

UNIVERSIDADE FEDERAL DE VIÇOSA

RAYANNE ANDRADE NUNES

**1,25-DIHYDROXYCHOLECALCIFEROL-GLYCOSIDE SUPPLEMENTATION FOR
LPS - CHALLENGED BROILER CHICKENS**

**VIÇOSA - MINAS GERAIS
2020**

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Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Zootecnia, para obtenção do título de *Magister Scientiae*.

Orientador: Arele Arlindo Calderano

Coorientadores: Márcio de Souza Duarte
Paulo Henrique R. F. Campos

**VIÇOSA - MINAS GERAIS
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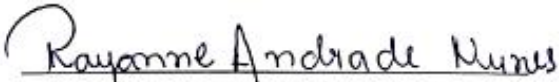
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APROVADA: 20 de julho de 2020.

Assentimento:


Rayanne Andrade Nunes
Autora


Arele Arlindo Calderano
Orientador

*Aos meus pais, Ana Amélia e Raimundo Nunes, por todo amor e ensinamento
A minha velhinha, Dona Ana (in memoriam) por acreditar em mim.*
Dedico

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BIOGRAFIA

Rayanne Andrade Nunes, filha de Ana Amélia Tavares Andrade e Raimundo de Jesus Nunes, nasceu no dia 23 de fevereiro de 1990, em Aracaju, Sergipe. Ingressou no curso Bacharel em Zootecnia na Universidade Federal de Sergipe, Campus São Cristóvão em julho de 2013 com término em junho de 2018. Em agosto deste mesmo ano ingressou no programa de pós-graduação em Zootecnia pela Universidade Federal de Viçosa, atuando na área de Nutrição e Produção de monogástricos, com linha de pesquisa em avicultura, submetendo-se à defesa da dissertação em 20 de julho de 2020.

“Bem sei eu que tudo podes, e que nenhum dos teus propósitos pode ser impedido.”

Jó 42:2

RESUMO

NUNES, Rayanne Andrade, M.Sc., Universidade Federal de Viçosa, julho de 2020. **Suplementação com 1,25-di-hidroxicolecalciferol-glicosídeo para frangos de corte desafiados por LPS.** Orientador: Arele Arlindo Calderano. Coorientadores: Marcio de Souza Duarte e Paulo Henrique Reis Furtado Campos.

Objetivou-se avaliar o efeito da suplementação dietética de 1,25-dihidroxicolecalciferol ($1,25(\text{OH})_2\text{D}_3$) glicosídeo sobre o desempenho, concentração de minerais na tíbia e resposta inflamatória de frangos de corte desafiados com lipopolissacarídeos (LPS). Aos 20 dias de idade, 144 frangos de corte machos foram pesados e distribuídos em delineamento inteiramente casualizado com 3 tratamentos, 8 repetições e 6 aves por unidade experimental. Os tratamentos foram: dieta controle e não administração de LPS (CON), dieta controle e administração de LPS (CON+LPS) e dieta controle suplementada com $5 \mu\text{g}$ de $1,25(\text{OH})_2\text{D}_3$ -glicosídeo/kg de dieta e administração de LPS (SUP+LPS). O desafio com LPS consistiu de aplicações intraperitoneais aos 21 e 23 dias de idade (1 e 1,12 mg/kg de peso corporal, respectivamente). Quatro horas após a primeira aplicação de LPS, uma ave por unidade experimental foi sacrificada para a coleta de amostras de sangue, baço, duodeno e jejuno. O desempenho das aves foi avaliado de 20 a 25 dias de idade. Ao final do experimento todas as aves foram abatidas para análises dos teores de cálcio e fósforos nas tíbias. Os dados foram submetidos à one-way ANOVA e as médias comparadas pelo teste de Tukey a 5% de significância. As aves do grupo CON+LPS apresentaram menor ganho de peso (GP) em relação as dos grupos CON e SUP+LPS ($P = 0,019$). Não houve efeito dos tratamentos sobre o consumo de ração, conversão alimentar, níveis de fosfatase alcalina óssea, cálcio e fósforo no sangue e de cálcio e fósforo na tíbia das aves ($P > 0,05$). O nível de *IL-10* no jejuno foi maior ($P = 0,011$) nas aves do grupo SUP+LPS em relação as dos grupos CON e CON+LPS. Entre as aves desafiadas com LPS, aquelas suplementadas com $1,25(\text{OH})_2\text{D}_3$ -glicosídeo apresentaram maior expressão de mRNA para *IL-10* ($P = 0,014$) e *IL-1 β* ($P = 0,045$) no baço, entretanto não foram observados efeitos sobre a expressão dessas citocinas no duodeno e jejuno das aves ($P > 0,05$). Conclui-se que a suplementação com $1,25(\text{OH})_2\text{D}_3$ -glicosídeo melhora o GP e altera a resposta inflamatória pelo aumento da produção de *IL-10* no jejuno e da expressão de mRNA para *IL-10* e *IL-1 β* no baço de frangos de corte desafiados com LPS. Entretanto, não foi observado efeito da suplementação com $1,25(\text{OH})_2\text{D}_3$ -glicosídeo sobre a mineralização óssea nas aves.

Palavras-chave: Inflamação. Desempenho. Aves. Vitamina D.

ABSTRACT

NUNES, Rayanne Andrade, M.Sc., Universidade Federal de Viçosa, July, 2020. **1,25-dihydroxycholecalciferol-glycoside supplementation for LPS - challenged broiler chickens.** Adviser: Arele Arlindo Calderano. Co-advisers: Marcio de Souza Duarte and Paulo Henrique Reis Furtado Campos.

This study evaluates the effects of dietary supplementation of 1,25-dihydroxycholecalciferol (1,25(OH)₂D₃) glycoside on performance, tibia mineral concentration, and inflammatory responses of broilers challenged with lipopolysaccharides (LPS). At 20 days old, 144 male broilers were weighed and distributed in a completely randomized design with three treatments, eight replicates, and six birds per experimental unit. The treatments consisted in a basal diet without LPS administration (CON), a basal diet with LPS administration (CON+LPS), and a basal diet supplemented with 5 µg of 1,25(OH)₂D₃-glycoside/kg of diet with LPS administration (SUP+LPS). The LPS challenge was administered as intraperitoneal injections at 21 and 23 days of age (1 and 1.12 mg/kg of body weight, respectively). Four hours after the first LPS injection, one bird per experimental unit was euthanized to collect blood, spleen, duodenum, and jejunum samples. Broilers performance was evaluated at 25 days of age, when all birds were slaughtered to analyze calcium and phosphorus concentrations in the tibia. Data were analyzed using a one-way ANOVA and adjusted means were compared using the Tukey test at 0.05 significance. The CON+LPS birds had less weight gain (WG) than CON and SUP+LPS groups ($P = 0.019$). Treatments had no effect on feed intake, feed conversion, serum levels of bone alkaline phosphatase, calcium, and phosphorus, and calcium and phosphorus concentration in the tibia ($P > 0.05$). The IL-10 level in the jejunum was higher ($P = 0.011$) in SUP+LPS birds than in the CON and CON+LPS groups. Among birds challenged with LPS, those supplemented with 1,25(OH)₂D₃-glycoside showed greater mRNA expression of IL-10 ($P = 0.014$) and IL-1 β ($P = 0.045$) in the spleen; however, no effect was observed on the expression of these cytokines in the duodenum and jejunum of the birds ($P > 0.05$). In conclusion, dietary supplementation with 1,25(OH)₂D₃-glycoside preserves WG and modulates the inflammatory response by increasing the production of IL-10 in the jejunum and the mRNA expression of IL-10 and IL-1 β in the spleens of broilers challenged with LPS, although bone mineralization is unaffected.

Keywords: Inflammation. Performance. Poultry. Vitamin D.

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1,25-dihydroxycholecalciferol-glycoside supplementation preserves weight gain and modulates the inflammatory response in lipopolysaccharide-challenged broiler chickens

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Abbreviations: 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; Ca, calcium; P, Phosphorus; LPS, lipopolysaccharides; WG, weight gain; DWG, daily weight gain; FI, feed intake; FC, feed conversion; BAP, bone alkaline phosphatase

1. Introduction

In broiler production systems, the high density of housing and poor hygiene conditions in the facilities can result in bacterial infection, inflammatory reactions, and impaired productive performance. During inflammation, nutrients are diverted to support the immune response while inflammatory mediators can suppress appetite, which further reduces nutrient availability for production and induces catabolism of host tissues (Takahashi et al., 2008; Broom and Kogut, 2018). In addition, pro-inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor, are known to increase bone resorption (Manolagas, 1998; Roux and Orcel, 2000).

In recent years, vitamin D₃ (cholecalciferol) and its metabolites have been studied in poultry production to understand the interaction between nutrition, performance and bone characteristics (Cheng et al., 2004; Brito et al., 2010; Han et al., 2018). Vitamin D₃ requires

two metabolic steps to become biologically active. Initially, 25-hydroxyvitamin D₃ [25(OH)D₃] is formed and stored in the liver. In a second step, the active metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] is formed (Norman, 2008).

Vitamin D₃ plays an essential role in maintaining calcium (Ca) and phosphorus (P) homeostasis and bone health. In broilers, the abundance of mRNA copies for calbindin, a protein responsible for intracellular transport of Ca in the intestine, increases rapidly in the duodenum after birds receive 1,25(OH)₂D₃ (Hsiao et al., 2018). In addition, vitamin D₃ has been linked to immunomodulatory effects. Lower mRNA expression of IL-1β was observed in the liver of broilers challenged with lipopolysaccharides (LPS) and supplemented with 25(OH)D₃ (Morris et al., 2014). However, the mRNA expression of IL-1β and IL-10 in the cecal tonsils of turkeys supplemented with 25(OH)D₃ increased after a coccidiosis challenge (Shanmugasundaram et al., 2019).

The use of synthetic 1,25(OH)₂D₃ in poultry nutrition has been studied for decades (Edwards Jr. and Hardy, 1989; Mitchell and Edwards Jr., 1996; Hsiao et al., 2018). However, few studies have examined the effects of 1,25(OH)₂D₃-glycoside on performance, bone mineralization, and inflammatory response in broilers (Alves et al., 2018; Castro et al., 2018). 1,25(OH)₂D₃-glycoside is a natural form identified in some plants belonging primarily to the *Solanaceae* family, such as *Solanum glaucophyllum* (Boland et al., 2003). Natural 1,25(OH)₂D₃-glycoside is absorbed in the intestines and eliminated from the body more slowly than the synthetic form, due to the slower release of 1,25(OH)₂D₃ (Bachmann et al., 2012). This is because 1,25(OH)₂D₃ derived from *Solanum glaucophyllum* is exclusively present in a glycosylated form. Deglycosylation is performed by intestinal enzymes before the uptake of 1,25(OH)₂D₃ through the intestinal wall, since only deglycosylated 1,25(OH)₂D₃ is absorbed by the system (Wasserman, et al., 1976; Napoli et al., 1977).

In this study, we examine the potential of dietary supplementation of 1,25(OH)₂D₃-glycoside to attenuate the negative effects of LPS inflammatory challenge on growth performance and Ca and P deposition in bones. We evaluate the effects of dietary supplementation of 1,25(OH)₂D₃-glycoside on the performance, tibia mineral concentrations, and inflammatory responses of LPS-challenged broiler chickens.

2. Materials and methods

2.1. Ethical Matters

The Animal Care and Use Committee of the Universidade Federal de Viçosa, Brazil, approved all animal-handling procedures (protocol no. 033/2019). The experiment was conducted according to the experimental protocols for the use of live birds from the Brazilian College of Animal Experimentation.

2.2. Birds, experimental design, and diets

One-day-old male broiler chickens (Cobb 500) were obtained from a commercial hatchery (Rivelli Alimentos SA, Matheus Leme, MG, Brazil). Chicks were vaccinated against bursal and Marek's disease (Serotype 3, Live Marek's Disease Vector, Merial Inc., Athens, GA). Birds were reared on floor pens (200 cm × 100 cm), according to genetic line recommendations. Until the beginning of the experiment, birds had free access to water and feed that consisted in a corn and soybean meal-based standard diet in mashed form, formulated to meet their nutritional requirements according to the NRC (1994).

At twenty days old, a total of 144 broilers (921.25 ± 44.35 g) were assigned based on body weight to a completely randomized design with three treatments, eight repetitions, and six birds per experimental unit. Birds were housed in 24 experimental units consisting of wire floor cages (500 cm²/bird) in a four-level battery equipped with a trough feeder and nipple drinker. Treatments consisted in a basal diet without LPS administration (CON), a basal diet with LPS

administration (CON+LPS), and a basal diet supplemented with 5 μg of 1,25(OH) $_2$ D $_3$ -glycoside/kg of diet with LPS administration (SUP+LPS). The basal diet was corn and soybean based formulated to meet nutritional recommendations of the NRC (1994; Table 1). For a source of 1,25(OH) $_2$ D $_3$ -glycoside, Panbonis[®] (Herbonis Animal Health GmbH, Switzerland) was used, which contains 10 ppm of 1,25(OH) $_2$ D $_3$ -glycoside. The SUP+LPS diet was supplemented with 0.5 g of product/kg of diet in replacement of starch. Antibiotics as growth promoters and anticoccidial were not used in the experimental diets. Diets were prepared in mashed form. Birds had free access to water and feed throughout the experimental period (20 to 25 days of age)

Table 1. Ingredients and nutrient composition of basal diet (g/kg)

<i>Ingredients</i>	20 - 25 days of age
Corn, 78,6 g/kg	531.7
Soybean meal, 450 g/kg	380.9
Soybean oil	41.9
Dicalcium phosphate	18.7
Limestone	10.7
Salt	4.7
DL-Methionine, 999 g/kg	3.5
L-Lysine HCl, 780 g/kg	2.3
Vitamin premix ¹	1.3
Trace mineral premix ²	1.3
Choline chloride, 600 g/kg	1.0
L-Threonine, 985 g/kg	1.0
L-Valine, 990 g/kg	0.5
Starch	0.5
<i>Calculated composition</i>	
Metabolisable energy, MJ/Kg	12,76
Crude protein	220,0
Calcium	10,0
Available phosphorous	4,5
Sodium	2,0
Digestible lysine	12,5
Digestible Methionine + Cysteine	9,3
Digestible valine	9,6
Digestible threonine	8,3
Digestible glycine + serine	17,5

¹Vitamin 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.

²Trace mineral premix provided per kg of diet: Mn, 75.87 mg; Zn, 70.47 mg; Fe, 54.18 mg; Cu, 10.80 mg; I, 1.096 mg; Se, 0.325 mg.

Birds submitted to CON+LPS and SUP+LPS treatments received intraperitoneal injections of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO; reconstituted in saline at a dose of 1.0 mg/mL) at 21 and 23 days of age. The initial dose of 1 mg/kg of body weight was increased by 12% at subsequent injection to circumvent endotoxin tolerance (Rakhshandeh and De Lange, 2011). Birds submitted to the CON treatment received injections containing similar amount of saline solution. Ambient temperature was maintained at 22°C and the birds were exposed to 18 hours of lighting during the entire experiment period.

2.3. Performance and sample collection

Broilers performance was evaluated from 20 to 25 days of age, determining weight gain (WG), daily weight gain (DWG), feed intake (FI), and feed conversion (FC).

At 21 days of age, one bird per pen, with body weight closest to the average weight of the pen, was selected for collecting samples. Four hours after the first LPS administration, blood was collected from the wing vein. Blood was centrifuged at $3,600 \times g$ at 4°C for 10 minutes for separation; serum samples were stored at -20°C until analysis of bone alkaline phosphatase (BAP), Ca, and P. After blood collection, the bird was euthanized by cervical displacement and slaughtered. The spleen and 2 centimeters of the duodenum and jejunum were collected and stored individually in cryogenic tubes, where they were kept in liquid nitrogen until the moment of the analyses.

At 25 days of age, all birds from each experimental unit were euthanized by cervical displacement and slaughtered. The liver, spleen, bursa, and breast of each bird were removed and weighed separately to determine their relative weights. The relative weights were

determined in relation to the animal's live weight. The left tibia of each bird was collected for analysis of Ca and P concentration.

2.4. Determination of tissue metabolite concentrations

Serum levels of BAP, Ca, and P were determined using an AU analyzer from Beckman Coulter (Brea, California, USA), using their kits.

Concentrations of Ca and P in the tibia were determined by spectrophotometry at 75 nm (method 968.08D; AOAC International, 2012).

The IL-10 level in the jejunum was measured with a Chicken IL-10 ELISA Kit (Elabscience Biotechnology Co., Ltd, USA). Analysis were performed according to the manufacturer's instructions, using an automated microplate washer (Biolisa Washer Plus, Bioclin, Belo Horizonte, Brazil) and a microplate reader (Biolisa Reader, Bioclin, Belo Horizonte, Brazil). The IL-10 content was determined according to a standard curve and recorded in picograms per milliliter (pg/mL).

2.5. Determination of mRNA content

To measure mRNA expression for IL-10, IL-1 β , and calbindin, total RNA were extracted from the duodenum, jejunum, and spleen samples using the Trizol[®] reagent (Invitrogen, Carlsbad, California, 279 USA) according to manufacturer's instructions, in the proportion of 1 mL for each 80 mg of tissue. RNA integrity was assessed on a 1% agarose gel stained with ethidium bromide (10 mg/mL) and visualized under ultraviolet light. For real-time PCR, we used the fluorescent dye SYBR GREEN (SYBR[®] GREEN PCR Master Mix, Applied Biosystems, USA). The amplification conditions in the thermocycler were initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute. The melting curves were performed to guarantee the specificity of the PCR products. The *β -actin* gene was used as an endogenous control (Table 2)

Table 2. Primer sequences for quantitative reverse transcription-PCR

Gene	GeneBank accession no.	Primer sequence (5'to3')
<i>IL-1β</i>	AJ245728	F: GCTCTACATGTCGTGTGTGATGAG R: TGTCGATGTCCC GCATGA
<i>IL-10</i>	AJ621614	F: CATGCTGCTGGGCCTGAA R: CGTCTCCTTGATCTGCTTGATG
<i>CaBP-D28k</i>	NM 205513.1	F: TTGGCACTGAAATCCC ACTGAA R: CATGCCAAGACCAAGGCTGA
<i>β-Actin</i>	NM_205518	F: ATTGTCCACCGCAAATGCTTC R: AAATAAAGCCATGCCAATCTCGTC

2.6. Statistical analyzes

Data were analyzed via one-way ANOVA using the PROC MIXED of SAS 9.4 (SAS Inst. Inc., Cary, North Carolina, USA), with subsequent comparison between treatment averages performed using the Tukey test at the 0.05 significance level.

3. Results

3.1. Performance

No mortality was recorded during the experimental period. Birds submitted to the control treatment and those challenged with LPS supplemented with 1,25(OH)₂D₃-glycoside showed higher WG ($P = 0.019$) and DWG ($P = 0.021$) than birds challenged without supplementation (Table 3). However, there was no treatment effect on FI, FC, and relative breast weight ($P > 0.05$).

Table 3. Growth performance of broiler chickens from 20 to 25 days of age

	CON	CON+LPS	SUP+LPS	SEM	<i>P</i> -value
WG (g/bird)	415.4 a	377.1 b	413.4 a	6.55	0.019
DWG (g/bird)	83.1 a	75.5 b	82.7 a	1.30	0.021
FI (g/bird)	566.1	548.5	560.9	5.25	0.388
FC	1.36	1.45	1.36	0.02	0.160
RBW (%)	24.4	24.1	24.2	0.25	0.832

CON: basal diet without LPS administration; CON+LPS: basal diet with LPS administration; SUP+LPS: basal diet supplemented with 5 μ g of 1,25(OH)₂D₃-glycoside/kg of diet and LPS administration.

SEM: standard error of the means (n = 8 for treatment).

WG: weight gain; DWG: daily weight gain; FI: feed intake; FC: feed conversion; RBW: relative breast weight.

Means on the same line, followed by different letters, differ from each other by the Tukey test ($P < 0.05$).

3.2. Organs relative weight

The LPS challenge, independent of the supplementation of 1,25(OH)₂D₃-glycoside, increased the relative spleen weight of the birds ($P = 0.001$; Table 4). The relative liver weight of birds challenged with LPS and not supplemented with 1,25(OH)₂D₃-glycoside was higher ($P = 0.028$) than that of the birds submitted to the control treatment. However, the relative bursa weight was unaffected ($P > 0.05$).

Table 4. Relative weight of breast and organs of broiler chickens at 25 days of age

	CON	CON+LPS	SUP+LPS	SEM	<i>P</i> -value
Spleen (%)	0.083 b	0.114 a	0.121 a	0.005	0.001
Bursa (%)	0.211	0.184	0.206	0.005	0.069
Liver (%)	2.21 b	2.48 a	2.35 ab	0.42	0.028

CON: basal diet without LPS administration; CON+LPS: basal diet with LPS administration; SUP+LPS: basal diet supplemented with 5 µg of 1,25(OH)₂D₃-glycoside/kg of diet and LPS administration.

SEM: standard error of the means (n = 8 for 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 and tibia minerals

There was no treatment effect on serum levels of BAP, P, and Ca or on P and Ca levels in the tibias ($P > 0.05$; Table 5).

Table 5. Serum metabolites at 21 days of age and calcium and phosphorus concentrations in the tibia at 25 days of age

	CON	CON+LPS	SUP+LPS	SEM	<i>P</i> -value
P serum (g/dL)	5.56	5.11	5.26	0.13	0.369
Serum calcium (g/dL)	5.78	6.50	6.03	0.28	0.590
BAP (g/dL)	7.70	6.57	8.15	0.56	0.516
P tibia (%)	8.95	8.70	8.88	0.10	0.615
Ca tibia (%)	14.83	14.76	14.63	0.16	0.882

CON: basal diet without LPS administration; CON+LPS: basal diet with LPS administration; SUP+LPS: basal diet supplemented with 5 µg of 1,25(OH)₂D₃-glycoside/kg of diet and LPS administration.

SEM: standard error of the means (n = 8 for treatment).

BAP: bone alkaline phosphatase.

3.4. IL-10 concentration in the jejunum

The level of IL-10 in the jejunum was higher (38.2 pg/ml; $P = 0.011$) in birds challenged with LPS that received 1,25(OH)₂D₃-glycoside compared to those that received a control diet, with or without challenge (9.7 pg/ml, on average; Figure 1).

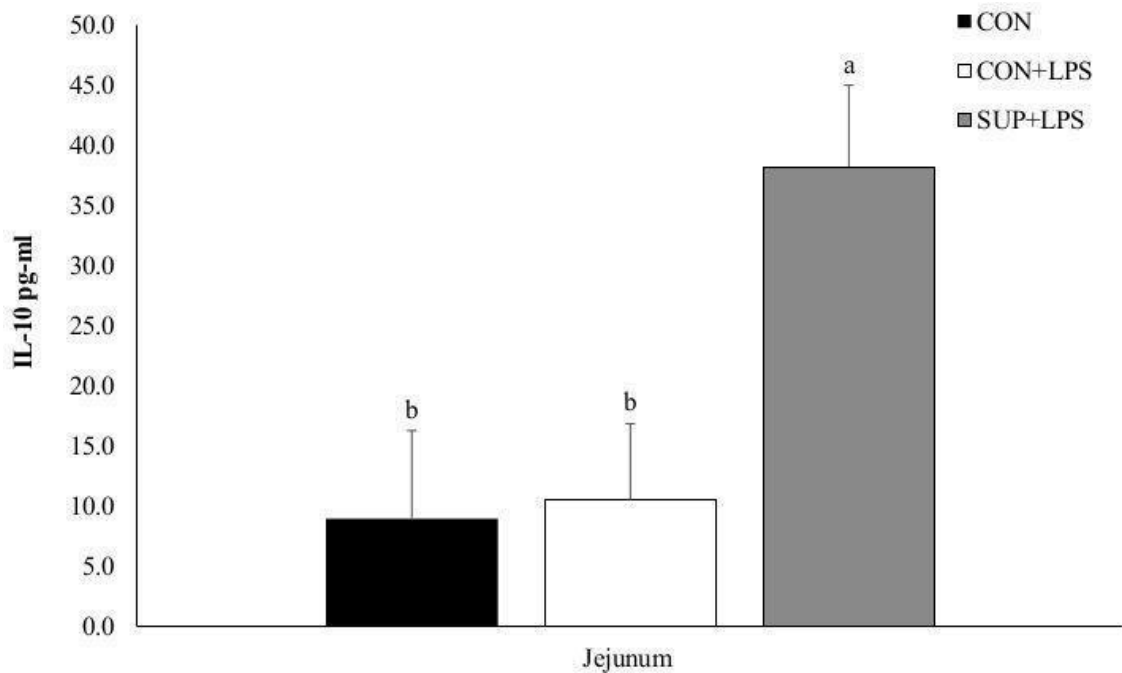


Figure 1. Levels of interleukin-10 (IL-10) in the jejunum of broilers at 21 days of age, 4 hours after the first administration of LPS. Comparisons were made between basal diet and no LPS administration (CON), basal diet and LPS administration (CON+LPS), and basal diet supplemented with 5 μ g of 1,25(OH)₂D₃-glycoside/kg of diet and LPS administration (SUP+LPS). $n = 8$ per treatment. Bars (+ standard error of the mean) with different letters differ from each other by the Tukey test. $P = 0.011$.

3.5. mRNA content

Among birds challenged with LPS, those supplemented with 1,25(OH)₂D₃-glycoside showed higher mRNA expression for IL-10 ($P = 0.014$) and IL-1 β ($P = 0.045$) in the spleen (Figures 2 and 3). However, mRNA expression for IL-10, IL-1 β , and calbindin in the duodenum and jejunum was not influenced by the treatments ($P > 0.05$; Figures 2, 3, and 4).

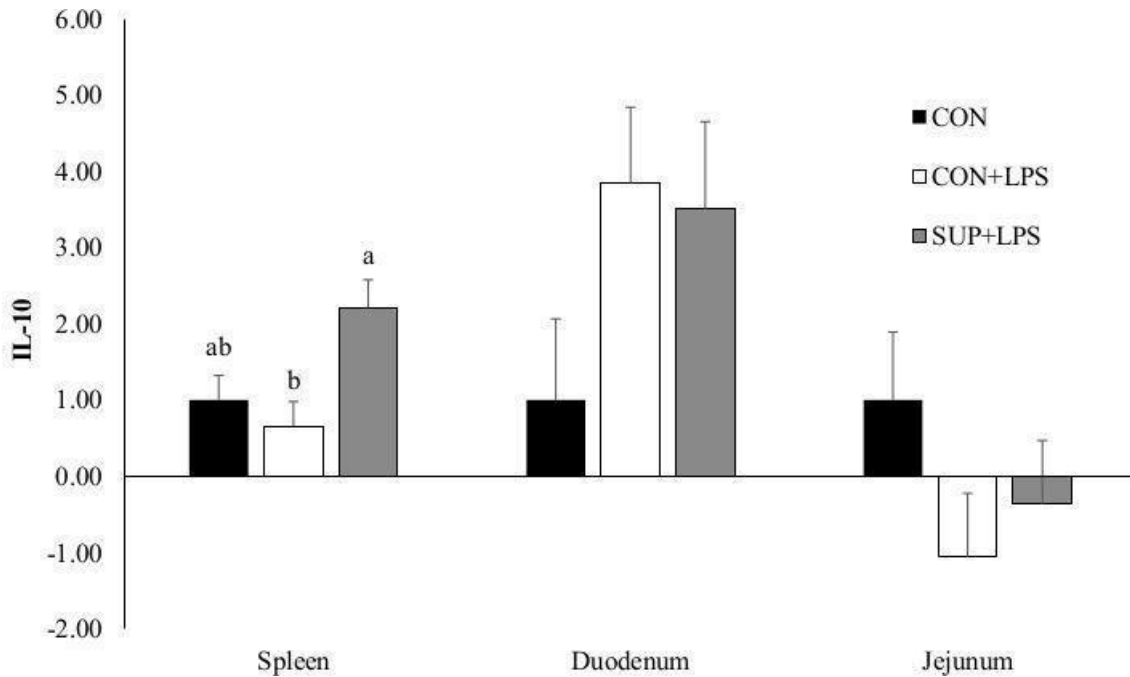


Figure 2. mRNA expression of IL-10 in the spleen, duodenum, and jejunum of broilers at 21 days of age, 4 hours after the first administration of LPS. Comparisons were made between basal diet and no LPS administration (CON), basal diet and LPS administration (CON+LPS) and basal diet supplemented with 5 μg of 1,25(OH) $_2$ D $_3$ -glycoside/kg of diet and LPS administration (SUP+LPS). $n = 8$ per treatment. Bars (+ standard error of the mean) with different letters differ from each other by the Tukey test. P -values: spleen $P = 0.014$; duodenum $P = 0.143$; jejunum $P = 0.256$.

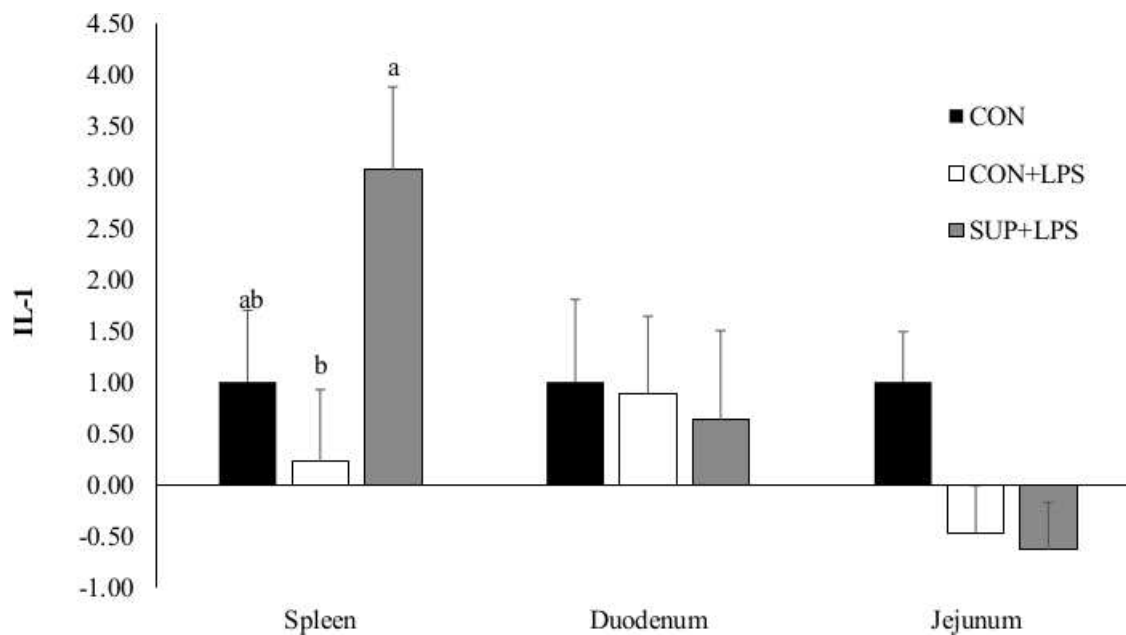


Figure 3. mRNA expression of IL-1 in the spleen, duodenum, and jejunum of broilers at 21 days of age, 4 hours after the first administration of LPS. Comparisons were made between basal diet and no LPS administration (CON), basal diet and LPS administration (CON+LPS)

and basal diet supplemented with 5 μg of 1,25(OH) $_2$ D $_3$ -glycoside/kg of diet and LPS administration (SUP+LPS). $n = 8$ per treatment. Bars (+ standard error of the mean) with different letters differ from each other by the Tukey test. P -values: spleen $P = 0.045$; duodenum $P = 0.710$; jejunum $P = 0.050$.

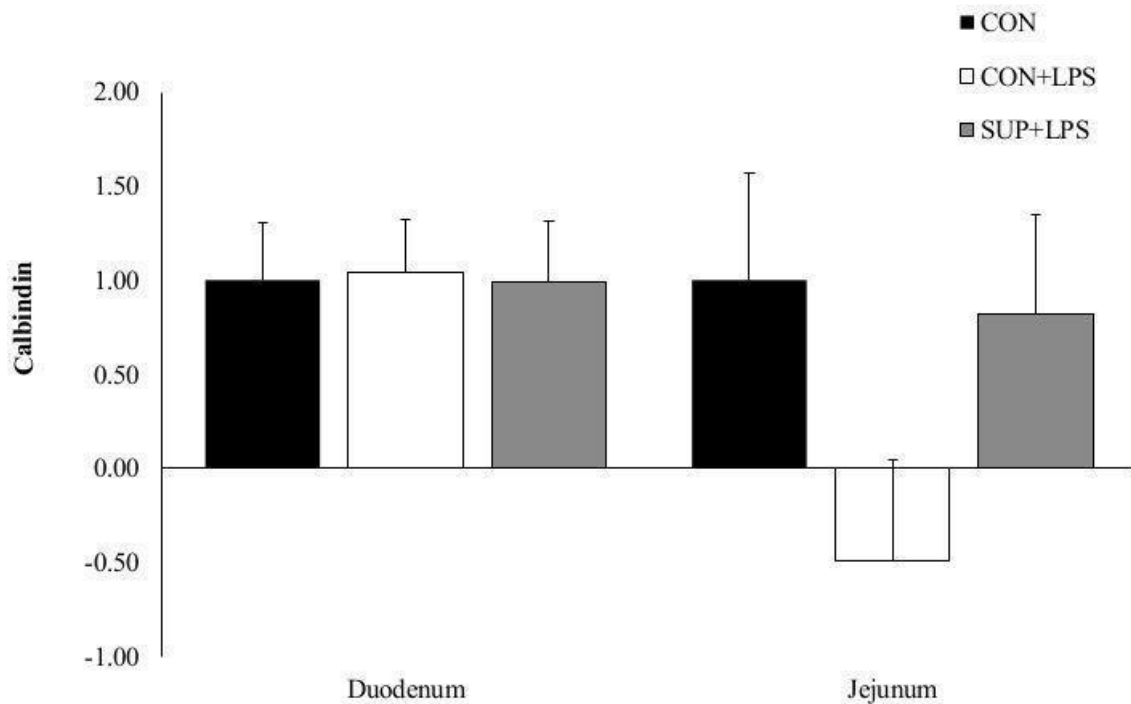


Figure 4. mRNA expression of calbindin in the duodenum and jejunum of broilers at 21 days of age, 4 hours after the first administration of LPS. Comparisons were made between basal diet and no LPS administration (CON), basal diet and LPS administration (CON+LPS) and basal diet supplemented with 5 μg of 1,25(OH) $_2$ D $_3$ -glycoside/kg of diet and LPS administration (SUP+LPS). $n = 8$ per treatment. Bars (+ standard error of the mean) with different letters differ from each other by the Tukey test. P -values: duodenum $P = 0.992$; jejunum $P = 0.180$.

4. Discussion

Our study hypothesis was that the reduction in performance and bone quality triggered by the inflammatory challenge could be attenuated by dietary supplementation of 1,25(OH) $_2$ D $_3$ -glycoside. The use of bacterial LPS as a model to induce inflammatory response in birds has been extensively used (Li et al., 2018; Zhang et al., 2019; Chen et al., 2020). This effect is evidenced by the greater nuclear accumulation of the nuclear factor kappa B (NF- κ B) in the protein content of TNF-alpha, and also by the abundance of mRNA copies of *IL-1 β* in the

jejunum of LPS-challenged broilers (Zhang et al., 2019). LPS also triggers physiological changes in birds, such as increased body temperature and the release of catabolic hormones, as well as reduced feed intake and growth (Adewole et al., 2016). Several studies have indicated worsening performance in chickens challenged with LPS, which is attributed primarily to the increased release of pro-inflammatory cytokines such as IL-1 β (Takahashi et al., 2008; Wang et al., 2016).

In our study, the administration of LPS reduced the birds WG. However, when the broilers received a diet with 1,25(OH) $_2$ D $_3$ -glycoside, there was no impairment of performance compared to those that received the control treatment. This effect may be related to the modulation of the inflammatory response caused by 1,25(OH) $_2$ D $_3$ -glycoside and less diversion of nutrients to support the immune response (Gao et al., 2008). During the 24 hours after administration of LPS, at 21 and 35 days of age, chickens supplemented with 25(OH)D $_3$ gained 2.5% and 3.8% more weight, respectively, than birds supplemented with cholecalciferol alone; this effect was attributed to modulation of the immune response in birds (Morris et al., 2014).

We observed that the relative weights of the spleen and liver were higher in birds that did not receive 1,25(OH) $_2$ D $_3$ -glycoside supplementation and were challenged by LPS, compared to those that received the control treatment. Recently, Chen et al. (2020) found a similar result, highlighting the importance of these organs and the increase in their metabolism during the acute-phase immune response. Takahashi et al. (1998) found that, after challenge with LPS, chicken livers showed an increase in the concentration of acute-phase proteins such as alpha-1-acid glycoprotein, as well as increased expression of various inflammatory factors such as MyD88, NF- κ B, TNF- α , IL-1 β , IL-6, IL-12, IL-18, iNOS, COX-2, and heat shock proteins HSP27, HSP60, HSP70, and HSP90 (Qu et al., 2020). Administering LPS also induces lipid accumulation in the liver (Ohhira et al., 2007).

Vitamin D₃ in its active form is essential for optimal absorption of intestinal Ca and P (Dusso et al., 2005). This relationship between 1,25(OH)₂D₃ and Ca absorption may be mediated by calbindin, a binding protein that plays a crucial role in the intracellular transport of Ca in the intestine. There is evidence that the expression of mRNA for calbindin in chicken intestines is stimulated by supplying 5 µg/kg of 1,25(OH)₂D₃ in the diet (Hsiao et al. 2018). However, we did not observe any treatment effect on the expression of the gene that encodes calbindin in the duodenum and jejunum of chickens.

There was no treatment effect on serum levels of Ca and P of the birds, suggesting that, at the time of collection, the homeostasis of these minerals was maintained independently of the challenge with LPS and supplementation with 1,25(OH)₂D₃-glycoside. Although an increase in serum Ca levels has already been observed in chickens challenged with LPS (Mireles et al., 2005), our results confirm previous observations made regarding giving chickens metabolites of vitamin D₃ 25(OH)D₃, 1α(OH)D₃, and 1,25(OH)₂D₃ (Hsiao et al., 2018; Tizziani et al., 2019). The serum concentration of Ca tends to remain within narrow limits through integrated hormonal regulation involving the intestine, kidneys, and bones, while serum P levels are maintained primarily via renal function (Sie et al., 1974).

The concentrations of Ca and P in the birds' tibias were also unaffected by the treatments. Thus, we could not confirm our initial hypothesis that the reduction in mineral deposition in the bones, triggered by the inflammatory challenge, could be attenuated by the nutritional supply of 1,25(OH)₂D₃-glycoside. Although LPS-challenged broilers have previously shown a reduction in the total level of Ca in the tibia (Mireles et al., 2005), it appears that the inflammatory challenge imposed on birds in our experiment was insufficient to trigger a loss in bone mineralization.

BAP is synthesized in osteoblasts and is involved in bone matrix calcification (Roudsari and Mahjoub, 2012). Consistent with the results observed for mineral deposition in the tibia,

there was also no treatment effect on the birds' serum BAP levels. It has been observed previously that supplementation with 25(OH)D₃ does not affect serum BAP concentrations in broilers (Tizziani et al., 2019).

IL-10 is a cytokine with a potent anti-inflammatory effect in birds (Arendt et al., 2019). In our study, we observed increased IL-10 concentrations in the jejunum of LPS-challenged broilers that received 1,25(OH)₂D₃-glycoside in their diet. A balance between pro- and anti-inflammatory responses is crucial for maintaining homeostasis. Thus, the effects observed on the production of IL-10 possibly have a direct relationship with WG preservation in birds challenged with LPS and supplemented with 1,25(OH)₂D₃-glycoside. The effect of 1,25(OH)₂D₃-glycoside on increasing IL-10 production has been well-described in humans (Dimeloe, et al., 2019; Liberto, et al., 2019). This effect seems to be directly linked to the presence of the nuclear vitamin D receptor (VDR) in immune system cells such as antigen-presenting cells, including macrophages, dendritic cells, and CD4⁺ and CD8⁺ T lymphocytes (Mathieu and Adorini, 2002). The VDR functions as a transcription factor that influences the rate of transcription mediated by RNA polymerase II in genes responsive to 1,25(OH)₂D₃ (Carlberg and Polly, 1998). This molecule promotes the differentiation of CD4⁺ T lymphocytes into T-reg and T-help2 cells, the primary sources of IL-10 (Van Etten and Mathieu, 2005; Baeke et al., 2010).

We observed that, among birds challenged with LPS, those supplemented with 1,25(OH)₂D₃-glycoside showed higher expression of mRNA for IL-10 and IL-1β in the spleen. In addition to lymphocytes, the red pulp of the spleen also contains macrophages (Jeurissen 1991). Morris and Selvaraj (2014) observed that macrophages and monocytes from birds challenged with LPS and treated with 25(OH)D₃ had more mRNA of IL-10 and IL-1 than cells treated with cholecalciferol alone. Turkeys supplemented with 25(OH)D₃ and subjected to coccidiosis infection also showed greater expression of mRNA for IL-1β and IL-10 in the cecal

tonsils (Shanmugasundaram et al., 2019). Rodriguez-Lecompte et al. (2016) observed that supplementation of high doses of vitamin D₃ induced the expression of both pro-inflammatory cytokines via Th1 (IL-12, IFN- γ , and IL-18) and anti-inflammatory cytokines via Th2 (IL-4, IL-10, and IL-13) in broiler spleens; however, they observed that mRNA expression for anti-inflammatory cytokines IL-10 and IL-4 increased more than expression of pro-inflammatory cytokines, suggesting that vitamin D₃ exerts a predominantly anti-inflammatory effect in this organ.

A prominent new finding from our study is that supplementation with 1,25(OH)₂D₃-glycoside preserves performance and modulates the inflammatory response in broilers submitted to an inflammatory challenge. Further research is needed to evaluate the effects of 1,25(OH)₂D₃-glycoside on bone mineralization in conditions of more prolonged inflammatory challenge, as well as other situations of immune challenge.

5. Conclusion

Supplementation with 1,25(OH)₂D₃-glycoside preserves WG and modulates the inflammatory response by increasing the production of IL-10 in the jejunum and the expression of mRNA for IL-10 and IL-1 β in the spleens of LPS-challenged broilers. However, supplementation with 1,25(OH)₂D₃-glycoside had no effect on bone mineralization.

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Conflict of Interest

The authors declare no conflict of interest.

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Viçosa, 18 de junho de 2019

CERTIFICADO

Certificamos que o projeto intitulado "Avaliação de duas fontes de zinco sobre o desempenho de frangos de corte, sobre a digestibilidade e do o balanço do zinco", protocolo nº 033/2019, sob a responsabilidade de **Luiz Fernando Teixeira Albino** - 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 17 de Mai. de 2019.

Finalidade: (X)Pesquisa ()Ensino

Vigência do Projeto: de 18 de junho de 2019 a 20 de Agosto de 2019

Espécie/linhagem: Frango de corte (*Gallus domesticus*) Nº de animais: 1000

Peso: 0,04 Kg Idade: 01 dia Sexo: Macho Origem: Incubatorio Rivelli Cnpj/CPF: 478.715.616 49 /Endereço: Ru a Leão José, 257 Mateus Leme, MG Responsável : Maria Cecília CRMV: 10595

CERTIFICATE

We certify that the project entitled "Evaluation of two sources of zinc on the performance of broiler chickens, on digestibility and on zinc balance", protocol nº 033/2019, under the responsibility of **Luiz Fernando Teixeira Albino** - 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 May, 17th, 2019.

Finality: (X)Research ()Education

Duration of the Project: from Jun, 18th, 2019 to Aug, 20th, 2019.

Species / strain: Broiler (*Gallus domesticus*) Nº of animals: 1000

Weight: 0,04 Kg Age: 01 day Sex: Male Source: Incubatorio Rivelli Cnpj/CPF: 478.715.616 49 /Endereço: Ru a Leão José, 257 Mateus Leme, MG Responsável : Maria Cecília CRMV: 10595

Luciana Navajas Rennó

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Coordenadora da CEUAP/UFV