# In ovo co-administration of vitamins (A and D) and probiotic lactobacilli modulates immune responses in broiler chickens

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**ABSTRACT** There is evidence that probiotic lactobacilli, in addition to essential vitamins, such as vitamin A and D, have immunomodulatory properties that enhance immune response of neonatal chickens against infections. The present study evaluated the effects of in ovo administration of retinoic acid (**RA**), 25-Hydroxyvitamin D$_3$ (**VitD**), and a lactobacilli cocktail on cytokine gene expression, antibody responses and spleen cell subsets in chickens. RA (90 $\mu$mol/egg) and VitD (0.6 $\mu$g/egg) were administered in ovo, either alone or in combination with lactobacilli ($10^7$ CFU/egg), at embryonic d 18. On d 5 and 10 posthatch, gene expression and cellular composition were analyzed in the bursa of Fabricius and spleen. Birds were immunized on d 14 and 21 posthatch with 2 T-dependent antigens, sheep red blood cells (**SRBC**) and keyhole limpet hemocyanin (**KLH**), to assess their antibody responses. Sera were collected from the immunized chickens on d 14, 21, 28, and 35 posthatch. The results demonstrated that lactobacilli treatment increased the number of monocyte/macrophages (KUL01$^+$) and CD3$^+$CD4$^+$ T cells in the spleen, and enhanced serum anti-KLH IgM and IgY on d 14 postprimary immunization ($P < 0.05$). RA significantly increased serum IgY and IgM titers to KLH and enhanced the expression of interferon (**IFN**)-$\alpha$, interleukin (**IL**)-1$\beta$, IL-6, IL-8, IL-12, IL-13, and transforming growth factor-$\beta$ (**TGF-$\beta$**) in the bursa of Fabricius ($P < 0.05$). The percentage of CD3$^+$CD8$^+$ T cells, and monocyte/macrophages (KUL01$^+$) was elevated in the spleen as well ($P < 0.05$). These findings reveal that prehatch administration of RA improves immunocompetency of neonatal chickens by increasing the production of cytokines that regulate innate immunity and through enhancing antibody-mediated response against T-dependent antigens.

Key words: retinoic acid, lactobacilli, cytokine, antibody, broiler chickens

## INTRODUCTION

Newly hatched chicks face stressful conditions during commercial hatchery procedures and may also experience delayed access to water and feed because of the variation in hatching time (Hollemans et al., 2018; Hedlund et al., 2019). These stressors can cause excessive secretion of corticosteroids, which in turn, suppress macrophage numbers and activity and decrease the number of lymphocytes (Yang et al., 2015). These events may lead to compromised immune function and possibly an increase the risk of infectious diseases in neonatal chicks (Simon, 2016).

Different strategies such as dietary intervention have been applied in the poultry industry to prevent the adverse effects of early postnatal stressors on the immune system of chickens (Taha-Abdelaziz et al., 2018). In ovo supplementation can be used as a strategy to deliver nutrients directly to chicken embryo to alleviate the adverse effects associated with commercial hatchery processes (Jha et al., 2019). The in ovo technology was initially developed for vaccination of chickens against Marek's disease (Longenecker et al., 1975). Following this breakthrough, further studies revealed that nutrients such as amino acids, minerals, and vitamins

can be administered through the in ovo route as well (Jha et al., 2019). Evidence suggests that some of these nutrients such as vitamin A and D have immunomodulatory activities and may improve posthatch development of the intestine and immune function of chickens (Mora et al., 2008; Sassi et al., 2018). Vitamin A is one of the essential vitamins that has a pivotal role in the immune system. Avian species are unable to synthesize vitamin A endogenously and need to obtain carotenoids from the diet (Sassi et al., 2018). Retinoic acid (**RA**), the active metabolite of vitamin A, is involved in many important physiological activities in the body, including maintaining epithelial integrity and development of the immune system (McCullough et al., 1999; Huang et al., 2018). RA plays regulatory roles in cell- and antibody-mediated immune responses by modulating T lymphocyte activation and proliferation as well as B lymphocyte proliferation and antibody production (Ertesvag et al., 2009; Bono et al., 2016). In addition, RA plays a key role in mediating innate defenses by promoting differentiation and maturation of epithelial cells and formation of the epithelial layer (Czarnewski et al., 2017). Along with RA, vitamin D is an essential vitamin that plays a crucial role in regulation and modulation of host immune function (Prietl et al., 2013). Deficiency of vitamin D has been found to be associated with impaired immune responses, and increased host susceptibility to many infections (Aranow, 2011). The cellular action of the active form of vitamin D ($1,25$-dihydroxyvitamin $D_3$) is mediated by the vitamin D receptor (**VDR**) which is expressed by majority of immune system cells (Cantorna, 2010; Aranow, 2011). Binding of vitamin D to its receptor initiates vitamin D bioactivity and regulates transcription of several genes involved in immune functions (Di Rosa et al. 2011). Immunomodulatory activities of vitamin D in chickens have been reported in some studies. Rodriguez and colleagues (2016) demonstrated that dietary supplementation of vitamin D increases the expression of host defense peptides and enhances T helper type 2 cytokine response in broiler chickens. In another study, treatment of chicken macrophages with $1,25$ $(OH)_2$ $D_3$ showed immunoregulatory activities by decreasing the number of $CD86^+$ and major histocompatibility complex (**MHC**)-$II^+$ cells as well as lowering the expression of CXCL8 and IL-$1\beta$ (Shojadoost et al., 2015). Although $1,25$-dihydroxyvitamin $D_3$ is the most active form of vitamin D in chickens, 25-dihydroxyvitamin $D_3$ is the major circulating metabolite that is available at higher concentrations with longer half-life and stability compared to $1,25$-dihydroxyvitamin $D_3$, making it a more desirable candidate for in ovo supplementation (Shojadoost et al., 2021b).

In addition to nutrients, prehatch administration of probiotic bacteria via in ovo delivery may strengthen resistance of chicks against early posthatch stressors and infectious agents (Pender et al., 2017; Alizadeh et al., 2020). Considering the potential interaction between gut microbiota and the host-immune system through pattern recognition receptors, early colonization of beneficial microbes via in ovo delivery can be important for modulation and development of the immune system in neonatal chickens (Broom and Kogut, 2018). Several studies have investigated the effects of dietary vitamins on immune responses in chickens, however, limited information is available regarding the in ovo administration of vitamins on posthatch immune function of chickens. Given the important role of vitamins (A and D) and probiotics in modulating immune system activity of chickens, the present study was conducted to determine the effects of in ovo inoculation of vitamins A and D either alone, or in combination with probiotic lactobacilli on chicken immune responses.

## MATERIALS AND METHODS

### Housing and Experimental Groups

Four hundred embryonated commercial broiler chicken eggs were obtained from the Arkell Poultry Research Station, University of Guelph. All embryonated eggs were incubated in the same incubator at the same hatchery. On embryonic d 18 (**ED18**), following candling and disinfection of eggs with 70% ethanol, eggs were punched by a hole puncher to inoculate vitamins and lactobacilli (200 $\mu$L) into the amniotic sac using a 23-gauge 2.5 cm needle. Eggs were distributed into 9 experimental groups outlined in Table 1. After hatching, chicks were group-housed in floor pens, each containing 40 birds. All experimental procedures were approved by the Animal Care Committee of the University of Guelph and conducted according to the guidelines of the Canadian Council on Animal Care guidelines

### Bacterial Culture and Vitamins Preparations

*Lactobacillus* spp (*L. crispatus-JB/SL-44*, *reuteri-JB/SL-42*, *L. johnsonii-JB/SL-39, L.*, and L. *salivarius-JB/SL-26*), were previously isolated from the intestines of healthy broiler chickens and have been characterized

**Table 1.** Experimental groups.

| Group | Abbreviated names | In ovo injection (ED18)[1] |
|---|---|---|
| 1 | RA[2] | 90 $\mu$mol retinoic acid (Sigma, Canada)/200 $\mu$L/egg |
| 2 | LAB[3] | $10^7$ CFU Lactobacilli/200 $\mu$L/egg |
| 3 | RA + LAB | [90 $\mu$mol of retinoic acid + $10^7$ CFU Lactobacilli]/200 $\mu$L/egg |
| 4 | Vit D[4] | 0.6 $\mu$g 25-Hydroxyvitamin $D_3$ (Sigma, Canada) |
| 5 | Vit D + LAB | [0.6 $\mu$g 25-Hydroxyvitamin $D_3$ + $10^7$ CFU Lactobacilli]/200 $\mu$L/egg |
| 6 | DMSO[5] | Dimethyl sulfoxide (retinoic acid diluent)/200 $\mu$L/egg |
| 7 | Ethanol | Absolute ethanol (25-hydroxyvitamin $D_3$ diluent)/200 $\mu$L/egg |
| 8 | PBS | Sterile phosphate-buffered saline (lactobacilli diluent)/200 $\mu$L/egg |
| 9 | UN[6] | None |

[1]Embryonic d 18.
[2]Retinoic acid.
[3]Lactobacilli.
[4]Vitamin D.
[5]Dimethyl sulfoxide untreated.
[6]Untreated.

(Brisbin et al., 2011; Brisbin et al., 2015). *Lactobacillus* spp were grown anaerobically at 37°C in MRS broth medium. The multi-strain *Lactobacillus* cocktail containing an equal amount of each individual strains ($10^7$ CFU/mL per strain) was prepared in PBS.

A stock solution of vitamin A (retinoic acid; RA; Sigma-Aldrich) was prepared by dissolving the vitamin in dimethyl sulfoxide (DMSO, retinoic acid diluent). A 3 mg/mL solution was prepared and stored in light-protected vials at -20°C. The stock solution was further diluted with PBS before use to reach the final dilution (90 $\mu$mol/200 $\mu$L). A stock solution of vitamin D3 (25-Hydroxyvitamin $D_3$; VitD; Sigma-Aldrich) was prepared by dissolving in absolute ethanol (vitamin D3 diluent) and stored in light protected vials at -20°C. The stock solution was further diluted in PBS before use to reach the final dilution (0.6 $\mu$g//200 $\mu$L). Appropriate controls were used in this experiment including DMSO, ethanol, and PBS at the same dilutions as used for the final dilution of the vitamins.

### Immunization and Sample Collection

Immunization and sample collection were performed as described previously (Alizadeh et al., 2020). Birds were immunized at 14- and 21-d posthatch via intramuscular injection with 100 $\mu$g of keyhole limpet hemocyanin (KLH; Sigma-Aldrich, Oakville, ON, Canada) and 0.25 mL of 2% sheep red blood cells (SRBC; PML Microbiologicals, Mississauga, ON, Canada) in 0.25 mL PBS. The negative control (untreated chickens) was injected with 0.25 mL of PBS. On d 14, 21, 28, and 35 posthatch blood samples were collected (via the wing vein) from 12 birds per treatment group. Blood samples were incubated at room temperature (RT) for 2 h and were centrifuged at 580 $\times$ g for 10 min for serum separation. Serum samples were then collected and stored at $-20$°C for antibody analysis. On d 5 and 10 posthatch, the bursa of Fabricius and spleen were collected (6 birds per treatment group) and kept in RNAlater (Invitrogen, Burlington, ON, Canada) and frozen at $-80$°C for subsequent RNA extraction. Spleen tissue samples were also collected in 1X Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY) and stored on ice for mononuclear cells isolation.

### Spleen Mononuclear Cell Preparation and Flow Cytometry Analysis

Mononuclear cells were prepared from the spleens of 6 birds per treatment group as previously described (Alizadeh et al., 2021). Briefly, tissue samples were rinsed 3 times with PBS and crushed through a 40-$\mu$m nylon cell strainer and resuspended in 5 mL of complete RPMI (Invitrogen, Burlington, ON, Canada) medium containing 10% fetal bovine serum and 1% Penicillin-Streptomycin (Gibco, Grand Island, NY). Solenocyte cell suspensions were overlaid on 4 mL Histopaque-1077 (Sigma, Oakville, ON) and centrifuged at 400 g for 20 min for density gradient separation. Mononuclear cells (buffy coat layer) were aspirated from the interface and washed (2x) in RPMI medium. Cells were counted using a hemocytometer and trypan blue and 100 $\mu$L of each cell suspension was seeded in 96-well plates at a density of $1 \times 10^6$ cells/well. Mononuclear cells were washed twice with fluorescent activated cell sorting (**FACS**) buffer and stained for 30 min at 4°C in the dark with monoclonal antibodies. Two different surface molecule staining panels were used in this study. Panel 1: mouse antichicken monocyte/macrophage-FITC (KUL01), mouse antichicken Bu-1-PB, and mouse antichicken IgM-APC-Cy7. Panel 2: mouse antichicken CD3-PB, mouse antichicken CD4-PE-Cy7, and mouse antichicken CD8-APC. Monoclonal antibodies were obtained from SouthernBiotech (Birmingham, AL). Dead cells were excluded using the fixable Live/Dead near- Infrared fluorescent reactive dye (Thermo Fisher Scientific, Mississauga, Canada). Subsequently, cells were washed (2x) in FACS buffer, resuspended and fixed in 2% paraformaldehyde for analysis. Flow cytometry was performed using a FACS Canto II flow cytometer (BD Bioscience, San Jose, CA) and data was processed by FlowJo Software (v.10).

### Serological Analysis

A direct hemagglutination assay was performed to detect antibody responses against SRBC in sera as previously described (Haghighi et al., 2005). Initially, serum samples were heat-inactivated at 56°C for 30 min. A 96-well V-bottom microplate was covered by PBS containing 0.05% bovine serum albumin. Serum samples (50 $\mu$L) were added to the wells and serially diluted (2-fold) in duplicates. Later, 50 $\mu$L of a 1% SRBC was added to each well. Plates were shaken for 1 min and incubated at 37°C for 24 h. A positive result was recorded when at least 50% of agglutination was observed.

Detection of KLH-specific IgG (IgY) and IgM titers in serum samples was performed by ELISA as previously described (Alizadeh et al., 2021). Briefly, 96-well flat-bottom Maxisorp high binding microplates were coated with 100 $\mu$L of a KLH solution (1 $\mu$g/mL KLH in 0.1 M $NaHCO_3$, pH 9.6 with 30 $\mu$g/mL BSA) and incubated overnight at 4°C. Plates were washed (4 times) with PBST (0.05% Tween 20; P137 Sigma-Aldrich Inc., St. Louis, MO) and blocked (2 h at RT in the dark) with 200 $\mu$L blocking buffer (PBST containing 0.25% of fish skin gelatin; Sigma-Aldrich, Oakville, ON). Plates were washed (4x) and 100 $\mu$L of serum samples (diluted 1/200) were added to the wells. Washing was repeated (4x) and 100 $\mu$L of detection antibodies (goat antichicken IgY or IgM conjugated to horseradish peroxidase) were added to the wells and incubated for 2h at RT in the dark. Finally, the plates were washed (4x) and 100 $\mu$L of substrate ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); Mandel Scientific, Guelph, ON, Canada) was added to each well. Absorbance was measured at 405 nm using a microplate reader

(Epoch, BioTek Instruments Inc., Winooski, VT) within 30 min of ABTS addition. To normalize plate-to-plate variation, negative (fetal bovine serum) and positive control (hyperimmune serum) were included in each plate. The following formula was used to calculate sample/positive (Sp) ratios: (mean of test sample - mean of negative control)/(mean of positive control - mean of negative control).

### RNA Extraction and cDNA Synthesis

Total RNA was extracted from the bursa of Fabricius and spleen using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Following treatment of RNA samples with DNase (DNA-free kit, Ambion, Austin, TX), the quantity, and quality of RNA samples were measured by a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse-transcription to cDNA was carried out using Superscript II First-Strand Synthesis kit (Invitrogen) based on the manufacturer's instructions.

### Real-Time Reverse Transcriptase Polymerase Chain Reaction

Quantitative real-time RT-PCR was performed using the LightCycler 480 II system (Roche Diagnostics GmbH, Mannheim, DE), as previously described (Alizadeh et al., 2020). The PCR cycling conditions included initial denaturation at 95°C, then 40 to 50 amplification cycles consisting of 95°C for 10 sec, annealing temperature ranged between 58 and 64°C (Table 2) for 5 sec, and extension at 72°C for 10 sec. Primers sequences and their accession numbers are listed in Table 2.

### Statistical Analysis

The expression levels of all genes were calculated relative to the housekeeping gene ($\beta$-actin) using the Light-Cycler 480 II advance relative quantification software (Roche Diagnostics). All data were analyzed using the generalized linear model (GLM) procedure of SAS (SAS Institute Inc., Cary, NC). Tukey's multiple comparison test was used to determine the significant differences among means. When error deviations did not have homogenous variance across treatments, the data was Log transformed. A $P$-value of <0.05 was considered statistically significant.
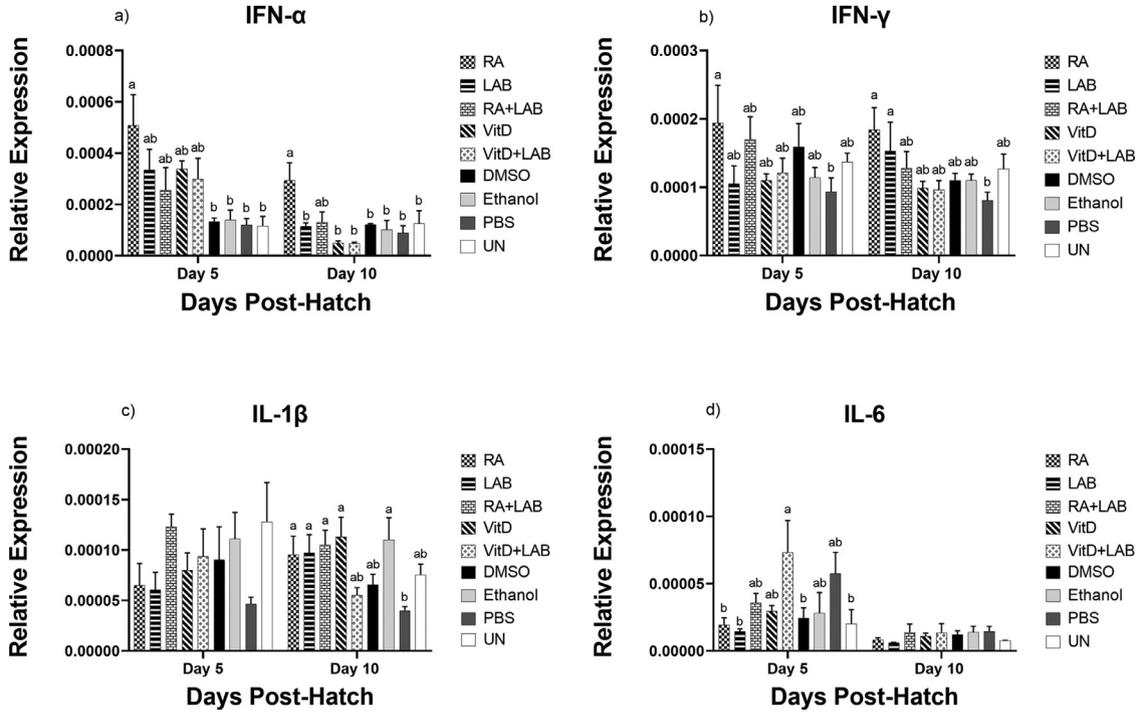
### RESULTS

### Hatchability

Embryonated eggs were inoculated via the amniotic sac at ED18, and hatchability was recorded on the d of hatch. In ovo inoculation did not affect the overall hatchability of chickens and 99.16% of eggs hatched following in ovo injection ($P > 0.05$).
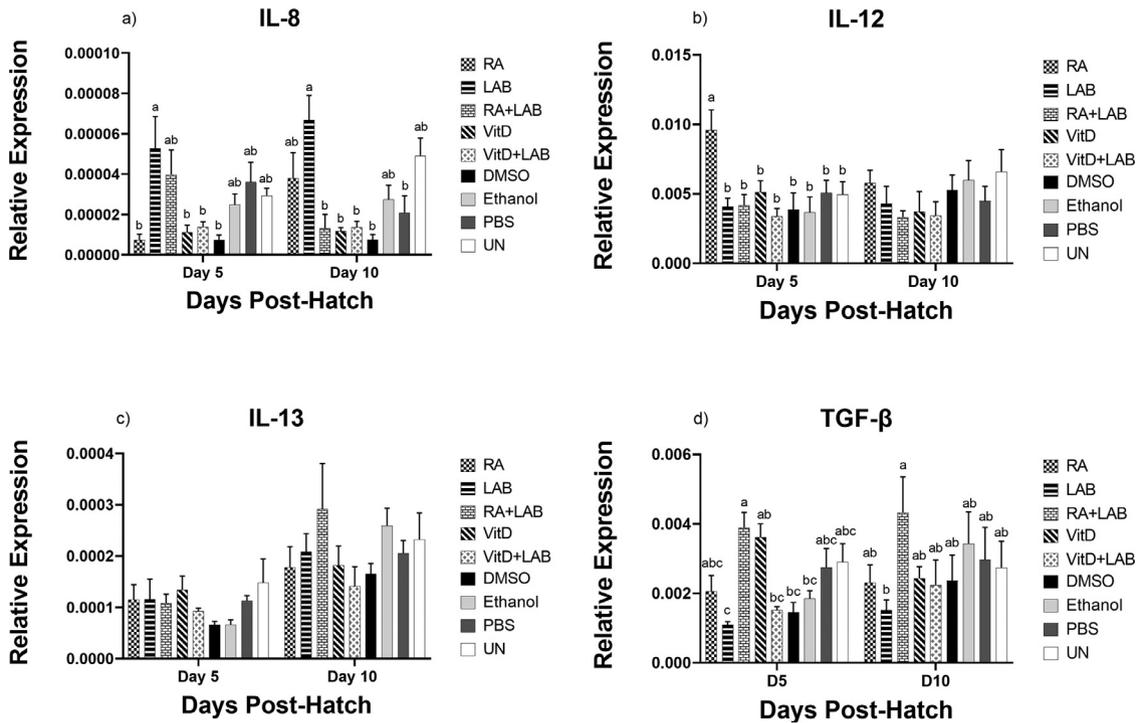
### Gene Expression in Spleen

The results for gene expression analysis in the spleen are presented in Figures 1 and 2. RA treatment significantly enhanced ($P < 0.05$) the expression of interferon (**IFN**)-$\alpha$ compared to all control groups (DMSO, ethanol, PBS, and Untreated) on d 5 and 10 posthatch (Figure 1A).

Expression of IFN-$\gamma$ was upregulated on d 10 posthatch in the group that received lactobacilli when compared to the PBS-injected control ($P < 0.05$), and RA treatment enhanced ($P < 0.05$) the expression of IFN-$\gamma$ compared to PBS-injected control at both time points (Figure 1B).

**Table 2.** Primer sequences used for real-time quantitative PCR.[1]

| Gene[2] | Primer sequence[3] (5'-3') | Annealing temperature | Gene Bank accession number |
|---|---|---|---|
| IFN-$\alpha$ | F: CGCTTAGGAGAGACAATCTGTGAA | 64 | AB021154 |
| | R: GCCTGTTTTAGGGATTTCAGAGAATTT | | |
| IFN-$\gamma$ | F: TGGCGGCGGGAGGAAAAGTG | 60 | NM_001030558 |
| | R: CACCGTGCTCCAGCTCAGGC | | |
| IL-1$\beta$ | F: GTGAGGCTCAACATTGCGCTGTA | 64 | Y15006 |
| | R: TGTCCAGGCGGTAGAAGATGAAG | | |
| IL-6 | F: CTGAAGAACTGGACAGAGAG | 60 | NM_204628.1 |
| | R: CACCAGCTTCTGTAAGATGC | | |
| IL-8 | F: CTGAAGGTGCAGAAGCAGAG | 64 | AJ009800 |
| | R: CCAGCTCTGCCTTGTAGGTT | | |
| IL-12p35 | F: AGCAGATCAAGGAGACGTTC | 60 | NM213588 |
| | R: ATCAGCAGGTACTCCTCGAT | | |
| IL-13 | F: ACTTGTCCAAGCTGAAGCTGTC | 60 | AJ621250.1 |
| | R: TCTTGCAGTCGGTCATGTTGTC | | |
| TGF-$\beta$[4] | F: CGGCCGACGATGAGTGGCTC | 60 | M31160.1 |
| | R: CGGGGCCCATCTCACAGGGA | | |
| $\beta$-Actin | F: CAACACAGTGCTGTCTGGTGGTA | 58 | X00182 |
| | R: ATCGTACTCCTGCTTGCTGATCC | | |

[1]The listed oligonucleotides were used to analyze gene expression via real-time quantitative PCR.
[2]IFN: interferon; IL: interleukin.
[3]F: forward; R: reverse.
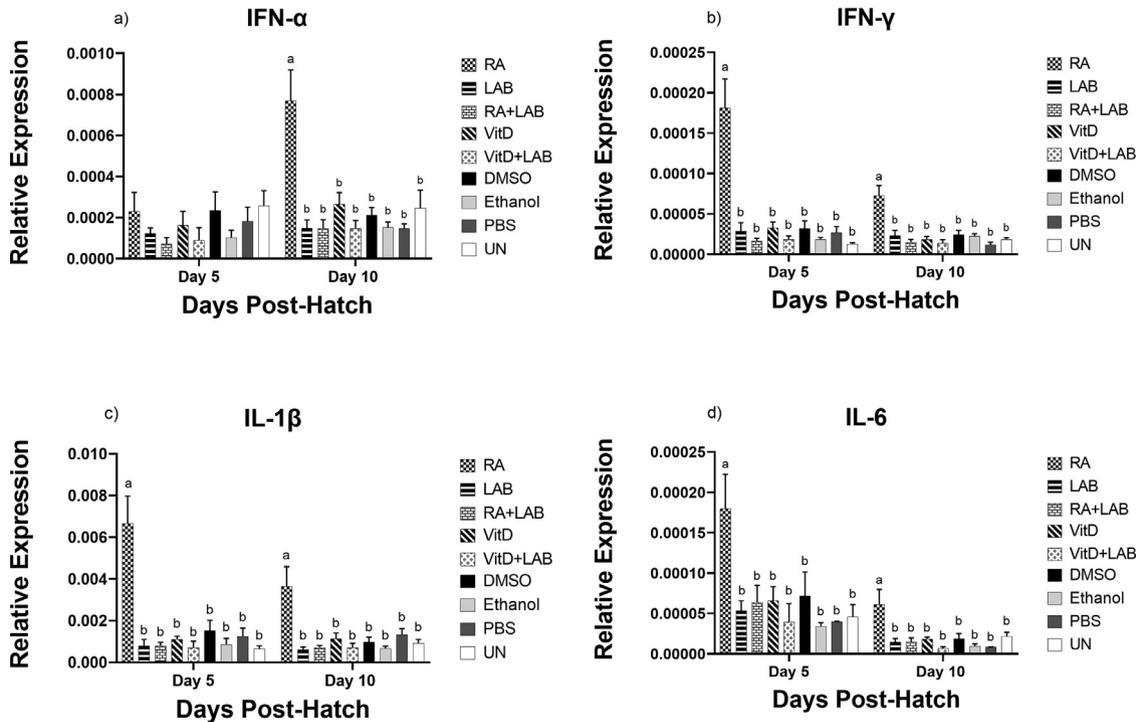[4]TGF-$\beta$: transforming growth factor beta.

**Figure 1.** Relative gene expression of cytokines (IFN-$\alpha$, IFN-$\gamma$, IL-1 $\beta$, IL-6) in the spleen.

Data represent the relative expression of cytokines (normalized to expression of $\beta$-actin) including IFN-$\alpha$ (A), IFN-$\gamma$ (B), IL-1$\beta$ (C), and IL-6 (D) in the spleen at d 5 and 10 posthatch. At embryonic d 18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P < 0.05$.



**Figure 2.** Relative gene expression of cytokines (IL8, IL-12. IL-13, TGF-$\beta$) in the spleen.

Data represent the relative expression of cytokines (normalized to expression of $\beta$-actin) including IL-8 (A), IL-12 (B), IL-13 (C), and TGF-$\beta$ (D) in the spleen at d 5 and 10 posthatch. At embryonic d18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P < 0.05$.

**Figure 3.** Relative gene expression of cytokines (IFN-$\alpha$, IFN-$\gamma$, IL-1 $\beta$, IL-6) in the bursa of Fabricius.

Data represent the relative expression of cytokines (normalized to expression of $\beta$-actin) including IFN-$\alpha$ (A), IFN-$\gamma$ (B), IL-1 (C), and IL-6 (D) in the bursa of Fabricius at d 5 and 10 posthatch. At embryonic d18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P < 0.05$.

Although the expression of IL-1$\beta$ on d 5 posthatch was not affected by any of the treatments ($P > 0.05$), the groups that received RA, lactobacilli (**LAB**), VitD, and ethanol showed an elevated expression ($P < 0.05$) of IL-1$\beta$ compared to PBS-injected control (Figure 1C). Concurrent administration of VitD and LAB increased expression of IL-6 (Figure 1D) on d 5 posthatch compared to the groups that received RA, LAB or DMSO ($P < 0.05$). However, the expression of IL-6 on d 10 was not altered by any of the treatments ($P > 0.05$). Lactobacilli treatment enhanced ($P < 0.05$) the expression of IL-8 compared to the groups that received RA, VitD, or DMSO at both time points (Figure 2A). Whereas expression of IL-12 was significantly upregulated in the RA group compared to all other groups on d 5 posthatch ($P < 0.05$), no significant difference was observed ($P > 0.01$) for IL-12 gene expression on d 10 posthatch (Figure 2B). Expression of IL-13 in the spleen was not altered ($P > 0.05$) by any of the treatments (Figure 2C). Expression of TGF-$\beta$ was upregulated ($P < 0.05$) in the group received concurrent administration of RA and LAB when compared to the group that only received LAB at both time points (Figure 2D).
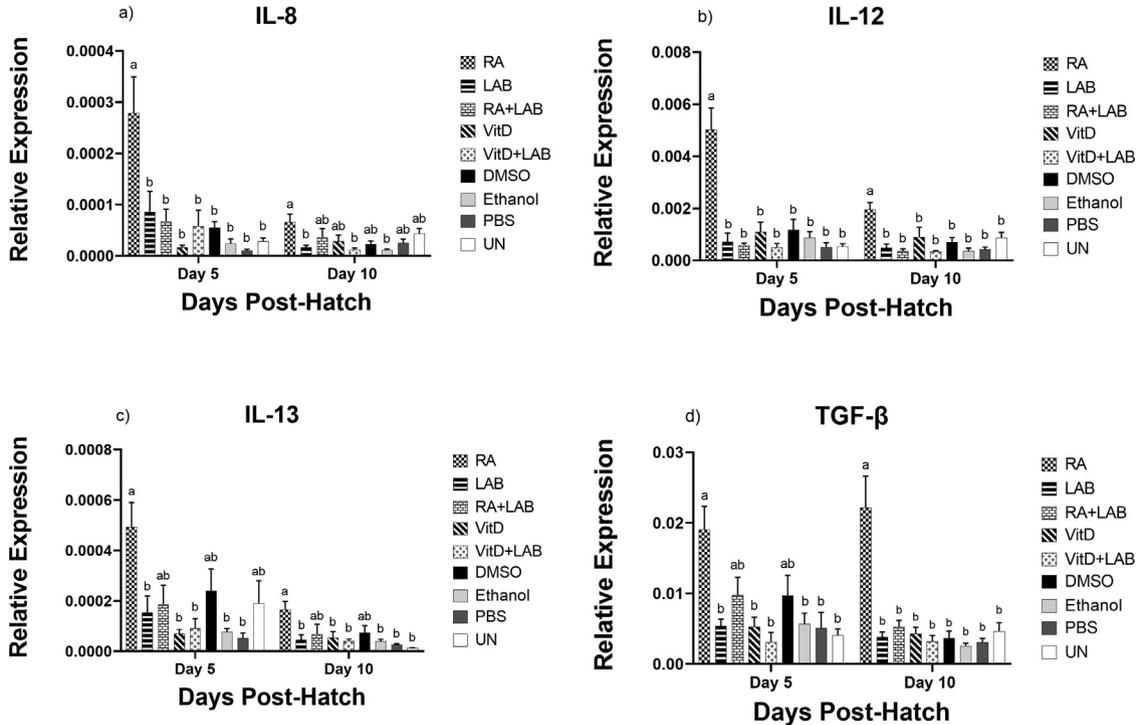
### Gene Expression in the Bursa of Fabricius

The results for gene expression analysis in the bursa of Fabricius are presented in Figures 3 and 4. Although the

expression of IFN-$\alpha$ on d 5 posthatch was not altered ($P > 0.05$) by any of the treatments, RA treatment increased ($P < 0.01$) IFN-$\alpha$ expression compared to all other groups on d 10 posthatch (Figure 3A). RA treatment enhanced the expression of IFN-$\gamma$ (Figure 3B), IL-1$\beta$ (Figure 3C), IL-6 (Figure 3D), and IL-12 (Figure 4B) compared to all other treatment groups at both time points ($P < 0.01$). Expression of IL-8 (Figure 4A) and TGF-$\beta$ (Figure 4D) was elevated in the RA-treated group when compared to all other groups on d 5 and 10 posthatch, respectively ($P < 0.01$). RA treatment increased expression ($P < 0.05$) of IL-13 when compared to the groups that received LAB, VitD, ethanol, or PBS at both time points (Figure 4C).

### Macrophage and Lymphocyte Populations

The results for macrophage and lymphocyte populations are presented in Figures 5 and 6. RA administration increased ($P < 0.05$) the percentage of monocyte/macrophages on d 5 posthatch compared to the group received concurrent administration of lactobacilli and VitD (Figure 5A). In addition, in ovo inoculation of LAB, VitD, and coadministration of LAB and RA increased ($P < 0.05$) the absolute numbers of monocyte/macrophages (KUL01$^{+}$) cells compared to the PBS-injected control on d 10 posthatch (Figure 5B). The percentage and the absolute numbers of Bu-1+IgM+ B cells

**Figure 4.** Relative gene expression of cytokines (IL8, IL-12, IL-13, TGF-$\beta$) in in the bursa of Fabricius.

Data represent the relative expression of cytokines (normalized to expression of $\beta$-actin) including IL-8 (A), IL-12 (B), IL-13 (C), and TGF-$\beta$ (D) in the bursa of Fabricius at d 5 and 10 posthatch. At embryonic d18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P < 0.05$.
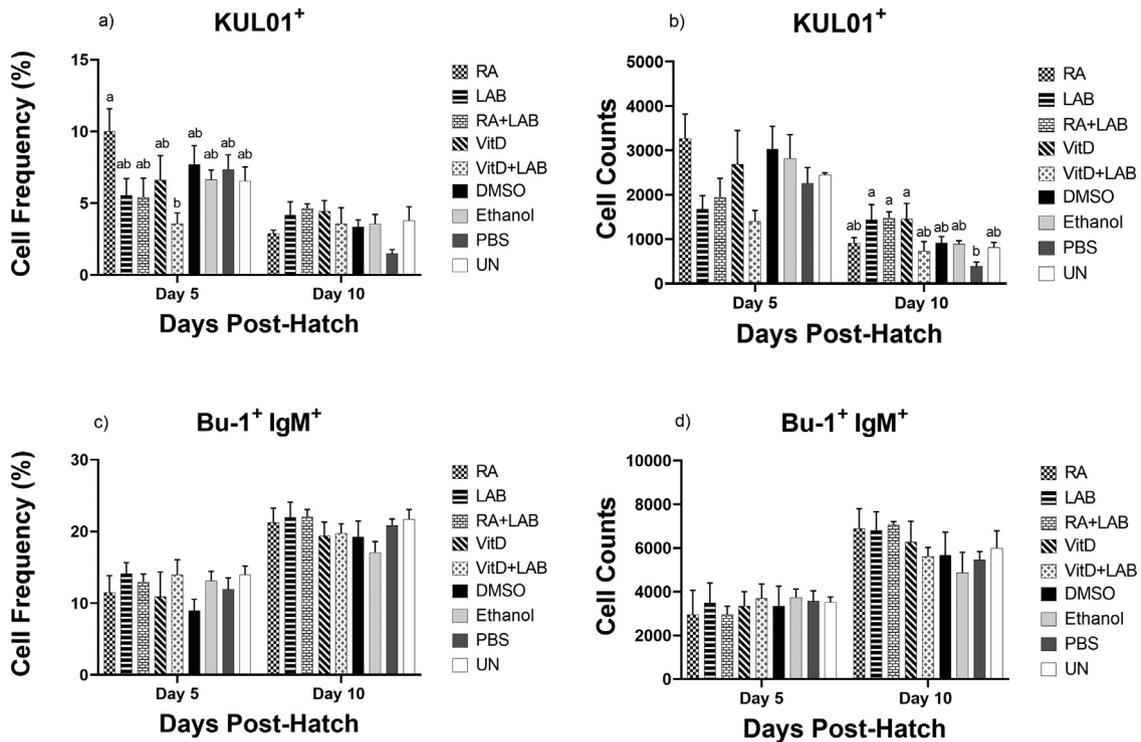


**Figure 5.** Changes in frequency and absolute numbers of monocyte/macrophages and B cells.

Data represent the frequency and absolute numbers of monocyte/macrophages (KUL01^{+}; A,B) and Bu-1^{+} IgM^{+} B cells (C,D) in the spleen at d 5 and 10 posthatch. At embryonic d18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P < 0.05$.
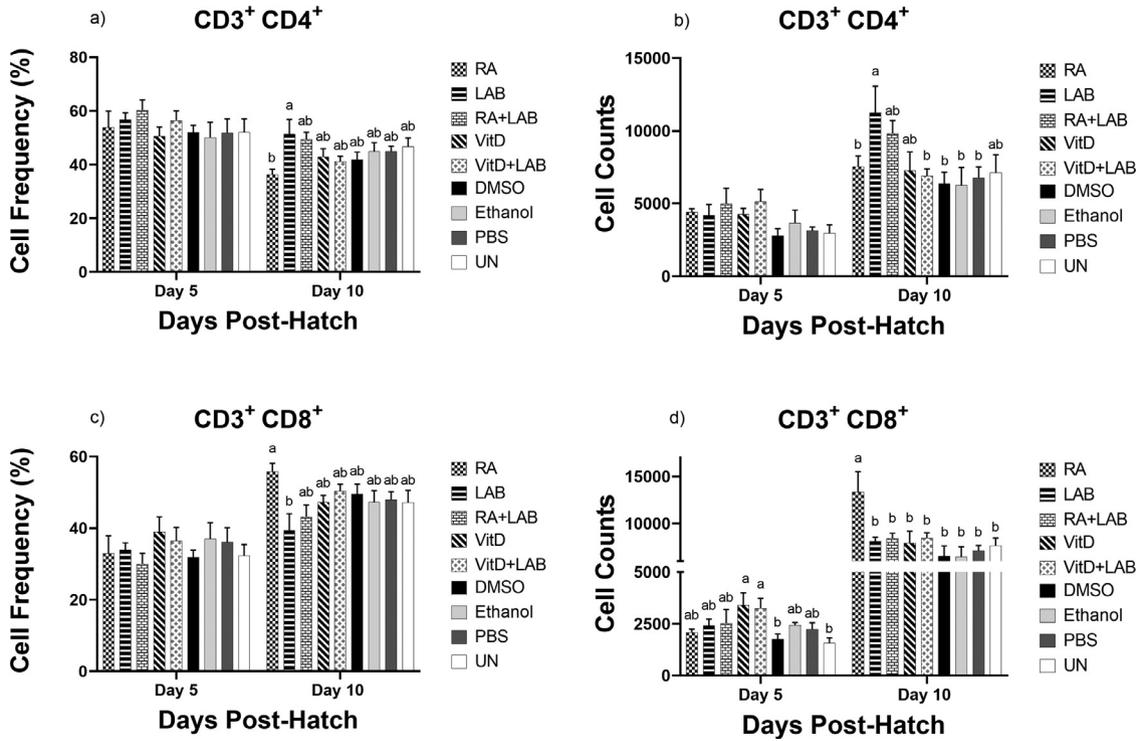
**Figure 6.** Changes in frequency and absolute numbers of monocyte/macrophages and T cells.

Data represent the frequency and absolute numbers of $CD3^+CD4^+$ (A,B) and $CD3^+CD8^+$ T cells (C,D) in the spleen at d 5 and 10 posthatch. At embryonic d18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P <$ 0.05.

were not affected ($P > 0.05$) by any of the treatment groups (Figure 5C,D). In ovo administration of LAB increased ($P < 0.05$) the percentage of $CD3^+CD4^+$ T cells compared to the RA-treated group on d 5 post-hatch (Figure 6A). The absolute number of $CD3^+CD4^+$ T cells was enhanced ($P < 0.05$) in birds that received LAB when compared to the RA group and controls

groups (Figure 6B). RA treatment significantly increased ($P < 0.05$) the percentage of $CD3^+CD8^+$ T cells compared to the LAB-treated group on d 5 post-hatch (Figure 6C). In ovo inoculation of VitD alone or in combination with LAB increased ($P < 0.05$) the absolute number of $CD3^+CD8^+$ T cells compared to the DMSO and untreated control groups on d 5 posthatch
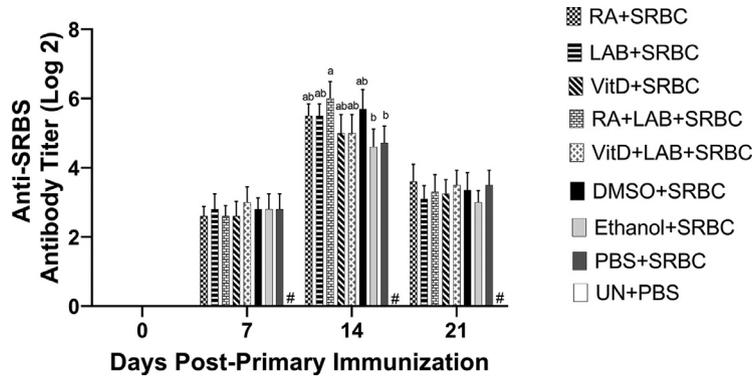


**Figure 7.** Serum anti-SRBC.

Data represent the serum anti-SRBC as determined by direct hemagglutination assay. At embryonic d18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. On d 14 and 21 posthatch, birds were immunized intramuscularly with 2% SRBC in 0.25 mL of mL PBS. The untreated group (UN) was inoculated intramuscularly with 0.25 mL of PBS and served as the negative control. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Bars with asterisks represent a significant difference among treatments. Bars with number sign (#) represent a significance compared to negative control (untreated and PBS injected) and experimental groups. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P < 0.05$.
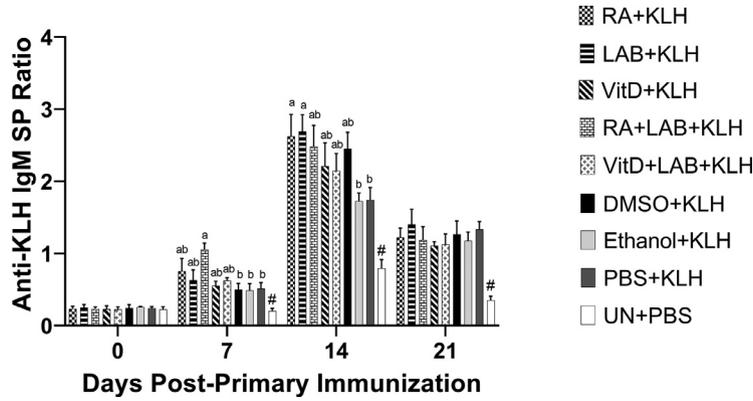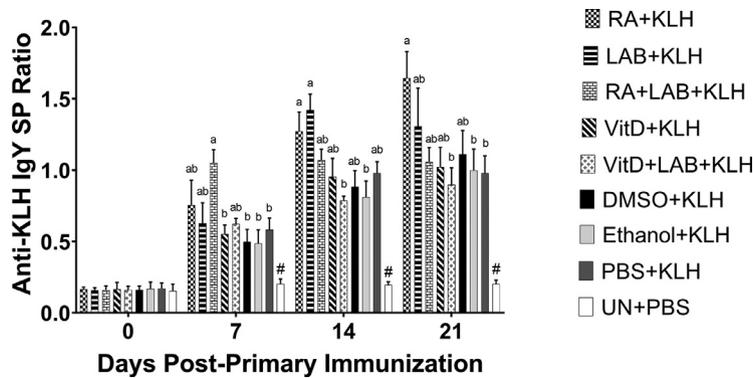
**Figure 8.** Serum anti-KLH IgM.
Data represent the serum anti-KLH IgM antibodies as determined by indirect ELISA. At embryonic d 18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. On d 14 and 21 posthatch, birds were immunized intramuscularly 100 $\mu$g KLH in 0.25 mL of mL PBS. Untreated group (UN) was inoculated intramuscularly with 0.25 mL of PBS and served as the negative control. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Bars with asterisks represent a significant difference among treatments. Bars with number sign (#) represent a significance compared to negative control (untreated and PBS injected) and experimental groups. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P < 0.05$.

(Figure 6D). In addition, RA increased ($P < 0.01$) the absolute number of CD3$^+$CD8$^+$ T cells compared to all other groups (Figure 6D).

### Antibody-Mediated Immune Responses

The results of antibody-mediated immune response against SRBC are presented in Figure 7. Immunization of birds with SRBC increased ($P < 0.01$) antibody titers against SRBC in all treatment groups when compared to the nonimmunized and nontreated group (negative control). Coadministration of RA and lactobacilli enhanced ($P < 0.05$) serum anti-SRBC titers compared to the group that in ovo injected with PBS and immunized with SRBC (positive control group) on d 14

postprimary immunization (Figure 7), however no significant difference was observed for anti-SRBC antibodies at other time points ($P > 0.05$). The results of antibody-mediated immune response against KLH are presented in Figures 8 and 9. Immunization with KLH increased IgM and IgY titers against KLH ($P < 0.01$) in all treatment groups compared to the nonimmunized and nontreated group (negative control group). Concurrent administration of RA and LAB increased ($P < 0.05$) serum IgM and IgY titers against KLH compared to control groups (in ovo injected with DMSO, ethanol, PBS, and immunized with KLH) on d 7 postprimary immunization (Figures 8 and 9). RA and LAB treatment enhanced ($P < 0.05$) serum IgM and IgY titers against KLH when compared to the positive



**Figure 9.** Serum anti-KLH IgY.
Data represent the serum anti-KLH IgY antibodies as determined by ELISA. At embryonic d18, eggs were randomly assigned to each experimental group and were injected with 200 $\mu$L of different treatments including retinoic acid (RA), 25-hydroxyvitamin D (VitD), lactobacilli, DMSO, ethanol, and PBS. The untreated control remained uninjected. On d 14 and 21 posthatch, birds were immunized intramuscularly with 100 $\mu$g KLH in 0.25 mL of mL PBS. The untreated group (UN) was inoculated intramuscularly with 0.25 mL of PBS and served as the negative control. GLM (General Linear Model) procedure of SAS was used for statistical analysis and difference among means was determined by Tukey's comparison. Bars with asterisks represent a significant difference among treatments. Bars with number sign (#) represent a significance compared to negative control (untreated and PBS injected) and experimental groups. Group means with no common letters differ significantly. Error bars represent the standard error of the mean. Results were considered statistically significant if $P < 0.05$.

control (PBS-injected and immunized birds) on d 14 postprimary immunization (Figures 8 and 9). In addition, LAB increased ($P < 0.05$) serum IgY titers against KLH compared to the positive control group (PBS-injected and immunized birds) on d 21 postprimary immunization (Figure 9).

## DISCUSSION

Evidence suggests that beneficial bacteria in addition to some vitamins can improve immunocompetence of neonatal chickens (Pender et al., 2016; Alizadeh et al., 2020; Shojadoost et al., 2021a). The present study evaluated the effects of in ovo supplementation of vitamins A and D either alone or in combination with probiotic bacteria on immune responses in chickens. Preliminary studies in our lab evaluated the effects of different doses of RA (10, 30, 90, 270 and 810 $\mu$mol/egg), VitD (1.8, 0.6, and 0.18 $\mu$g 200 $\mu$L/egg) and lactobacilli ($1 \times 10^5$, $1 \times 10^6$, and $1 \times 10^7$ CFU/egg) on expression of cytokine genes in chicken embryos. The results demonstrated that inoculation of eggs with 90 $\mu$mol/egg of RA, 0.6 $\mu$g/egg VitD, and $1 \times 10^7$ CFU/egg lactobacilli showed the greater immunomodulatory responses (data not shown). Therefore, these doses were selected for the present study.

Here, expression of cytokines in chicken lymphoid organs was evaluated as markers of induction of immune responses. Cytokines are signaling molecules that are released by cells of the immune system and play a critical role in initiating and regulation of innate and inflammatory responses and in maintaining immune hemostasis (Paul and Seder, 1994). In the current study, expression of most of the measured cytokines in the group that received RA was significantly increased compared to the controls and other treatment groups (especially in the bursa of Fabricius). Although the role of RA in modulating immune responses in mammals is well documented, little is known about its role in modulating chicken immune responses, especially during the early stage of the chicken life. RA deficiency can lead to high susceptibility to infections in different species (Schuster et al., 2008; McGill et al., 2019). It has been shown that pretreatment of mouse macrophages with RA modifies cytokine production in favor of T helper-type II (Th2) cytokines following lipopolysaccharide (**LPS**) stimulation. Further, in activated macrophages, RA significantly inhibits IL-12 production and their capacity to induce IFN$-\gamma$ production in antigen-primed CD4$^+$ T cells, whereas enhancing IL-4 production (Kang et al., 2000). Similarly, Ma and colleagues (2005) demonstrated that dietary supplementation of RA in mice reduces T helper-type I (Th1) cytokines (IFN-$\gamma$ and IL-12) whereas expression of Th2 cytokines and the ratio of IL-4: IFN-$\gamma$ were elevated. Here, we showed that in ovo supplementation of RA significantly enhanced the expression of Th1 (IFN-$\gamma$ and IL-12) and Th2 cytokines (IL-13) in the bursa of Fabricius, indicating the immunomodulatory activities of RA. In addition,

a recent study by our group, investigated the effects of different levels of RA (30, 90, and 270 $\mu$mol/egg) on chicken embryo immune function and found that while the expression of IFN-$\alpha$, IFN-$\gamma$, IL-1$\beta$, IL-2, IL-8, IL-12, and IL-13 was downregulated with 270 $\mu$mol/egg of RA, the expression of some of the above genes was upregulated in the embryos that received 90 $\mu$mol/egg of RA (Shojadoost et al., 2021a). These results suggest that RA modulates immune parameters of chickens in a dose-dependent manner. In the present study, while RA increased expression of some cytokines, including IFN-$\gamma$, IL-1 $\beta$, IL-6, IL-8 and IL-12, no synergistic effect was observed for cytokine expression in the group that received concurrent administration of RA and probiotic lactobacilli. This might be related to the immunoregulatory effects of lactobacilli and their role in maintaining a state of immune system homeostasis. Indeed, some of the strains used in the present study, such as *L. reuteri*, appear to have immunoregulatory activities by reducing cytokine and nitric oxide production in macrophages (Brisbin et al., 2015). Previous in ovo studies have also shown that in ovo administration of lactobacilli downregulated expression of cytokines in the bursa of Fabricius of chickens (Alizadeh et al., 2020).

In addition to investigating their effect on cytokine gene expression in lymphoid organs, the effects of RA, LAB, and VitD on cellular composition of the spleen were assessed in various treatment groups by analyzing the absolute number and percentage of macrophages, B, and T cells. Macrophages are professional antigen-presenting cells that play a critical role in innate immunity through phagocytosis of pathogens and secretion of inflammatory and anti-inflammatory mediators (Franken et al., 2016). In line with our previous finding (Alizadeh et al., 2021), the results of our study showed that in ovo inoculation of lactobacilli alone or in combination with RA increased the number of KUL01$^+$ cells in the spleen of treated birds. Higgins et al. (2008) also showed that Lactobacillus-based probiotic supplementation increases the number of macrophages compared to the untreated control in the cecum of chickens (Higgins et al., 2008). We observed that the absolute number of KUL01$^+$ cells was increased by VitD inoculation on d 10. It has been reported that vitamin D induces differentiation of monocytes into mature phagocytic macrophages (Kreutz et al., 1993; Hewison, 2012).

Alternatively, our study revealed that concurrent administration of vitamin D and lactobacilli decreased the percentage of KUL01$^+$ cells compared to the RA group suggesting an immunoregulatory function of VitD and lactobacilli when they are administered together. The immunoregulatory role of vitamin D and its beneficial effects on prevention of autoimmune disease has been reported (Arnson et al., 2007). Vitamin D exerts anti-inflammatory effects by downregulating the expression of proinflammatory cytokines (Gunville et al, 2013). For example, Shojadoost et al. (2015) demonstrated that although treatment of chicken macrophages with vitamin D has no effect on nitric oxide production and MHC-II and CD86 expression, it

downregulates expression of proinflammatory cytokines (CXCL8 and IL-1$\beta$) following LPS treatment.

This study showed that the percentage and absolute number of Bu-1$^+$ IgM$^+$ cells were not changed by in ovo inoculation of lactobacilli, RA, or VitD. A previous study also demonstrated that in ovo administration of different doses of lactobacilli may not change the percentage and absolute number of B cells in the spleen (Alizadeh et al., 2021). It appears that RA has an important role in development and differentiation of B cells rather than B cell proliferation. Accordingly, Blomhoff et al. (1992) showed that RA inhibits proliferation of B cells in human peripheral blood. In another study, RA at a physiological concentration was seen to inhibit B cell proliferation in mice and humans (Fahlman et al., 1995). Results of the present study demonstrated that RA treatment induced a significant increase in the percentage of CD3$^+$CD8$^+$ T cells in the spleen, while lactobacilli inoculation increased the percentage of CD3$^+$CD4$^+$ T cells compared to the RA group and enhanced the absolute number of these cells in comparison to all control groups. The mechanism by which these treatments altered T cell populations remains unclear. Although not assessed in this study, a possible explanation is that RA and lactobacilli treatments might have induced T cell differentiation in the thymus. It is also possible that these treatments may have had a local effect on recruitment and proliferation of certain subsets of T cells. The elevated number of CD3$^+$CD8$^+$ T cells in splenocytes of birds treated with RA could be related to the enhanced proliferation of these cells mediated by RA as suggested in a study conducted by Engedal et al. (2006).

Treatment with lactobacilli increased the absolute number of CD3$^+$CD4$^+$ T cells compared to the control groups on d 10 posthatch. These results are in line with our previous finding that in ovo supplementation of lactobacilli at 10$^7$ CFU increased the percentage of CD3$^+$CD4$^+$ T helper cells in the spleen (Alizadeh et al., 2021). Other studies have also demonstrated that dietary supplementation of *L. acidophilus* and *L. fermentum* increased the number of CD3$^+$CD4$^+$ T cells in peripheral blood and intestinal intraepithelial lymphocytes of chickens, respectively (Bai et al., 2013; Asgari et al., 2016). However, the exact mechanism underlying the immunomodulatory effect of lactobacilli on CD3$^+$CD4$^+$ T helper cells is not completely understood.

To assess antibody-mediated immune responses, chicks were immunized with SRBC and KLH that both are considered thymus-dependent antigens, which require the presence of T helper cell for B cell activation and antibody production (Gehad et al., 2002). Lactobacilli and vitamin (A and D) have been shown to modulate antibody-mediated immune responses to various antigens in chickens (Haghighi et al., 2005; Alizadeh et al., 2020; Shojadoost et al., 2021a). The results of the current study demonstrated that in ovo administration of lactobacilli and RA significantly increased serum anti-KLH IgY and IgM titers at d 14

postprimary immunization. In addition, concurrent administration of vitamin A and probiotic lactobacilli synergistically enhanced serum anti-SRBC titer, and anti-KLH IgY and IgM levels on d 7 postprimary immunization. These results confirm and extend our earlier finding that in ovo inoculation of probiotic lactobacilli increased antibody responses against SRBC and KLH in newly hatched chicks (Alizadeh et al., 2021). Evidence suggests that RA induces development of T cell-dependent antibody response and facilitates differentiation of activated B cells to antibody-secreting plasma cells (Ross et al., 2011). It has also been reported that supplementation of vitamin A (13,200 IU/kg) in chickens enhances antibody production against $\beta$-casein (Sklan et al., 1994). In another study, supplementation of RA in mice significantly enhanced antibody production (IgG) against a T cell-dependent antigen (tetanus toxoid), suggesting the beneficial effects of vitamin A for enhancing host immunity (Ma and Ross, 2005).

Considering the role of cytokines in the regulation of antibody production, the enhanced antibody response in the RA-treated group might be related to the ability of vitamin A to modulate cytokine production (IL-13). The bursa of Fabricius is a primary immune organ in chickens and is considered as a primary B cell development site. Further this organ may act as a secondary lymphoid organ and be involved in antibody production (Ekino et al., 1985; Loken et al., 2020). The elevated expression of IL-13, which plays a crucial role in the proliferation and differentiation of B cells, in the bursa of Fabricius of the RA-treated group might have contributed to the enhanced production of anti-KLH antibodies observed in this group (Wynn, 2003). Further, the elevated antibody response in the RA-treated group could be attributable to the ability of RA to stimulate B cell maturation. Chen and Ross (2007) reported that RA can promote B cell maturation toward a more differentiated phenotype by enhancing a proportion of B cells that express higher level of activation-induced deaminase (**AID**) and B lymphocyte-induced maturation protein-1 (Blimp-1) which may account for enhanced antibody production.

Although in our study treatment with lactobacilli increased antibody response against KLH, in ovo inoculation of vitamin D alone or in combination with lactobacilli did not change antibody response to different antigens. This could be related to the immunoregulatory activities of vitamin D that inhibits further activation of the immune system against foreign antigens. In contrast, Vazquez et al. (2018) demonstrated that dietary supplementation of vitamin D (5,000 IU/kg) increases serum antibody titers against Newcastle disease virus (**NDV**) and increases intestinal IgA levels in broiler chickens. The conflicting results observed between these studies might be explained by the difference in antigens (KLH vs NDV), in addition to the dosage and route of administration of vitamin D$_3$. Altogether, this suggests that one-time in ovo inoculation of vitamin D at 0.6 $\mu$g/egg may not deliver the same magnitude of

immunomodulatory effects compared to daily dietary intake.

In summary, while in ovo supplementation of probiotic lactobacilli modulates some aspects of immune responsiveness in newly hatched chicks, overall greater responses in terms of cytokine gene expression, lymphocyte population and antibody production were observed in the group that received RA. These finding suggest that in ovo supplementation of vitamin A might help chickens to develop immune competence. However, whether the induced responses would confer protection against subsequent infections requires further investigation.

## ACKNOWLEDGMENTS

## DISCLOSURES

Jake Astill was employed by the company "Artemis Technologies Inc".

The remaining authors declare that the research was conducted in the absence of any commercial of financial relationship that could be construed as potential conflict of interest.

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