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Original Article

Expression of TGF-β/Smads in Cecum and Spleen of Chicken Infected with E. Tenella

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ABSTRACT

Chicken coccidiosis is a common and severe parasitic disease caused by infection from *Eimeria* spp., which affects the integrity of the intestinal mucosa. TGF-β has been shown to play an important role in the healing of intestinal mucosas, immunity, and the maintenance of bowel mucosa integrity. Very little is known about the presence of the components of TGF-β/Smads signaling pathway of chicken at different times following coccidian infection. In the present study, we measured the levels of TGF-β2, 3, 4, receptor TβRI, II, down-stream Smad 2, 3, 7 in cecum and spleen of chicken at different times after inoculation with *Eimeria tenella* using quantitative real-time PCR. The results showed that the TGF-β/Smads signaling pathway was not activated in cecum in the early stage of infection. However, on the 8th day, the expression of TGF-β2, 4, down-stream protein Smad 2, 7 were significant up-regulated in the spleen, which indicated that the TGF-β/Smads signaling was changed in the *E. tenella* infection and was differentially expressed in various tissues in the early stages of infection.

INTRODUCTION

Coccidiosis is one of the most costly diseases for the modern poultry industry worldwide (Kim *et al.*, 2014; Grenier *et al.*, 2016). It is a protozoan disease caused by parasites of the *Eimeria* species, which multiply within the intestinal tract, cause destruction of intestinal mucosa, and can induce a severe inflammatory response (Abdel-Latif *et al.*, 2016). Transforming growth factor-β (TGF-β) has been known not only for repairing mucosal injuries, but also for preserving the integrity of the intestinal mucosa (Massague, 1990). Research results have suggested that the development of immunity against *Eimeria* in chicken may be associated with the release of TGF-β in chicken's spleens, cecal tonsils and duodena, presumably as part of an anti-inflammatory response following coccidian infection (Jakowlew *et al.*, 1997; Choi *et al.*, 1999; Wigley & Kaiser, 2003; Song *et al.*, 2010). Transforming growth factor-β is used as an adjuvant in immunization with coccidial DNA vaccine to prevent coccidiosis and has been proven to decrease fecal oocyst shedding (Min *et al.*, 2002).

Transforming growth factor-β predominately signals through the Smad family of proteins. The actions of TGF-β are mediated by binding to cell surface receptors. Most cells have three types of transmembrane serine/threonine kinase receptor proteins: TGF-β receptor I (TβRI), II (TβRII) and III (TβRIII)(Kubiczkova *et al.*, 2012; Rasal *et al.*, 2015). Both TβR and Smad play critical roles in signal transduction for the biological activities of TGF-β. While the contribution of TGF-β/Smads signalingduring *Eimeria tenella* infection is unknown, understanding the interplay between host and parasites in the intestine is crucial for designing new approaches

against coccidiosis (Yun *et al.*, 2000). In the current study, chickens artificially infected with *E. tenella* were used to study the relationship between TGF-β/Smads signaling pathway and bowel mucosa damages. We systematically investigated the expression of TGF-βs, TβRs, and Smads in the cecum and spleen at different times post-infection (P.I).

MATERIALS AND METHODS

Parasites

Parental *E. tenella* was graciously provided by the Institute of Animal Health, Guangdong Academy of Agricultural Sciences. The coccidia were developed and maintained at the Animal Science Department of Henan University of Technology. Sporulated oocysts for experimental infections were counted in a hemocytometer.

Animals and Diets

Day-old male egg-type Roman chicken were obtained from our lab hatchery, kept in wire cages, and reared in a coccidia-free lab with feed and water provided *ad libitum*. Room temperature was maintained at 36 °C (beginning) and 34 °C (end) through the experiment. All birds were fed with commercial corn-soybean basal diets, which had no anti-coccidian drugs (Table 1). Constant light was provided during the entire experimental period.

Table 1 – Nutrient content of commercial feed used in the experiment.

Ingredient	Content (%)
Crude Protein	≥18.0
Coarse Fibre	≤8.0
Crude Ash	≤9.0
Calcium	0.6-1.3
Total Phosphorus	≥0.5
NaCl	0.3-0.8
Lysine	≥0.85
Methionine	0.36-0.9
Moisture	≤14.0

Experimental Procedures

Sixty 16-day-old healthy male chickens were selected and randomly assigned to two groups (experimental and control group). Chickens in the experimental group were each inoculated orally with 1×10^5 sporulated *E. tenella* oocysts. The uninfected control group received the same volume of physiological saline. Clinical observations were carried out daily to monitor the health and mortality of experimental chicken. At 6hr and 3, 5, 6, 8 days P.I, six live chicks from each group were euthanized. Cecal and spleen tissues were collected for pathological assessment and study of related gene expression. Cecal fragments were washed with ice-cold saline to remove intestinal contents and immediately put in liquid nitrogen before quantifying the expression of selected genes.

Quantitative Real-time PCR Analysis of Gene Expression

Total RNA was isolated from the ceca and spleen samples of chicken using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA integrity was assessed *via* agarose gel (1.2 %) electrophoresis while RNA concentration and purity were spectrophotometrically determined using A_{260} and A_{280} measurements in a Biophotometer (Eppendorf, Germany). Total RNA was reversely transcribed into cDNA using First Strand cDNA Synthesis Kit (Dingguo Changsheng, China). Polymerase chain reaction (PCR) was performed with GoTaq® qPCR Master Mix (Promega, Belgium). Primers were designed using NCBI Primer BLAST. Primer used for qRT-PCR is shown in Table 2. Thermal cycling parameters were as follows: 1 cycle at 95 °C for 3 min, and then 40 cycles at 95 °C for 30s, 62 °C for 30s, 72 °C for 20s on Mastercycler ep RealplexReal-Time PCR Detection System (Eppendorf, Germany). Fluorescent data were used to derive the *C(t)* or the PCR cycle at a threshold that is noted as the first significant deviation in fluorescence from a base line value. Analyses were performed in duplicates. The resultant value was expressed relative to GAPDH (control gene), which showed the most stability. Relative gene expression was analyzed using the $2^{-\Delta\Delta C(t)}$ method (Livak & Schmittgen, 2001).

Data Analyses

Statistical analyses were performed using the Predictive Analytics Software (PASW) version 18.0. T-test was used to assess the differences between the *E. tenella* infected group and the control group. Values were reported as mean ± standard error (SE). Differences between the two groups were considered statistically significant at $p<0.05$.

RESULTS

Clinical Signs of Chickens Infected with *E. tenella*

The chickens were assessed daily for 8 days following *E. tenella* challenge at 16 days old. No unusual clinical signs were found in the control group throughout

Table 2 – Sequences of PCR primers in this study.

Gene[1]	GenBank number	Primer sequence (5′-3′)	Orientation	Product size(bp)
TGF-β2	NM_001031045.3	TATCATCACCAGGACAGCGT	Forward	177
		ACCTTGTGGCTTAGGGTCTG	Reverse	
TGF-β3	NM_205454.1	ACCTTGTGGCTTAGGGTCTG	Forward	211
		ATTCCTTGCCTTCCCAGTTC	Reverse	
TGF-β4	JQ423909.1	CGGGACGGATGAGAAGAAC	Forward	258
		CGGCCCACGTAGTAAATGAT	Reverse	
TβRI	NM-204246.1	GCTGTGGTTGGTGTCAGATT	Forward	156
		GGTTTGCCTTGTGTGCCTAC	Reverse	
TβRII	NM-205428.1	GACCACCGCCAAGTAGCAT	Forward	129
		TGACAGCCTCAGTTCTCCAG	Reverse	
Smad2	NM-204561.1	GTCATCCATTCTGCCATTCA	Forward	100
		ATTCTGCTCACCACCACCA	Reverse	
Smad3	NM-204475.1	GAGCCGCAGAGCAACTACAT	Forward	135
		CGGAGACATAGGATTTGGTGAT	Reverse	
Smad7	XM_427238.7	CTCTGTGCCTTCCTCCACTG	Forward	244
		CTGGCTTCTGTTGTCCGAGT	Reverse	
GAPDH	K01458	GGTGGTGCTAAGCGTGTTAT	Forward	264
		ACCTCTGTCATCTCTCCACA	Reverse	

[1] TGF-β2, 3, 4 = Transforming growth factor-β2, 3, 4; TβR I,II = Transforming growth factor-β receptor I,II; Smad2, 3, 7 = drosophila mothers against decapentaplegic protein 2, 3, 7; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase.

the experiment. As shown in Table 3, chicken in the infected group had lower body weight than those in the control group at five days after infection. The difference was significant at 8d PI (p<0.01).

Table 3 – Body weight (g) of chicken after infection.

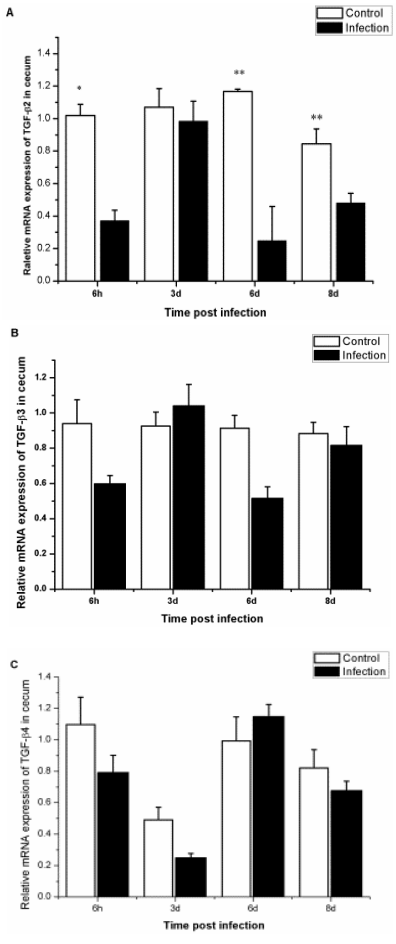
Group	5d(PI)	6d(PI)	8d(PI)
Control	212.22±8.35	232.13±4.49	263.05±5.58
Infected	196.60±3.56	219.53±6.54	214.18±7.99**

Values are means ± S.E. ** p≤0.01.

No clear pathological changes were observed in the control group throughout the experiment. By contrast, the chicks infected with the recovered *E. tenella* demonstrated loss of appetite, listlessness, bloody diarrhoea, ruffled and tarnished feathers. At 5 days P.I, the clinical signs became more severe, and large amount of bloody stools were found. At the 8th day P.I, there no new bloody stools were observed, but the ceca became crispy and there was loss of elasticity. The cecum wall was covered with bleeding spots and filled caseous contents. Mortality was 0%.

mRNA Expression of TGF-β/Smads Related Genes in Cecum of Chickens Infected with *E. tenella*

The influence of *E. tenella* in cecum of chicken on the expression of TGF-β/Smads related genesis presented in Fig. 1. Compared with the chicken in the control group, TGF-β2gene expression of the infected birds was significantly down-regulated at 6 hr, 6d, 8d after infection (p<0.05). The expression of TGF-β Receptor receptor II (TβRII) was down-regulated at 6 hr, 3d and 6d pi (p<0.05) (Fig. 3, E*)*. The expression of TGF-β3 and 4, TGF-βreceptor I (TβRI), and Smad2, 3, 7 showed no significant differences (p>0.05).



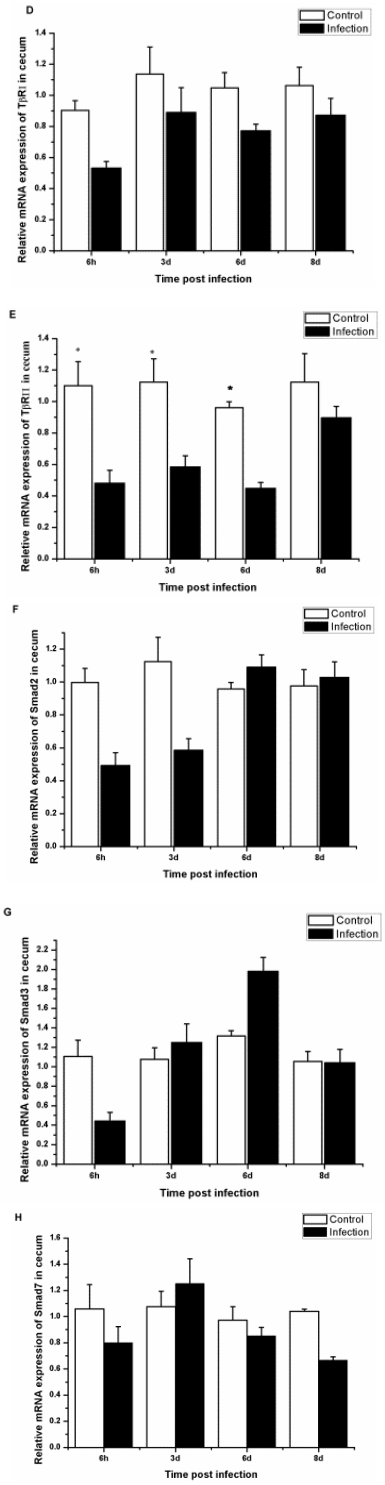
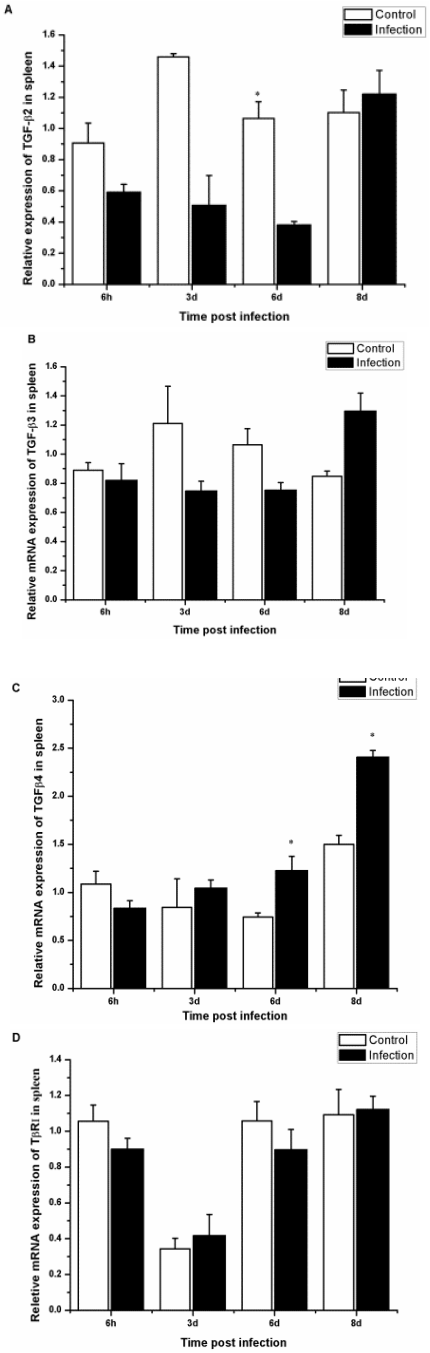


Figure 1– Relative expression of TGF-β/smad related genes in the cecum of *E. tenella* infected chicken. A, TGF-β2; B, TGF-β3; C, TGF-β4; D, TβRI; E, TβRII; F, Smad 2; J, Smad 3; H, Smad 7. Data were expressed as means ± SE. Differences were considered significant at $^{*}p<0.05$, $^{**}p<0.01$.

mRNA Expression of TGF-β/Smads Related Genes in Spleen of Chickens Infected with *E. tenella*

In chicken spleen, the influence of *E. tenella* on the expression of TGF-β/Smads related genesis presented in Fig. 2. The expression of TGF-β2 was down-regulated at 6d P.I ($p<0.05$) and had no significant difference at other time point ($p>0.05$). Expression of TGF-β4 was moderately up-regulated at 6 and 8d after infection ($p<0.05$). The expression of TGF-β3, TβR, and TβRII showed no significant differences ($p>0.05$). The expression of Smad 2 was down-regulated at 6h, but up-regulated at 6d P.I ($p<0.05$). The expression of Smad3 decreased slightly at 6hr P.I. The expression of Smad 7 increased significantly 8d P.I ($p<0.05$).



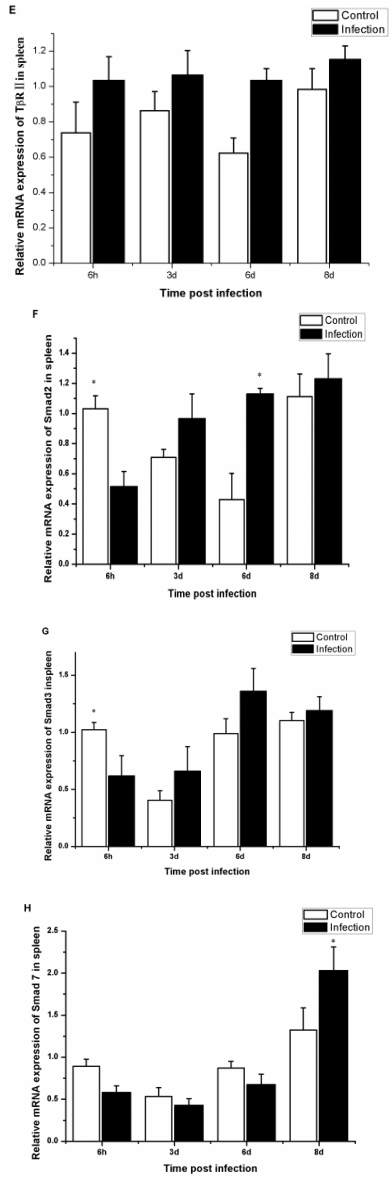


Figure 2 – mRNA expressions of TGF-β/Smad related genes in spleen of chickens infected with *E. tenella*. A, TGF-β2; B, TGF-β3; C, TGF-β4; D, TβRI; E, TβRII; F, Smad 2; J, Smad 3; H Smad 7. Data were expressed as means ± SE. Differences were considered significant at $^{*}p<0.05$.

DISCUSSION

Coccidiosis is a major intestinal parasitic disease in poultry characterized by damages in the intestinal mucosa, including inflammation and villous atrophy (Dalloul & Lillehoj, 2006). Disruption of the intestinal barrier affects the absorption of nutrients, and possibly makes the bird more susceptible to diseases. In our study, we observed weight loss, listlessness, loss of appetite, bloody diarrhoea, and huddling in the infected chicken; findings that were also described before (Lillehoj *et al.*, 2004). Weight loss in the infected group was much higher than that in the control group ($p<0.01$) (Table 3). TGF-β is important for maintaining normal intestinal homeostasis and mucosaintegrity (Lillehoj *et al.*, 2004). In the present study, the expression of TGF-β 2 (Fig. 1A) was down-regulated and no significant difference in the expression of TGF-β 3 (Fig. 1B) was found in birds challenged with *E. tenalla* in the cecum tissue ($p<0.05$). Jakowlew *et al.* (1997) showed that expression of mRNAs for TGF-β 2 and 3 remained constant after infection with coccidian parasites in l-month-old chicken. Mediation of TGF-β signaling is complex, being either stimulatory or inhibitory, depending on the differentiation state of the cell and cues from the surrounding environment (Omer *et al.*, 2000). Different stages of *E. tenella* life cycle led to different lesions in chickens. Thus, the expression of TGF-β signaling showed distinction between the different evolutive stages of *E. tenella*. Similar models of intracellular parasitic infection in mice showed that IEL produced a low level of TGF-β in *Eimeria spp.* Infection, and necrotizing enterocolitis was associated with decreased tissue expression of TGF-β 2 in intestinal epithelial cells (Inagaki-Ohara *et al.*, 2006; Maheshwari *et al.*, 2011). The expression of TGF-β 2 was regulated in epithelial cells in an autocrine fashion and enteral supplementation with recombinant TGF-β 2 was protective (Namachivayam *et al.*, 2013). Immunogenicity antigen is different at different stages of *E. tenella* life cycle, leading to different immune responses. The early endogenous stages of the *Eimeria* life cycle are considered to be more immunogenic than the later sexual stages, suggesting that some of the effects of immunity take place before penetration into the surface enterocytes (Yun *et al.*, 2000). The first few days post-infection is the time when the pro-inflammatory capacity of TGF-β 2 would influence the developing immune response (Dalloul & Lillehoj, 2006; Namachivayam *et al.*, 2013). These results suggest that TGF-β2 is also important in the induction of immune effector responses to *Eimeria* infections in chickens.

TGF-β 4 has been found to be important in regulating immune function in coccidia-infected IECs of chicken (Jin, 2020). Jakowlew et al. (1997) reported that expression of TGF-β 4 mRNAs increased 2.5 folds in spleen cells. As expected, the result of the present study showed that TGF-β 4 expression was increased at 8d in the spleens of birds challenged with *E. tenalla* (Fig. 2C). This result is consistent with the report by Karaffová *et al.* (2015), who found that TGF-β4 expression was mainly enhanced at the late phase of infection. Production of TGF-β 4 is highest in the tips of intestinal villi, and might participate in modulating growth of the intestinal villus need to be repaired at the late phase of infection.

Both TβR and Smads are important factors for TGF-βs signaling. Our study showed that the expression of TβRII in the cecum of the coccidian infected chicken was inhibited in the first few days (Fig. 1D). Brown *et al.* (1999), in a study in mice, suggested that TGF-β signaling was important for blood vessel development, following a finding where endothelial cells of TβRII receptor-null mice were not closely associated with each other or with the surrounding stromal cells, leading to vessel rupture and systemic edema. Furthermore, Deheuninck & Luo (2009) suggested that Smads were critical mediators of the growth inhibitory signals of TGF-β in epithelial cells. Yan *et al.* (2009) found that Smad7 played a key role in regulating signal transduction of TGF-β family cytokines. In the present study, while the expression of Smad 2/3/7 in the cecum of the infected group showed no significant differences in the infection group (Fig. 1 F, G and H) (p>0.05), the mRNA expression of Smad7 in the spleen was significantly increased on the 8th day after infection (Fig. 2 H) (p<0.05).

In the present study, the expression of TGF-β/Smads signaling in the spleen was not consistent with their expression in the cecum, which responds to both inflammation and physical damages caused by *E. tenella* infection. Song *et al.* (2010) reported that TGF-β 4 expression in chicken spleen increased by 3 times after coccidia infection. That result is consistent with our findings in the present study. Expression of TGF-β/Smads signaling elements in the spleen increased significantly, indicating that the spleen was trying to down regulate the inflammatory response to cecum injuries.

CONCLUSION

Our study demonstrated that the expression of TGF-β/Smads signaling pathway was changed in chicken infected with *E. tenella*. The expression of TGF-β/Smads signaling was up-regulated in the spleen and the expression in the spleen was not consistent with that in the cecum during the early stage of *E. tenella* infection. Further investigations into the effects of other elements in TGF-β signaling and its inhibition effects on different cell types might be necessary. A better understanding of the interactions between host cytokines and parasites is important for developing new strategies to cope with the disease.

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REFERENCES

Abdel-Latif M, Abdel-Haleem HM, Abdel-Baki AAS. Anticoccidial activities of Chitosan on *Eimeria* papillata-infected mice. Parasitology Research 2016;1-8.

Brown CB, Drake CJ, Barnett JV. Antibodies directed against the chicken type II TGFβ receptor identify endothelial cells in the developing chicken and quail. Developmental Dynamics 1999;215(1):79-85.

Choi K, Lillehoj H, Zalenga D. Changes in local IFN-γ and TGF-β4 mRNA expression and intraepithelial lymphocytes following *Eimeria acervulina* infection. Veterinary Immunologyand Immunopathology 1999;71(3):263-75.

Dalloul RA, Lillehoj HS. Poultry coccidiosis:recent advancements in control measures and vaccine development. Expert Review of Vaccines 2006;5(1):143-63.

Deheuninck J, Luo, K. Ski and SnoN, potent negative regulators of TGF-β signaling. Cell Research 2009;19(1):47-57.

Grenier B, Dohnal I, Shanmugasundaram R, Eicher SD, Selvaraj RK, Schatzmayr G, Applegate TJ. Susceptibility of broiler chickens to coccidiosis when fed subclinical doses of deoxynivalenol and fumonisins—special emphasis on the immunological response and the mycotoxin interaction. Toxins 2016;8(8):231.

Inagaki-Ohara K, Dewi FN, Hisaeda H, Smith AL, Jimi F, Miyahira M, et al. Intestinal intraepithelial lymphocytes sustain the epithelial barrier function against *Eimeria* vermiformis infection. Infection and Immunity 2006;74(9):5292-301.

Jakowlew SB, Mathias A, Lillehoj HS. Transforming growth factor-β isoforms in the developing chicken intestine and spleen:increase in transforming growth factor-β4 with coccidia infection. Veterinary Immunology and Immunopathology 1997;55(4):321-39.

Jin H, Haicheng Y, Caiyun Z, Yong Z, Jinrong W. The expression of NF-kB signaling pathway was inhibited by silencing TGF-b4 in Chicken IECs infected with *E. tenella*. Brazilian Journal of Poultry Science 2020;22(4):1-8.

Karaffová V, Bobíková K, Husáková E, Levkut M, Herich R, Revajová V, et al. Interaction of TGF-β4 and IL-17 with IgA secretion in the intestine of chickens fed with E. faecium AL41 and challenged with S. Enteritidis. Research in Veterinary Science 2015;100:75-9.

Kim WH, Jeong J, Park AR, Yim D, Kim S, Chang HH, e al. Downregulation of chicken interleukin-17 receptor A during *Eimeria* infection. Infection and Immunity 2014;82(9):3845-54.

Kubiczkova L, Sedlarikova L, Hajek R, Sevcikova S. TGF-β–an excellent servant but a bad master. Journal of Translational Medicine 2012;10(1):1.

Lillehoj H, Min W, Dalloul R. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. Poultry Science 2004;83(4):611-23.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2– ΔΔCT method. Methods 2001;25(4):402-8.

Maheshwari A, Kelly DR, Nicola T, Ambalavanan N, Jain SK, Murphy–Ullrich J, et al. TGF-β 2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. Gastroenterology 2011;140(1):242-53.

Massague J. The transforming growth factor-beta family. Annual Review of Cell Biology 1990;6(1):597-641.

Min W, Lillehoj HS, Burnside J, Weining KC, Staeheli P, Zhu JJ. Adjuvant effects of IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γTGF-β4 and lymphotactin on DNA vaccination against *Eimeria acervulina*. Vaccine 2002;20(1):267-74.

Namachivayam K, Blanco CL, MohanKumar K, Jagadeeswaran R, Vasquez M, McGill-Vargas L, et al. Smad7 inhibits autocrine expression of TGF-β2 in intestinal epithelial cells in baboon necrotizing enterocolitis. American Journal of Physiology-Gastrointestinal and Liver Physiology 2013;304(2):G167-G180.

Omer F, Kurtzhals J, Riley E. Maintaining the immunological balance in parasitic infections:a role for TGF-β? Parasitology Today 2000;16(1):18-23

Rasal KD, Shah TM, Vaidya M, Jakhesara SJ, Joshi CG. Analysis of consequences of non-synonymous SNP in feed conversion ratio associated TGF-β receptor type 3 gene in chicken. Meta Gene 2015;4:107-17.

Song H, Song X, Xu L, Yan R, Shah MAA, Li X. Changes of cytokines and IgG antibody in chickens vaccinated with DNA vaccines encoding *Eimeria acervulina* lactate dehydrogenase. Veterinary Parasitology 2010;173(3):219-27.

Wigley P, Kaiser P. Avian cytokines in health and disease. Revista Brasileira de Ciência Avícola 2003;5(1):1-14.

Yan X, Liu Z, Chen Y. Regulation of TGF-β signaling by Smad7. Acta Biochimica et Biophysica Sinica 2009;41(4):263-72.

Yun C, Lillehoj H, Lillehoj E. Intestinal immune responses to coccidiosis. Developmental & Comparative Immunology 2000;24(2):303-24.