The immunomodulatory effect of vitamin D in chickens is dose-dependent and influenced by calcium and phosphorus levels

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ABSTRACT Vitamin D requirement is estimated to be higher than recommended values for the first two weeks of a broiler chicken's life, and is heavily dependent on the concentrations of Ca and P in the diet. There are data indicating the beneficial effect of higher vitamin D levels on performance and overall health of the chickens. However, data on the role of higher vitamin D levels on the innate immune response of chickens are limited. Therefore, in the current study, we examined the effect of higher doses of vitamin D supplementation on the innate immune response in broiler chickens receiving optimal or calcium (Ca) and phosphorus (P) deficient diets. Three hundred Ross-308 male broiler chicks were randomly allocated into 60 cages with 5 birds per cage in a 3 × 2 factorial design with three levels of vitamin D and two levels of Ca/P with each experimental diet fed to 10 cages (10 replicates). Quantitative reverse transcription PCR (n = 5) was used to assess Toll-like receptor (TLR2b and 4), cytokine/chemokine (IL-12, IFN-γ, IL-10, IL-4, IL-13, IL-18, CxCLi2) and cathelicidin (CATH1, CATHB1, CATH3) transcription levels in peripheral blood mononuclear cells (PBMCs), spleen, and bursa of Fabricius. Vitamin D supplementation of the Ca and P deficient diet considerably augmented transcription of TLR2b, TLR4, CATH1, and CATHB1 and predominantly Th2 cytokines in spleen. Supplementation of the control diet with vitamin D downregulated TLR4 transcription, and dose-dependently increased CATH1, CATHB1, Th1, and Th2 cytokine transcription (Th2>Th1). All diets downregulated CATH3 transcription. In conclusion, vitamin D or its derivative 25-OH-D_3 both have a robust immunomodulatory property with a more favorable Th2 response, while at the same time enhancing observed Th2 cytokine responses under both optimal and lower Ca and P inclusion levels in the diets of broiler chickens.

Key words: Vitamin D, TLRs, cytokines, antimicrobial peptides, cathelicidins

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INTRODUCTION

Vitamin D, a fat-soluble vitamin that is critical in the regulation of calcium (**Ca**) and phosphorus (**P**) homeostasis, circulates throughout the bloodstream in its major form, 25-hydroxycholecalciferol (25-OH-D_3). Dietary vitamin D is absorbed by the small intestine and is rapidly taken up by the liver where it is hydrolyzed by the enzyme 25-hydroxlyase to 25-OH-D_3, the storage form of vitamin D in the body, which is further converted by 1-α-hydroxylase in the kidney to its biologically active form, 1,25$(OH)_2D_3$. This pleiotropic hormone exerts a variety of biological effects including the regulation of bone and mineral metabolism as well as the modulation of the immune response (Schwarz et al., 2012). 1-α-hydroxylase is expressed by different immune cells such as macrophages and dendritic cells, and high concentrations of 1,25$(OH)_2D_3$ can be found in lymphoid microenvironments (Overbergh et al., 2000; Veldman et al., 2000; Chen et al., 2007). Hereby its specific action is increased and potentially undesirable systemic effects, such as hypercalcaemia and increased bone resorption are limited.

In its biologically active form, vitamin D has multiple effects on both the innate and adaptive immune systems. 1,25$(OH)_2D_3$ primed human monocytes respond less effectively to bacterial cell wall components, most likely due to decreased levels of Toll-like receptor (**TLR**)2 and TLR4 (Sadeghi et al., 2006). Recently, Shojadoost et al. (2015) showed that 1,25$(OH)_2D_3$ has an immunomodulatory property in chicken macrophages, while in the absence of Lipopolysaccharides (LPS) and Pam_3CSK_4, mainly a

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downregulation in CD86, MHC-II, CXCL8, and IL-1β was observed. TLRs are key players in the innate immune response to microbial pathogens by early detection of microbial-associated molecular patterns (**MAMPs**) followed by a cascade of events resulting in clearance of infection, and subsequently activating the adaptive immune response (Opal and Huber, 2002; Eriksson et al., 2003). 1,25$(OH)_2D_3$ exerts an inhibitory effect on adaptive immune cells in vitro, e.g., by inhibiting T cell proliferation and the production of interleukin (IL)-12 and interferon-gamma (IFN-γ) by T cells (Mora et al., 2008). This reduction of IFN-γ and IL-12 also followed a concomitant increased production of IL-4 and IL-10 thereby skewing the T cell differentiation towards T-helper (Th) 2 and regulatory T cells (Mathieu and Adorini, 2002; van Etten and Mathieu, 2005; Baeke et al., 2010). After exposure to a physiological level of 1,25$(OH)_2D_3$, human and C57BL/6 mice dendritic cell (**DC**) maturation was inhibited due to decreased expression of MHC class II, CD40, CD80, CD86, and IL-12 (Griffin et al., 2001; van Etten and Mathieu, 2005; Sochorova et al., 2009). Treatment of macrophages with 1,25$(OH)_2D_3$ induced expression of the cathelicidin LL-37 and bacterial killing (Liu et al., 2006, 2007; Berrington and Hawn, 2007). Importantly, expression of the sole human cathelicidin LL37 was induced by vitamin D through an active vitamin D receptor element (VDRE) in the promoter of its gene (Wang et al., 2004). Up to date four chicken cathelicidins have been described: CATH1-3 and CATHB1. Even though knowledge about the functions of the avian cathelicidins is limited, preliminary results point towards activities comparable to mammalian homologs (Cuperus et al., 2013).

In chickens, varying levels of the vitamin D metabolite, 25-OH-D_3 on bone, kidney, and intestinal health have been studied extensively (Yarger et al., 1995; Mitchell et al., 1997). When Ca and P are deficient in the diet, vitamin D acts on the kidney and bone for new mineralization; but when Ca and P levels in diet are sufficient, vitamin D acts on the intestine as a result of suppressed parathyroid hormone due to the actions of both Ca and 1,25$(OH)_2D_3$ in the parathyroid gland thereby conserving bone resorption and bone Ca (Plum and DeLuca, 2009). The requirement for vitamin D of starting broilers has been estimated to be higher than that recommended by NRC (5 μg/kg or 200 IU/kg), and requirements can vary depending on the concentrations of dietary Ca and P (Waldroup et al., 1965; Kasim and Edwards Jr, 2000). This could have wider implications from bone mineral density to immunity of broiler chickens. Indeed, higher concentrations of vitamin D have been shown to prevent tibial dyschondroplasia (**TD**), and vitamin D requirement of broiler chickens up to 14d of age was reported to be in the range of 35 to 50 μg/kg at optimal Ca and P concentrations in the diet (Whitehead et al., 2004). However, there is scant information on the effect of higher concentrations of vitamin D in the diet of broiler chickens on the innate immune response both under sufficient and deficient levels of Ca and P in the diet. Therefore, the objective of the current study was to evaluate the effects of higher concentrations of 25-OH-D_3 at optimal, and low Ca and P levels in the diet on the innate immune responses of unchallenged broiler chickens.

MATERIALS AND METHODS

Birds and Experimental Design

All experimental procedures were reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and chickens were handled according to the guidelines described by the Canadian Council on Animal Care (CCAC).

Animal Trial

In total, 300 day-old, unvaccinated, Ross 308 male broiler chicks (Carlton Hatchery, Grunthal, Manitoba, Canada) were randomly allocated into 60 cages with 5 birds per cage. Each experimental diet was fed to 10 cages (10 replicates). A 3 $\times$ 2 factorial design with three levels of vitamin D and two levels of Ca/P was used with animal as an experimental unit. Treatments were; (1) control diet, (2) low Ca and P diet (LCP), (3) LCP plus 2,760 IU/kg of additional 25-OH-D_3 (25(OH)D-Min-LCP), (4) LCP plus 9,800 IU/kg of additional 25-OH-D_3 (25(OH)D-Max-LCP), (5) control with 2,760 IU/kg of additional 25-OH-D_3 (25(OH)D-Min), and (6) control with 9,800 IU/kg of additional 25-OH-D_3 (25(OH)D-Max). All dietary treatments contained 200 IU/kg of 25-OH-D_3, the standard concentration of vitamin D in broiler chicken feed. Birds received water and feed ad libitum. At the end of the trial (d 21), blood from the wing veins, and bursa of Fabricius and spleen were collected from the same 5 birds per treatment. Sampling time (d 21) was selected to study the effect of high vitamin D3 requirement in the first 3 weeks of age on the innate immune response of chickens. Samples were snap frozen in liquid nitrogen and stored at -80°C until further processing for gene expression analysis.

PBMCs Isolation

Isolation of PBMCs was conducted using density gradient centrifugation with Ficoll-Paque Plus (GE Health care, Piscataway, NJ) following the manufacturer's procedure. Briefly, the blood from K_2EDTA vacutainer tubes was diluted 1:1 with Dulbecco's PBS and placed on ice. The blood was carefully layered into a tube containing an equal volume of Ficoll (Histopague-1077, Sigma-Aldrich, St. Louis, MO) to form a distinct layer above the Ficoll. Tubes were centrifuged at 400 $\times$ g for 30 min at room temperature and the mononuclear cell layer (buffy coat) were removed and transferred to different centrifuge tubes until RNA extraction.

Table 1. Chicken toll-like receptors, cytokines, and cathelicidin primer and probe sequences. Listed oligonucleotides were used to analyse gene expression via quantitative real-time polymerase chain reaction (**qRT-PCR**).

Gene	Primer sequence (5′-3′)	Annealing temp (°C)	Accession number
β-actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	61	X00182
GAPDH	F: GTAAACCATGTAGTTCAGATCGATGA R: GCCGTCCTCTCTGGCAAAG Pr: AGTGGTGGCCATCAATGATCCC	60	NM204305
TLR2b	F: CGCTTAGGAGAGACAATCTGTGAA R:GCCTGTTTTAGGGATTTCAGAGAATTT	59	NM204278
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	60	AY064697
IL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	59	AJ309540
IL-12p35	F: CTGAAGGTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAG	64	NM213588
IL-18	F: CTGCCTAAACCCCAAGGAA R: AACCCTACACCAGACCCACA	59	NP_001025729
CxCLi2	F: CCAAGCACACCTCTCTTCCA R: GCAAGGTAGGACGCTGGTAA	55	NM_205498
IFN-γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	60	X99774
IL-4	F: TGTGCCCACGCTGTGCTTACA R: CTTGTGGCAGTGCTGGCTCTCC	55	NM_001007079
IL-13	F: ACTTGTCCAAGCTGAAGCTGTC R: TCTTGCAGTCGGTCATGTTGTC	60	NM_001195791
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	55	AJ621614
CATH1	F: GTCGACCCTGTCCGCGTCA R: GAGGTTGTATCCTGCAATCAC Pr: CCTGATGACCAGCGGC	60	DQ092351
CATH3	F: CCATGGCTGACCCTGTCC R: TGATGGCTTTGTAGAGGTTGATG Pr: CGCAGCCACCGTGTTG	60	DQ092353
CATHB1	F: GTGTCCATAGAGCAGCAG R: ATTCAACCACTCCCAGATG Pr: TCCACCAGTTGCGGAT	65	AB307733

Total RNA Extraction and Reverse Transcription

Extraction of total RNA from PBMCs, bursa of Fabricus, and spleen samples was performed using Trizol Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) as instructed in the manufacturer's protocol. Processing of RNA and reverse transcription was carried out as described previously (Rodríguez-Lecompte et al., 2012).

Quantitative Reverse Transcription PCR

Quantitative Reverse Transcription PCR (**qRT-PCR**) for all target genes but the cathelicidins was performed using the Step One thermo cycler (Applied Biosystem, Mississauga, Ontario, Canada). A detailed description of the methodologies and the different parameters used has been described previously (Yitbarek et al., 2012). For the cathelicidins, qRT-PCR was carried out using the CFX Connect Real Time PCR Detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands) on a 96-well plate with 25 μL of total reaction volume and with each PCR run including a no-template control and a standard curve generated from serially diluted pooled cDNA of all experimental samples. iQ Supermix (Bio-Rad Laboratories, Veenendaal, The Netherlands) was used according to the manufacturer's instruction. The thermal cycling protocol consisted of initial denaturation at 95°C for 5 minutes, followed by amplification for 40 cycles consisting of 20 seconds at 95°C, 30 seconds at annealing temperature (see Table 1) and 40 seconds at 72°C. Sequences to designed primers for β-actin, GAPDH, TLR2b, TLR4, IL-6, IL-10, IL-12p35, IL-13, IL-18, chemokine CxCLi2 (previously IL-8), IFN-γ and the chicken cathelicidins CATH1, −3, and −B1 were obtained from GenBank (Table 1). Probes (for GAPDH and cathelicidins only) were designed with a 5′-FAM^{TM} label and LNA^{TM} bases for enhanced specificity.

Statistical Analysis

The Proc Mixed Procedure of SAS (SAS institute, Cary, NC) was used to analyze CT values for all genes based on a 3 × 2 factorial design with three levels of vitamin D and two levels of Ca/P and animal as an experiment unit. Levels of expression for all genes were calculated relative to β-actin (TLRs and cytokines) or GAPDH (cathelicidins), and gene expression was presented as fold changes relative to the control diet. Gene expression fold change, standard error and statistical significance were calculated using REST 2009 (Qiagen,

Valencia, CA; Pfaffl, 2001) where all data were considered significantly different at $P < 0.05$.

RESULTS

All gene expression analysis results are presented as fold changes compared to the control diet. No Ca and P level × vitamin D dose interaction effect was observed in the expression of TLR2b and TLR4 in the PBMCs and bursa of Fabricius. Expression of TLR2b and TLR4 in PBMCs was not affected by treatments, except for a downregulation of TLR4 expression in the 25(OH)D-Min-LCP treatment ($P = 0.043$) (Figure 1a). In the spleen, a Ca and P level × vitamin D dose interaction effect was observed in TLR2b and TLR4 ($P = 0.002$ and 0.0264, respectively). Expression of TLR2b in the spleen was upregulated in birds that received the LCP ($P = 0.041$), 25(OH)D-Min-LCP ($P = 0.042$), and 25(OH)D-Max-LCP ($P = 0.000$) treatments, whereas it was downregulated in the 25(OH)D-Min treatment ($P = 0.030$) (Figure 1b). Expression of TLR4 in the spleen was upregulated in the 25(OH)D-Max-LCP ($P = 0.031$) and 25(OH)D-Min ($P = 0.000$) treatments and downregulated in the 25(OH)D-Max treatment ($P = 0.046$) (Figure 1b). Both TLR2b and TLR4 expressions in the bursa of Fabricius showed no significant difference among all treatments, except for a downregulation of TLR4 expression in the 25(OH)D-Min treatment ($P = 0.000$) (Figure 1c).

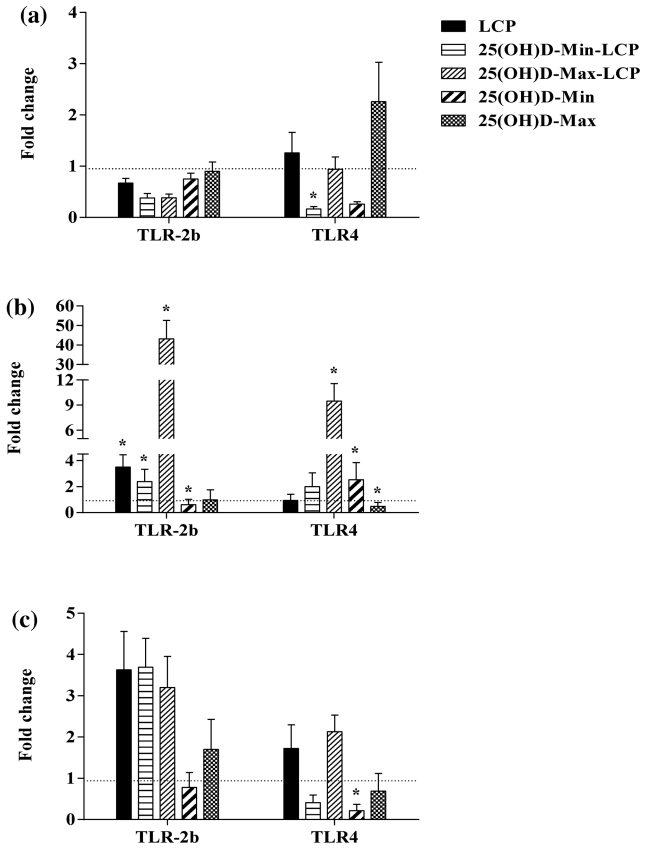

Figure 1. TLR2b and TLR4 gene expression in PBMCs (a), spleen (b) and bursa of Fabricius (c) of broiler chickens fed diets differing in Ca, P and 25-OH-D_3 levels. Tissue samples were collected at end of trial (d21 of age) and analysed using qRT-PCR normalized against β-actin (housekeeping gene) expression. Data are presented as fold changes compared to control treatment (dotted line) (n = 5). *An asterisk indicates that the bars differ significantly from the control treatment at $P < 0.05$.

Cytokine analysis in PBMCs showed that there was no significant up or dowregulation in all the genes analyzed in all treatments, except in the 25(OH)D-Min-LCP and 25(OH)D-Min treatments where significant downregulation of both IL-18 and CxCLi2 was observed ($P < 0.001$) (Figure 2). Furthermore, No Ca/P × vitamin D interaction effect was observed in the expression of all cytokines in the PBMCs ($P > 0.05$). In the spleen, expression of IL-12p35 was upregulated in all treatments ($P < 0.046$, Figure 3a). Expression of IL-18 was upregulated in the LCP and 25(OH)D-Max-LCP treatments ($P < 0.032$), while it was downregulated in the 25(OH)D-Min-LCP and 25(OH)D-Min treatments ($P < 0.007$) (Figure 3b). Expression of IFN-γ was upregulated in all LCP treatments ($P < 0.043$) while it was downregulated in the 25(OH)D-Min treatment ($P = 0.000$) (Figure 3c). Expression of IL-10 was upregulated in all treatments ($P < 0.050$), except in the 25(OH)D-Min treatment where no significant change was observed (Figure 3d). Expression of IL-4 was upregulated in all LCP treatments ($P < 0.012$) (Figure 3e). Expression of IL-13 was upregulated in the LCP and 25(OH)D-Max-LCP ($P = 0.031$ and 0.003, respectively) (Figure 3f). Expressions of CxCLi2 was upregulated in the LCP, 25(OH)D-Max and 25-OH-D3-Max-LCP treatments compared to control ($P < 0.032$), while it was downregulated in the 25(OH)D-Min and 25(OH)D-Min-LCP treatments ($P < 0.007$) (Figure 3g). A significant Ca and P level × vitamin D dose interaction effect was observed in the expression of only IL-18 and CxCLi2 of all the cytokines in the spleen ($P = 0.0004$ and 0.032, respectively).

In the bursa of Fabricius, expression of IL-12p35 was upregulated in all treatments ($P < 0.05$), except in the 25(OH)D-Min where no change was observed (Figure 4a). Higher expression of IFN-γ was observed in the LCP ($P = 0.042$) and 25(OH)D-Max-LCP ($P = 0.039$) treatments (Figure 4c). Significant upregulation of IL-10 was observed in all treatments ($P < 0.045$) (Figure 4d). Both IL-4 and IL-13 showed similar trends where an upregulation of both cytokines was observed in the LCP, 25(OH)D-Min-LCP and 25(OH)D-Max-LCP treatments ($P < 0.037$) (Figure 4e and f). A downregulation of CxCLi2 in the 25(OH)D-Min treatment was observed ($P < 0.05$) (Figure 4g). No Ca and P level × vitamin D dose interaction effect was observed in the expression of all the cytokines, except for CxCLi2 ($P = 0.0124$).

For all the cathelicidins, no significant up or downregulation in PBMCs was observed (Figure 5a). In the spleen, CATH-1 was upregulated in the 25(OH)D-Min-LCP, 25(OH)D-Max-LCP and 25(OH)D-Max treatments ($P < 0.048$), while it was downregulated in the

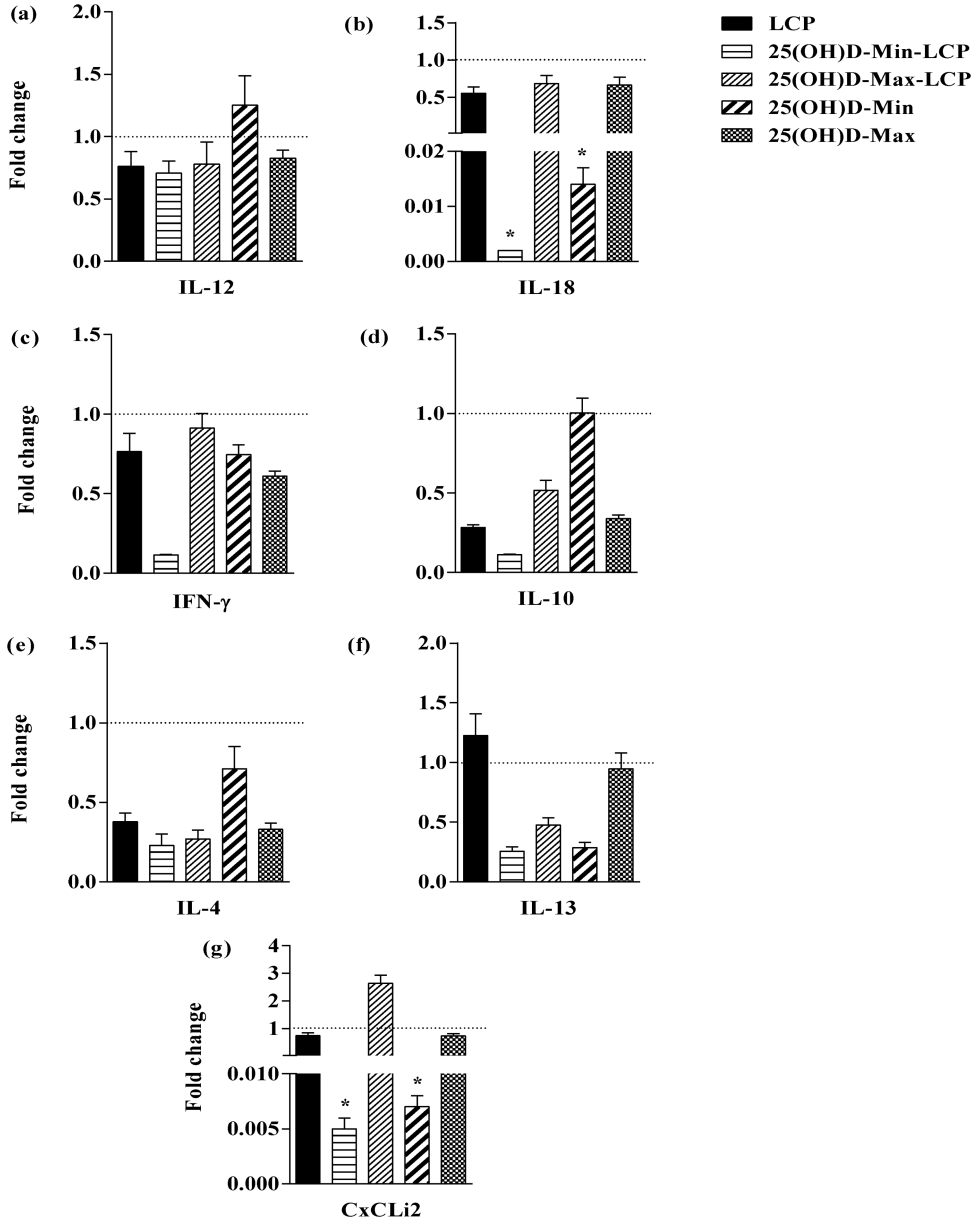

Figure 2. Cytokine IL-12 (a), IL-18 (b), IFN-γ (c), IL-10 (d), IL-4 (e), IL-13 (f), and chemokine CxCLi2 (g) gene expression in PBMCs of broiler chickens fed diets differing in Ca, P and 25-OH-D_3 levels. Tissue samples were collected at end of trial (d 21 of age) and analysed using qRT-PCR normalized against β-actin (housekeeping gene) expression. Data are presented as fold changes compared to control treatment (dotted line) (n = 5). *An asterisk indicates that the bars differ significantly from the control treatment at $P < 0.05$.

LCP treatment ($P = 0.014$) (Figure 5b-i). Expression of CATH-B1 was upregulated in the 25(OH)D-Min-LCP, 25(OH)D-Max-LCP and, 25(OH)D-Max treatments ($P < 0.016$) (Figure 5b-ii). Expression of CATH-3 in the spleen was downregulated in all treatments ($P < 0.049$) (Figure 5b-iii). Except for an upregulation of CATH-1 in the 25(OH)D-Max treatment ($P = 0.040$), no treatment effect in cathelicidin's expression was observed in the bursa of Fabricius ($P = 0.040$) (Figure 5c). Furthermore, no Ca and P level × vitamin D dose interaction effect was observed in the expression of all cathelicidins in all tissues except for the expression of CATH-3 in the spleen ($P = 0.016$).

DISCUSSION

Knowledge about how the chicken immune system responds to vitamin D and its metabolite 25-OH-D_3 is far from complete. The present study evaluated the influence of vitamin D on the gene expression of TLRs, cytokines, a chemokine, and cathelicidins in PBMCs, spleen, and bursa of Fabricius of broiler chickens under non-challenged conditions and with different levels of Ca and P in the diet. Supplementation of the control diet with a high concentration of vitamin D resulted in downregulation of TLR4 expression in spleen tissue, while TLR2b expression was not affected. This is in line

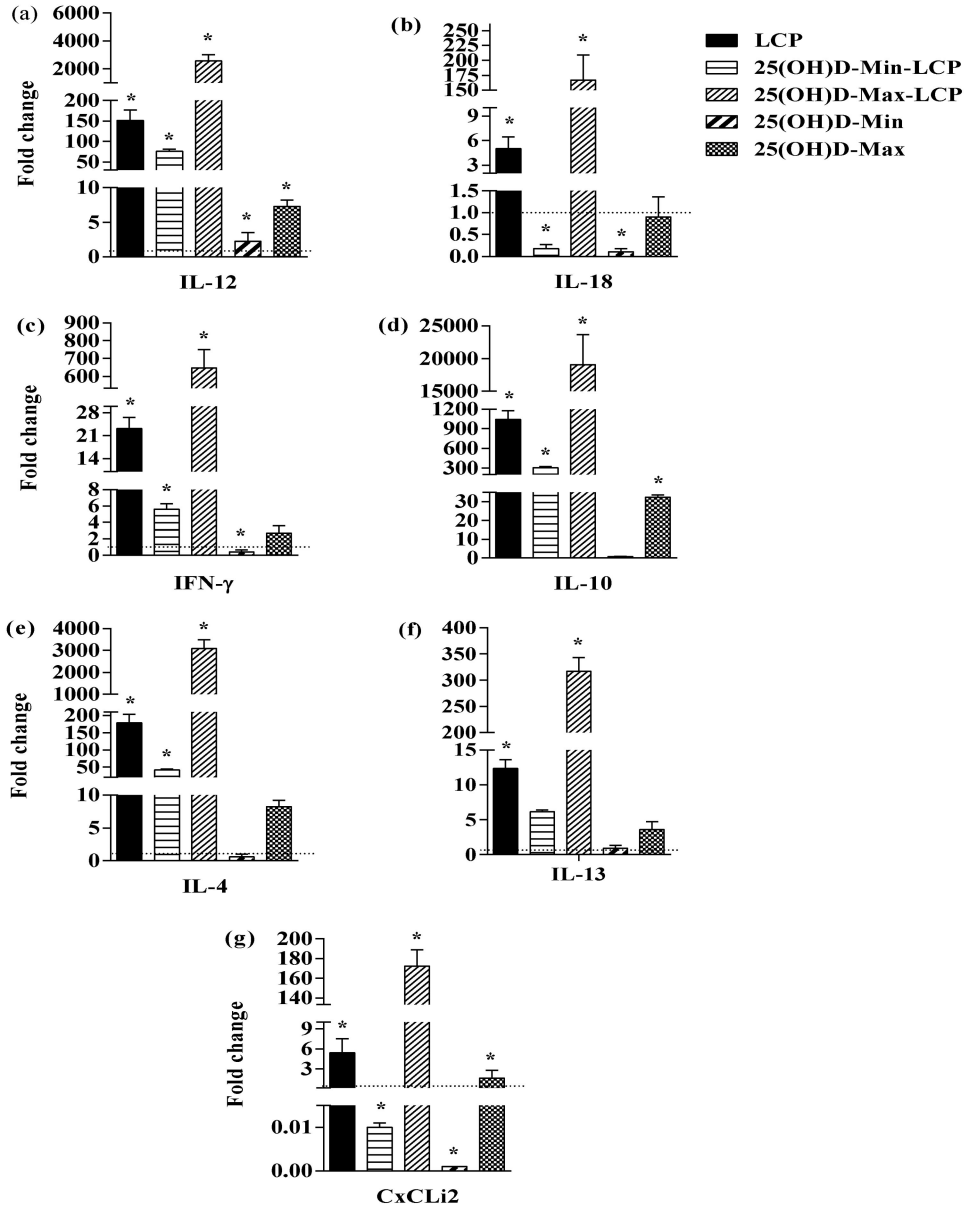

Figure 3. Cytokine IL-12 (a), IL-18 (b), IFN-γ (c), IL-10 (d), IL-4 (e), IL-13 (f), and chemokine CxCLi2 (g) relative gene expression in the spleen of broiler chickens fed diets differing in Ca, P and 25-OH-D_3 levels. Tissue samples were collected at end of trial (d 21 of age) and analysed using qRT-PCR normalized against β-actin (housekeeping gene) expression. Data are presented as fold changes compared to control treatment (dotted line) (n = 5). *An asterisk indicates that the bars differ significantly from the control treatment at $P < 0.05$.

with other studies that have shown that 1,25$(OH)_2D_3$ exerts its effect by downregulating TLR2 and TLR4 thereby inducing a hypo-responsive effect to MAMPs (Sadeghi et al., 2006). However, supplementation of the Ca/P reduced diet with the same dose of vitamin D resulted in an upregulation of TLR2b expression, and to a lesser extent TLR4 in spleen tissue. Activation of both TLR2 and TLR4 has been shown to increase the expression of VDR and 1-α-hydroxylase (Liu et al., 2006; Adams and Hewison, 2008). Thus, additional examination is warranted to further elucidate the relationship between Ca and P homeostasis and TLR2 and TLR4 expression.

Previous studies suggested that the main immunomodulatory property of vitamin D would be to inhibit pro-inflammatory cytokine production while enhancing the expression of anti-inflammatory cytokines by acting directly on T lymphocytes or antigen presenting cells (Lemire et al., 1995; Boonstra et al., 2001). Control diet (200 IU/kg) supplementation with vitamin D up to 2,760 IU/kg resulted in downregulation of CXCLi2 and IL-18 in the spleen and PBMCs, with CxCLi2 also being downregulated in the bursa of Fabricius, suggesting a predominant anti-inflammatory action of vitamin D at this concentration. CXCLi2, one of the two human CXCLi8 orthologs found in chickens, predominantly attracts monocytes (Kaiser and Stäheli, 2013). In macrophages, VDR signaling limits inflammatory responses by targeting the miR-155-SOCS1 pathway, an important regulator of innate immunity and

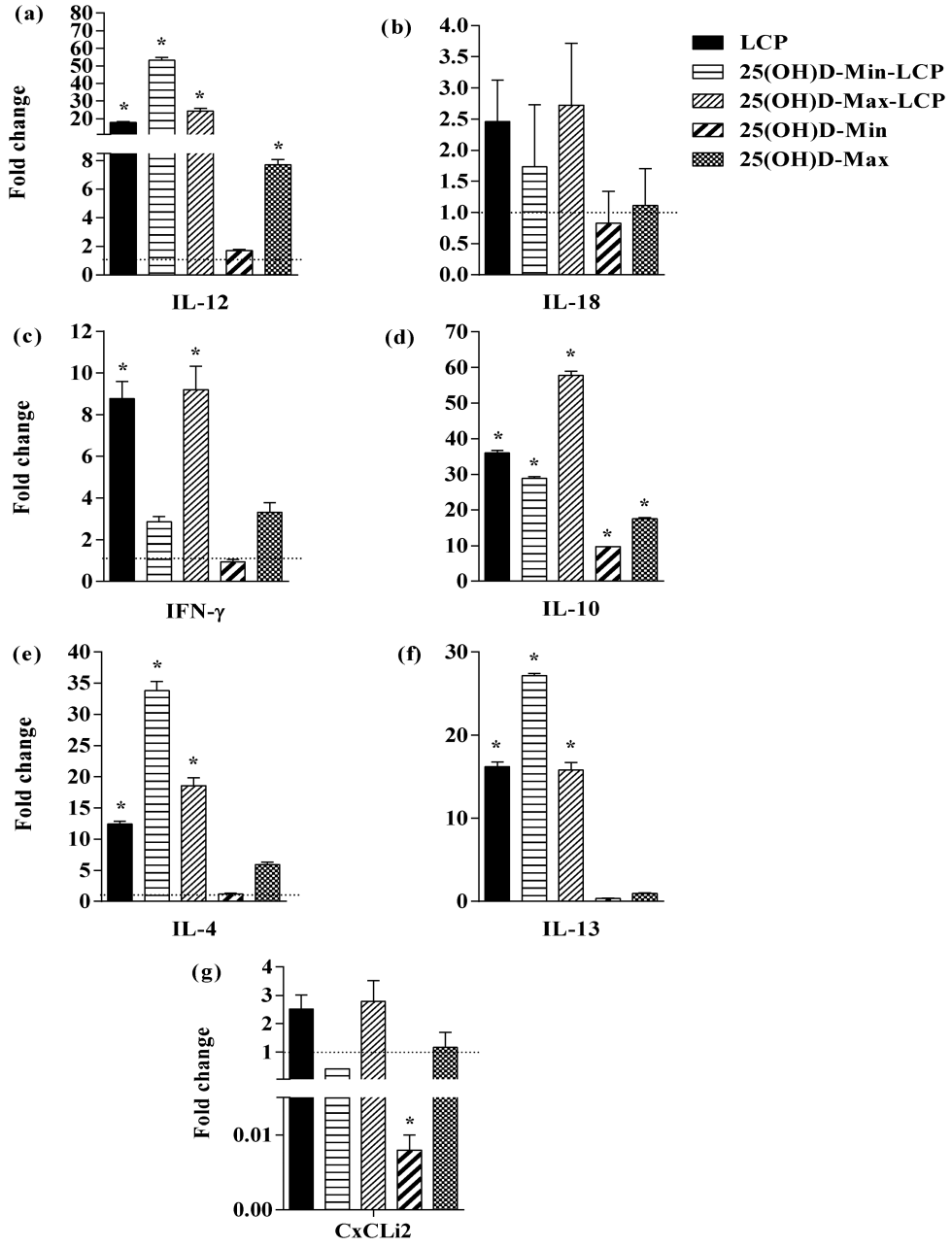

Figure 4. Cytokine IL-12 (a), IL-18 (b), IFN-γ (c), IL-10 (d), IL-4 (e), IL-13 (f), and chemokine CxCLi2 (g) relative gene expression in the bursa of Fabricius of broiler chickens fed diets differing in Ca, P and 25-OH-D_3 levels. Tissue samples were collected at end of trial (d 21 of age) and analysed using qRT-PCR normalized against β-actin (housekeeping gene) expression. Data are presented as fold changes compared to control treatment (dotted line) (n = 5). *An asterisk indicates that the bars differ significantly from the control treatment at $P < 0.05$.

TLR signaling pathways (O'Neill et al., 2011; Chen et al., 2013). In this way, a more Th2 favoring environment may be formed. Whether Th1 or Th2 cells will predominate is mainly determined by the microenvironment in which these naive T-helper cells develop (Cantorna et al., 2004). Supplementation of the control diet with a higher dose of vitamin D (9,800 IU/kg) resulted in CXCLi2 and IL-18 expression in spleen and PBMCs as well as CXCLi2 in bursa of Fabricius comparable to base line.

Reduction of dietary Ca and P levels resulted in overall enhanced cytokine expression in spleen, but with an IL-12/IL-10 ratio in favor of an anti-inflammatory status. Supplementation of this diet with 2,760 IU/kg of vitamin D reduced in spleen the expression of CXCLi2 and all tested cytokine genes. At the highest dose, vitamin D supplementation of the low Ca and P diet resulted in higher Th1 (IL-12, IFN-γ and IL-18) and Th2 (IL-4, IL-10 and IL-13) cytokines in the spleen and bursa of Fabricius, and a higher CXCLi2 in the spleen. IL-12 and IL-18 are known to trigger signaling pathways leading to the transcription of pro-inflammatory cytokines. For instance, IFN-γ induction via activation of STAT4 and AP-1 promotes the recruitment of inflammatory cells and killing of pathogens (Nakahira et al., 2002). However, the more pronounced Th2

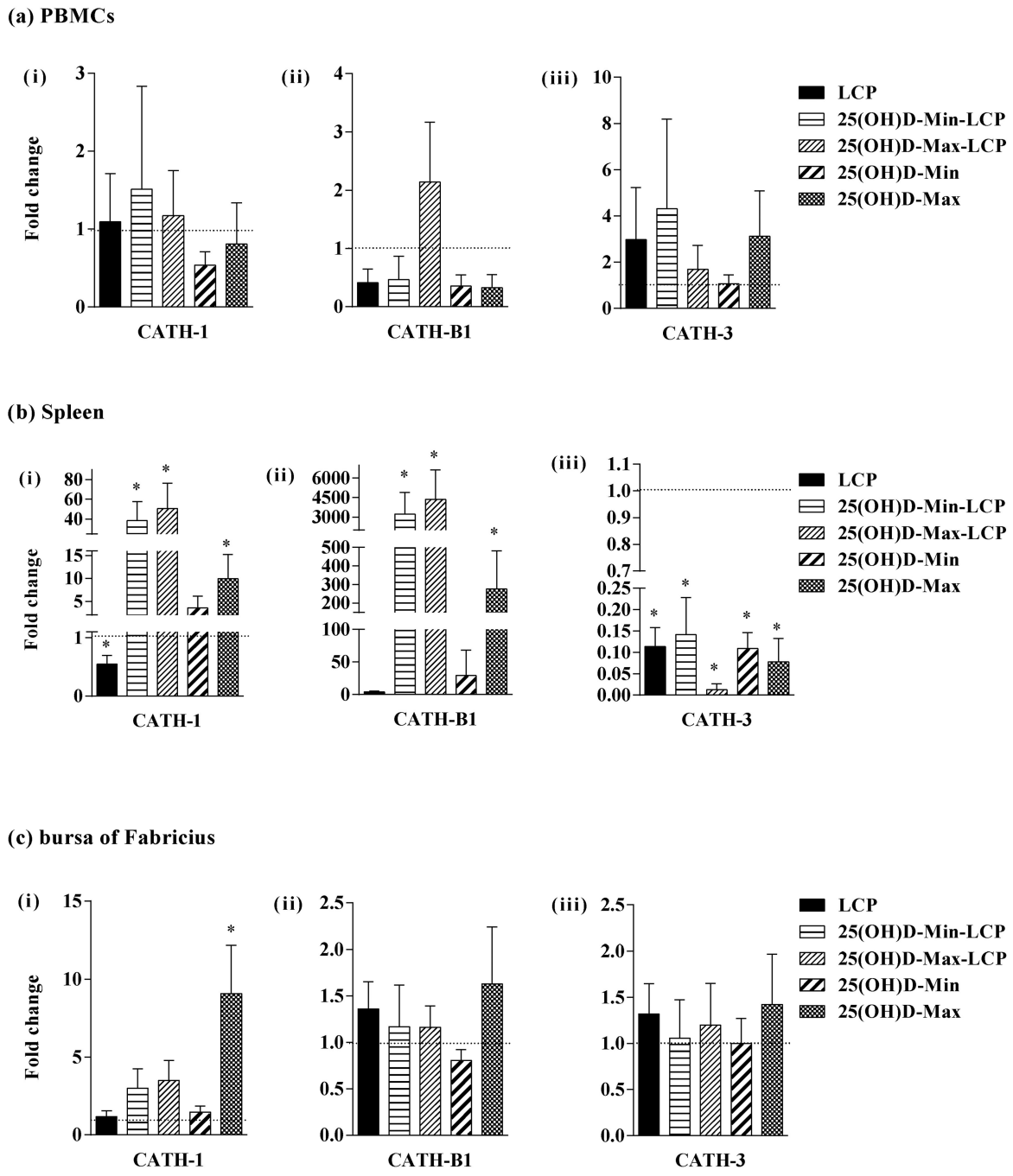

Figure 5. CATH-1 (i), CATH-B1 (ii), and CATH-3 (iii) gene expression in PBMCs (a), spleen (b), and bursa of Fabricius (c) of broiler chickens fed diets differing in Ca, P and 25-OH-D_3 levels. Tissue samples were collected at end of trial (d 21 of age) and analysed using qRT-PCR normalized against GAPDH (housekeeping gene) expression. Data are presented as fold changes compared to control treatment (dotted line) (n = 5). *An asterisk indicates that the bars differ significantly from the control treatment at $P < 0.05$.

cytokine response in spleen suggests a more anti-inflammatory mode of action of vitamin D in this organ.

Cathelicidins are a group of Host Defense Peptides (**HDPs**), multifunctional peptides with both antimicrobial and immunomodulatory functions. Among the diverse functions described for mammalian cathelicidins are chemotaxis, stimulation of phagocytosis, activation and differentiation of immune cells and inhibition of LPS mediated effects (Zanetti, 2005). In our study, the three chicken cathelicidins were found to respond differently to vitamin D. Increased bursal expression of CATH1 has been reported by others (Zhang et al., 2011). In their study, upregulation of CATH1 expression was also seen in the thymus, but not examined in spleen tissue, where we see the strongest increase. Interestingly, in contrast to the considerable upregulation of CATH1 and CATHB1 in spleen by vitamin D supplementation of the low Ca/P diet, we see a downregulation of CATH3 in this organ for all treatment groups relative to the control diet. This might

point to a negative feedback loop within the cathelicidin cluster. As it is still mostly unclear in which cells the chicken cathelicidins are expressed, the observed increased mRNA expression could indicate induction of local gene expression as well as an influx of cathelicidin expressing cells. Immunohistochemistry studies with cathelicidin specific antibodies could answer this question. Although the overall effect of increased cathelicidin expression cannot easily be predicted, because of the multitude of effects these peptides have, augmentation of immunity against bacterial pathogens is to be expected. In fact, evidence is accumulating that vitamin D mediated modulation of cathelicidin expression may play a pivotal role in the outcome of some bacterial infections, such as tuberculosis. It has been shown that, $25(OH)_2D$ modulates the expression of human cathelicidin LL-37 in monocytes when challenged with M. tuberculosis (Rivas-Santiago et al., 2008).

In chickens, the spleen acts both as reservoir and activation site for leukocytes and, therefore, splenic gene expression reflects systemic immune function (Redmond et al., 2010). In general, while vitamin D induced expression of both pro- and anti-inflammatory cytokines was upregulated, the increased expression levels for anti-inflammatory cytokine IL-10 and the Th2 cytokine IL-4 were far greater than those of the pro-inflammatory cytokines. Furthermore, the most profound effects of dietary vitamin D3 supplementation were observed for spleen tissue of birds fed a low Ca/P diet. Under these circumstances vitamin D supplementation will result in the induction of Ca-binding protein in the intestine, and increase calcium absorption and retention. The simultaneously augmented TLR2b and TLR4 expression levels reflect an increased sensory status of the innate immune system and may in part have to do with an attempt to maintain an immunological balance. However, this could also result in an exaggerated immune response and not benefit the birds overall health. In conclusion, we have shown that the presence of high doses of vitamin D_3 or its derivative 25-OH-D_3 above the recommended concentrations has a robust systemic influence in shifting the immune system from Th1 towards Th2 response and that this is most evident in the spleen particularly when dietary levels of calcium are low.

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