Research Article

Intra-Amnionic Threonine Administered to Chicken Embryos Reduces *Salmonella* Enteritidis Cecal Counts and Improves Posthatch Intestinal Development

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This study assessed the effect of *in ovo* threonine supplementation on the response of broiler chicks challenged with *Salmonella* Enteritidis, considering bacterial counts in cecal contents, intestinal morphology, body weight, and weight gain. Fertilized eggs were inoculated in the amniotic fluid with saline (NT) or 3.5% threonine (T) solution at day 17.5 of incubation. At hatch, chicks were individually weighed and cloacal swabs were screened for *Salmonella*. At 2 days of age, half of the birds from each *in ovo* treatment were given either 0.5 mL of nutrient broth (sham-inoculated) or nalidixic acid-resistant *Salmonella* Enteritidis (SE Nal^R) in nutrient broth (8.3×10^7 colony forming units (CFU) SE Nal^R/mL). The birds were distributed using a completely randomized design with four treatments after the *Salmonella* challenge: no *in ovo* Thr supplementation and sham-inoculated in the posthatch challenge (NT-SHAM), *in ovo* Thr supplementation and sham-inoculated (T-SHAM), no *in ovo* Thr supplementation and SE Nal^R-challenged (NT-SE), and *in ovo* Thr supplementation and SE Nal^R-challenged (T-SE). *In ovo* threonine supplementation reduced *Salmonella* Enteritidis colonization 168-hour postinoculation and reduced the negative effects associated with *Salmonella* infection on intestinal morphology and performance, with results similar to those of the sham-inoculated birds. *In ovo* Thr supplementation increased the expression of *MUC2* at hatch and the expression of *MUC2* and *IgA* at 2 days of age and 168-hour postinoculation. Our results suggest that providing *in ovo* threonine promotes intestinal health in broilers challenged with *Salmonella* Enteritidis in the first days of life.

1. Introduction

Important transformations occur posthatch that are essential for chick survival and determinant for the broiler. The most important of these transformations is the rapid development of the gastrointestinal tract, which is necessary for enabling nutrient assimilation. During this stage, broilers are exposed to several types of micro-organisms, but their defense mechanisms are minimally developed and are limited to the innate immune system and maternal antibodies.

The hatchling's gastrointestinal tract contains a small number of microorganisms [1], which facilitate pathogen colonization [2]. Susceptibility to *Salmonella* infections depends on age; younger animals up to 3 days old are more vulnerable to intestinal infections and more prone to developing inflammatory reactions and intestinal lesions than adult birds [3].

The mucosal layer is the first line of defense against bacteria and other pathogens [4]. Mucin is the main component of the protective mucosal layer, which protects the intestinal epithelium and prevents damage and infections by pathogenic bacteria. Mucin production is closely related to dietary threonine levels [5].

Evidence suggests that threonine (Thr) supplied to broilers in early life stages, e.g., during embryogenesis, may improve their immune response to *Salmonella* during post-hatch. For example, *in ovo* Thr supplementation increased mucin (MUC2) expression in broiler chickens [6] and quails [7], thus increasing protection of the intestinal mucosa against pathogens and improving digestion and nutrient absorption.

Studies have shown that *in ovo* nutrition benefits the intestinal development [8, 9], increases the intestinal expression of glucose and amino acid transporters [9, 10], increases mucin synthesis in the intestinal epithelium [6, 7], improves the humoral immune response [11], and increases interleukin expression [6]. The present study evaluated the effect of *in ovo* Thr supplementation on the response of two-day-old broilers challenged with *Salmonella* Enteritidis.

2. Materials and Methods

All management, slaughter, and sampling procedures were approved by the Ethical Committee of Animal Use in Research of the Federal University of Paraíba (Comissão de Ética no Uso de Animais da Universidade Federal da Paraíba) under the protocol number CEUA 078/2017. The study was conducted in the Departamento de Zootecnia, Centro de Ciências Agrárias da Universidade Federal da Paraíba, Brazil.

2.1. Incubation and In Ovo Supplementation. Three hundred fertile eggs were obtained from a commercial hatchery (Guaraves Alimentos Ltda., Guarabira, PB, Brazil), with a mean weight of 67.0 ± 1.56 g. The eggs originated from Cobb500 breeders aged 44 weeks and were distributed into three artificial incubators (IP130, Premium Ecológica Ltda., Belo Horizonte, MG, Brazil). All incubators were maintained under standard incubation conditions from one to seventeen days (D17) at 37.7°C and 60% relative humidity, with automatic turning every two hours. On the 11th day of incubation (D11), nonfertile eggs were discarded after candling.

The eggs were randomly distributed into two treatments ($n = 150$ per group) based on the Thr concentration to be injected *in ovo* (NT: 0.0% Thr; T: 3.5% Thr) on D17.5 according to Uni and Ferket [12]. Briefly, all eggs were cleaned with 70% ethanol and punctured at the air chamber end. Nutritive solution (1.0 mL) was warmed to 30°C and injected through the puncture in the amniotic fluid using 1 mL syringes and 21 G needles. Inoculated eggs were placed in hatching trays and incubated at 36.7°C. After hatching, chicks were weighed individually and incubation was assessed by chick weight at hatching, total hatchability, fertile hatchability, pipped eggs, and embryo mortality.

2.2. Treatments and Management. At hatch, chicks were identified with leg bands and individually weighed and cloacal swabs were sampled from 20 birds per incubator to screen for *Salmonella*. The birds were then distributed into a completely randomized experimental design with 4 treatments of 36 birds (repetitions) per treatment kept in boxes. Each box was equipped with a feeder and a drinker and covered with nylon mesh to avoid contamination between boxes by vectors such as flies.

A corn-soybean meal diet was formulated for the initial phase based on Rostagno et al. [13]. Levels were 22.20% CP, 2950 kcal/kg ME, 1.31% digestible lysine, 0.94% digestible methionine + cystine, and 0.852% digestible threonine. Birds were weighed individually at the beginning and end of the experimental period. Initial and final body weights were used to calculate weight gain.

2.3. Inoculation. The birds were challenged with *Salmonella* at two days old. Inoculum was prepared using one colony of nalidixic acid-resistant *Salmonella* Enteritidis (SE Nal^R) in nutrient broth (Acumedia, USA) containing nalidixic acid (100 μg/mL) for 24 h at 37°C, and then, an aliquot (0.1 mL) was cultured again for four hours at 37°C. Inoculum concentration was determined by plating a serial dilution (10^{-1} to 10^{-6}) on brilliant green agar plates (Acumedia) with nalidixic acid (100 μg/mL) and incubating at 37°C. *Salmonella* colonies were counted after 24 hours. All birds in each box were inoculated with 0.5 mL of *Salmonella* Enteritidis Nal^R (8.3×10^7 CFU/mL) into the crop, except the sham-inoculated control birds, who received only nutrient broth.

The birds were distributed in a completely randomized design with four treatments after being challenged with *Salmonella*: no *in ovo* Thr supplementation and sham-inoculated in the posthatch challenge (NT-SHAM), *in ovo* Thr supplementation and sham-inoculated (T-SHAM), no *in ovo* Thr supplementation and SE Nal^R-challenged (NT-SE), and *in ovo* Thr supplementation and SE Nal^R-challenged (T-SE).

2.4. Microbial Analysis. At 24-, 96-, and 168-hour postinoculation (hpi) with *Salmonella* or at 3, 6, and 9 days of age, six birds per treatment were killed by cervical dislocation. Cecal contents were collected from all birds in each treatment ($n = 6$) and weighed before being serially diluted (10^{-1} to 10^{-6}) with peptone water (Acumedia). Twenty microliter aliquots was cultured on brilliant green agar with nalidixic acid (100 μg/mL) and incubated at 37°C for 24 hours. Colony counts were performed, and values were expressed in colony-forming units per gram of cecal content (CFU/g).

2.5. Histology Analysis. Four birds per treatment were killed, and the medial regions of the duodenum, jejunum, and ileum were sampled for histological analyses. Samples were rinsed with sterile saline, immersed in buffered formalin for 24 hours, and rinsed in 70% ethanol. Slides were mounted with 5 μm sections and stained with hematoxylin and eosin. Two slides with five to seven sections were mounted per bird. Photomicrographs of the duodenal mucosa were taken using a digital camera with 12.1 megapixels (Sony Inc.) connected to a light microscope, with 1.7 optical zoom and 10x objective. The images were analyzed using ImageJ software [14]. Villus height (VH), crypt depth (CD), villus width at the apex (AW),
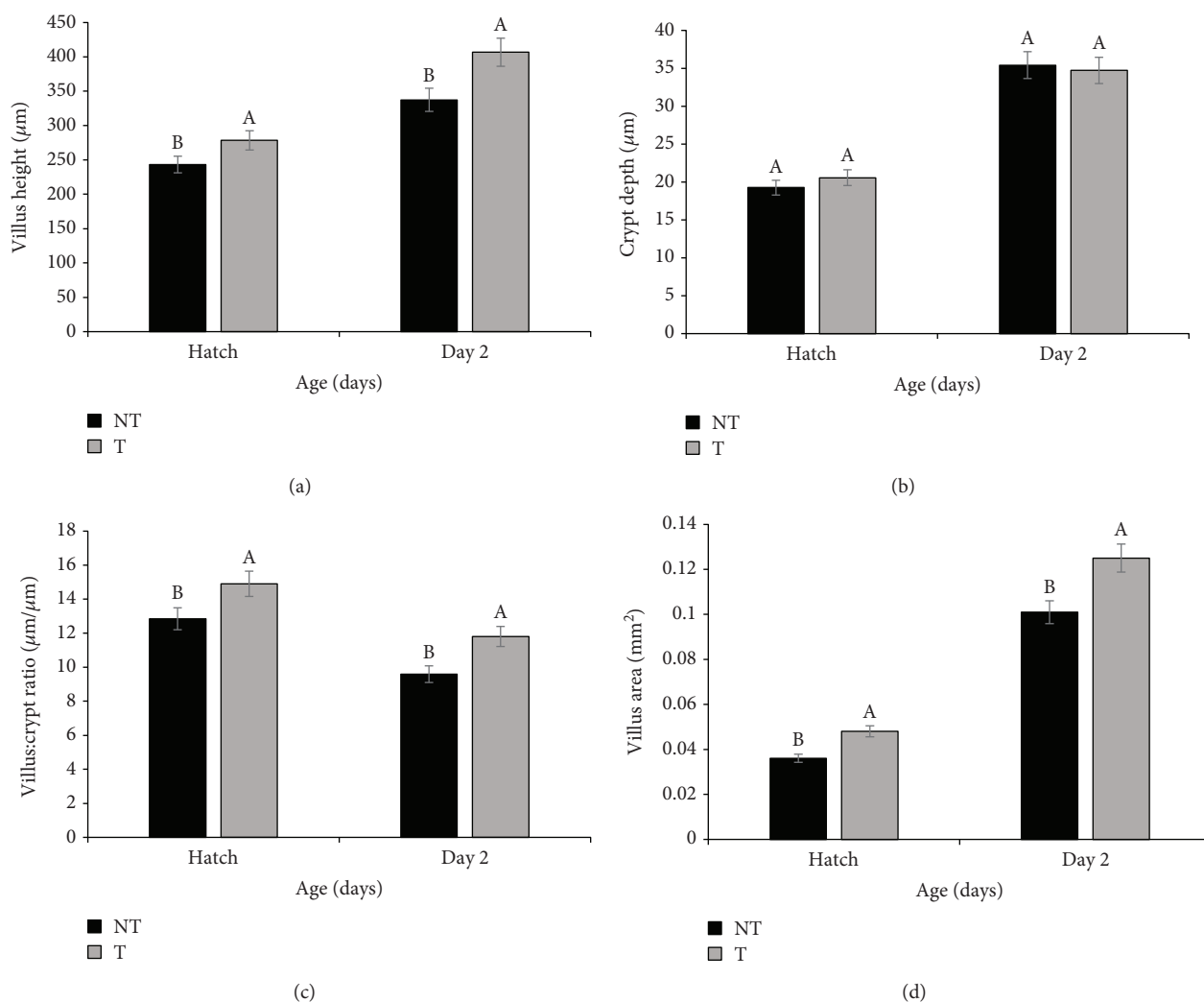
(a)

(b)

(c)

(d)

FIGURE 1: Villus height (a), crypt depth (b), villus : crypt ratio (c), and villus area (d) in the ileum at hatch and at two days of age in broilers supplemented with Thr *in ovo* (NT: 0.0% Thr; T: 3.5% Thr). The error bars represent the standard deviation.

and base width (BW) were measured. Ten measurements were made per bird, with a total of 60 measurements per variable per treatment. The mean value was calculated per variable per bird. The villus : crypt ratio was calculated (VH/CD), and villus area was calculated according to Sakamoto et al. [15]: $(2\pi)(AW/2)(VH)$.

2.6. MUC2 and IgA mRNA Expression. Total RNA was isolated from four ileum samples per treatment (500 μg) using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's guidelines, and the quality and purity were assessed using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA) based on 260/280 and 260/230 ratios. Reverse transcription was performed using an AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's guidelines.

Relative quantification by real-time polymerase chain reaction (PCR) was performed using the Brilliant III Ultra-Fast SYBR QPCR Master Mix (Agilent Technologies). Cycling was performed using a Stratagene Mx3005P (Agilent Technologies). MUC2 primer sequences were 5′-ATGTTTTTGCA TCCCATTGC-3′ (forward) and 5′-TGCGGTGGATTGTC AGAATA-3′ (reverse); IgA primer sequences were 5′-ACCA CGGCTCTGACGTACAC-3′ (forward) and 5′-CGATCG TCTCCTTCACAGCA-3′ (reverse), and β-actin primer sequences were 5′-ACCACTGGCATTGTCATGGACTCT-3′ (forward) and 5′-TCCTTGATGTCACGGACGATTTCC-3′ (reverse). Primers were designed using Primer Express (version 3, Applied Biosystems, Foster City, CA, USA).

Relative MUC2 and IgA mRNA abundance was determined using the $2^{-\Delta\Delta Ct}$ method [16]; Ct values for each sample were standardized for β-actin RNA.

2.7. Statistical Analysis. Microbial data were analyzed using a completely randomized design with two treatments, considering only treatments T-SE and NT-SE, with six replicates per treatment and sampling age (24, 96, and 168 hpi). Morphometry, gene expression, and performance data were analyzed using a completely randomized design with two treatments for samplings at posthatch and two days of age and four treatments for samplings at 24, 96,
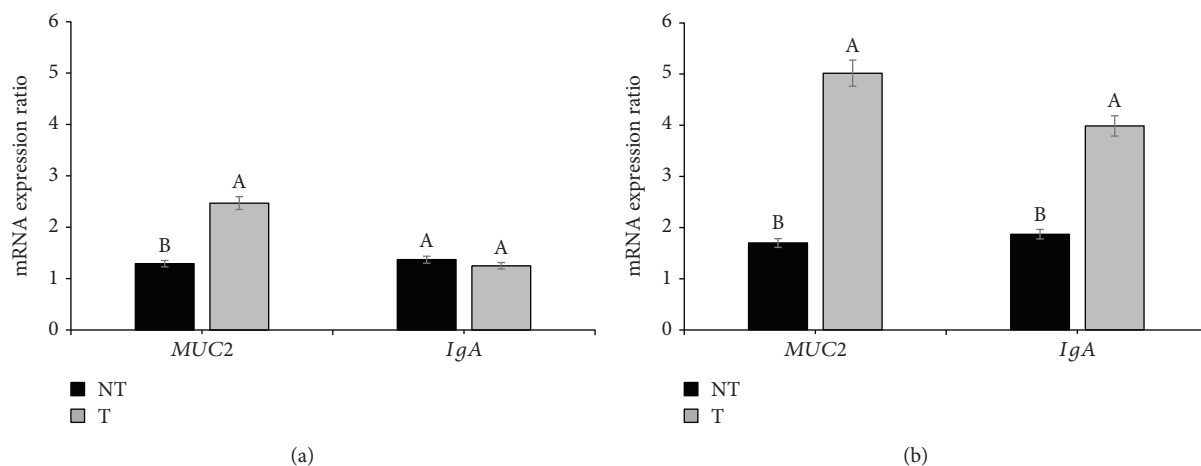
(a)

(b)

FIGURE 2: Relative expression of mucin (MUC2) and immunoglobulin A (IgA) mRNA at hatch (a) and two days of age (b) in broilers supplemented with Thr in ovo (NT: 0.0% Thr; T: 3.5% Thr). The error bars represent the standard deviation.
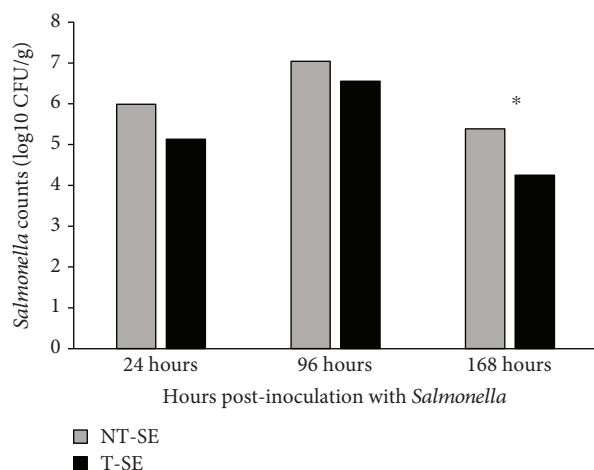

FIGURE 3: Bacterial counts in cecal contents (CFU/g) in broilers challenged with Salmonella Enteritidis (SE) and supplemented with Thr in ovo. NT-SE = no in ovo Thr supplementation and SE Nal^R-challenged; T-SE = in ovo Thr supplementation and SE-challenged.

and 168 hours after inoculation with Salmonella. When significant differences were found, means were compared using Tukey's test and F test at $p \leq 0.05$. Analyses were calculated using SAS [17].

3. Results

In ovo Thr supplementation did not affect hatchability (83% vs. 80%) or embryo mortality (17% vs. 20%). In ovo supplementation with 35 mg of Thr resulted in higher weight ($p \leq 0.05$) at hatch when compared to birds not supplemented in ovo (53.36 g vs. 51.19 g, respectively).

Villus height, crypt depth, villus:crypt ratio, and villus area in the ileum showed similar results at hatch and at two days of age (Figure 1). All studied parameters were higher for animals supplemented with Thr in ovo (T) ($p \leq 0.05$), but crypt depth was not different ($p > 0.05$). This was similar in the duodenum and jejunum, except for villus

height at hatch and villus area at both ages in the jejunum, which did not significantly differ between treatments ($p > 0.05$) (Supplementary Table 1).

In ovo Thr supplementation affected the relative expressions of MUC2 and IgA at hatch and two days of age (Figure 2). Thr supplementation resulted in increased expression of MUC2 at hatch ($p \leq 0.05$), but not of IgA, which did not significantly differ from the control group ($p > 0.05$). At two days of age, MUC2 and IgA expressions were higher with Thr supplementation than for the control group ($p \leq 0.05$).

In ovo Thr supplementation had no effect ($p > 0.05$) on Salmonella cecal counts at 24 and 96-hour postchallenge; however, Salmonella cecal counts were lower with Thr supplementation at 168-hour postchallenge ($p \leq 0.05$) (Figure 3). Salmonella was not recovered from sham-inoculated control birds.

The changes in villus height, crypt depth, villus:crypt ratio, and villus area in the ileum at 24, 96, and 168 hpi were similar (Figure 4). Villus height, villus:crypt ratio, and villus area were higher for T-SHAM, and crypt depth was higher ($p \leq 0.05$) for NT-SE at 24, 96, and 168 hpi, but crypt depth at 96 hpi was not different between T-SHAM, T-SE, and NT-SHAM ($p > 0.05$). The T-SE group presented a higher or similar villus height and area than NT-SHAM in the three periods (Figure 4).

Similar results were observed in the duodenum and jejunum at 24 hpi (Supplementary Table 2). Villus height, villus:crypt ratio, and villus area were higher for T-SHAM ($p \leq 0.05$), and the groups NT-SHAM and T-SE showed similar results. At 96 hpi, duodenal and jejunal villus height, villus:crypt ratio, and villus area were higher for broilers from unchallenged treatments (NT-SHAM and T-SHAM) ($p \leq 0.05$) (Supplementary Table 3). Interestingly, the group supplemented with Thr in ovo and challenged with Salmonella (T-SE) presented similar villus heights and areas to the unchallenged groups. Crypt depth for both intestinal segments was higher for the treatment challenged with Salmonella and not supplemented with Thr in ovo ($p \leq 0.05$).
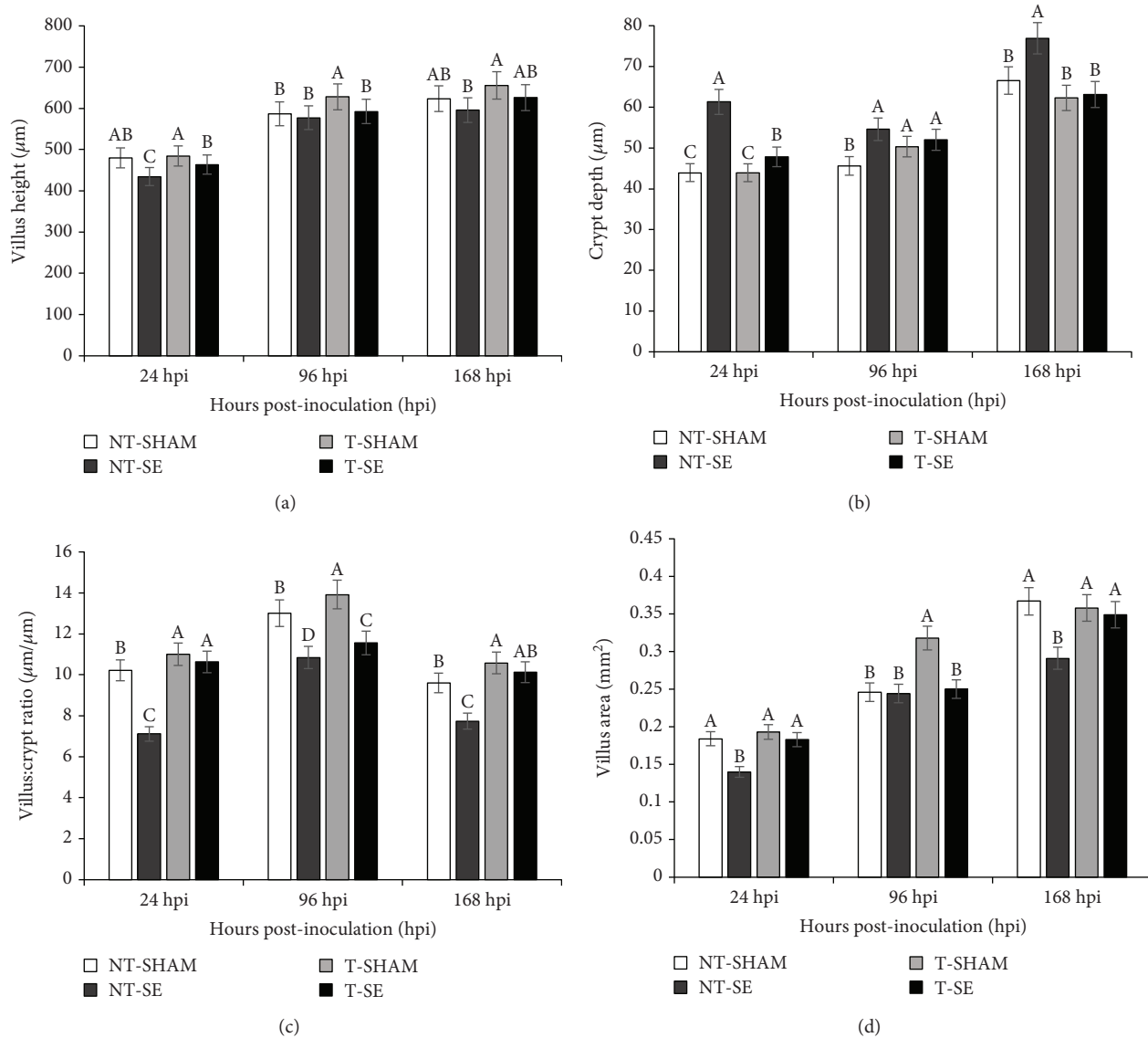
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FIGURE 4: Villus height (a), crypt depth (b), villus : crypt ratio (c), and villus area (d) in the ileum in broilers at 24, 96, and 168-hour postinoculation (hpi) with *Salmonella*. NT-SHAM: no *in ovo* Thr supplementation and sham-inoculated in the posthatch challenge; T-SHAM: *in ovo* Thr supplementation and sham-inoculated; NT-SE: no *in ovo* Thr supplementation and SE Nal[R]-challenged; and T-SE: *in ovo* Thr supplementation and SE Nal[R]-challenged. The error bars represent the standard deviation.

At 168 hpi (Supplementary Table 4), villus height, villus : crypt ratio, and villus area in the duodenum and jejunum were higher in the T-SHAM group ($p \leq 0.05$). Duodenal and jejunal villus height and villus area were similar between T-SHAM, NT-SHAM, and T-SE. The NT-SE group presented the highest crypt depth and lowest villus area and villus : crypt ratio ($p \leq 0.05$). The lowest crypt depths were seen in T-SHAM and T-SE birds.

Relative expression of mucin (*MUC2*) in the ileum at 168 hpi was highest for T-SE ($p < 0.01$), followed by T-SHAM. Relative expression of immunoglobulin A (*IgA*) was also higher for T-SHAM and T-SE ($p \leq 0.05$). For both genes, expression was lower for NT-SHAM and NT-SE (Figure 5).

At 24 hpi, no significant differences in initial weight were observed between the four treatments ($p > 0.05$). Final weight and weight gain (Figure 6) were higher for T-SHAM

($p \leq 0.05$) and not different from NT-SHAM. NT-SE presented the lowest final weight and weight gain ($p \leq 0.05$).

Similar results were observed at 96 and 168 hpi (Figure 6); mean final weight and weight gain were highest for T-SHAM ($p \leq 0.05$). However, at 168 hpi, T-SHAM was not different from NT-SHAM. Similar to what was observed at 24 hpi, NT-SE presented the lowest final weight and weight gain at 96 and 168-hour postinoculation. T-SE presented a similar final weight and weight gain to NT-SHAM in all sampling times ($p > 0.05$).

4. Discussion

The present results show that *in ovo* Thr supplementation increased *MUC2* and *IgA* expressions, positively affected histological and performance parameters, and decreased
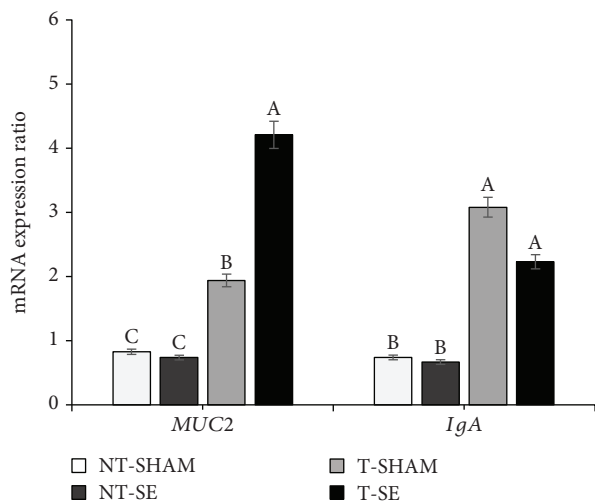
FIGURE 5: Relative expression of mucin (MUC2) and immunoglobulin A (IgA) mRNA in broilers at 168-hour (9 days of age) postinoculation with Salmonella. NT-SHAM: no in ovo Thr supplementation and sham-inoculated in the posthatch challenge; T-SHAM: in ovo Thr supplementation and sham-inoculated; NT-SE: no in ovo Thr supplementation and SE Nal^R-challenged; and T-SE: in ovo Thr supplementation and SE Nal^R-challenged. The error bars represent the standard deviation.

Salmonella Enteritidis cecal counts (168 hpi). Furthermore, in ovo Thr supplementation did not affect hatchability and supplemented chicks weighed more at hatch.

The benefit of in ovo supplementation on live weight at hatch may be related to the use of energy reserves during embryogenesis. Gluconeogenesis from protein catabolism may be only source of glucose for the embryo at hatch, thus negatively affecting broiler growth and development and likely leading to decreased body weight [8]. In the present study, this negative effect may have been alleviated by in ovo Thr supplementation because Thr is a gluconeogenesis substrate. Therefore, in ovo Thr supplementation may have increased the embryo's energy status by decreasing muscle protein depletion, thereby increasing body weight at hatch.

Similarly, higher body weight at hatch was seen after supplementing 30 mg and 35 mg Thr in ovo with only a small decrease in hatchability when different levels of Thr supplementation were used [18]. No negative effects on hatchability and beneficial effects on hatchling weight were observed when Thr [11, 19] or other amino acids [20, 21] were supplemented in ovo. Several factors related to the method of in ovo nutrient injection, including volume, temperature, concentration, site, embryo age, and egg handling, may affect the hatch rate and other parameters.

Our results also showed that in ovo Thr supplementation benefitted gastrointestinal tract (GIT) development. Thr-supplemented animals presented higher villus height, villus : crypt ratio, villus area, and MUC2 and IgA expressions on the day of hatch and at two days of age. Previous studies have suggested improved intestinal development in response to in ovo nutrient supplementation, possibly related to the nutrients stimulating the intestinal mucosa at an earlier stage [9, 22, 23]. In ovo supplementation

guarantees availability of nutrients and cofactors necessary to sustain and even accelerate enteric development, which may otherwise be limited by low nutrient availability at the end of incubation [11].

Kadam et al. [11] tested different levels of in ovo Thr supplementation and observed higher feed intake in broilers supplemented with Thr. This may be related to better functional development of the GIT with greater density of intestinal goblet cells in the intestines of animals supplemented with Thr, because Thr is an essential component of enzymes and mucin [11]. Tahmasebi and Toghyani [19] observed longer jejunum and ileum in animals supplemented in ovo with Thr, arginine (Arg), and Thr + Arg than in nonsupplemented animals. Regarding intestinal morphology parameters, the authors observed increased ileum villus height in animals supplemented with Thr and Arg.

Dietary supplementation with both Thr and mannan oligosaccharide (MOS) [5] or only Thr [24] has been reported to decrease the number of Salmonella-positive animals and bacterial counts in the cecal contents, similar to the present finding with in ovo Thr supplementation. Some factors may have contributed to decreased Salmonella cecal counts in the Thr-administered group at 168-hour postinoculation (9 days of age). The first is the development of the gut-associated lymphoid tissue (GALT). GALT remains immature up to three weeks posthatch, and intestinal protection is conferred by innate immunity and maternal antibodies, which may prevent or control pathogen translocation or intestinal spread [25].

Another important factor is the establishment of the commensal microbiota that becomes similar to that of the adult chickens around the second-week posthatch. Commensal intestinal bacteria protect the host from pathogen colonization by competing with the pathogens for epithelial binding sites and available nutrients and by producing bacteriocins, strengthening the intestinal immune response [26]. Commensal intestinal bacteria can also inhibit pathogenic bacteria from adhering to the intestinal mucosa by increasing intestinal mucin production [4]. In addition, Faure et al. [27] showed that some specific amino acids, including Thr, can modulate the intestinal microbiota, promoting the multiplication of commensal microorganisms and that the mechanisms involved in this modulation are likely related to mucin synthesis. Mucin production is closely related to dietary Thr levels, and high dietary Thr levels help protect the intestinal epithelium [5]. Furthermore, mucins are rich in Thr and limited dietary Thr may result in decreased mucin biosynthesis, affecting the mucosal protective layer [4]. Similar to previous results reported with dietary supplementation with Thr, supplementation in ovo in the present study resulted in greater MUC2 expression, which may have contributed to the establishing commensal microbiota and increased protection against Salmonella infection. Higher MUC2 expression after in ovo Thr supplementation was previously reported in chicks [6] as well as in quails [7].

Infectious processes on the intestinal mucosal surface may result in rapidly releasing of stored mucin granules,
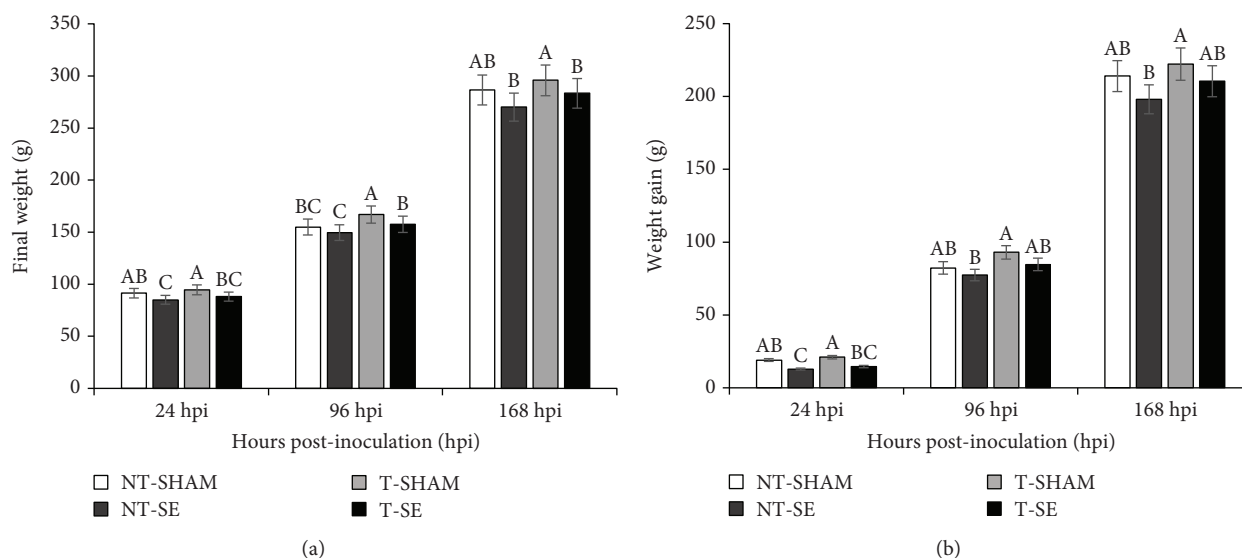
(a)

(b)

FIGURE 6: Final weight (3, 6, and 9 d) and weight gain in broilers supplemented *in ovo* with Thr and challenged with *Salmonella* Enteritidis posthatch at 24, 96, and 168-hour postinoculation (hpi). NT-SHAM: no *in ovo* Thr supplementation and sham-inoculated in the posthatch challenge; T-SHAM: *in ovo* Thr supplementation and sham-inoculated; NT-SE: no *in ovo* Thr supplementation and SE Nal[R]-challenged; and T-SE: *in ovo* Thr supplementation and SE Nal[R]-challenged. The error bars represent the standard deviation.

thus strengthening the protection provided by the intestinal barrier [28]. Mucin is the main component of the protective mucosal layer and acts as a protective layer of the intestinal epithelium, preventing damage and infection by pathogenic bacteria, and as a fixation substrate for commensal bacteria [29]. In addition, the mucosal layer supplies nutrients for establishing commensal microbiota, strengthening intestinal protection through competitive exclusion [30]. The mucin layer supplies an adequate environment for brush border enzymes to function, thus aiding nutrient absorption [25].

This rationale supports our findings, where *in ovo* Thr supplementation resulted in increased mucin (*MUC2*) and immunoglobulin A (*IgA*) expression, which may have conferred higher protection to animals from the T-SE group. Kadam et al. [11] observed that *in ovo* Thr supplementation improved the humoral immune response, indicating that Thr supplementation resulted in increased immunoglobulin synthesis.

In the present study, broiler chicks from the T-SE group showed similar performance compared to animals from T-SHAM and NT-SHAM. These results might be associated with preserved intestinal mucosal integrity since the T-SE animals presented similar morphometry to unchallenged animals (T-SHAM and NT-SHAM), while chicks from nonsupplemented groups presented compromised mucosal integrity at all times evaluated (24, 96, and 168 hpi). Improved intestinal integrity was also observed in *Salmonella*-challenged chicks fed Thr in the diet [24]. These results indicate that *in ovo* Thr supplementation reduced the burden of *Salmonella* toxins in the gut of broiler chicks. Those toxins have been reported to affect mucin and antimicrobial molecule production and cause cell lysis, apoptosis, rupture of cell junctions, and triggering of inflammatory signaling [4].

5. Conclusions

In ovo Thr supplementation ameliorated the detrimental effects of *Salmonella* Enteritidis infection, decreased bacterial counts in the cecal content (168 hpi or 9 days of age), and preserved intestinal mucosal integrity, resulting in higher final weight and weight gain. The processes through which *in ovo* Thr supplementation improves broiler response to being challenged with *Salmonella* likely involve increased intestinal mucin synthesis and mucosal antibodies (IgA) and faster maturation of the gastrointestinal tract during embryogenesis.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

Conflicts of Interest

The authors declare that there is no conflict of interests.

Authors' Contributions

Alexandre L. de B. Moreira Filho, Patrícia E. N. Givisiez, Celso J. B. de Oliveira, and Bryan White conceived and designed the experiments. Alexandre L. de B. Moreira Filho, Candice M. C. G. de Leon, Mauro de M. S. Saraiva, and Maria de F. S. Andrade performed the experiments and lab analyses. Alexandre L. de B. Moreira Filho, Patrícia E. N. Givisiez, Celso J. B. de Oliveira, Candice M. C. G. de Leon, and Oliveiro C. de Freitas Neto analyzed the data. Patrícia E. N. Givisiez, Celso J. B. de Oliveira, Oliveiro C. de Freitas Neto, and Bryan White provided reagents/materials/analysis tools. Alexandre L. de B. Moreira Filho, Patrícia E. N. Givisiez, Celso J. B. de Oliveira, and Oliveiro C. de Freitas Neto wrote the paper.

Acknowledgments

Supplementary Materials

Supplementary Table 1: villus height (VH), crypt depth (CD), villus : crypt ratio (V : C), and villus area (area) of broilers at hatch and at two days of age following *in ovo* feeding with threonine. Supplementary Table 2: villus height (VH), crypt depth (CD), villus : crypt ratio (V : C), and villus area (area) of broilers supplemented with threonine *in ovo*, 24-hour postinoculation with *Salmonella* Enteritidis. Supplementary Table 3: villus height (VH), crypt depth (CD), villus : crypt ratio (V : C), and villus area (area) of broilers supplemented with threonine *in ovo*, 96-hour postinoculation with *Salmonella* Enteritidis. Supplementary Table 4: villus height (VH), crypt depth (CD), villus : crypt ratio (V : C), and villus area (area) of broilers supplemented with threonine *in ovo*, 168-hour postinoculation with *Salmonella* Enteritidis. *(Supplementary Materials)*

References

[1] A. A. Pedroso, A. B. Batal, and M. D. Lee, "Effect of in ovo administration of an adult-derived microbiota on establishment of the intestinal microbiome in chickens," *American Journal of Veterinary Research*, vol. 77, no. 5, pp. 514–526, 2016.

[2] H. Juricova, P. Videnska, M. Lukac et al., "Influence of *Salmonella enterica* serovar enteritidis infection on the development of the cecum microbiota in newly hatched chicks," *Applied and Environmental Microbiology*, vol. 79, no. 2, pp. 745–747, 2013.

[3] A. L. Smith, C. Powers, and L. R. Beal, "The avian enteric immune system in health and disease," in *Avian Immunology*,
K. A. Schat, B. Kaspers, and P. Kaise, Eds., pp. 227–250, Academic Press, London, 2014.

[4] M. A. McGukin, S. K. Lindén, P. Sutton, and T. H. Florin, "Mucin dynamics and enteric pathogens," *Nature Review Microbiology*, vol. 9, no. 4, pp. 265–278, 2011.

[5] E. G. Santos, F. G. P. Costa, J. H. V. Silva et al., "Protective effect of mannan oligosaccharides against early colonization by *Salmonella* Enteritidis in chicks is improved by higher dietary threonine levels," *Journal of Applied Microbiology*, vol. 114, no. 4, pp. 1158–1165, 2013.

[6] S. K. Bhanja, M. Sudhagar, A. Goel et al., "Differential expression of growth and immunity related genes influenced by in ovo supplementation of amino acids in broiler chickens," *Czech Journal of Animal Science*, vol. 59, no. 9, pp. 399–408, 2014.

[7] H. Kermanshahi, A. Daneshmand, N. K. Emami et al., "Effect of *in ovo* injection of threonine on Mucin2 gene expression and digestive enzyme activity in Japanese quail (*Coturnix japonica*)," *Research in Veterinary Science*, vol. 100, pp. 257–262, 2015.

[8] Z. Uni and R. P. Ferket, "Methods for early nutrition and their potential," *World's Poultry Science Journal*, vol. 60, no. 1, pp. 101–111, 2004.

[9] E. Tako, P. R. Ferket, and Z. Uni, "Changes in chicken intestinal zinc exporter mRNA expression and small intestinal functionality following intra-amniotic zinc-methionine administration," *The Journal of Nutritional Biochemistry*, vol. 16, no. 6, pp. 339–346, 2005.

[10] O. T. Foye, C. Ashwell, Z. Uni, and P. R. Ferket, "The effects of intra-amnionic feeding of arginine and/or ß-hyroxy-ß-methyl-butyrate on jejunal gene expression in the turkey embryo and hatchling," *International Journal of Poultry Science*, vol. 8, no. 5, pp. 437–445, 2009.

[11] M. M. Kadam, S. K. Bhanja, A. B. Mandal et al., "Effect of in ovo threonine supplementation on early growth, immunological responses and digestive enzyme activities in broiler chickens," *British Poultry Science*, vol. 49, no. 6, pp. 736–741, 2008.

[12] Z. Uni and P. R. Ferket, "Enhancement of development of oviparous species by in ovo feeding," US. Patent US6592878B2, 2003.

[13] H. S. Rostagno, L. F. T. Albino, J. L. Donzele et al., *Tabelas Brasileiras para Aves e Suínos: Composição de Alimentos E Exigências Nutricionais*, UFV, Viçosa, 3rd edition, 2011.

[14] M. D. Abramoff, P. Magalhaes, and S. J. Ram, "Image processing with ImageJ," *Biophotonics International*, vol. 11, pp. 36–42, 2004.

[15] K. Sakamoto, H. Hirose, A. Onizuka et al., "Quantitative study of changes in intestinal morphology and mucus gel on total parenteral nutrition in rats," *The Journal of Surgical Research*, vol. 94, no. 2, pp. 99–106, 2000.

[16] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT-PCR," *Nucleic Acids Research*, vol. 29, no. 9, pp. 45e–445, 2001.

[17] SAS Institute Inc, *SAS/STAT® 9.2 User's Guide*, SAS Inst. Inc., Cary, NC, USA, 2008.

[18] M. Salmanzadeh, Y. Ebrahimnezhad, H. A. Shahryar, and A. Lotfi, "The effects of in ovo injection of L-threonine in broiler breeder eggs on characters of hatching and growth performance broiler chickens," *European Journal of Experimental Biology*, vol. 1, pp. 147–151, 2011.

[19] S. Tahmasebi and M. Toghyani, "Effect of arginine and threonine administered *in ovo* on digestive organ developments and subsequent growth performance of broiler chickens," *Journal of Animal Physiology and Animal Nutrition*, vol. 100, no. 5, pp. 947–956, 2016.

[20] K. M. Gaafar, S. A. Selim, and S. S. El-Ballal, "Effect of in-ovo administration with two levels of amino acids mixture on the performance of Muscovy ducks," *Emirates Journal of Food and Agriculture*, vol. 25, no. 1, pp. 58–65, 2013.

[21] T. M. Shafey, A. H. Mahmoud, A. A. Alsobayel, and M. A. Abouheif, "Effects of *in ovo* administration of amino acids on hatchability and performance of meat chickens," *South African Journal of Animal Science*, vol. 44, no. 2, pp. 123–130, 2014.

[22] E. Tako, P. R. Ferket, and Z. Uni, "Effects of in ovo feeding of carbohydrates and betahydroxy-beta-methylbutyrate on the development of chicken intestine," *Poultry Science*, vol. 83, no. 12, pp. 2023–2028, 2004.

[23] A. Smirnov, E. Tako, P. R. Ferket, and Z. Uni, "Mucin gene expression and mucin content in the chicken intestinal goblet cells are affected by in ovo feeding of carbohydrates," *Poultry Science*, vol. 85, no. 4, pp. 669–673, 2006.

[24] A. L. de Barros Moreira Filho, C. J. B. de Oliveira, H. B. de Oliveira et al., "High incubation temperature and threonine dietary level improve ileum response against post-hatch *Salmonella* Enteritidis inoculation in broiler chicks," *PloSOne*, vol. 10, no. 7, article e0131474, 2015.

[25] E. Bar-Shira, I. Cohen, O. Elad, and A. Friedman, "Role of goblet cells and mucin layer in protecting maternal IgA in precocious birds," *Developmental and Comparative Immunology*, vol. 44, no. 1, pp. 186–194, 2014.

[26] I. Gabriel, M. Lessire, S. Mallet, and J. F. Guillot, "Microflora of the digestive tract: critical factors and consequences for poultry," *World's Poultry Science Journal*, vol. 62, no. 3, pp. 499–511, 2006.

[27] M. Faure, C. Mettraux, D. Moennoz et al., "Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium-treated rats," *The Journal of Nutrition*, vol. 136, no. 6, pp. 1558–1564, 2006.

[28] C. W. Davis and B. F. Dickey, "Regulated airway goblet cell mucin secretion," *Annual Review of Physiology*, vol. 70, no. 1, pp. 487–512, 2008.

[29] S. K. Linden, P. Sutton, N. G. Karlsson, V. Korolik, and M. A. McGuckin, "Mucins in the mucosal barrier to infection," *Mucosal Immunology*, vol. 1, no. 3, pp. 183–197, 2008.

[30] D. Pan and Z. Yu, "Intestinal microbiome of poultry and its interaction with host and diet," *Gut Microbes*, vol. 5, no. 1, pp. 108–119, 2014.