Article

Improvement in the Immunity- and Vitamin D₃-Activity-Related Gene Expression of Coccidiosis-Challenged Ross 708 Broilers in Response to the In Ovo Injection of 25-Hydroxyvitamin D₃ †

Seyed Abolghasem Fatemi 1,*, Kenneth S. Macklin 2, Li Zhang 1, Ayoub Mousstaaid 1, Sabin Poudel 1, Ishab Poudel 1 and Edgar David Peebles 1

1 Department of Poultry Science, Mississippi State University, Mississippi State, MS 39762, USA
2 Department of Poultry Science, College of Agriculture, Auburn University, Auburn, AL 36849, USA
* Correspondence: sf1006@msstate.edu
† This publication is a contribution of the Mississippi Agriculture and Forestry Experiment Station. This publication does not imply endorsement by Mississippi Agricultural and Forestry Experiment Station of these products, nor similar ones not mentioned.

Simple Summary: Coccidiosis is still considered one of the main diseases affecting the performance and health of poultry reared under intensive production systems. Vitamin D₃ sources have been shown to reduce the negative effects of a coccidiosis infection with a subsequent improvement in live performance and intestinal immunity. Therefore, the aim of the current research was to explore molecular mechanisms that may play role in an improvement in immunity and vitamin D activity in response to the in ovo injection of vitamin D₃ (D₃) and 25-hydroxyvitamin D₃ (25OHD₃) alone or in combination in broilers subjected to a coccidiosis infection. In this study, it was shown that the expression of genes linked to an anti-inflammatory response increased and a pro-inflammatory response decreased in the jejunum of 28-day-old broilers (2 weeks after coccidiosis infection) after an in ovo injection of 2.4 µg of 25OHD₃. Additionally, the in ovo administration of 2.4 µg of 25OHD₃ increased the expression of genes linked to D₃ function. In conclusion, the in ovo administration of 2.4 µg of 25OHD₃ at 18 days of incubation can improve the immunity as well as the D₃ activity of broilers challenged with coccidiosis.

Abstract: Effects of the in ovo administration of two vitamin D₃ sources (vitamin D₃ (D₃) and 25-hydroxyvitamin D₃ (25OHD₃)) on the expression of D₃ activity- and immunity-related genes in broilers subjected to a coccidiosis infection were investigated. At 18 d of incubation (doi), five in ovo injection treatments were administered to live embryonated Ross 708 broiler hatching eggs: non-injected (1) and diluent-injected (2) controls, or diluent injection containing 2.4 µg of D₃ (3) or 2.4 µg of 25OHD₃ (4), or their combination (5). Birds in the in ovo-injected treatments were challenged at 14 d of age (doa) with a 20 × dosage of a live coccidial vaccine. At 14 and 28 doa, the expression of eight immunity-related genes (IL-2, IL-6, IL-10, TLR-4, TLR-15, MyD88, TGF-β4, and IFN-γ) and four D₃ activity-related genes (1α-hydroxylase, 25-hydroxylase, 24-hydroxylase, and VDR) in the jejunum of one bird in each treatment–replicate group were evaluated. No significant treatment effects were observed for any of the genes before challenge. However, at 2 weeks post-challenge, the expression of 1α-hydroxylase, TGF-β4, and IL-10 increased in birds that received 25OHD₃ alone in comparison to all the other in ovo-injected treatment groups. Additionally, the expression of 24-hydroxylase and IL-6 decreased in birds that received 25OHD₃ in comparison to those injected with diluent or D₃ alone. It was concluded that the in ovo injection of 2.4 µg of 25OHD₃ may improve the intestinal immunity as well as the activity of D₃ in Ross 708 broilers subjected to a coccidiosis challenge.

Keywords: 25-hydroxyvitamin D₃; broilers; D₃ activity-related genes; immunity-related genes; in ovo injection
1. Introduction

Cholecalciferol or vitamin D₃ (D₃) is a common supplemental source of vitamin D in poultry diets. Vitamin D₃ is hydroxylated twice to become the functionally active form of vitamin D. Vitamin D₃ is converted to 25-hydroxycholecalciferol (25OHD₃) by the action of 25-hydroxylase in the liver and is then hydroxylated by renal cells to 1,25-dihydroxycholecalciferol [1,25-(OH)₂D₃] by 1 α-hydroxylase [1,2]. In the chicken, the conversion of 25OHD₃ to active form (1,25-(OH)₂D₃) is tightly regulated by several factors including vitamin D receptor (VDR) activity, 1,25-(OH)₂D₃ concentration, and calcium serum levels [3,4]. In comparison to D₃ at the same level of inclusion, dietary supplementation of 25OHD₃ more strongly enhances broiler breast meat yield [5,6], protein synthesis rate [6,7], and the density of satellite cells in breast muscle [7]. Additionally, 25OHD₃ has been shown to more effectively reduce the inflammatory response and increase the humoral immunity of broiler chickens that were challenged with pathogens [8,9]. The expression of 1 α-hydroxylase in the livers of lipopolysaccharide-injected birds increased when they were fed 25OHD₃, but not when they were fed D₃ [10]. This may result in increased 1,25-(OH)₂D₃ levels in the liver and the suppression of the inflammatory response in 25OHD₃-fed birds [11]. The expression of 1 α-hydroxylase and VDR occurs in the macrophages and in the livers of chickens [12,13]. Therefore, increased biosynthesis of 1,25-(OH)₂D₃ from 25OHD₃ can increase the expression of 1 α-hydroxylase and subsequently increase the expression of VDR [13].

In ovo injection techniques in broilers have emerged to allow for the direct administration of particular nutrients or vaccines to embryos, which provides an early stimulation of their immune responses [14]. Additionally, in ovo injection provides a cost-effective and uniform delivery of vaccines with limited contamination issues [15]. Improvements in chicken embryonic and post-hatch development and immune status in response to the in ovo injection of various injected materials, including vitamins, have been observed in previous studies. These observed improvements have been particularly evident in poultry reared under intensive production conditions [16,17]. It is well documented that the in ovo injection of 0.6 to 2.4 µg of 25OHD₃ can increase broiler breast meat yield [18–20] and live performance [18–20]. It can also improve the characteristics of hatching [21], bone quality [22,23], immunity [18,24,25], and small intestine morphology [25] of broilers not challenged with pathogens. Furthermore, the in ovo injection of 2.4 µg of 25OHD₃ has been shown to increase BWG and breast meat yield [26], and to improve the small intestine morphology and immunity [27] of broilers subjected to a coccidiosis infection. However, the molecular mechanisms by which the in ovo injection of vitamin D₃ sources affect chicken intestinal histomorphology, muscle formation, or immunity have not been previously investigated. Therefore, the objective of this study was to investigate the impacts of the in ovo injection of D₃ and 25OHD₃, alone or in combination on the expression of genes linked to immunity and D₃ activity in Ross 708 broilers subjected to a coccidiosis infection.

2. Materials and Methods

2.1. Experimental Design, Egg Incubation, and Coccidial Infection

Fifty Ross 708 broiler hatching eggs were randomly set in each of 5 treatment groups in each of 6 replicate trays (1500 total eggs) in a Chick Master Incubator (Chick Master Incubator Company, Medina, OH, USA). The conditions and arrangement of the treatment-replicate groups of eggs in the incubator were as described by Fatemi et al. [26]. In a previous companion study [26] in which the same incubation regimens were used, no significant treatment effects were found for percentage egg weight loss from 0 to 18 day (d) of incubation (doi), indicating uniform incubational conditions for all prespecified in ovo injection treatment groups. At 18 doi, a Zoetis Inovoject m (Zoetis Animal Health, Research Triangle Park, NC, USA) multi-egg in ovo injection machine was used to deliver a 50 µL solution volume into each egg. The in ovo injection treatments were: non-injected (1) and diluent injected (2) controls, or diluent injection containing 2.4 µg D₃ (3) or 2.4 µg 25OHD₃ (4), or their combination (5). All in ovo injection solutions were freshly prepared in
accordance to the procedure described by Fatemi et al. [21,22]. At hatch, chicks belonging to the same treatment group across replicate hatching baskets were pooled prior to placement. A total of 20 birds from each pooled treatment group were randomly placed in each of 2 separate isolation rooms containing wire-floored battery cages (0.76 m × 0.46 m (0.35 m²)). Four birds were placed in each of 8 replicate cages in each of 5 in ovo injection treatment groups (4 birds × 8 replicates × 5 treatments = 160 total birds in each room). The replicate cages for each of the in ovo injection treatment groups were randomly arranged within each isolation room and room was considered as a blocking factor. A Mississippi State University basal corn-soybean diet as described by Fatemi et al. [18,19,26] was used throughout the 41 d of age (doa) study period (Fatemi et al., 2021 [18,19,26]. A coccidial challenge infection was performed at 14 doa according to the method described by Fatemi et al. [26] and Poudel et al. [28,29]. Chicks were left unchallenged in the non-injected treatment group.

2.2. Tissue Collection, Total RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

At 14 and 18 d of age (doa), one bird in each room from each treatment-replicate cage was randomly selected for sampling. Approximately 10 to 20 g of the medial side of each jejunum sample was immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated using the TRIZol® procedure (Invitrogen, Carlsbad, CA, USA) using 1 mL TRIZol® to every 30 mg of tissue according to the manufacturer’s recommendations. RNA quantification was performed using A NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA) and agarose electrophoresis. RNA samples were stored at −80 °C until cDNA synthesis. cDNA synthesis was performed using 200 ng of each total RNA sample according to High Capacity cDNA Reverse Transcription Kit protocol (Applied Biosystems, Foster City, CA, USA) [30]. The resulting cDNA was stored at −20 °C. RT-qPCR reactions were conducted in a 20 µL reaction system containing 10 µL of SYBR Green premix, 1.2 µL of forward primer (10 µM), 1.2 µL of reverse primer (10 µM), 2.0 µL of 5× diluted cDNA, and 5.6 µL of RNase-free water. The program was set at 95 °C for 60 s, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Melting curve analysis was performed to analyze the specificity of the primers according to protocol described by Poudel et al. [31]. In total, two endogenous or “housekeeping” genes, plus 20 target genes associated with immune response and D₃-related activity were identified for RT-qPCR (Table 1). The forward and reverse primers of house-keeping genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] and 18S ribosomal RNA [18S]); genes related to immunity including interleukin (IL)-2, IL-6, IL-10, interferon gamma (IFN-γ), myeloid differentiation factor 88 (MyD88), transforming growth factor beta 4 (TGF-β4), Toll-like receptor (TLR)-4, and TLR-15; and genes linked to vitamin D activity including 1α-hydroxylase, 24-hydroxylase, 25-hydroxylase, and VDR were designed according to the procedure described by Fatemi [31] and Poudel et al. [32]. NormFinder software version 20 was used to normalize the target gene data [33], and the normalization suitability of the expression of GAPDH and 18S genes were tested. The GAPDH was an endogenous gene that was most suitable for normalization of the data for the target genes. The fold-change differences were calculated according to the method described by Livak and Schmittgen [34].
Table 1. Real-time PCR primers and GenBank accession numbers of chicken housekeeping genes, and target genes associated with immunity and vitamin D-related activity.

| Gene Symbol | Accession No | Type                  | Orientation | Sequence (5’ to 3’)                      | Length (nt) | Amplicon Size (bp) | Reference                  |
|-------------|--------------|-----------------------|-------------|------------------------------------------|-------------|--------------------|---------------------------|
| RNA 18S     | M59389.1     | Housekeeping          | Forward     | GCCAACAGAGAGAAGATGACAC                   | 22          | 140                |                           |
|             |              |                       | Reverse     | GTAACACCATCACCAGAGTCTGGA                | 22          | 72                 |                           |
| GAPDH       | NM204305     | Housekeeping          | Forward     | GCCGTCCTCTCTGGCAAAG                     | 19          |                    |                           |
|             |              |                       | Reverse     | AGTTTACAGGCTCTAAAAATCCAC                 | 24          | 102                |                           |
| IL-2        | AY386204     | Immunity-related      | Forward     | CACAAAGTTGCTGTCATG                      | 22          |                    |                           |
| IL-6        | AB559572     | Immunity-related      | Forward     | GCCGAAGAAGCATGGAGATA                    | 20          | 143                | Al-Zghoul et al. [35]     |
|             |              |                       | Reverse     | AGTGTCTGAAAGGCGGAAAC                    | 20          | 103                |                           |
| IL-10       | AJ621614     | Immunity-related      | Forward     | ATCGAGCAGTCCTCTCGAT                     | 20          |                    |                           |
|             |              |                       | Reverse     | GTGAAGAGGTTGAAAATACATATG                 | 19          | 71                 |                           |
| IFN-γ       | AJ001678     | Immunity-related      | Forward     | GGGTCTTACAGCTGAGC                       | 20          | 119                | Brisbin et al. [36]       |
| TGF-β4      | M31160.1     | Immunity-related      | Forward     | GGTGAAATGCTCCTGTGTGTAG                  | 20          |                    |                           |
| TLR-4       | AY064697     | Immunity-related      | Forward     | AGTCTGAAATTTGCTGACTAAAT                 | 24          | 190                |                           |
| TLR-15      | NM_001037835 | Immunity-related      | Reverse     | GGTGTTGTATGTGAAAGT                     | 20          | 113                |                           |
| MyD88       | NM_001030962 | Immunity-related      | Reverse     | ATCGTGCCTGCTGTAGA                       | 18          |                    |                           |
| 1α-hydroxylase | XM_422077 | Vitamin D activity-related | Forward     | TCGTGGCAGAAATACAGAGA                    | 20          | 125                |                           |
|             |              |                       | Reverse     | ACTGCCACATCTTTGGTT                      | 20          |                    |                           |
| 25-hydroxylase | NM_001277354 | Vitamin D activity-related | Forward     | GCTGTCATCTGAGATTTTGTC                   | 21          | 160                | Shanmugasundaram and selvaraj [12] |
|             |              |                       | Reverse     | CCAACGGAAGGACACAGGT                     | 20          | 133                | Shanmugasundaram and selvaraj [12] |
| 24-hydroxylase | AF019142.1 | Vitamin D activity-related | Forward     | ATGCGATGAAAGGAGGATTAGTC                 | 20          | 157                |                           |
|             |              |                       | Reverse     | GAGTCCAGGGTTGGAAC                      | 20          |                    |                           |
| VDR         | AF011356.1   | Vitamin D activity-related | Forward     | CAGTGGCAAGGAGGATTAGTC                  | 20          |                    |                           |
|             |              |                       | Reverse     | GAGTCCAGGGTTGGAAC                      | 20          |                    |                           |
2.3. Statistical Analysis

Each wire-floored battery cage served as an experimental unit, with all 5 treatments randomly represented in each of 8 replicate cages. A completely randomized experimental design was employed within each room and room served as a blocking factor. A non-injected treatment group was kept in a separate part of the battery cages to eliminate its exposure to coccidial oocysts. Furthermore, a treatment group of birds that have received an in ovo injection solution as well as a coccidial challenge were included. However, in the analysis of effects of coccidial challenge on 28 doa, the non-injected treatment was not included. All house-keeping and target genes were tested for normality using PROC UNIVARIATE and were determined as being normally distributed. Prior to (14 doa) and 2 wk after (28 doa) coccidiosis infection, all gene expression data between and across in ovo injection treatment were analyzed by one-way ANOVA using the procedure for linear mixed models (PROC GLIMMIX) of SAS©, version 9.4 (SAS Institute Inc., Cary, NC, USA). Differences were deemed significant at \( p \leq 0.05 \), and Tukey’s least square means comparison was used for means separation [37].

3. Results

No significant treatment differences were observed for the immunity- and vitamin D3 activity-related genes at 14 doa (Tables 2 and 3). However, there were significant treatment effects for both genes involved in the immunity and vitamin D3 functions of the broilers at 28 doa (Tables 2 and 3). The in ovo injection of D3 significantly increased the expression of IL-6 at 28 doa in comparison to the diluent and 25OHD3 injection treatment group. Conversely, the expression of IL-10 was increased in the 25OHD3 alone injection treatment group in comparison to that of all other injection treatment groups, and the expression of IL-10 was higher in the D3 + 25OHD3 treatment as compared to the diluent-injected and D3 alone treatment groups. The expression of TGF-β4 was also higher in the 25OHD3 alone injection treatment when compared to all other injection treatment groups (Table 2). Moreover, the in ovo injection of 25OHD3 resulted in a higher expression of 1α-hydroxylase as compared to all other in ovo treatments and resulted in a numerically \( (p = 0.072) \) lower expression of 24-hydroxylase as compared to the D3 + 25OHD3 and D3 alone treatment groups at 2 wk post infection (Table 3). Across in ovo treatment (Table 4), the expressions of IL-2, IL-6, IL-10, TGF-β4, TLR-4, 1α-hydroxylase, and VDR were significantly higher at 2 wk post-infection (28 doa) in comparison to their expression levels before coccidiosis infection (14 doa).

Table 2. Effects of in ovo injection treatment (non-injected, diluent-injected (50 µL), and 50 µL of diluent containing 2.4 µg of vitamin D3 (D3), 2.4 µg of 25-hydroxycholecalciferol (25OHD3), or 2.4 µg of D3 and 2.4 µg of 25OHD3 (D3 + 25OHD3)) on the fold change expression of immune-related genes at 14 and 28 d of age (doa).

| Treatment | n | IL-2 | IL-6 | IL10 | IFN-γ | TGF-β4 | TLR4 | TLR-15 | MyD88 |
|-----------|---|------|------|------|-------|--------|------|--------|-------|
| 14 doa    |   |      |      |      |       |        |      |        |       |
| Non-injected 1 | 8 | 0.90 | 1.53 | 1.14 | 0.87  | 0.91   | 0.93 | 1.53   | 1.10  |
| Diluent 2 | 8 | 1.04 | 1.07 | 1.04 | 1.06  | 1.06   | 1.06 | 1.01   | 1.12  |
| D3 3      | 8 | 1.12 | 1.69 | 1.27 | 1.49  | 1.03   | 1.20 | 1.38   | 1.31  |
| 25OHD3 4 | 8 | 1.05 | 1.06 | 1.33 | 0.91  | 2.16   | 0.92 | 0.95   | 1.17  |
| D3 + 25OHD3 5 | 8 | 1.00 | 1.03 | 1.11 | 0.80  | 1.14   | 1.11 | 1.01   | 1.11  |
| SEM       |   | 0.195| 0.435| 0.309| 0.196 | 0.522  | 0.178| 0.277  | 0.153 |
| p-value   |   | 0.758| 0.489| 0.869| 0.103 | 0.274  | 0.522| 0.556  | 0.763 |
| 28 doa    |   |      |      |      |       |        |      |        |       |
| Diluent 6 | 8 | 1.05 | 1.06 | 1.18 | 1.10  | 1.03   | 1.17 | 1.17   | 1.06  |
| D3 7      | 8 | 1.25 | 4.41 | 1.70 | 0.89  | 1.53   | 1.34 | 0.96   | 1.31  |
| 25OHD3 8 | 8 | 1.41 | 0.93 | 9.00 | 0.46  | 4.70   | 1.56 | 0.97   | 1.34  |
Table 2. Cont.

| Treatment          | n  | IL-2 | IL-6 | IL10 | IFN-γ | TGF-β4 | TLR4 | TLR-15 | MyD88 |
|--------------------|----|------|------|------|-------|--------|------|--------|-------|
| D₃ + 25OHD₃        | 8  | 1.50 | 2.52 | 3.40 | 1.03  | 2.29   | 1.47 | 1.50   | 1.40  |
| SEM                |    | 0.471| 0.832| 0.553| 0.223 | 0.505  | 0.279| 0.223  | 0.218 |
| p-value            |    | 0.012| 0.001| 0.198| <0.001| 0.777  | 0.294| 0.660  |       |

* Treatment means within the same column with no common superscripts are significantly different (*p ≤ 0.05).*

1. Eggs that were not injected with any solution and also were not challenged with coccidiosis vaccine at 14 doa.
2. Eggs injected with 50 µL of commercial diluent at d 18 of incubation and that were not challenged with coccidiosis vaccine at 14 doa.
3. Eggs injected with 50 µL of commercial diluent containing vitamin 2.4 µg of D₃ at d 18 of incubation and that were also challenged with coccidiosis vaccine at 14 doa.
4. Eggs injected with 50 µL of commercial diluent containing 2.4 µg of 25OHD₃ at d 18 of incubation and that were also challenged with coccidiosis vaccine at 14 doa.
5. Eggs injected with 50 µL of commercial diluent containing 2.4 µg of D₃ and 2.4 µg of 25OHD₃ at d 18 of incubation and also were challenged with coccidiosis vaccine at 14 doa.

Table 3. Effects of in ovo injection treatment (non-injected, diluent-injected (50 µL), and 50 µL of diluent containing 2.4 µg of vitamin D₃ (D₃), 2.4 µg of 25-hydroxycholecalciferol (25OHD₃), or 2.4 µg of D₃ and 2.4 µg of 25OHD₃ (D₃ + 25OHD₃) on the fold change expression of vitamin D activity-related genes at 14 and 28 d of age (doa).

| Treatment          | n  | 1α-hydroxylase | 25-hydroxylase | 24-hydroxylase | VDR   |
|--------------------|----|----------------|----------------|----------------|-------|
| 14 doa             |    |                |                |                |       |
| Non-injected       | 8  | 1.01           | 0.89           | 2.02           | 0.91  |
| Diluent            | 8  | 1.02           | 1.02           | 2.02           | 1.14  |
| D₃                 | 8  | 1.34           | 1.17           | 2.64           | 1.32  |
| 25OHD₃             | 8  | 1.34           | 1.07           | 1.49           | 1.19  |
| D₃ + 25OHD₃        | 8  | 1.16           | 1.03           | 2.94           | 2.77  |
| SEM                | 0.246| 0.580| 0.606| 0.195|       |
| p-value            | 0.549| 0.299| 0.381|       |       |
| 28 doa             |    |                |                |                |       |
| Diluent            | 8  | 1.04           | 1.02           | 2.03           | 1.25  |
| D₃                 | 8  | 1.16           | 1.13           | 2.02           | 2.82  |
| 25OHD₃             | 8  | 3.43           | 1.25           | 0.47           | 1.56  |
| D₃ + 25OHD₃        | 8  | 1.80           | 1.91           | 2.08           | 2.08  |
| SEM                | 0.583| 0.329| 0.437| 0.274|       |
| p-value            | 0.001| 0.423| 0.972| 0.312|       |

* Treatment means within the same column with no common superscripts are significantly different (*p ≤ 0.05).*

1. Vitamin D receptor.

Table 4. Effects of coccidiosis infection on the fold change expression of genes linked to immunity and vitamin D activity before and after coccidiosis infection.

| Genes            | n  | 14 doa | 28 doa | SEM  | p-Value |
|------------------|----|--------|--------|------|---------|
| IL-2             | 16 | 1.017  | 1.30   | 0.130| 0.031   |
| IL-6             | 16 | 1.34   | 2.48   | 0.508| 0.028   |
| IL10             | 16 | 1.21   | 3.82   | 0.381| 0.002   |
| IFN-γ            | 16 | 1.08   | 0.88   | 0.139| 0.135   |
| TGF-β4           | 16 | 1.29   | 2.39   | 0.410| 0.010   |
| TLR4             | 16 | 1.02   | 1.39   | 0.156| 0.021   |
| TLR-15           | 16 | 1.25   | 1.12   | 0.185| 0.509   |
| MyD88            | 16 | 1.15   | 1.27   | 0.124| 0.380   |
| 1α-hydroxylase   | 16 | 1.18   | 1.86   | 0.260| 0.011   |
| 25-hydroxylase   | 16 | 1.04   | 1.33   | 0.177| 0.110   |
When compared to the diluent and D3 alone treatment. The conversion of 25OHD3 to the active form of vitamin D (1,25-(OH)2D3) is facilitated by 1α-hydroxylase in the kidney [12], intestine [12], breast and thigh muscles [12], and immune cells including macrophages and T-cells [10]. Moreover, by the action of 24-hydroxylase, 25OHD3 can also be converted to the inactive form of vitamin D (24,25-(OH)2D3) in many cells in which there are vitamin D receptors. Therefore, an increase in the expression of 1α-hydroxylase or a decrease in the expression of 24-hydroxylase may directly affect vitamin D3 activity. In previous companion studies, these current treatments effectively down-regulated the inflammatory response in the 25OHD3 cell proliferation and cytokine production [46]. Thus, these results indicate that dietary 25OHD3 treatment group could be due to the up-regulation of genes linked to vitamin D activity. However, in ovo administration of 2.4 µg of 25OHD3 alone increased the expression of anti-inflammatory response genes (IL-10 and TGF-β), and deceased the expression of a pro-inflammatory response gene (IL-6) during the coccidiosis infection. In comparison to D3 at the same level of supplemental dietary inclusion, it has likewise been shown that the expression of IL-10 increased and IL-1β decreased in response to 2760 IU/kg of 25OHD3 in broilers challenged with coccidiosis [8]. Additionally, dietary 25OHD3 at 100 µg/kg increased CD4+CD25+ cells and deceased CD8+CD25+ cells in coccidiosis-infected broilers [10] and turkeys [39]. It is well documented that the CD4+CD25+ cells [37,41], IL-10 [43,44] and TGF-β [45] are associated with regulatory T cell (Tregs) formation that is facilitated by an up-regulation of the expression of the aforementioned genes. The Tregs are immunity suppression cells that act to inhibit T cell proliferation and cytokine production [46]. Thus, these results indicate that dietary 25OHD3 induces an adaptive immune response, and more specifically the activation of an anti-inflammatory response during systemic and chronic infections including coccidiosis. The down-regulation of an inflammatory response in the 25OHD3 alone in ovo-injected treatment group could be due to the up-regulation of genes linked to vitamin D3 activity. When compared to the diluent and D3 alone injected treatment groups in their study, the objective of this study was to determine the effects of in ovo administration of D3 and 25OHD3 on the gene expression of broilers subjected to a coccidiosis infection. It is well documented that a coccidial infection generates a chronic intestinal infection as a result of a robust up-regulation of pro-inflammatory cytokines linked to the innate [30,38] or adaptive [8,10,39] immune systems. Among genes that have been reported to be expressed in association with innate immunity responses in chickens infected by coccidiosis are TLR-4, TLR-15, MyD88, and nuclear factor kappa B (NF-κB). Zhou et al. [30] reported that broilers subjected to an E. tenella infection had a higher level of expression of the TLR-4 and TLR15 genes. In addition, layers challenged with E. tenella have been shown to exhibit a 2 fold increase in the up-regulation of TLR15, NF-κB, and MyD88 gene expression in their ceca between 4 and 24 h post infection [38]. Receptors such as TLR are capable of recognizing conserved pathogen-associated molecular patterns [40]. These TLR are present in all the developmental stages of the life cycle of various Eimeria species [41]. The MyD88 protein is a downstream adaptor for TLR and is curial for many of the functions of TLRs. Intrinsic to those functions, LR-MyD88 signaling has been shown to trigger inflammatory responses to interior pathogens [42]. In the current study, none of the genes associated with innate immunity were differentially expressed, while the expression of those genes linked to adaptive immunity were altered by in ovo injection treatment. The findings in the current study showed that the in ovo injection of D3 alone or in combination with 25OHD3 did not alter the expression of genes that were linked to either an immune response or to vitamin D activity. However, in ovo administration of 2.4 µg of 25OHD3 alone increased the expression of anti-inflammatory response genes (IL-10 and TGF-β), and deceased the expression of a pro-inflammatory response gene (IL-6) during the coccidiosis infection. In comparison to D3 at the same level of supplemental dietary inclusion, it has likewise been shown that the expression of IL-10 increased and IL-1β decreased in response to 2760 IU/kg of 25OHD3 in broilers challenged with coccidiosis [8]. Additionally, dietary 25OHD3 at 100 µg/kg increased CD4+CD25+ cells and deceased CD8+CD25+ cells in coccidiosis-infected broilers [10] and turkeys [39]. It is well documented that the CD4+CD25+ cells [37,41], IL-10 [43,44] and TGF-β [45] are associated with regulatory T cell (Tregs) formation that is facilitated by an up-regulation of the expression of the aforementioned genes. The Tregs are immunity suppression cells that act to inhibit T cell proliferation and cytokine production [46]. Thus, these results indicate that dietary 25OHD3 induces an adaptive immune response, and more specifically the activation of an anti-inflammatory response during systemic and chronic infections including coccidiosis. The down-regulation of an inflammatory response in the 25OHD3 alone in ovo-injected treatment group could be due to the up-regulation of genes linked to vitamin D3 activity. When compared to the diluent and D3 alone injected treatment groups in their study, the expression of 1α-hydroxylase increased, and 24-hydroxylase decreased in response to the 25OHD3 alone treatment. The conversion of 25OHD3 to the active form of vitamin D (1,25-(OH)2D3) is facilitated by 1α-hydroxylase in the kidney [12], intestine [12], breast and thigh muscles [12], and immune cells including macrophages and T-cells [10]. Moreover, by the action of 24-hydroxylase, 25OHD3 can also be converted to the inactive form of vitamin D (24,25-(OH)2D3) in many cells in which there are vitamin D receptors. Therefore, an increase in the expression of 1α-hydroxylase or a decrease in the expression of 24-hydroxylase may directly affect vitamin D3 activity. In previous companion studies, these current treatments

| Genes        | n  | 14 doa  | 28 doa  | SEM    | p-Value |
|--------------|----|---------|---------|--------|---------|
| 24-hydroxylase VDR | 16 | 1.82    | 1.42    | 0.377  | 0.285   |
| 24-hydroxylase VDR | 16 | 1.11 b  | 1.58 a  | 0.153  | 0.003   |

a,b Treatment means within the same column within effect with no common superscripts are significantly different (p ≤ 0.05). 1 14 d of age, when all birds were unchallenged. 2 28 d of age (14 days post-infection), when birds were challenged with coccidiosis vaccine.
employed were also investigated. In comparison the injection of diluent or D₃ alone, the in ovo administration of 2.4 μg of 25OHD₃ improved BW gain from 0 to 28 doa [26], meat yield [26], small intestine morphology [27], and the inflammatory response [27] of broilers subjected to a coccidial infection. Therefore, these morphological and serological improvements observed in previous studies may be due to the stimulation of genes linked to vitamin D activity as well as those eliciting an anti-inflammatory response during a coccidiosis challenge.

Across in ovo treatment, the expression of major vitamin D activity genes including VDR and 1α-hydroxylase, that converts 25OHD₃ to 1,25-(OH)₂D₃, was significantly increased in the birds subjected to a coccidial infection. The 25OHD₃ interacts with VDR in various organs and tissues such as the intestines [4,47], muscle cells, bone, kidney, parathyroid gland, pancreas, pituitary, chorioallantoic membrane, and the egg shell gland [48,49]. However, its efficacy is associated with VDR and 1α-hydroxylase, that converts 25OHD₃ to 1,25-(OH)₂D₃ [4]. It is well-documented that hydroxylation in the liver is reduced when chickens are subjected to stressful conditions [50], mycotoxicosis [51], and E-coli infections [52]. Additionally, hydroxylation in the liver is decreased during severe coccidiosis infections [53]. Thus, these results indicate that the intestinal absorption of D₃ and its functionality may be reduced during a coccidiosis infection, which may subsequently lead to the additional use of supplemental dietary D₃ or other D₃ sources, such as 25OHD₃ to support of vitamin D function. Furthermore, the expression of IL-2, IL-6, and TLR-4 involved in pro-inflammatory responses significantly increased during the coccidiosis infection in this study. Several studies have reported the elevation in the expression of IL-2 and IL-6 during various avian Emeria infections [54–56]. Likewise, TLR-4 categorized as a pathogenic infection indicator, has been shown to be up-regulated during an E. tenella infection [30]. Therefore, the broilers in the current study that experienced a successful Emeria infection exhibited a measurable immune reaction.

5. Conclusions

In conclusion, the aim in the current research was to identify the genes that are linked to an immune response and to vitamin D activity in the jejunum of the small intestine of broilers subjected to an Emeria infection after having received individual or combinational in ovo injection of 2 vitamin D₃ sources. Beside upon the findings in this study, it is suggested that the coccidiosis infection had significant effects on the expression of genes involved in vitamin D, function (1α-hydroxylase and VDR), and a pro-inflammatory response (IL-2, IL-6 and TLR-4). Furthermore, the in ovo administration of 2.4 μL of 25OHD₃ resulted in the up-regulation of genes linked to an anti-inflammatory response (IL-10 and TGF-β4). Moreover, the expression of 1α-hydroxylase increased and 24-hydroxylase deceased in response to the in ovo injection of 2.4 μL of 25OHD₃. However, the other in ovo-injection treatments investigated in this study did not display significant effects on the expression of genes associated with either an inflammatory reaction or to vitamin D function. These results, therefore, demonstrate that the amniotic in ovo administration of 25OHD₃ at 18 doi may more quickly ameliorate the negative effects of a coccidiosis infection by means of its effects on intestinal vitamin D activity or an inflammatory reaction. Further research is required to determine the regulatory effects of the in ovo administration of different vitamin D₃ sources on the immune response and intestinal development of broilers subjected a coccidiosis infection.

Author Contributions: Conceptualization, S.A.F.; methodology, S.A.F., A.M. and K.S.M.; software, S.A.F.; validation, S.A.F., A.M., L.Z. and E.D.P.; formal analysis, S.A.F.; investigation, S.A.F.; resources, E.D.P.; data curation, S.A.F., I.P., S.P., A.M. and E.D.P.; writing—original draft preparation, S.A.F.; writing—review and editing, S.A.F., L.Z., S.P., K.S.M. and E.D.P.; visualization, S.A.F.; supervision, E.D.P.; project administration, S.A.F.; funding acquisition, E.D.P. All authors have read and agreed to the published version of the manuscript.
Animals 2022, 12, 2517

Funding: This research was supported by the DSM Nutritional Products Inc., Zoetis Animal Health Co., United States Department of Agriculture (USDA agreement no. 58-6406-4-016) and Merial Select Inc.

Institutional Review Board Statement: The experimental procedure was accepted by the Institutional Animal Care and Use Committee of Mississippi State University (Protocol #IACUC-20-248).

Informed Consent Statement: Not applicable.

Data Availability Statement: None of the data were deposited in an official repository.

Acknowledgments: The authors express their appreciation for the assistance of the graduate and undergraduate students of the Poultry Science Department of Mississippi State University. Our special thanks are also extended to April Levy for her invaluable assistance.

Conflicts of Interest: The authors indicate that they have no conflict of interest. The authors alone are responsible for the content and writing of this article.

References
1. Henry, H.L. Measurement of the chicken kidney 25-hydroxyvitamin D3 1-hydroxylase and 25-hydroxyvitamin D3 24-hydroxylase. Methods Enzymol. 1980, 67, 445–449. [PubMed]
2. Booth, B.E.; Tsai, H.C.; Morris, R.C., Jr. Vitamin D status regulates 25-hydroxyvitamin D3-1 alpha-hydroxylase and its responsiveness to parathyroid hormone in the chick. J. Clin. Investig. 1985, 75, 155–161. [CrossRef] [PubMed]
3. Russell, J.; Bar, A.; Sherwood, L.M.; Hurwitz, S. Interaction between calcium and 1,25-dihydroxyvitamin D3 in the regulation of preproparathyroid hormone and vitamin D receptor messenger ribonucleic acid in avian parathyroids. Endocrinology 1993, 132, 2639–2644. [CrossRef] [PubMed]
4. De Matos, R. Calcium metabolism in birds. Vet. Clin. N. Am. Exot. Anim. Pract. 2008, 11, 59–82. [CrossRef]
5. Yarger, J.G.; Quarles, C.L.; Hollis, B.W.; Gray, R.W. Safety of 25-hydroxycholecalciferol as a source of cholecalciferol in poultry rations. Poult. Sci. 1995, 74, 1437–1446. [CrossRef]
6. Vignale, K.; Greene, E.S.; Caldas, J.V.; England, J.; Boonsinchai, N.; Sodsee, P.; Pollock, E.D.; Dridi, S.; Coon, C.N. 25-Hydroxycholecalciferol enhances male broiler breast meat yield through the mTOR pathway. J. Nutr. 2015, 145, 855–863. [CrossRef]
7. Hutton, K.C.; Vaughn, M.A.; Litta, G.; Turner, B.J.; Starkey, J.D. Effect of vitamin D status improvement with 25-hydroxycholecalciferol on skeletal muscle growth characteristics and satellite cell activity in broiler chickens. J. Anim. Sci. 2014, 92, 3291–3299. [CrossRef]
8. Morris, A.; Shanmugasundaram, R.; Lilburn, M.S.; Selvaraj, R.K. 25-Hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection. Poult. Sci. 2014, 93, 1951–1956. [CrossRef]
9. Chou, S.H.; Chung, T.K.; Yu, B. Effects of supplemental 25-hydroxycholecalciferol on growth performance, small intestinal morphology, and immune response of broiler chickens. Poult. Sci. 2009, 88, 2333–2341. [CrossRef]
10. Morris, A.; Shanmugasundaram, R.; McDonald, J.; Selvaraj, R.K. Effect of in vitro and in vivo 25-hydroxyvitamin D treatment on macrophages, T cells, and layer chickens. J. Anim. Sci. 2015, 93, 2894–2903. [CrossRef]
11. Christakos, S.; Ajibade, D.V.; Dhawan, P.; Fechner, A.J.; Mady, L.J. Vitamin D: Metabolism. Endocrinol. Metab. Clin. N. Am. 2010, 39, 243–253. [CrossRef] [PubMed]
12. Shanmugasundaram, R.; Selvaraj, R.K. Vitamin D-1alpha-hydroxylase and vitamin D-24-hydroxylase mRNA studies in chickens. Poult. Sci. 2012, 91, 1819–1824. [CrossRef] [PubMed]
13. Shojadoost, B.; Behboudi, S.; Villanueva, A.I.; Brisbin, J.T.; Ashkar, A.A.; Sharif, S. Vitamin D3 modulates the function of chicken macrophages. Res. Vet. Sci. 2015, 100, 45–51. [CrossRef] [PubMed]
14. Williams, C.J. In ovo vaccination and chick quality. Int. Hatch. Prac. 2011, 19, 7–13.
15. Williams, C.J. In ovo vaccination for disease prevention. Int. Poult. Prod. 2007, 15, 7–9.
16. Peebles, E.D. In ovo applications in poultry: A review. Poult. Sci. 2018, 97, 2322–2338. [CrossRef]
17. Mousstaaid, A.; Fatemi, S.A.; Elliott, K.E.C.; Alqhtani, A.H.; Peebles, E.D. Effects of the in ovo injection of L-ascorbic acid on broiler hatching performance. Animals 2022, 12, 1020. [CrossRef]
18. Fatemi, S.A.; Alqhtani, A.H.; Elliott, K.E.C.; Bello, A.; Zhang, H.; Levy, A.W.; Peebles, E.D. Improvement in the performance and inflammatory reaction of Ross 708 broilers in response to the in ovo injection of 25-hydroxyvitamin D3. Poult. Sci. 2015, 100, 135–146. [CrossRef]
19. Fatemi, S.A.; Elliott, K.E.C.; Bello, A.; Zhang, H.; Alqhtani, A.H.; Peebles, E.D. Effects of the in ovo injection of vitamin D3 and 25-hydroxyvitamin D3 in Ross 708 broilers subsequently fed commercial or calcium and phosphorous-restricted diets: I. performance, carcass characteristics, and incidence of woody breast myopathy. Poult. Sci. 2021, 100, 10122. [CrossRef]
20. Fatemi, S.A.; Elliott, K.E.C.; Bello, A.; Durojaye, O.; Zhang, H.; Turner, B.; Peebles, E.D. The effects of in ovo-injected vitamin D3 sources on the eggshell temperature and early post-hatch performance of Ross 708 broilers. Poult. Sci. 2020, 99, 1357–1362. [CrossRef]
21. Fatemi, S.A.; Elliott, K.E.C.; Bello, A.; Durojaye, O.; Zhang, H.; Turney, B.; Peebles, E.D. Effects of source and level of in ovo-injected vitamin D₃ on the hatchability and serum 25-hydroxycholecalciferol concentrations of Ross 708 broilers. Poult. Sci. 2020, 99, 3877–3884. [CrossRef] [PubMed]

22. Bello, A.; Hester, P.Y.; Gerard, P.D.; Zhai, W.; Peebles, E.D. Effects of commercial in ovo injection of 25-hydroxycholecalciferol on bone development and mineralization in male and female broilers. Poult. Sci. 2014, 93, 2734–2739. [CrossRef] [PubMed]

23. Yair, R.; Shahar, R.; Uni, Z. In ovo feeding with minerals and vitamin D₃ improves bone properties in hatchlings and mature broilers. Poult. Sci. 2015, 94, 2695–2707. [CrossRef] [PubMed]

24. Abbas, T.; Shakeri, M.; Zargar, M.; Kohram, H. Growth performance parameters, bone calcification and immune response of in ovo injection of 25-hydroxycholecalciferol and vitamin K3 in male Ross 308 broilers. Theriogenology 2017, 90, 260–265. [CrossRef]

25. Fatemi, S.A.; Elliott, K.E.C.; Bello, A.; Zhang, H.; Peebles, E.D. Effects of the in ovo injection of vitamin D₃ and 25-hydroxyvitamin D₃ in Ross 708 broilers subsequently fed commercial or calcium and phosphorous-restricted diets: II. Immunity and small intestine morphology. Poult. Sci. 2021, 100, 101240. [CrossRef] [PubMed]

26. Fatemi, S.A.; Elliott, K.E.C.; Bello, A.; Peebles, E.D. Effects of the in ovo injection of vitamin D₃ and 25-hydroxyvitamin D₃ in Ross 708 broilers subsequently challenged with coccidiosis: I. performance, meat yield and intestinal lesion. Poult. Sci. 2021, 100, 101382. [CrossRef]

27. Fatemi, S.A.; Elliott, K.E.C.; Bello, A.; Macklin, K.S.; Peebles, E.D. Effects of the in ovo injection of vitamin D₃ and 25-hydroxyvitamin D₃ in Ross 708 broilers subsequently challenged with coccidiosis: II. Immunological and inflammatory responses and small intestine histomorphology. Animals 2022, 12, 1027. [CrossRef]

28. Poudel, S.; Zhang, L.; Tabler, G.T.; Lin, J.; Zhai, W. Effects of riboflavin and Bacillus subtilis on internal organ development and intestinal health of Ross 708 male broilers with or without coccidial challenge. Poult. Sci. 2021, 100, 100973. [CrossRef]

29. Zhou, Z.; Wanga, Z.; Cao, L.; Hua, S.; Zhang, Z.; Qin, B.; Guo, Z.; Nie, K. Upregulation of chicken TLR4, TLR15 and MyD88 mRNA expression of interleukin-6 and genes involved in its induction pathways in 2 broiler chicken breeds. Poult. Sci. 2019, 98, 1805–1819. [CrossRef] [PubMed]

30. Zhou, Z.; Wanga, Z.; Cao, L.; Hua, S.; Zhang, Z.; Qin, B.; Guo, Z.; Nie, K. Upregulation of chicken TLR4, TLR15 and MyD88 mRNA expression of interleukin-6 and genes involved in its induction pathways in 2 broiler chicken breeds. Poult. Sci. 2019, 98, 1805–1819. [CrossRef] [PubMed]

31. Alder, C.L.; Jensen, J.L.; Ørntoft, T.F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004, 64, 5245–5250. [CrossRef] [PubMed]

32. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001, 25, 402–408. [CrossRef] [PubMed]

33. Al-Zghoul, M.B.; Saleh, K.M.; Ababneh, M.M.K. Effects of pre-hatch thermal manipulation and post-hatch acute heat stress on the mRNA expression of interleukin-6 and genes involved in its induction pathways in 2 broiler chicken breeds. Poult. Sci. 2019, 98, 1805–1819. [CrossRef] [PubMed]

34. Bristol, J.T.; Gong, J.; Parvizi, P.; Sharif, S. Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. Clin. Vaccine Immunol. 2010, 17, 1337–1343. [CrossRef]

35. Steel, R.G.D.; Torrie, J.H. Principles and Procedures of Statistics: A biometrical Approach, 2nd ed.; McGraw-Hill: New York, NY, USA, 1980.

36. Shannugasundaram, R.; Morris, A.; Selvaraj, R.K. Regulatory T cell properties of chicken CD4⁺ CD25⁺ Cells. J. Immunol. 2011, 186, 1997–2002. [CrossRef] [PubMed]

37. Shannugasundaram, R.; Selvaraj, R.K. Regulatory T cell properties of chicken CD4⁺ CD25⁺ Cells. J. Immunol. 2011, 186, 1997–2002. [CrossRef] [PubMed]

38. Morris, A.; Selvaraj, R.K. In vitro 25-hydroxycholecalciferol treatment of lipopolysaccharide-stimulated chicken macrophages increases nitric oxide production and mRNA of interleukin-1 beta and 10 during a coccidial challenge. J. Anim. Sci. 2014, 93, 2894–2903. [CrossRef] [PubMed]

39. Wan, Y.Y.; Richard, A.F. ‘Yin-Yang’ functions of transforming growth factor-beta and T regulatory cells in immune regulation. Immunol. Rev. 2007, 220, 199–213. [CrossRef]
46. Sojka, D.K.; Huang, Y.H.; Fowell, D.J. Mechanisms of regulatory T-cell suppression—A diverse arsenal for a moving target. *Immunology*. 2008, 124, 13–22. [CrossRef]

47. Nemere, I.; Dormanen, M.C.; Hammond, M.W.; Okamura, W.H.; Norman, A.W. Identification of a specific binding protein for 1 alpha, 25-dihydroxyvitamin D3 in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. *J. Biol. Chem.* 1994, 269, 23750–23756. [CrossRef]

48. Norman, A.W. Studies on the vitamin D endocrine system. *Avian. J. Nutr.* 1987, 117, 797–807. [CrossRef]

49. Elaroussi, M.A.; Prahl, J.M.; DeLuca, H.F. The avian vitamin D receptors: Primary structures and their origins. *Proc. Natl. Acad. Sci. USA* 1994, 91, 11596–11600. [CrossRef]

50. Thaxton, J.P.; Puvadolpirod, S. Model of physiological stress in chickens. 5. Qualitative evaluation. *Poult. Sci.* 2000, 79, 391–395. [CrossRef] [PubMed]

51. Yarru, L.P.; Settivari, R.S.; Antoniou, E.; Ledoux, D.R.; Rottinghaus, G.E. Toxicological and gene expression analysis of the impact of aflatoxin B1 on hepatic function of male broiler chicks. *Poult. Sci.* 2009, 88, 360–371. [CrossRef] [PubMed]

52. Peighambari, S.M.; Julian, R.J.; Gyles, C.L. Experimental *Escherichia coli* respiratory infection in broilers. *Avian. Dis.* 2000, 44, 759–769. [CrossRef] [PubMed]

53. Ali, B.H. The hepatic and duodenal activities of some drug metabolizing enzymes in chickens: Influence of infection with *Escherichia coli* endotoxin and coccidiosis. *Eur. J. Drug Metab. Pharm.* 1997, 22, 223–227. [CrossRef] [PubMed]

54. Lynagh, G.R.; Bailey, M.; Kaiser, P. Interleukin-6 is produced during both murine and avian *Eimeria* infections. *Vet. Immunol. Immunopathol.* 2000, 76, 89–102. [CrossRef]

55. Hong, Y.H.; Lillehoj, H.S.; Lillehoj, E.P.; Lee, S.H. Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria* maxima infection of chickens. *Vet. Immunol. Immunopathol.* 2006, 114, 259–272. [CrossRef] [PubMed]

56. Yu, H.; Zou, W.; Wang, X.; Dai, G.; Zhang, T.; Zhang, G.; Xie, K.; Wang, J.; Shi, H. Research Note: Correlation analysis of interleukin-6, interleukin-8, and C-C motif chemokine ligand 2 gene expression in chicken spleen and cecal tissues after *Eimeria tenella* infection in vivo. *Poult. Sci.* 2020, 99, 1326–1331. [CrossRef]