

Effects of dietary nucleotide supplementation on growth performance and physiology of broiler chickens under pre- and post-inflammatory challenge

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ABSTRACT - The objective of this study was to evaluate the effects of dietary nucleotide supplementation on growth performance, serum immunoglobulin G (IgG) and uric acid levels, immune-related organs, and intestinal morphometric parameters of broiler chickens under pre- and post-inflammatory challenge with lipopolysaccharide (LPS). Ninety-six seven-day-old male broiler chicks were randomized in a 2x2 factorial design composed of two dietary types (supplemented with 0 and 0.3 g of purified nucleotides/kg of feed) and two inflammatory conditions (with and without LPS administration) with eight replicates per treatment and three birds per cage. The experimental period was divided into two phases: 7-20 days of age (pre-LPS challenge) and 21-35 days of age (post-LPS challenge). Data were analyzed using one-way and two-way ANOVA for the pre- and post-challenge phases, respectively. During the pre-challenge phase, dietary nucleotide supplementation improved body weight (982 vs. 1009 g/bird), daily weight gain (58.1 vs. 60.1 g/bird), feed conversion rate (1.28 vs. 1.25 g/g), and serum concentration of IgG (1.17 vs. 1.76 mg/mL) of broilers. During the post-challenge phase, nucleotide supplementation increased serum IgG of broilers at 28 days of age (3.01 vs. 4.34 mg/mL). For the intestinal morphometric parameters, nucleotide supplementation increased villus height (299.3 vs. 315.6 µm). Dietary nucleotide supplementation improves the performance of broilers from seven to 20 days of age (pre-LPS challenge). However, from 21 to 35 days of age, supplementation of 0.3 g of purified nucleotides/kg of feed, independent of inflammatory challenge, does not contribute to improve performance of broilers. Additionally, nucleotide supplementation increases IgG production and villus height in the jejunum of broilers.

Keywords: immunoglobulin, feed additive, nutrition, poultry

1. Introduction

Nucleotides are the fundamental molecules of nucleic acids and play crucial roles in storage and transfer of genetic information, cell division, and protein synthesis. In healthy animals, the endogenous *de novo* synthesis of nucleotides and salvage pathways are considered sufficient to fulfill cellular requirements (Sanchez-Pozo and Gil, 2002). However, these nutrients may become occasionally essential under certain conditions, such as rapid growth or immunological challenge (Leung et al., 2019a). Rapidly proliferating cells and tissues, such as those in the immune system and the gastrointestinal tract, have high demands for DNA and RNA synthesis (Gil, 2002). Thus, exogenous supplementation of these compounds may be essential to support growth and maintain functions.

The positive effects of nucleotide supplementation on performance and physiology in pigs are already documented (Weaver and Kim, 2014; Waititu et al., 2017; Jang and Kim, 2019). However, in broilers, these effects remain unclear. Recently, Leung et al. (2019a) observed improved feed conversion during pre-challenge and increased body weight gain (BWG) and villus height (VH) in *Eimeria*-challenged broilers fed diet supplemented with nucleotides. In a subsequent study, Leung et al. (2019b) concluded that, regardless of *Eimeria* challenge, supplementation with nucleotides had no effect on growth performance and intestinal function. Several other previous studies reported increases in BWG, VH, and IgA activity in the jejunum of broilers (Daneshmand et al., 2017a,b), while others showed no effect of nucleotide supplementation on growth performance (Alizadeh et al., 2016a,b). Different management and environmental factors such as age, strain, source and level of nucleotides, and type of challenge may explain the contradictory results by different researchers (Daneshmand et al., 2017a) and suggest the importance of further study regarding nucleotide supplementation for broilers. Moreover, there is limited information on nucleotide supplementation and gastrointestinal and immune parameters in poultry under inflammatory challenge.

In light of the previously mentioned benefits of nucleotides, it was hypothesized that dietary nucleotide supplementation could improve growth performance, gastrointestinal development, and immune function of broilers. Thus, the objective of this study was to evaluate the effects of dietary nucleotide supplementation on growth performance, serum IgG and uric acid levels, immune-related organs, and intestinal morphometric parameters of broiler chickens under pre- and post-inflammatory challenge.

2. Material and Methods

2.1. Ethical matters

The institutional Animal Care and Use Committee approved all animal handling procedures (case no. 57/2017), and the experiment was conducted according to the experimental protocol for use of live birds from the Brazilian College of Animal Experimentation.

2.2. Broiler chickens, experimental diets, and challenge

The experiment was conducted in Viçosa, MG, Brazil (20°45'57.19" S, 42°51'35.42" W, and 682 m altitude). 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 one day old until the beginning of the experiment, the birds were reared on floor pens (200 × 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 seven days old, a total of 96 broiler chickens (168.5±1.18 g) were randomly selected. A 2×2 factorial completely randomized design was applied, composed of two diet types (supplemented with 0 and 0.3 g of purified nucleotides per kg of feed) and two inflammatory conditions (without and with lipopolysaccharide [LPS] administration) with eight replicates per treatment and three birds per cage. The nucleotide supplement (ASCOGEN, Chemoforma, Switzerland) contained 15% purified nucleotides and was added to the basal diet in place of starch at a dose of 2 g/kg. Birds were housed in wire floor 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 mash form. Free access to water and feed was provided throughout the experimental period.

Table 1 - Composition of basal diets (as fed basis)

Item	Starter (7-20 d)	Grower (21-35 d)
Ingredient (g/kg)		
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 ¹	1.00	1.00
Mineral premix ²	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 ³	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

¹ Vitamin 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 g; vitamin B5, 10.0 g; vitamin B6, 2,800 mg; vitamin B9, 700 mg; vitamin B12, 12 mg; biotin, 70 mg.

² Mineral 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.

³ Nucleotides (2 g/kg) were added as a replacement for starch.

Birds were exposed to 23 h of light from one to seven 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 by 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 (pre-LPS challenge) and 21-35 days of age (post-LPS challenge).

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 a dose of 1.0 mg/mL) at 21, 23, 25, and 27 days 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 De Lange, 2012). Animals without LPS administration received an injection of a similar amount of a saline solution.

2.3. Performance

Body weight and feed intake of each experimental unit were recorded on days 20 and 35 to calculate the daily weight gain (DWG), daily feed intake (DFI), and feed conversion rate (FCR).

2.4. Sample collection and procedure

At 20, 28, and 35 days of age, one bird per experimental unit with an average weight was selected for blood collection from the wing vein. Blood was centrifuged at $3,600 \times g$ at 4°C for 10 min for separation, and serum samples were stored at -20°C until analysis. After the blood collection on day 35, birds were euthanized by cervical displacement and slaughtered. The liver, spleen, and bursa of Fabricius were removed from each bird 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 a 2-cm section of jejunal tissue (midway between the Meckel's diverticulum and the entrance of the bile ducts) was collected for morphometric evaluation.

2.5. Serum parameter measurement

Levels of IgG in serum samples were measured using a commercial ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) specific to chickens. The analysis methods 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). Dilutions of 1:100,000 of the serum samples were used. The IgG content was determined according to a standard curve and recorded in nanograms per milliliter (ng/mL).

Serum uric acid (UA) was determined using commercial kits (K139; 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.

2.6. Intestinal morphometry

The 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 in liquid paraffin at 60°C . Paraffin blocks were fixed on a rotary microtome (Spencer® model 19459, USA), and cross sections were sliced to $5\text{-}\mu\text{m}$ thickness. Cuts were made semi-serially with one in each of ten sections used to avoid repeating the analyses in the same histological area. Six sections were placed on each glass slide and dyed with Hematoxylin and 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 the villus measurements was expressed as a mean for each bird ($n = 8$ birds/treatment). The slides were examined using an optical microscope (EVOS® XL Core Imaging System, Thermo Fisher Scientific Inc., Bothell, WA) at 10X magnification. Morphometric analysis was performed using ImageJ software (National Institutes of Health, USA). Villus height 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 between villus height and crypt depth (VH:CD) was calculated.

2.7. 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 effect of nucleotides. A one-way ANOVA was performed according to the following general model:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}, \quad (1)$$

in which 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 the experiment (21-35 days), a two-way ANOVA was used to measure the main effects of nucleotides, LPS, and their interaction. Additionally, initial BW was used as a covariate according to the following general model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}, \quad (2)$$

in which 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 the mean \pm standard error of the mean (SEM). The significance level of the F test was defined as $P < 0.05$.

3. Results

3.1. Pre-challenge

During the first phase (7-20 days), dietary nucleotide supplementation improved ($P < 0.05$) BW, DWG, and FCR of broilers (Table 2). However, DFI was not affected ($P > 0.05$) by treatments. Birds supplemented with nucleotides also showed increased ($P < 0.05$) serum IgG concentration.

3.2. Post-challenge

No interaction ($P > 0.05$) was observed between nucleotide supplementation and LPS challenge on growth performance of broilers from 21-35 days of age (Table 3). Nucleotide supplementation had no effect ($P > 0.05$) on performance. However, LPS administration resulted in a reduction ($P < 0.05$) in DWG and DFI of broilers.

No interaction ($P > 0.05$) between nucleotides and LPS challenge was found for serum IgG and UA, relative weight of immune organs, and jejunum morphometric parameters of broilers (Table 4). At

Table 2 - Effects of nucleotide supplementation on growth performance from 7-20 days and serum immunoglobulin G (IgG) at 20 d of broiler chickens.

Nucleotides	BW (g)	DWG (g/d/b)	DFI (g/d/b)	FCR (g/g)	IgG (mg/mL)
0.0	982	58.1	74.5	1.28	1.17
0.3	1009	60.1	75.2	1.25	1.76
SEM	6.63	0.47	0.86	0.02	0.18
P-value	0.007	0.007	0.541	0.049	0.031

BW - body weight; DWG - daily weight gain; DFI - daily feed intake; FCR - feed conversion ratio; SEM - standard error of the means ($n = 16$ for each treatment).

Table 3 - Effects of nucleotide supplementation on growth performance of broiler chickens from 21-35 days

Main effect		BW (g)	DWG (g/bird)	DFI (g/bird)	FCR (g/g)
Nucleotides	Challenge				
0.0		2365	99.1	156.8	1.58
0.3		2353	96.0	154.6	1.61
	Control	2398	101.2a	159.3a	1.57
	LPS	2320	93.9b	152.1b	1.62
SEM		41.39	2.90	3.35	0.02
	P-value				
Nucleotides		0.778	0.283	0.514	0.138
Challenge		0.073	0.017	0.041	0.054
Nucleotides \times challenge		0.612	0.502	0.990	0.152

LPS - lipopolysaccharide; BW - body weight; DWG - daily weight gain; DFI - daily feed intake; FCR - feed conversion ratio; SEM - standard error of the means ($n = 8$ for each treatment).

a,b - Within a column, means with different letters are significantly different at $P < 0.05$.

Table 4 - Effects of nucleotide supplementation on serum immunoglobulin G (IgG) and uric acid at 28 and 35 days and relative weight of immune organs and jejunum morphometric parameters at 35 days

Main effect		IgG 28 d	IgG 35 d	UA 28 d	UA 35 d	Bursa of	Spleen	Liver	VH	CD	VH/CD
Nucleotides	Challenge	(mg/mL)	(mg/mL)	(mg/dL)	(mg/dL)	Fabricius (%)	(%)	(%)	(μ m)	(μ m)	
0.0		3.01a	4.52	2.62	1.59	0.188	0.124	4.972	299.3a	44.0	6.80
0.3		4.34b	5.71	3.14	1.71	0.165	0.154	4.566	315.6b	46.4	6.80
	Control	3.00a	3.85a	2.79	1.50	0.177	0.124	4.787	309.9	45.0	6.89
	LPS	4.35b	6.39b	2.97	1.80	0.176	0.154	4.751	305.1	45.4	6.72
SEM		0.546	1.07	0.25	0.23	0.23	0.016	0.261	7.80	1.80	0.28
P-value											
Nucleotides		0.022	0.278	0.063	0.599	0.344	0.077	0.131	0.046	0.198	0.488
Challenge		0.021	0.027	0.492	0.212	0.762	0.076	0.885	0.556	0.843	0.364
Nucleotides \times Challenge		0.559	0.493	0.209	0.432	0.980	0.282	0.185	0.918	0.444	0.734

LPS - lipopolysaccharide; UA - uric acid; VH - villus height; CD - crypt depth; VH:CD - villus height to crypt depth ratio; SEM - standard error of the means (n = 8 for each treatment).

a,b - Within a column, means with different letters are significantly different at $P < 0.05$.

28 days of age, nucleotide supplementation or LPS administration increased ($P < 0.05$) serum IgG concentration. However, at 35 days of age, only birds injected with LPS showed increased ($P < 0.05$) serum IgG. Nucleotide supplementation and LPS challenge had no effect on UA or the relative weights of the bursa, spleen, and liver. Nucleotide supplementation increased ($P < 0.05$) VH when compared with the control group, but no effects ($P > 0.05$) were observed for CD or the VH:CD ratio. The LPS administration did not affect ($P > 0.05$) jejunum morphometric parameters.

4. Discussion

The hypothesis for this study was that nucleotide dietary supplementation could improve growth performance, gastrointestinal development, and immune function of broilers. During the pre-inflammatory challenge phase (7-20 days), it was confirmed that nucleotide supplementation improved BWG and FCR of broilers. It is known that the dramatic increase in growth rate of broilers is manifested primarily in the first three weeks after hatching. Under rapid growth conditions, nucleotides may become conditionally essential nutrients (Hess and Greenberg, 2012; Leung et al., 2019a). Accordingly, beneficial effects of nucleotides or nucleosides on growth performance have been reported in broilers (Daneshmand et al., 2017a,b).

One possible explanation for the above beneficial effects 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. Increased glutamine availability is particularly important for high-glutamine-demand processes and organs such as the *pectoralis major* muscle (0.06 g glutamine/g protein of muscle; Hu et al., 2016).

The initial hypothesis of this study regarding the post-inflammatory challenge phase (21-35 days) was not confirmed. During this phase, nucleotide supplementation had negligible effects on growth performance. One possible explanation is that the amount of nucleotides given (0.03%) may not have been sufficient to mitigate the negative effects of inflammatory challenge. In fact, the challenged animals had reduced feed intake and, consequently, lower nucleotide intake. Data obtained in previous studies regarding the effects of nucleotides on performance of broilers are inconsistent. One such study reported that supplementation with 0.1% nucleotides to broilers can improve growth parameters (Leung et al., 2019b), while other investigators showed that this supplementation did not influence BW, weight gain, or feed intake (Alizadeh et al., 2016a,b; Leung et al., 2019b). Different management and environmental factors such as animal model, age, strain, source and level of nucleotides, type of challenge, and stressors may explain the inconsistencies observed by different researchers (Daneshmand et al., 2017a).

As expected, LPS challenge reduced DFI and DWG of broilers in 4.5 and 7.2%, respectively. This effect has already been observed in other studies with broilers and is dependent on the LPS dose, number of applications, and time between application and weighing of the birds. For example, Li et al. (2018), after applying 0.6 mg of LPS/kg of BW at 24, 25, and 26 days of age, observed a reduction of 15.4% in feed intake and of 16.2% in weight gain in the period of 22 to 28 days of age. Zhang et al. (2020) observed that 24 h after the application of 5.0 mg of LPS/kg of BW at 21 days of age resulted in a 44.6% reduction in weight gain compared with the control group. This may be explained by changes in the partitioning of nutrients away from growth and toward processes associated with the acute-phase response. Lipopolysaccharide is a molecule present in the outer membrane of gram-negative bacteria and has commonly been used to induce an inflammatory response in broilers in experimental conditions (Lieboldt et al., 2017; Li et al., 2018; Zhang et al., 2020). Lipopolysaccharide 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., 2008), which activate neutrophils, monocytes, and macrophages to initiate killing of bacteria and tumor cells 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 (Balaji et al., 2002; Adewole et al., 2016).

It was observed on days 20 (pre-challenge) and 28 (post-challenge) that nucleotide supplementation increased serum IgG levels of broilers. The change in IgG levels in nucleotide-fed broilers 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 nucleotide and nucleoside supplementation increased the production of immunoglobulins in pigs and birds (Jang et al., 2013; Trckova et al., 2014; Daneshmand et al., 2017b). Lymphocyte B produces immunoglobulins (Ochsenbein and Zinkernagel, 2000) and requires a high number of nucleotides, which are synthesized *de novo* by other organs (primarily the liver), for maintenance and fast proliferation (Rudolph et al., 1990; Jyonouchi et al., 1994). 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).

The LPS challenge also 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 level of immune reactivity or sensitivity to vaccine antigens. Lai et al. (2011) observed that LPS enhanced primary total immunoglobulin and IgG antibody responses of broilers to human serum albumin antigen.

It has been reported that surplus nucleotides in excess of what is required by enterocytes might be converted into UA and transported through the bloodstream to the liver for further metabolism or utilization by hepatocytes (Daneshmand et al., 2017b). In the present study, we did not observe an effect of nucleotide supplementation on serum UA, indicating that the level of supplementation did not exceed the cellular requirement. This suggests that during the post-challenge phase, the level of supplemented nucleotides was insufficient to mitigate the negative effects of inflammatory challenge.

The bursa and spleen contain different immune system cells that require sufficient DNA and RNA for maintenance and growth. The liver is important for acute-phase protein and cytokine production. Thus, the relative weights of these organs are important immunological indices. Despite the nucleotide effect observed on serum IgG, there was no effect on relative weights of the bursa, spleen, and liver of broilers at 35 days of age. Previous studies reported similar results for the relative weight of the spleen, but an increase in the relative weight of the bursa was observed (Daneshmand et al., 2017a,b, Leung et al. 2019b). The high bursa weight has been related to possible migration of exogenous nucleotides to supply more nucleosides for the synthesis of cells in this organ.

In the present study, dietary nucleotide supplementation increased VH in the jejunum of birds at 35 days of age. This is in accordance with other studies carried out with birds and pigs (Moore et al., 2011; Jung and Batal, 2012; Wu et al., 2018). Avian species have a high growth rate capacity that is characterized by rapid early development of the digestive tract (Lilja, 1983), which requires a high

number of nucleotides. Complete small intestine structure plays a fundamental role in nutrient digestive and absorptive functions and is a crucial barrier to pathogens and toxins in young birds (Bartell and Batal, 2007). Thus, the increase in VH proportions by nucleotide supplementation may have contributed to the improved performance observed in the first phase of this study.

Nucleotide supplementation improved poultry performance in the pre-inflammatory phase (7-21 days) in this study, but not in post-challenge conditions (21-35 days) despite the improvement in VH observed. Based on other works (Alizadeh et al., 2016a,b; Leung et al., 2019b) and our results, the level of nucleotides offered may not be sufficient to mitigate the negative effects of inflammatory challenge. Thus, further studies with higher levels of nucleotide supplementation are encouraged.

5. Conclusions

Our findings confirmed that dietary nucleotide supplementation improves the body weight gain and feed conversion ratio of broilers from 7 to 20 days of age. However, in the post-inflammatory challenge phase (21-35 days of age), supplementation of 0.3 g of purified nucleotides/kg of feed, independent of inflammatory challenge, does not contribute to improved performance of broilers. Additionally, nucleotide supplementation increases immunoglobulin G production and villi height in the jejunum of broilers.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: B.S. Kreuz, G.C. Rocha and A.A. Calderano. Data curation: A.A. Calderano. Formal analysis: B.S. Kreuz, F.S. Silva and A.A. Calderano. Investigation: B.S. Kreuz, S.O. Borges and A.A. Calderano. Methodology: G.C. Rocha, M.I. Hannas and A.A. Calderano. Project administration: A.A. Calderano. Resources: M.I. Hannas, L.F.T. Albino and A.A. Calderano. Supervision: G.C. Rocha, L.F.T. Albino and A.A. Calderano. Writing-original draft: B.S. Kreuz. Writing-review & editing: G.C. Rocha, P.H.R.F. Campos, F.S. Silva and A.A. Calderano.

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