have also observed that birds supplemented with natural capsaicin extract had higher levels of serum total antioxidantcapacity and SOD and lower levels of the pro-inflammatory cytokines IL-1β.

5. Conclusion

Supplementation with 1 mg CAP/kg diet improved the growth performance and intestinal morphometry of LPS-challenged broiler chickens. However, this level ofsupplementation did not influence the expression of genes related to oxidative and inflammatory stress in the jejunum of these birds.

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General Conclusion

Supplementation with 5, 10, or 15 g of purified lignin per kg of diet did not improve the performance or antioxidant response of broiler chickens subjected to heat stress. Conversely, supplementation with 1 mg of capsaicinoids per kg of diet improved the growth performance and intestinal morphometry of LPS-challenged broiler chickens. However, this level of supplementation did not influence the expression of genes related to oxidative and inflammatory stress in the jejunum of these birds.

ANEXOS

UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
CEUAP/UFV

Campus Universitário – Viçosa, MG – 36570-900 – Telefone:(31) 3899.3275 – e-mail: ceuap@ufv.br – site: www.ceuap.ufv.br

Viçosa, 26 de Agosto de 2020

CERTIFICADO

Certificamos que o projeto intitulado **"Suplementação de compostos fenólicos para frangos de corte submetidos a estresse por calor"**, protocolo **nº 038/2020**, sob a responsabilidade de **Arele Arlindo Calderano** - 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) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi apreciado pela Comissão de Ética no Uso de Animais de Produção da Universidade Federal de Viçosa (CEUAP-UFV) em reunião de **18 de ago. de 2020.**

Finalidade: **(x)Pesquisa ( )Ensino** Vigência do Projeto: de **01 de novembro de de 2020** a **30 de outubro de 2022** Espécie/linhagem: **Frango de corte *(Gallus galus domesticus)*** Nº de animais: **280**
Peso: **1,0 Kg** Idade: **22 dias** Sexo: **Macho** Origem: **Incubatorio Rivelli Cnpj/CPF: 478.715.616-49 Endereço: Rua Leão José, 257 Mateus Leme, MG Responsável : Maria Cecília CRMV: 10595**

CERTIFICATE

We certify that the project entitled **"Supplementation of phenolic compounds for broilers subjected to heat stress",** protocol **nº 038/2020**, under the responsibility of **Arele Arlindo Calderano** - which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **Aug. 18th, of 2020**.

Finality: **(x)Research ( )Education**
Duration of the Project: from **Nov. 01st, of 2020** to **Oct. 30th, of 2022**.
Species / strain: **Broiler *(Gallus galus domesticus)*** Nº of animals: **280**
Weight: **1,0 Kg** Age: **22 days** Sex: **Male** Source: **Incubatorio Rivelli Cnpj/CPF: 478.715.616-49 Endereço: Rua Leão José, 257 Mateus Leme, MG Responsável : Maria Cecília CRMV: 10595**

Luciana Navajas Rennó
Coordenadora da CEUAP/UFV

UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
CEUAP/UFV

Campus Universitário – Viçosa, MG – 36570-900 – Telefone:(31) 3899.3275 – e-mail: ceuap@ufv.br – site: www.ceuap.ufv.br

Viçosa, 06 de Jul, de 2021

CERTIFICADO

Certificamos que o projeto intitulado **"Efeitos da Capsaicina sobre o desempenho, metabolizabilidade de nutrientes, resposta inflamatória e morfometria intestinal de frangos de corte desafiados com LPS"**, protocolo **nº 039/2021**, sob a responsabilidade de **Arele Arlindo Calderano** - 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) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi apreciado pela Comissão de Ética no Uso de Animais de Produção da Universidade Federal de Viçosa (CEUAP-UFV) em reunião de **09 de Julho de 2021.**
Finalidade: **(x)Pesquisa ( )Ensino** Vigência do Projeto: de **01 de Ago. 2021** a **30 de Jul. de 2022**
Espécie/linhagem: **Frango de corte *(Gallus gallus domesticus)*** Nº de animais: **192**
Peso: **0,225 kg** Idade: **08 dias** Sexo: **Macho** Origem: **: Incubatorio Rivelli Cnpj/CPF: 478.715.616-49 Endereço: Rua Leão José, 257 Mateus Leme, MG Responsável : Maria Cecília CRMV: 10595**

CERTIFICATE

We certify that the project entitled **"Effects of Capsaicin on performance, nutrient metabolizability, inflammatory response and intestinal morphometry of LPS-challenged broilers",** protocol **nº 039/2021**, under the responsibility of **Arele Arlindo Calderano** - which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **Jul. 09th, of 2021**.
Finality: **(x)Research ( )Education**
Duration of the Project: from **Aug. 01st of, 2021** to **Jul. 30th, of 2022**.
Species / strain: ***Broiler (Gallus gallus domesticus)*** Nº of animals: **192**
Weight: **0,225 kg** Age: **08 days** Sex: **Male** Source: **: Incubatorio Rivelli Cnpj/CPF: 478.715.616-49 Endereço: Rua Leão José, 257 Mateus Leme, MG Responsável : Maria Cecília CRMV: 10595**

Luciana Navajas Rennó
Coordenadora da CEUAP/UFV