

BRUNA STRIEDER KREUZ

**EFFECTS OF DIETARY NUCLEOTIDES SUPPLEMENTATION ON
PHYSIOLOGICAL AND GROWTH PERFORMANCE RESPONSES OF LPS -
CHALLENGED BROILER CHICKENS**

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.

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APROVADA: 23 de julho de 2018.



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Aos meus pais, Altair e Coreti, e aos meus irmãos, Débora e João Leopoldo, por me proporcionarem os momentos mais felizes da minha vida.

Dedico.

"O período de maior ganho em conhecimento e experiência é o período mais difícil da vida de alguém."

Dalai Lama.

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Biografia

Bruna Strieder Kreuz, filha de Altair Andrioli Kreuz e Coreti Neli Strieder Kreuz, nasceu em 15 de fevereiro de 1992, em Roque Gonzales, Rio Grande do Sul. Ingressou no curso de Zootecnia na Universidade Federal de Pelotas em março de 2010. Em março de 2012, transferiu sua graduação para a Universidade Federal de Viçosa. De março de 2014 a julho de 2015 foi bolsista pelo programa Ciência sem Fronteiras, cursando parte da graduação na Montana State University – Bozeman – Montana – EUA. Graduou-se em Zootecnia pela Universidade Federal de Viçosa em 29 de julho de 2016. Em agosto do mesmo ano iniciou o Mestrado em Zootecnia, na mesma instituição, realizando suas pesquisas na área de Nutrição e Produção de Monogástricos e submetendo-se à defesa da dissertação em 23 de julho de 2018.

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Abstract

KREUZ, Bruna Strieder, M.Sc., Universidade Federal de Viçosa, July, 2018. **Effects of dietary nucleotides supplementation on physiological and growth performance responses of LPS - challenged broiler chickens.** Adviser: Arele Arlindo Calderano. Co-advisers: Melissa Izabel Hannas and Luiz Fernando Teixeira Albino.

Nucleotides play crucial roles in the storage and transfer of genetic information, cell division and protein synthesis. The aim of this study was to evaluate the effects of dietary nucleotides supplementation on growth performance, body temperature, serum biochemical parameters, immunoglobulin G (IgG) levels, immune organ weight, and intestinal morphometry of broiler chickens challenged with *Escherichia coli* lipopolysaccharide (LPS). Ninety-six 7-day-old broiler chicks (Cobb 500) were randomized in a 2 x 2 factorial design composed of two dietary types (supplemented with 0 and 0.3 g of free nucleotides/kg of feed) and two inflammatory conditions (without and with LPS administration), with 8 replicates per treatment and 3 birds per cage. The experimental period was divided into two phases: 7-20 days (initial; before LPS challenge), and 21-35 days of age (final). The LPS challenge consisted of repeated intraperitoneal injection of LPS at 21, 23, 25, and 27 days of age. The initial dose of 1 mg/kg of body weight was increased by 12% at each subsequent injection to circumvent endotoxin tolerance. Data were analyzed using a one-way and two-way ANOVA for initial and final phases, respectively. During the initial phase, nucleotides supplementation resulted in greater ($P < 0.05$) body weight and average daily gain (ADG), and better ($P < 0.05$) feed conversion ratio. In addition, birds supplemented with nucleotides had greater ($P < 0.05$) serum IgG concentrations. During the final phase, LPS challenge resulted in decreased ($P < 0.05$) ADG and average daily feed intake, and in greater ($P < 0.05$) body temperature. Nucleotides supplementation increased ($P < 0.05$) serum IgG concentration on day 28 and LPS challenge increased ($P < 0.05$) serum IgG concentration on day 28 and 35. At 35 days of age, broilers supplemented with nucleotides had greater ($P < 0.05$) villus height than their control counterparts. In summary, dietary nucleotides supplementation improves growth performance and serum IgG level in healthy young broilers, and improves gut integrity in 35-day-old broilers.

Resumo

KREUZ, Bruna Strieder, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Efeitos da suplementação dietética de nucleotídeos sobre respostas fisiológicas e de desempenho produtivo de frangos de corte desafiados com LPS.** Orientador: Arele Arlindo Calderano. Coorientadores: Melissa Izabel Hannas e Luiz Fernando Teixeira Albino

Os nucleotídeos desempenham papéis cruciais no armazenamento e transferência de informação genética, divisão celular e síntese proteica. O objetivo deste estudo foi avaliar os efeitos da suplementação de nucleotídeos sobre o desempenho produtivo, temperatura corporal, parâmetros bioquímicos séricos, níveis de imunoglobulina G (IgG) no soro sanguíneo, peso de órgãos imunes e morfometria intestinal de frangos de corte desafiados com lipopolissacarídeo de *Escherichia coli* (LPS). Noventa e seis frangos de corte (Cobb 500) com 7 dias de idade foram distribuídos aleatoriamente em um experimento do tipo fatorial 2 x 2 composto por duas dietas (suplementados com 0 e 0,3 g de nucleotídeos livres/kg de ração) e duas condições inflamatórias (sem e com administração de LPS), com 8 repetições por tratamento e 3 aves por unidade experimental. O período experimental foi dividido em duas fases: 7-20 dias (inicial; antes do desafio com LPS) e 21-35 dias de idade (final). O desafio inflamatório consistiu em injeção intraperitoneal repetida de LPS aos 21, 23, 25 e 27 dias de idade. A dose inicial de 1 mg/kg de peso corporal de LPS foi aumentada em 12% a cada injeção subsequente para contornar a tolerância à endotoxina. Os dados foram analisados usando ANOVA unidirecional e bidirecional para as fases inicial e final, respectivamente. Durante a fase inicial, a suplementação de nucleotídeos resultou em maior ($P < 0,05$) peso corporal e ganho médio diário (GMD), e melhor ($P < 0,05$) conversão alimentar. Além disso, as aves suplementadas com nucleotídeos tiveram maiores concentrações séricas de IgG ($P < 0,05$). Durante a fase final, o desafio com LPS resultou em diminuição ($P < 0,05$) no GMD e consumo médio diário de ração, e em maior ($P < 0,05$) temperatura corporal. A suplementação com nucleotídeos aumentou ($P < 0,05$) a concentração sérica de IgG aos 28 dias de vida dos animais e o desafio com LPS aumentou ($P < 0,05$) os níveis de IgG nos dias 28 e 35. Aos 35 dias, frangos suplementados com nucleotídeos apresentaram maior ($P < 0,05$) altura de vilosidades do que os animais do tratamento controle. Desta forma, a suplementação de nucleotídeos na dieta melhora o desempenho produtivo e o nível sérico de IgG em frangos jovens saudáveis e melhora a integridade intestinal em frangos com 35 dias de idade.

METABOLISM AND NUTRITION

DIETARY NUCLEOTIDES FOR BROILERS

Effects of dietary nucleotides supplementation on physiology and growth performance of LPS - challenged broiler chickens¹

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ABSTRACT Nucleotides play crucial roles in the storage and transfer of genetic information, cell division and protein synthesis. The aim of this study was to evaluate the effects of dietary nucleotides supplementation on growth performance, body temperature, serum biochemical parameters, immunoglobulin G (IgG) levels, immune organ weight, and intestinal morphometry of broiler chickens challenged with *Escherichia coli* lipopolysaccharide (LPS). Ninety-six 7-day-old broiler chicks (Cobb 500) were randomized in a 2 x 2 factorial design composed of two dietary types (supplemented with 0 and 0.3 g of free nucleotides/kg of feed) and two inflammatory conditions (without and with LPS administration), with 8 replicates per treatment and 3 birds per cage. The

experimental period was divided into two phases: 7-20 days (initial; before LPS challenge), and 21-35 days of age (final). The LPS challenge consisted of repeated intraperitoneal injection of LPS at 21, 23, 25, and 27 days of age. The initial dose of 1 mg/kg of body weight was increased by 12% at each subsequent injection to circumvent endotoxin tolerance. Data were analyzed using a one-way and two-way ANOVA for initial and final phases, respectively. During the initial phase, nucleotides supplementation resulted in greater ($P < 0.05$) body weight and average daily gain (ADG), and better ($P < 0.05$) feed conversion ratio. In addition, birds supplemented with nucleotides had greater ($P < 0.05$) serum IgG concentrations. During the final phase, LPS challenge resulted in decreased ($P < 0.05$) ADG and average daily feed intake, and in greater ($P < 0.05$) body temperature. Nucleotides supplementation increased ($P < 0.05$) serum IgG concentration on day 28 and LPS challenge increased ($P < 0.05$) serum IgG concentration on day 28 and 35. At 35 days of age, broilers supplemented with nucleotides had greater ($P < 0.05$) villus height than their control counterparts. In summary, dietary nucleotides supplementation improves growth performance and serum IgG level in healthy young broilers, and improves gut integrity in 35-day-old broilers.

Key words: feed additive, growth performance, inflammation, intestinal morphometry

Introduction

Nucleotides are the fundamental molecules of nucleic acids playing crucial roles in the storage and transfer of genetic information, cell division, and protein synthesis (Rodwell, 2006). They can be obtained via de novo endogenous biosynthesis, salvage pathways, or exogenous dietary sources (Hess and Greenberg, 2012). Because of their endogenous synthesis and salvage pathways, nucleotides are not nutritionally essential. However, they may become conditionally essential under rapid growth, stress, immunological challenges and disease state conditions (Carver, 1994).

Rapidly proliferating cells and tissues, such as those in the immune system and the gastrointestinal tract, have high demands for DNA and RNA synthesis that are not met by de novo synthesis of nucleotides; therefore, they must rely on the salvage pathway (Gil, 2002). Thus, exogenous supplementation of these compounds may be essential to support growth and to maintain cell and tissue functions. Accordingly, studies have demonstrated that the use of nucleotides as functional ingredients in pigs' and chickens' diets improved the growth performance, intestinal morphometry, and immune responses of the animals (Maribo, 2003; Domeneghini et al., 2004; Che et al., 2016; Daneshmand et al., 2017). In addition, nucleotides act to increase humoral immunity and cell-mediated immunity, resulting in improved host resistance to bacterial infections (Maldonado et al., 2001; Frankič, et al., 2006; Hess and Greenberg, 2012).

In commercial conditions, birds are exposed to high stocking density and poor hygiene conditions resulting in bacterial contamination, inflammatory (Takahashi et al., 2008) and immune system activation, and impaired growth performance (Xie et al., 2000). The lipopolysaccharide (**LPS**) is a molecule present in the outer membrane of gram-negative bacteria commonly used to induce an inflammatory response in experimental conditions (Chapman et al., 2005). LPS injections induce the synthesis and

release of pro-inflammatory cytokines such as tumor necrosis factor- α , interleukin-1 β (**IL-1 β**) and IL-6 (Yang et al., 2007), which activate neutrophils, monocytes, and macrophages to initiate bacterial and tumor cell killing and stimulate T and B lymphocyte proliferation (Calder, 2001). In addition, they mediate the systemic effects of inflammation such as fever, weight loss, and acute-phase protein synthesis in the liver (Korver and Klasing, 1997; Balaji et al., 2002).

In regard to the key biological functions of nucleotides, it was hypothesized that the dietary supplementation would improve growth performance, gastrointestinal development, and inflammatory/immune functions of broilers submitted to an inflammatory challenge. Thus, the aim of this study was to evaluate the effects of dietary nucleotides supplementation on growth performance, body temperature, serum biochemical parameters, serum IgG levels, immune related organs, and intestinal morphology of broiler chickens challenged with repeated administration of LPS.

Material and Methods

The experimental protocol was reviewed and approved by the Universidade Federal de Viçosa Institutional Animal Care and Use Committee (protocol no. 57/2017) and followed principles established by the Canadian Council on Animal Care Guidelines and Policies (CCAC, 1993).

Broiler Chickens, Experimental Diets and Challenge

Male broiler chickens (Cobb 500) used in the experiment were obtained from a commercial hatchery (Rivelli Alimentos SA, Matheus Leme, MG, Brazil). The chicks were vaccinated against Bursal Disease and Marek's Disease (Serotype 3, Live Marek's

Disease Vector, Merial Inc. Athens, GA). From 1-day-old until the beginning of the experiment, the birds were reared on floor pens (200 cm × 100 cm) equipped with two nipple drinkers and a feed dispenser. They had free access to water and were fed ad libitum a corn-soybean meal-based standard diet formulated to meet their nutritional requirements according to Rostagno et al. (2017).

At 7-day-old, a total of ninety-six broiler chickens (168.5 ± 1.18 g) were randomized, on the basis of their body weight, in a 2 x 2 factorial completely randomized design composed of two dietary types (supplemented with 0 and 0.3 g of free nucleotides/kg of feed) and two inflammatory conditions (without and with LPS administration), with 8 replicates per treatment and 3 birds per cage. The source of nucleotides contained 15% free nucleotides (Ascogen[®], Nutriquest Technofeed, Brazil). Birds were housed in wire floored cages (500 cm²/bird) in a four-level battery equipped with a trough feeder and a nipple drinker. Two corn-soybean meal-based standard diets were formulated to meet the nutritional requirements of broilers during the starter (7-20 days) and grower (21-35 days) phases according to Rostagno et al. (2017; Table 1). Diets were prepared in a mash form. Free access to water and feed was provided during the entire experimental period.

Birds were exposed to 23 h of light from 1 until 7 days-old, after which an 18 h light:6 h dark cycle was implemented until the end of the experiment. Ambient temperature at the beginning of the experiment was maintained at approximately 28 °C and gradually reduced to 22 °C at 21 days of age, after which this temperature was held until the end of the experiment. The experimental period was divided into two phases: 7-20 days of age (initial; before LPS challenge), and 21-35 days of age (final).

The LPS challenge consisted of repeated intraperitoneal injections of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO; reconstituted in saline

at the dose of 1.0 mg/mL) at 21, 23, 25, and 27 d of age. The initial dose of 1 mg/kg of body weight (**BW**) was increased by 12% at each subsequent injection to circumvent endotoxin tolerance (Rakhshandeh and Lange, 2011). Animals without LPS administration received a similar amount of a saline solution.

Body Temperature and Performance

Body temperature was measured using a digital veterinary thermometer (Inco term 6200.03; accuracy 0.1 ° C) inserted 5 cm into the cloaca of one animal per experimental unit, previously identified, before and at 2, 4, 6, 8 and 10 h after each LPS or saline injection. **BW** and feed intake of each experimental unit were recorded on d 20 and 35 to calculate the average daily gain (**ADG**), average daily feed intake (**ADFI**) and feed conversion ratio (**FCR**). Mortality was recorded daily.

Sample Collection and Procedure

One bird per experimental unit was chosen within the unit average weight and identified 24 h before the first LPS administration for sample collection. Blood was collected, from the wing vein, of the same animals before (20 d of age) and during (28 and 35 d of age) the LPS challenge. Blood was centrifuged at 3,000 rpm for 10 min for separation and serum samples were stored at -20° C until analysis. After the final blood collection, birds were euthanized by cervical displacement and slaughtered. Liver, spleen and bursa of Fabricius from each bird were removed and weighed separately on a digital scale (0,0001 g) to determine the relative organ weight. Relative organ weight (g/kg **BW**) was calculated according to the method described by Li et al. (2010). Intestinal contents were flushed out and 2 cm of jejunal (midway between the Meckel's diverticulum and the entrance of the bile ducts) tissue was collected for morphometric evaluation.

Serum Parameters Measurement

Immunoglobulin G (**IgG**) levels were measured on serum samples by an antibody commercial ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) specific to chickens. The methods of 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). A 1:100,000 dilution of the serum samples was used. Contents of IgG were determined according to a standard curve and expressed as nanogram per milliliter (ng/mL).

Serum concentrations of total protein (**TP**), albumin (**ALB**), and uric acid (**UA**) were determined using commercial kits (K031, K040, K139, respectively; Bioclin, Belo Horizonte, Brazil) and performed in an automated clinical chemistry analyzer (BS200E, Shenzhen Mindray Bio-Medical Electronics Co. Ltd., China), following the manufacturer's instructions. Serum globulin (**GLOB**) levels were determined by subtracting serum albumin levels from total serum proteins.

Intestinal Morphometry

Two cm jejunal samples were washed in saline solution and kept in a 10% formaldehyde phosphate buffer for 48 h. Then, cross sections were made and the segments were dehydrated through a graded series of ethanol, diaphanized with xylol and embedded with liquid paraffin at 60°C. Paraffin blocks were fixed on a rotary microtome (Spencer® model 19459, USA) and cross sections were sliced to a thickness of 5 µm (cuts were made semi-serially, with 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 dyed 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) and the average of villi

measurements was expressed as a mean for each bird (n=8 birds/treatment). The slices were examined using an optical microscope (EVOS[®] XL Core Imaging System, Thermo Fisher Scientific Inc., Bothell, WA), with 10X magnification. Morphometric analysis was made on ImageJ software (National Institutes of Health, USA). Villus height (**VH**) was measured from the top of the villus to the villus-crypt junction, and crypt depth (**CD**) was measured from the base of the villus to the sub-mucosa. The relationship villi: crypt was calculated.

Statistical Analysis

The first period of the experiment (7-20 days), before the challenge with LPS, was analyzed as a completely randomized design, with 16 replicates per treatment, to measure the main effects of nucleotides. A one-way ANOVA was performed 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 nucleotides (not supplemented and supplemented), and ε_{ij} is the random error.

For the second period of experiment (21-35 days), a two-way ANOVA was used to measure the main effects of nucleotides, LPS, and their respective interaction. Additionally, the initial body weight was used as covariate according to the following general model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij},$$

where: Y_{ij} is the measured dependent variable, μ is the overall mean, α_i is the effect of nucleotides, β_j is the effect of LPS, $(\alpha\beta)_{ij}$ is the interaction between the levels i and j of the respective factors, and ε_{ij} is the random error.

All analyses were carried out in R software (R Core Team, 2018) using the car package (Fox and Weisberg, 2019). Data were presented as mean \pm standard error of the mean (**SEM**). Significance levels of F test were defined as follows: $P < 0.05$ (significant), and $0.05 < P < 0.10$ (tendency towards significance).

Results

Before LPS Challenge

Growth Performance. Dietary nucleotides improved ($P < 0.05$) BW, ADG, and FCR of broilers during the starter period (7-20 days; Table 2). ADFI was not affected ($P > 0.05$) by treatments.

Serum Metabolites. Dietary nucleotides did not affect ($P > 0.05$) UA, TP, and GLOB serum concentrations at 20 days of age (Table 3). However, nucleotides tended to increase ($P = 0.080$) ALB when compared to the control group. Birds supplemented with nucleotides also showed increased ($P < 0.05$) serum concentration of IgG when compared to the control birds.

During LPS Challenge

Body Temperature. Irrespective of LPS administration, no interaction ($P > 0.05$) was found between nucleotides supplementation and LPS (Table 4). After the first LPS administration, LPS-treated chickens had greater ($P < 0.05$) body temperature 4 and 8 h post injection. Nucleotide supplementation tended ($P = 0.09$ and $P = 0.06$) to increase body temperature of broilers 4 and 6 h post injection, when compared to the control group. After the second LPS administration, LPS increased ($P < 0.05$) bird body temperature 6 and 10 h post injection compared to the saline group. Nucleotide supplementation also

increased ($P < 0.05$) body temperature of animals 2 h following the injection (Figure 1). On the third day of the challenge, LPS-treated chickens showed higher ($P < 0.05$) body temperature 4, 6 and 8 h post injection and LPS tended ($P = 0.07$) to increase body temperature 10 h post LPS or saline administration. Nucleotides also increased ($P < 0.05$) the body temperature of birds 6 h post injection. In the fourth day of challenge, LPS induced higher ($P < 0.05$) body temperature on broilers at 4, 6, 8 and 10 h post injection (Figure 2).

Growth performance. No interaction ($P > 0.05$) was observed between nucleotides supplementation and LPS challenge for growth performance from 21-35 days of age (Table 5). Nucleotide supplementation had no effect ($P > 0.05$) on performance during the grower phase. LPS injection resulted in a reduction ($P < 0.05$) in ADG and ADFI, and tended to decrease ($P = 0.07$) BW and worsen ($P = 0.05$) FCR of broilers.

Serum Metabolites and IgG. No interaction ($P > 0.05$) between nucleotides and LPS challenge was found for serum metabolites at 28 and 35 days of age (Table 6). Nucleotide supplementation had no effect ($P > 0.05$) on UA, TP, ALB, or GLOB at 28 and 35 days of age. LPS administration did not affect ($P > 0.05$) UA, TP, or ALB at 28 and 35 days of age. A tendency towards increased ($P = 0.08$) serum globulin was observed on day 28 of age in response to LPS. No interaction ($P > 0.05$) between nucleotides and LPS challenge was found for serum concentrations of IgG at 28 or 35 days of age. However, at 28 days of age, nucleotide supplementation and LPS administration increased ($P < 0.05$) serum IgG concentration (Figure 3). At 35 days of age, birds injected with LPS also showed increased ($P < 0.05$) serum IgG whereas nucleotides were not affected ($P > 0.05$).

Relative Organs Weight and Intestinal Morphometry. No interaction ($P > 0.05$) between nucleotides and LPS challenge was observed for relative weights of bursa, spleen, and liver at 35 days of age (Table 7). Nucleotides and LPS challenge had no significant effects ($P > 0.05$) on relative weight of the bursa of Fabricius and liver at 35 days of age. However, nucleotides and LPS tended ($P = 0.08$ and $P = 0.08$, respectively) to increase the relative weight of the spleen (Figure 4).

No interaction ($P > 0.05$) between nucleotides and LPS challenge was found on the morphological parameters of the jejunum at 35 days of age (Table 7). Nucleotide supplementation increased ($P < 0.05$) villus height when compared to the control group, but no effects ($P > 0.05$) were observed for CD and VH/CD ratio. LPS injection did not affect ($P > 0.05$) gut morphology.

Discussion

According to our results, nucleotides supplementation significantly increased body weight gain and feed efficiency during the starter period. Under standard conditions, animals' nucleotide requirements are met via endogenous production and salvage pathways (Sanchez-Pozo and Gil, 2002). However, in some specific conditions such as rapid growth phases, gut injury, immunosuppression, or decreased protein intake, exogenous demand for nucleic acids could be increased (Hess and Greenberg, 2012). In such cases, nucleotides became conditionally essential, and exogenous sources may benefit growth performance. Accordingly, beneficial effects of nucleotides or nucleosides on growth performance have been reported in broilers (Esteve-Garcia et al., 2007; Superchi et al., 2012; Daneshmand et al., 2017) and pigs (Waititu et al., 2016, 2017). According to Daneshmand et al. (2017), a hypothesis explaining this improvement is that

nucleotide supplementation may conserve amino acids such as glutamine, aspartate and glycine for de novo synthesis resulting in the use of these amino acids for growth purposes. De novo pathways can absorb about 1% of the glutamine flux (Grimble, 1996), while dietary nucleotides reduce the de novo pathways increasing glutamine availability to glutamine-high-demand processes and organs such as the Pectoralis major muscle (0.06g glutamine/g protein of muscle; Hu et al., 2016).

In response to LPS-challenge, nucleotides supplementation had negligible effects on growth performance. As expected, increasing amounts of LPS injection reduced ADFI and ADG. The inflammatory response led to changes in the partitioning of nutrients away from growth and toward processes associated with the acute-phase response. This may explain the reduced ADG. LPS is a potent inflammatory mediator and has been used to induce symptoms of bacterial infection and immunological stress in laboratory animals (Yang et al., 2008). An immunological challenge can result in a series of physiological changes including increased body temperature, depressed feed intake, changes in plasma acute phase protein concentration, activation of the immune system, activation of the hypothalamic-pituitary-adrenal axis, and inhibition of the somatotrophic axis (Hashimoto et al., 1988; Johnson, 1997; Adewole et al., 2016). Toll-like receptor 4 (TLR-4) recognizes LPS of gram-negative bacteria such as *E. coli* and contributes to the initiation of inflammatory responses mediated by pro-inflammatory cytokines (Kogut et al., 2005). The upregulation of TLR-4 may imply a robust inflammatory response and could be associated with the upregulation of IL-1 β (Staeheli et al., 2001). IL-1 β , a pro-inflammatory cytokine, is involved in the stimulation of T cells and macrophages, induction of fever, triggering of acute-phase responses, and activation of the vascular endothelium (Dinarello, 2005). Several studies have indicated that the worst performances of LPS-challenged broilers are attributed to the release of proinflammatory

cytokine, such as IL-1 β (Parmentier et al., 1998; Takahashi et al., 2008; Wang et al., 2016). In our findings, LPS upregulated the body temperature of the birds. The course of the body temperature was characterized by an initial normal temperature followed by a fever phase in LPS-treated chickens. However, nucleotide supplementation also increased body temperature after injections. According to Che et al. (2016), dietary nucleotides markedly increased gene expressions of TLR-4. Thus, they can stimulate the subsequent larger cytokine cascade.

Nucleotides supplementation increased serum immunoglobulins G levels on day 20 and 28, regardless of LPS challenge on day 28. The change in IgG levels in nucleotide-fed birds implies that an impact on humoral immunity occurred in response to vaccination on the first day of life. This result agrees with previous studies in which nucleotides and nucleosides are found to increase the production of immunoglobulins in birds (Jang et al., 2013; Trckova et al., 2014; Daneshmand et al., 2017). Lymphocytes are essential members of the immune system and require a high number of nucleotides, which are synthesized de novo by other organs (primarily by the liver), for maintenance and fast proliferation (Rudolph et al., 1990; Jyonouchi, 1994). Lymphocyte B produces natural antibodies (Ochsenbein et al., 1999; Ochsenbein and Zinkernagel, 2000). Chicken IgG is the predominant natural antibody against systemic infection and its increased concentration in serum could assist in early recognition and clearance of invading pathogens in the body (Dankowiakowska et al., 2013). In our study, LPS challenge increased IgG concentration in the serum. Similarly, Yang et al. (2008) reported that LPS challenge increased lymphocyte proliferation and humoral response in chickens. This increase can be explained by an enhanced status of immune reactivity or sensitivity to vaccine antigen. Lai et al. (2011) observed that LPS enhanced primary total immunoglobulin and IgG antibody responses to human serum albumin antigen,

suggesting the involvement of CD4+T cells. The inflammatory response to LPS involves the production of acute phase proteins, such as alpha-1 acid glycoprotein (Takahashi et al., 1998). This, along with the higher IgG production, might explain the tendency of increased serum GLOB on day 28 of age.

A tendency of increase in the relative spleen weight due to nucleotide supplementation and LPS injection was observed. Our findings were similar to the results previously described by Wang et al. (2016), where LPS resulted in greater spleen weight. Lymphoid organs such as bursa of Fabricius and spleen are responsible for the body's defenses against different types of pathogens and diseases, which can drastically increase the rate of cell turnover in the immune system (Daneshmand et al., 2017). The spleen contains different immune compounds and plasma cells, which need high amounts of DNA and RNA for maintenance and growth (Zhou et al., 2015). The elevated relative weight of spleen observed in challenged birds may have resulted from LPS-induced systemic inflammation and recruitment of inflammatory cells to the spleen (Lin et al., 2007) and dietary nucleotides were able to provide enough DNA and RNA for rapid cell turnover.

The present findings revealed that feeding nucleotides increased VH in the jejunum of birds at 35 days of age, which is in accordance with other studies (Moore et al., 2011; Jung and Batal, 2012; Wu et al., 2018). Nucleotides can exert beneficial effects on intestinal morphometry and improved growth performance could be achieved by rapid digestive tract development in growing birds. Avian species have a high growth rate capacity that is characterized by a rapid early development of the digestive tract (Lilja, 1983), which requires a high number of nucleotides. Complete small intestinal structure plays a fundamental role in the nutrient digestive and absorptive functions, and is a crucial barrier to preventing pathogens and toxins in young birds (Bartell and Batal, 2007). The

development of villi and epithelial cells relies on the availability of genomic materials such as nucleosides in cellular nucleotide pools in order to maintain DNA and RNA for rapid growth (Sato et al., 1999; Daneshmand et al., 2017). Dietary nucleotides support rapid epithelial growth, leading to the enhancement of villi and epithelial cells development, nutrient absorption, and consequently greater growth performance in broilers. In conclusion, our findings demonstrate that dietary nucleotide supplementation improves the growth performance and immunity of broiler chickens, enabling them to mount a faster and stronger antibody response by enhancing IgG production. Our results also show that dietary nucleotides can promote intestinal development in broilers, and thus could be utilized as an important additive for the poultry industry.

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Tables

Table 1. Composition of diets (as fed basis)¹

Ingredients (g/Kg)	Starter (7-20 d)	Grower (21-35 d)
Corn (7,86%)	518.7	586.7
Soybean meal (45%)	396.2	329.2
Soybean oil	43.6	46.2
Dicalcium phosphate	16.9	14.9
Limestone	8.45	7.12
Salt	5.16	4.93
Vitamin Premix ²	1.00	1.00
Mineral premix ³	1.00	1.00
DL-Methionine	3.38	2.96
L-Lysine	1.94	2.26
L-Threonine	0.74	0.70
L-Valine	0.35	0.43
Choline Cl-60%	0.50	0.50
Antioxidant	0.10	0.10
Starch	2.00	2.00
Calculated provisions (g/Kg, unless shown)		
Metabolizable energy, MJ/Kg	12.77	13.19
Crude protein	225.0	200.0
Calcium	8.78	7.58
Available phosphorous	4.19	3.74
Sodium	2.18	2.08
Potassium	8.91	7.90
SID Met	6.31	5.62
SID Met + Cys	9.29	8.32
SID Lys	12.56	11.24
SID Arg	14.08	12.23
SID Thr	8.29	7.42

¹Nucleotides (2 g/kg) were added as a replacement for starch.

²Vitamin premix per kilogram contained vitamin A, 7,500,000 IU; vitamin D3, 1,900,000 IU; vitamin E, 28,000 IU; vitamin K3, 1,500 mg; vitamin B1, 2,000 mg; vitamin B2, 5,000 mg; vitamin B3, 30.0 g; vitamin B5, 10.0 g; vitamin B6, 2,800 mg; vitamin B9, 700.0 mg; vitamin B12, 12 mg; biotin, 70.0 mg.

³Mineral premix per kilogram contained Mn, 58.36 g; Zn, 54.21 g; Fe, 41.68 g; Cu, 8.31 g; I, 843.0 mg; Se, 250.0 mg.

Table 2. Effects of nucleotides on growth performance of broiler chickens from 7-20 days of age

	Diet		SEM ²	P-value
	Control	Nucleotides		
BW (g) ¹	982	1009	6.63	0.007
ADG (g/d/b)	58.1	60.1	0.47	0.007
ADFI (g/d/b)	74.5	75.2	0.86	0.541
FCR (g/g)	1.29	1.25	0.02	0.049

¹BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio; g/d/b: grams per day per bird.

²SEM: standard error of means (results are given as means (n = 16) for each treatment).

Table 3. Effects of nucleotides on serum metabolites of broiler chickens at 20 d of age

	Diet		SEM ²	P-value
	Control	Nucleotides		
UA ¹ (mg/dL)	3.00	3.55	0.31	0.225
TP (g/dL)	2.86	2.80	0.06	0.556
ALB (g/dL)	1.60	1.54	0.02	0.080
GLOB (g/dL)	1.27	1.26	0.04	0.853
IgG (mg/ml)	1.17	1.76	0.18	0.031

¹UA: uric acid; TP: total protein; ALB: albumin; GLOB: globulin; IgG (mg/ml): immunoglobulin G.

²SEM: standard error of means (results are given as means (n = 16) for each treatment).

Table 4. Body temperature (°C) of broiler chickens supplemented with nucleotides after LPS¹ injections

LPS	Control		Nucleotides		SEM ³	P-value		
	-	+	-	+		N	LPS	N x LPS
21 d								
Before ²	41.30	41.14	41.10	41.28	0.10	0.765	0.952	0.115
4 h	41.26	41.56	41.41	41.66	0.08	0.115	0.002	0.778
6 h	41.18	41.34	41.41	41.55	0.12	0.061	0.204	0.915
8 h	41.29	41.65	41.46	41.74	0.09	0.151	0.001	0.626
10 h	41.21	41.46	41.38	41.46	0.11	0.454	0.126	0.454
23 d								
Before	41.30	41.31	41.40	41.19	0.11	0.907	0.352	0.296
4 h	41.53	41.53	41.67	41.85	0.07	0.002	0.198	0.216
6 h	41.38	41.69	41.48	41.80	0.10	0.313	0.005	0.952
8 h	41.18	41.40	41.09	41.24	0.10	0.210	0.064	0.703
10 h	41.23	41.40	41.06	41.33	0.07	0.107	0.005	0.529
25 d								
Before	41.39	41.20	41.23	41.23	0.08	0.388	0.242	0.242
4 h	40.93	41.15	40.99	41.43	0.12	0.178	0.011	0.392
6 h	41.01	41.48	41.27	41.54	0.06	0.014	<0.0001	0.119
8 h	41.16	41.36	41.23	41.43	0.08	0.434	0.017	0.999
10 h	41.20	41.25	40.99	41.21	0.07	0.104	0.075	0.249
27 d								
Before	41.29	41.16	41.20	41.16	0.07	0.534	0.248	0.534
4 h	41.18	41.39	41.20	41.61	0.10	0.209	0.003	0.312
6 h	41.18	41.49	41.24	41.44	0.09	0.947	0.010	0.549
8 h	41.26	41.33	41.03	41.33	0.05	0.696	0.010	0.247
10 h	41.04	41.11	40.96	41.15	0.06	0.181	0.038	0.252

¹Birds were intra-abdominally injected with LPS (1, 1.12, 1.25, 1.40 mg/kg of BW) or saline solution at 21, 23, 25, and 27 d of age.

²Before LPS or saline injection; 4, 6, 8 and 10 hours after injection.

³SEM: standard error of the means (results are given as means (n = 8) for each treatment).

Table 5. Effects of nucleotides on growth performance of broiler chickens challenged with lipopolysaccharide (LPS)¹ from 21-35 days of age

LPS	Control		Nucleotides		SEM ³	P-value		
	-	+	-	+		N	LPS	N x LPS
BW (g) ²	2393	2336	2402	2303	41.39	0.778	0.073	0.612
ADG (g/d/b)	101.8	96.4	100.6	91.3	2.90	0.283	0.017	0.502
ADFI (g/d/b)	160.4	153.2	158.2	151.0	3.35	0.514	0.041	0.990
FCR (g/g)	1.58	1.59	1.58	1.66	0.02	0.138	0.054	0.152

¹Birds were intra-abdominally injected with LPS (1, 1.12, 1.25, 1.40 mg/kg of BW) or saline solution at 21, 23, 25, and 27 d of age.

²BW: body weight; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio; g/d/b: grams per day per bird.

³SEM: standard error of the means (results are given as means (n = 8) for each treatment).

Table 6. Effects of nucleotides and LPS¹ challenge on serum metabolites and on concentrations of immunoglobulin G (mg/ml) of broiler chickens at 28 and 35 d of age

LPS	Control		Nucleotides		SEM ³	N	P-value		
	-	+	-	+			LPS	N x LPS	
28 d									
UA ² (mg/dL)	2.35	2.88	3.22	3.06	0.25	0.063	0.492	0.209	
TP (g/dL)	3.10	3.13	3.12	3.25	0.09	0.433	0.350	0.561	
ALB (g/dL)	1.55	1.48	1.46	1.51	0.04	0.553	0.780	0.134	
GLOB (g/dL)	1.54	1.65	1.54	1.65	0.06	0.984	0.084	0.996	
IgG (mg/ml)	2.49	3.52	3.50	5.17	0.546	0.022	0.021	0.559	
35 d									
UA (mg/dL)	1.53	1.64	1.47	1.95	0.23	0.599	0.212	0.432	
TP (g/dL)	3.11	3.12	3.08	2.89	0.14	0.364	0.541	0.476	
ALB (g/dL)	1.57	1.52	1.50	1.47	0.05	0.192	0.388	0.804	
GLOB (g/dL)	1.56	1.60	1.60	1.53	0.11	0.859	0.915	0.618	
IgG (mg/ml)	3.63	5.41	4.07	7.35	1.07	0.278	0.027	0.493	

¹Birds were intra-abdominally injected with LPS (1, 1.12, 1.25, 1.40 mg/kg of BW) or saline solution at 21, 23, 25, and 27 d of age.

²UA: uric acid; TP: total protein; ALB: albumin; GLOB: globulin.

³SEM: standard error of the means (results are given as means (n = 8) for each treatment).

Table 7. Effects of nucleotides on the relative weight (% organ weight to body weight) of immune organs and on jejunum morphology (μm) of broiler chickens challenged with lipopolysaccharide (LPS)¹ at 35 days of age

LPS	Control		Nucleotide		SEM ³	P-value		
	-	+	-	+		N	LPS	N x LPS
<i>Relative organs weight (%)</i>								
Bursa	0.191	0.185	0.163	0.167	0.019	0.344	0.762	0.980
Spleen	0.118	0.130	0.130	0.177	0.016	0.077	0.076	0.282
Liver	5.166	4.777	4.407	4.724	0.261	0.131	0.885	0.185
<i>Jejunum morphology (μm)</i>								
VH ²	302.0	296.5	317.5	313.6	7.80	0.046	0.556	0.918
CD	43.1	44.9	46.9	45.9	1.80	0.198	0.843	0.444
VH/CD	7.1	6.8	6.8	6.7	0.28	0.488	0.364	0.734

¹Birds were intra-abdominally injected with LPS (1, 1.12, 1.25, 1.40 mg/kg of BW) or saline solution at 21, 23, 25, and 27 d of age.

²VH: villus height; CD: crypt depth; VH/CD: the ratio of villus height to crypt.

³SEM: standard error of the means (results are given as means (n = 8) for each treatment).

Figures

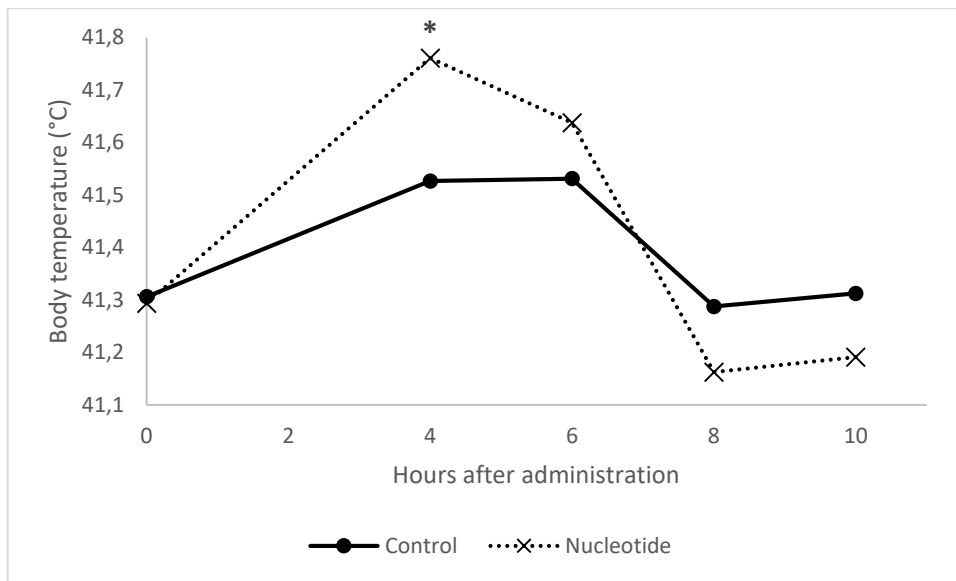


Figure 1. Body temperature versus time curve of nucleotide supplemented chickens (n = 16) and control chickens (n = 16), after the second day of LPS or saline injection (23 d of age). The nucleotide supplemented chickens showed fever after injection. (*) Indicate a significant difference ($P < 0.05$) among the groups.

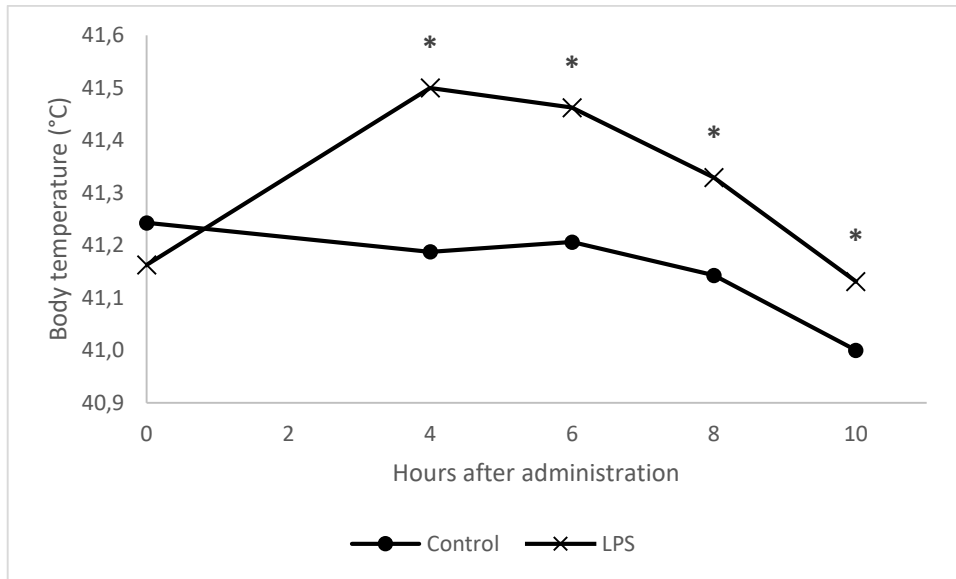


Figure 2. Body temperature versus time curve of LPS-treated chickens (n = 16) and control chickens (n = 16) after the fourth day of injection (27 d of age). The LPS-treated chickens showed fever after injection. (*) Indicate a significant difference ($P < 0.05$) among the groups.

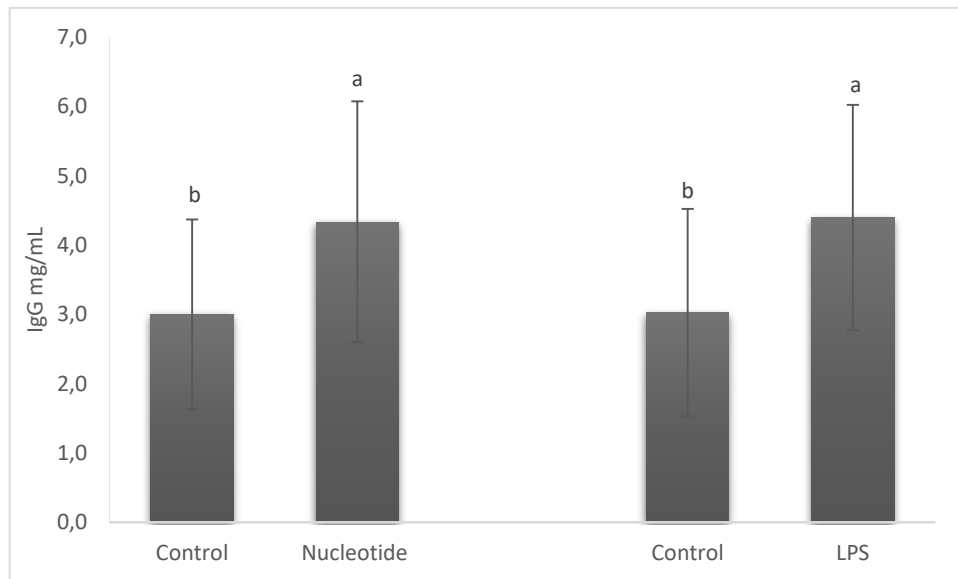


Figure 3. Effect of nucleotide supplementation and LPS challenge on serum concentrations of immunoglobulin G at 28 d of age. Data are presented with means \pm S.D. (standard deviation) of eight replicates. Different letters (a-b) indicate a significant difference ($P < 0.05$) among the groups.

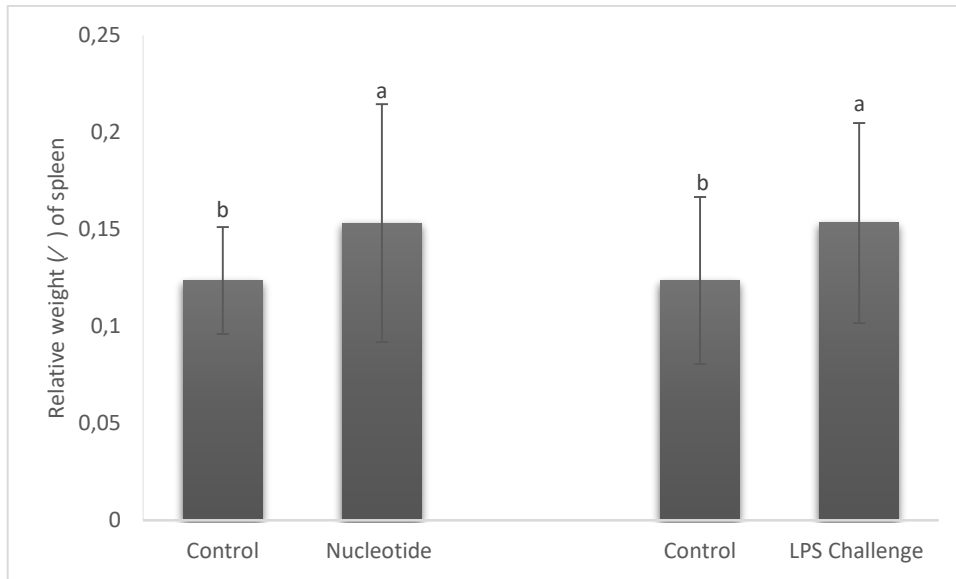


Figure 4. Effect of nucleotide supplementation and LPS challenge on relative weight of spleen at 35 d of age. Data are presented with means \pm S.D. (standard deviation) of eight replicates. Different letters (a-b) indicate a tendency of significant difference ($P < 0.08$) among the groups.

Anexo



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, 17 de Nov. de 2017

CERTIFICADO

Certificamos que o projeto intitulado "Efeitos da suplementação de nucleotídeos na ração de frangos de corte sob desafio imune", protocolo nº 057/2017, 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 aprovado pela comissão de ética no uso de animais de produção da universidade federal de viçosa (ceuap-ufv) em reunião de 17 de Nov. de 2017.

Finalidade: (X)Pesquisa ()Ensino

Vigência do Projeto: de 17 de Nov. de 2017 a 31 de Jan. de 2018

Espécie/linhagem: Frango (*Gallus gallus domesticus*) Nº de animais: 96

Peso: 225 g Idade: 8 dias Sexo: Macho Origem: Incubatorio Rivelli - Cnpj/CPF: 478.715.616-49 -

Responsável: Maria Cecilia CRMV: 10595

CERTIFICATE

We certify that the project entitled "Effects of dietary nucleotide supplementation on broilers in response to an immune challenge", protocol nº 057/2017, 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 Nov, 17th, 2017.


Finality: (X)Research ()Education

Duration of the Project: from Nov, 17th, 2017 to Jan, 31th, 2018.

Species / strain: Broiler (*Gallus gallus domesticus*) Nº of animals: 96

Weight: 225 g Age: 8 days Sex: Male Source: Incubatorio Rivelli - Cnpj/CPF: 478.715.616-49 -

Responsável: Maria Cecilia CRMV: 10595


Luciana Navajas Rennó
Coordenadora da CEUAP/UFV