# Effects of vitamin D3 on expression of defensins, Toll-like receptors, and vitamin D receptor in liver, kidney, and spleen of Silky Fowl

S.M. Li[1], L.H.Ouyang[1], D.G. Zhou[2]

[1]Institute of Animal Husbandry and Veterinary, Jiangxi Academy of Agricultural Science, Nanchang, P.R. China
[2]College of Animal Science and Technology, Sichuan Agricultural University, Yaan, P.R. China

**ABSTRACT**: The expression of avian β-defensins (*AvBDs*), Toll-like receptors (*TLRs*), and vitamin D receptor (*VDR*) following *in vivo* vitamin $D_3$ injection was studied. Healthy 90-day Silky Fowls were abdominally injected with vitamin $D_3$ or untreated. Real-time PCR analyses revealed that injection of vitamin $D_3$ significantly ($P < 0.05$) up-regulated the expression of *TLR*s (*TLR2*, *TLR5*), *VDR*, *AvBD*s (*AVBD-6*, *GAL-1*), and 24-hydroxylase (*CYP24A1*) in the tissues (liver, spleen, and kidney) at various times 8–24 h post injection. These results suggest that expression of *VDR*, *AvBDs*, and *TLRs* seems to be induced by vitamin $D_3$ and it was concluded that the tissues expressing *TLRs* and *VDR* respond to vitamin $D_3$ and in turn upregulate these tissues cellular functions to synthesize *AvBDs*. Intraperitoneal injection of vitamin $D_3$ likely resulted in enhancing the expression of *AvBDs*, *TLRs*, and *VDR*, which provided insight into factors important for the control of the innate immune response in the chickens.

**Keywords**: vitamin $D_3$; induction; chicken; avian β-defensins; real-time PCR

Black-Bone Silky Fowl (*Gallus gallus domesticus* Brisson), called a marvel of traditional Chinese medicine, has been well known in Asia. It originates from Taihe County, east of Wushan Mountain in Jiangxi Province, P.R. China.

A major concern for healthy breeding in both developed and developing countries is the alarming increase of antibiotic resistance to bacteria. This impending crisis has spurred the search for new therapeutic agents to combat antibiotic resistance. One potential solution lies within the system all animals are "born with," the innate immune system responsible for keeping animal health (van Dijk et al., 2008). The innate immunity found both in plants and animals is phylogenetically ancient (Sugiarto and Yu, 2004). It provides animals the capacity to repel assaults quickly from numerous infectious agents including bacteria, viruses, fungi, and parasites (Sugiarto and Yu, 2004; Kaiser, 2007). Because bacteria have difficulty in developing resistance against β-defensins and are quickly killed by them, this class of antimicrobial agents is being commercially developed as a source of peptide antibiotics (Zhao et al., 2001; Milona et al., 2007). The majority of the pharmaceutical effort has concentrated on the development of topically applied agents. The expense and difficulty of preparing large amounts of peptides and the uncertainty in their systemic use have slowed down their development beyond topical treatments.

Recent insights into the functions of $1,25(OH)_2$ vitamin $D_3$ (1,25D3) as an immune-modifying agent have illuminated a large body of previously unexplained associations between alterations in

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vitamin $D_3$ (Weber et al., 2005). Elevated 1,25D3 and hypercalcemia have been associated with active pulmonary tuberculosis (Liu et al., 2006), and lower serum concentrations of the 1,25D3 precursor 25OH vitamin $D_3$ (25D3) in African Americans correlates with increased susceptibility to infection (Liu et al., 2006). An explanation for these events has been provided by observations that stimulation of Toll-like receptors (*TLRs*) increases production of 1,25D3 in monocytes, which in turn leads to an increase in the production of antimicrobial peptides (AMPs) (Liu et al., 2006). Results of recent studies support a role of vitamin $D_3$ in the regulation of innate immune functions (Yim et al., 2007).

Activation of vitamin $D_3$ to 1,25D3 requires 2 major hydroxylation steps, the first by 25-hydroxylase (*CYP27A1*) and the second by 1α-hydroxylase (*CYP27B1*), enzymes located mainly in the human liver and kidney, respectively. However, some 1,25D3-targeted organs such as the epidermis also possess the enzymes to produce 1,25D3 (Evans et al., 2006; Schauber et al., 2007). Upon binding to the vitamin D receptor (*VDR*), 1,25D3 activates target genes through vitamin D-responsive elements (VDREs) in the gene promoter (Schauber et al., 2007). Simultaneously, 1,25D3 induces the vitamin $D_3$ catabolic enzyme 24-hydroxylase (*CYP24A1*), thereby initializing its own degradation. Control of 1,25D3-producing and catabolizing enzymes therefore determines the level of bioactive hormone.

Previous findings that 1,25D3 regulates the expression and activation of AMPs in monocytes and keratinocytes in the epidermis (Liu and Modlin, 2008) suggest that in addition to its effects on differentiation and formation of a physical barrier, 1,25D3 also provides a stimulus for rapid production of a chemical antimicrobial shield. In particular, 1,25D3 induces the expression of *LL-37*, a human AMP belonging to the cathelicidin family (Schauber et al., 2007; Liu et al., 2009). With the observation that cathelicidin is increased with increasing concentrations of 1,25D3 (Liu et al., 2007), the importance of vitamin $D_3$ for immune defense warrants renewed interest.

AMPs include defensins family and cathelicidin family. Molecular mechanisms of vitamin D controlling the expression of avian β-defensins (*AvBDs*) are still poorly understood. Moreover, little is known about the expression relationship of *AvBDs*, vitamin D, *TLRs*, and *VDR in vivo* of poultry. Control of *AvBDs* expression follows a pattern consistent with expectations for a gene required for innate immune response, we hypothesized that vitamin $D_3$ signaling may be activated during intraperitoneal injection of vitamin $D_3$. In this study, we investigated the expression of genes influenced by vitamin $D_3$. It has been shown, for the first time to our knowledge, that intraperitoneal injection of vitamin $D_3$ resulted in enhanced expression of *AvBDs*, *TLRs*, and *VDR*, which provided insight into factors important for the control of the innate immune response in chickens.

## MATERIAL AND METHODS

### Experimental birds and tissue collection

32 healthy Taihe Silky Fowls equally divided between male and female not significantly different from each other (mean body weight (BW) at 90 days was 1.32 ± 0.05 kg) were used. They were maintained under a light regimen of 14 h light : 10 h dark in individual cages and provided with feed and water *ad libitum* (Sezer and Tarhan, 2005). The birds were equally divided into two groups (male = female each) with or without vitamin $D_3$ (Sigma-Aldrich (Shanghai) Trading Co. Ltd., Shanghai, P.R. China) injection: (1) control birds which were not injected, (2) intraabdominally vitamin $D_3$-injected birds. The birds were injected in the belly cavity with vitamin $D_3$ (concentration 1 mg/ml) at a dose of 1 ml/kg body weight before tissues collection ($n$ = 4 each). To examine the general change of Avian *AvBDs*, *VDR*, and *TLRs* expression, the liver, spleen, and kidney of Taihe Silky Fowl were collected from birds of both groups. This study was carried out in accordance with the Guideline for Animal Experimentation, Sichuan Agricultural University, P.R. China.

### RNA extraction and RT-PCR

For extraction of total RNA, approximately 100 mg of tissues were homogenized in 1 ml of Trizol (Takara Bio Inc., Otzu, Japan) and processed for extraction according to the manufacturer's instructions. The quality and quantity of RNA in each sample was assessed at A260/280 nm and the resulting samples were treated with DNAase (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Total RNA samples were

reverse transcribed using RT-PCR kit (Takara Bio Inc., Otzu, Japan) according to the manufacturer's protocol, PCR reactions were carried out with the program as follows: 37°C reverse transcribed reaction for 15 min, followed by 85°C inactivation of reverse transcriptase for 5 s, to standardize the amount of template in each PCR reaction. Each of the 10 µl RNA amplification reaction mixture contained 0.5 µl PrimeScript™ RT Enzyme Mix I, 0.5 µl Oligo dT Primer (50µM), and 0.5 µl Random 6 mers (100µM) (Takara Bio Inc., Otzu, Japan). The resulting cDNA was diluted to 2 ng/ml, and the samples were stored at –80°C until further analysis.

## Quantitative real-time PCR

Real-time RT-PCR (SYBR Green I) analysis was performed on six differentially expressed genes, the liver, spleen, and kidney of Taihe Silky Fowl. Primers of target sequences were designed using Primer3 and synthesized by Takara Bio Inc., Otzu, Japan (Table 1). A two-step reverse transcription PCR method (Bílek et al., 2008) was used to generate cDNA using SYBR PrimeScript RT-PCR kit (Takara Bio Inc., Otzu, Japan) according to the manufacturer's protocol. Real-time fluorescent measuring was conducted on the iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, USA). Each of the 25 µl cDNA amplification reaction mixture contained 12.5 µl SYBR® *Permix Ex Taq*™ (2 ×), 0.5 µl sense primer (10µM), 0.5 µl antisense primer (10µM), 9.5 µl PCR water, and 2 µl cDNA template. All real-time PCR reactions were carried out with the same program as follows: 95°C cDNA initial denaturation for 2 min, followed by 45 cycles of 95°C denaturation for 15 s, 60°C annealing and extension for 1 min, and fluorescence measured after annealing and extension, and melting curve program (60–95°C with a heating rate of 0.1°C per s and a continuous fluorescence measurement) and finally a cooling step to 40°C. A 10-fold dilution series of cDNA were included in each run to determine PCR efficiency by constructing a relative standard curve. PCR $R^2$ values were consistently > 0.99 and they were used to convert the cycle threshold (Ct) values into raw data. All experiments contained a negative control and samples were analyzed in independent runs.

## Relative copy number calculation

Details of the following procedures ($2^{-\Delta\Delta Ct}$) are published, so the following materials and methods descriptions are abbreviated for the sake of brevity.

## Statistical analysis

Statistical analysis of the relative copy numbers of gene products was performed using SPSS software, Version 11.5 for MS Windows. Data were expressed as the mean ± SE. Significance of differences in *TLRs*, *VDR*, and *AvBDs* expressions, and vitamin $D_3$ treated groups were examined by one-way ANOVA, followed by Duncan's multiple range test. Differences were considered significant when $P$ values were < 0.05.

# RESULTS

## Confirmation of primer specificity

Specificity of RT-PCR products was documented with high resolution gel electrophoresis and re-

Table 1. Primer sequences specific for real-time PCR and for conventional PCR

| Target gene | Forward primer (5'-3') | Reverse primer (5'-3') | Accession numbers | Product length (bp) |
|---|---|---|---|---|
| *β-actin* | TCACCAACTGGGATGATATGGA | TTGGCTTTGGGGTTCAGG | NM_205518 | 118 |
| *GAL-1* | GCTGTTCTTGGTGGGGTTCTT | AGGATGAAGGGGAGGAGCA | NM_204993 | 120 |
| *AVBD-6* | AAATGGCCTCTCTGGCACTC | AGTTTTGGTGGTGATGTCTGGTT | NM_001001193 | 143 |
| *TLR2* | TCCATTGAGAAGAGCCACAAGA | AAAAGGCGAAAGTGCGAGAA | NM_204278 | 101 |
| *TLR5* | CCAGGTGTGCAGTATCTCCTCTT | CACATCCAAACATAAACCTCTCTCC | NM_001024586 | 150 |
| *VDR* | CAATGGTGGGAGGTTGGAG | AGTGGGGCTGATTGTGGTG | NM_205098 | 95 |
| *CYP24A1* | TGGAAAGCCTATCGGGACTATC | CTCCTTGGGTTTCATCAGTTTCTT | XM_428199 | 114 |

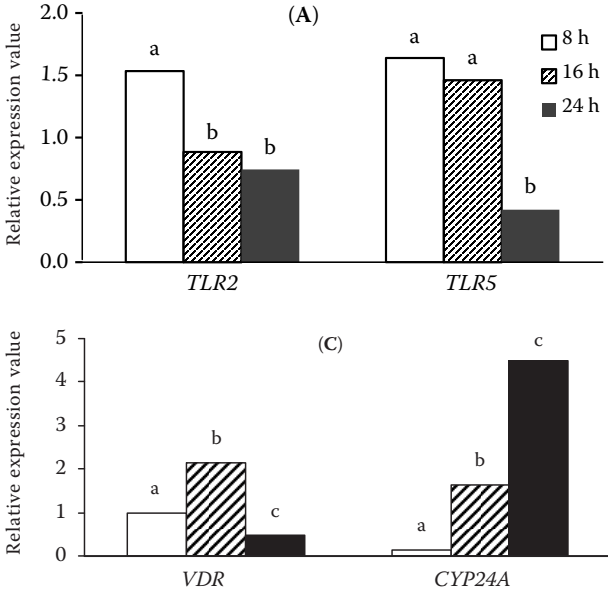


Figure 1. The effect of vitamin $D_3$ on the relative expression values of *TLRs*, *AvBDs*, *VDR*, and *CYP24A1* in liver during 8–24 h after injection. Bars with different letter sare significantly different at $P < 0.05$

sulted in a single product with the desired length (Table 1). In addition, the iQ5 real-time PCR detection system melting curve analysis was performed which resulted in single product specific melting temperatures. No primer-dimers were generated during the applied 45 real-time PCR amplification cycles.

## Vitamin $D_3$ induction of the gene expression

Within 24 h, vitamin $D_3$ induces an increase in the expression of *AvBDs*, *TLR2*, *TLR5*, *VDR*, and *CYP24A1* in the liver, kidney, and spleen of Silky Fowl. Figure 1 shows the effects of vitamin $D_3$ injected intraabdominally on the expression of *TLR2*, *TLR5*, *VDR*, *CYP24A1*, *AvBD-6*, and avian β-defensins gallinacin 1 (*GAL-1*) in the liver. The expression of *TLR2* and *TLR5* in the liver was rapidly increased and achieved peak at 8 h after injection (Figure 1A), and subsequently the expression of *GAL-1*, *AvBD-6* (Figure 1B), and *VDR* (Figure 1C) achieved peak at 16 h, at last the expression of *CYP24A1* achieved peak at 24 h (Figure 1C). We also hypothesized that *GAL-1* and *AvBD-6* expression may be regulated by vitamin $D_3$. To test this, the response of kidney and spleen *in vivo* to vitamin $D_3$ was studied.

Figure 2 shows the effects of vitamin $D_3$ on the expression of the six genes in kidney. Significant changes in the expression were observed for these

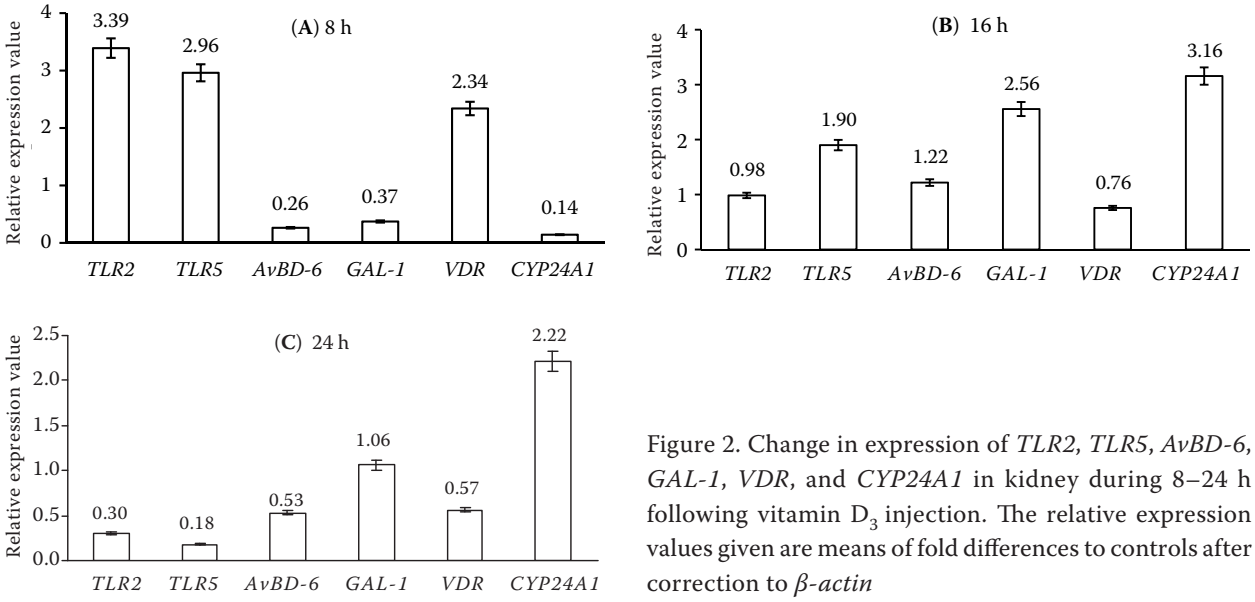


Figure 2. Change in expression of *TLR2*, *TLR5*, *AvBD-6*, *GAL-1*, *VDR*, and *CYP24A1* in kidney during 8–24 h following vitamin $D_3$ injection. The relative expression values given are means of fold differences to controls after correction to *β-actin*

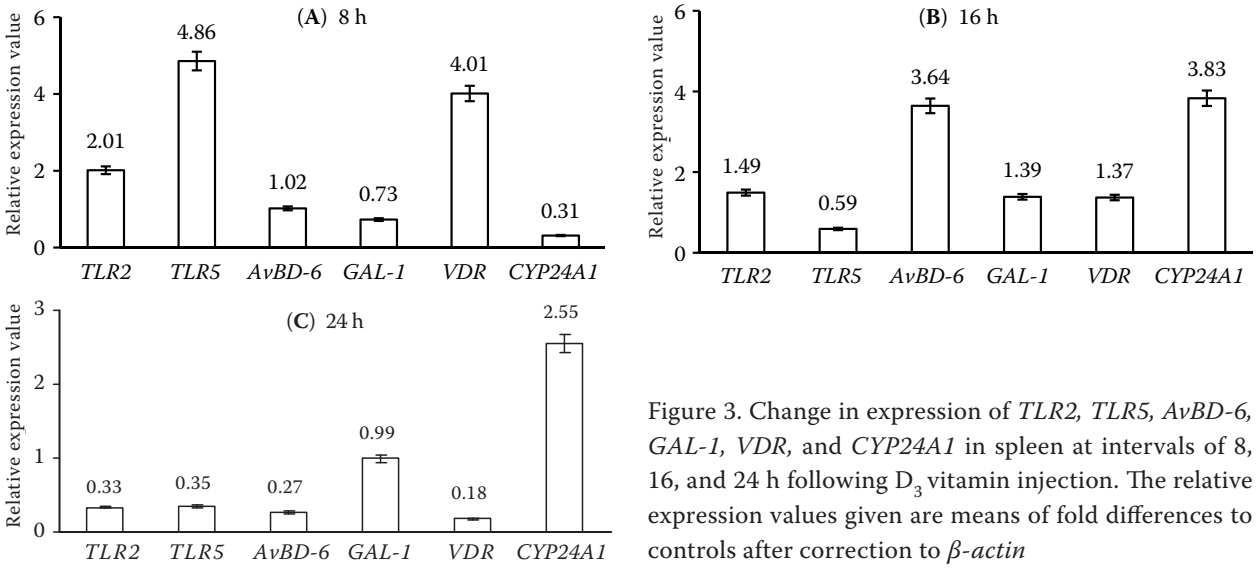


Figure 3. Change in expression of *TLR2, TLR5, AvBD-6, GAL-1, VDR,* and *CYP24A1* in spleen at intervals of 8, 16, and 24 h following $D_3$ vitamin injection. The relative expression values given are means of fold differences to controls after correction to *β-actin*

genes in the post-treatment from 8 to 24 h, relative to untreated controls. A significant increase of *TLR2*, *TLR5*, and *VDR* expression was observed at 8 h after injection (Figure 2A), and a similar response was not seen (Figures 2B and 2C). The *GAL-1* and *AvBD-6* also showed significant increase in expression for the first 16 h after injection (Figure 2B), and subsequently the expression of *CYP24A1* also achieved significant increase at 16 and 24 h (Figures 2B and 2C). The relative expression values for the six genes showed an increase tendency in *TLR2*, *TLR5*, and *VDR* at first 8h after injection, and subsequently the expression of *GAL-1* and *AvBD-6* achieved rapid increase at 16 h, at last the expression of *CYP24A1* also achieved the increase.

Similarly to the response seen *in vivo* following vitamin $D_3$ injection, levels of *TLR2, TLR5*, *VDR*, *CYP24A1*, *AvBD-6*, and *GAL-1* mRNA increased in spleen at different times (Figure 3), and the expression tendency of the six genes was similar in the tissues of kidney. Therefore, these observations show that vitamin $D_3$ at first induces *TLR2*, *TLR5*, and *VDR* in the tissues of liver, kidney, and spleen *in vivo* and then the same set of recognition and response elements of innate immunity is induced by vitamin $D_3$.

## DISCUSSION

Avian β-defensins, like other antimicrobial peptides (AMP), is considered to be a defense molecule by virtue of its antibacterial properties and is believed to contribute to innate immunity following exocytosis from the granular cells in the presence of bacteria (van Dijk et al., 2008). Recently, 1,25D3 was found to induce human AMP expression in keratinocytes *in vitro* and *in vivo* (Schauber et al., 2007) and an observed increase in cathelicidin expression after UVB irradiation was supposed to be caused by an increase in 1,25D3 (Weber et al., 2005). These observations led us to examine whether the regulation of vitamin $D_3$ is involved in the innate immunity response in chickens. We found multiple genes under the influence of vitamin $D_3$, and *AvBD-6* and *GAL-1*, to our knowledge previously unknown to be inducible by vitamin $D_3$, were induced in the tissues of liver, kidney, and spleen after injection. Our results suggest that vitamin $D_3$ after intraabdominal injection may stimulate the tissues of liver, kidney, and spleen to increase the metabolic conversion of 25D3 to 1,25D3, thus driving the expression and function of *AvBDs*, *VDR*, *TLR2*, and *TLR5* complex. The increase in *VDR* and *TLRs* enabled the tissues cells *in vivo* to further enhance the defensins expression, while also amplifying the generation of active vitamin $D_3$. The increase in the level of active vitamin $D_3$ also enhances the *CYP24A1* expression which increases the metabolic conversion of active vitamin $D_3$ to avoid excessive levels of vitamin $D_3$. To our knowledge, this elegant system of control of innate immunity by vitamin $D_3$ was previously unknown in chickens.

*In vivo*, chickens lacking the *CYP27B1* enzyme can respond to an increase in vitamin $D_3$-regulated *VDR* expression, however further investigations

are needed to confirm *in vivo* that the expression relationship of vitamin $D_3$ and *VDR* would be helpful. However, data derived from the results of injection of excess vitamin $D_3$ to Silky Fowl did confirm that vitamin $D_3$ can act *in vivo* to induce *TLR2*, *TLR5*, *VDR*, *AvBD-6*, and *GAL-1*. These observations complement, but are distinct from recent work in monocytes showing that activation of *TLR2* leads to an increase in 1,25D3 (Evans et al., 2006; Liu et al., 2006; Schauber et al., 2007). Notwithstanding, avian β-defensins expression in the Silky Fowl does vary with tissues, as shown in the vitamin $D_3$ challenge only experiment of the present study, where a significant up-regulation was seen. It confirms that treatment with vitamin $D_3$ in the present study provoked a distinct immune response. It is conceivable that particular *AvBDs* may respond differently to vitamin $D_3$ challenge. Unfortunately, we cannot tell at present if differential expression of the *AvBDs* occurs.

The link between vitamin $D_3$ and immune function in the liver, kidney, and spleen was further underlined by correlation with expression of *VDR*, *TLR2*, and *TLR5*. These genes were expressed at similar levels in the first 8 h in these tissues, and significant correlation with *AvBD-6* and *GAL-1* expression was observed in the tissues of liver, kidney, and spleen. Relatively little is known about the expression and function of *TLRs* and *VDR* in immune regulation of *AvBDs* by vitamin $D_3$. *TLRs* and associated signaling peptides have been shown to be functionally active in human immune cells (Liu et al., 2006). The principal ligand for *TLRs*, *VDR*, can act as a potent stimulator of human AMP expression in macrophages (Krutzik et al., 2008), epithelial cells (Yim et al., 2007; Schauber et al., 2008). It therefore seems likely that a similar mechanism for induction of *AvBDs* synthesis is present in liver, kidney, and spleen of Silky Fowl, although the specific stimulus for *TLRs*, *VDR*, and the target tissues expressing this receptor has yet to be determined *in vivo* and *ex vivo*.

In conclusion, the results of this study suggest that the genes of *VDR*, *TLR2*, *TLR5*, *AvBD-6*, *GAL-1*, and *CYP24A1* are induced expression by vitamin $D_3$ in the liver, kidney, and spleen of Silky Fowl during various times. The tissues expressing *TLRs* and *VDR* respond to vitamin $D_3$, likely leading to upregulation of avian β-defensins *AvBD-6* and *GAL-1*. Such function of vitamin $D_3$ in the Silky Fowl tissues may play essential roles in the control of the innate immune response *in vivo*.

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*Corresponding Author*

Prof. Dinggang Zhou, Sichuan Agricultural University, College of Animal Science and Technology, Yaan, Sichuan 625014, P.R. China
Tel. +86 08 352 886 103, e-mail: lisiming16@yahoo.com.cn