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## Arginine and Vitamin E Improve the Cellular and Humoral Immune Response of Broiler Chickens

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**Abstract:** Two experiments were conducted to determine the effects of vitamin E (VE) and Arginine (ARG) on humoral and cellular immunity of broiler chickens, with 2 levels of ARG: normal (NARG, 1.2% in the feed) and high ARG (HARG, additional 0.3% ARG in the drinking water); and 3 levels of VE (40, 80, and 400 IU / kg feed). A corn-soybean meal diet and water were provided *ad libitum* throughout the study. Humoral immunity was measured as antibody responses to an i.p. injection of sheep red blood cells (SRBC, 10% solution) at d 25 followed by agglutination assay 4, 8, and 16 d after injection (Experiment 1). Cell-mediated immune response was assessed by the cutaneous basophil hypersensitivity test to phytohemagglutinin (PHA)-P at d 17 (Experiment 1) and PHA-M at d 41 (Experiment 1 and 2). Four d after SRBC injection the antibody titers ( $\log_2$ ) were higher in HARG than in NARG birds ( $P < 0.01$ ), and in VE-80 compared with VE-40 and VE-400 birds ( $P < 0.001$ ). At 8 and 16 d the antibody titers were not different between ARG levels, but the VE-80 birds maintained higher antibody titers ( $P < 0.001$ ) than VE-40 and VE-80 birds. Naïve birds fed HARG showed a higher response than NARG birds ( $P < 0.05$ ) to PHA-P at d 17 and to PHA-M at d 41, but after a second exposure ARG levels did not have a significant effect. Birds fed VE-400 had a lower response to PHA than birds fed VE-40 and VE-80 birds ( $P < 0.001$ ) at d 41. These results show that high levels of ARG accelerate antibody production whereas VE at 80 IU kg / feed maintains a proper immune function over time, suggesting that ARG and VE may have complimentary effects on the immune function and health of broiler chickens.

**Key words:** Arginine, vitamin E, humoral immunity, cell-mediated immunity

### Introduction

Poultry possess limited natural resistance against colonization or infection by pathogenic organisms. For this reason, the poultry industry has relied on the use of antibiotics to improve flock health and productivity. However, the use of antibiotics in animal production is under severe public scrutiny because livestock production practices have been linked to the development of antibiotic-resistant bacteria within the human population (Phillips *et al.*, 2004; Ratcliff, 2000). Consequently, the use of antibiotics in livestock production is being subjected to intense public scrutiny and consumers are showing disapproval for the use of antibiotics in animal agriculture. The poultry industry is hence compelled to find alternatives to the use of antibiotics in order to maintain and enhance markets for poultry products. It has been known that nutrition plays a significant role in the modulation and function of the immune response of chickens (Klassing, 1998; Kidd, 2004). Arginine (ARG), an essential amino acid for avian species, and vitamin E (VE) have been shown to directly influence the immune system of birds under several experimental models (Kidd *et al.*, 2001; Friedman *et al.*, 1998).

In chickens, an ARG deficient diet has been associated with poor development of the thymus and spleen (Kwak *et al.*, 1999), thymus size being a sensitive indicator of health and of acute and chronic stress response (Shelat

*et al.*, 1997). Arginine modulates or boosts humoral and cellular immune response to experimental infection challenges. The number of heterophyls and the heterophyll/lymphocyte ratio in the blood of chickens challenged with infectious bronchitis virus is increased with high dietary ARG (Lee *et al.*, 2002). Dietary ARG proportionally affects NO production and acute phase inflammatory response following lipopolysaccharide (LPS) injections and marginal deficiency of dietary arginine reduce splenocyte proliferative responses (Takahashi *et al.*, 1999). In mammalian models ARG has been reported to enhance wound healing response as assessed by wound breaking stress and collagen synthesis (Shi *et al.*, 2002). In the diet of humans and injured animals arginine enhances T lymphocyte responses, accelerates wound healing, and improves survival (Barbul *et al.*, 1990; Kirk *et al.*, 1993).

On the other hand, VE supplementation increases the response of anti-T toxoid IgA antibody titers in intestinal scraps (Muir *et al.*, 2002), modulate CD4<sup>+</sup>CD8<sup>-</sup> T cell subset populations (Erf *et al.*, 1998), and increases lymphocyte proliferative responses to concavalin A and Salmonella typhimurium LPS during heat stress (Puthongsiriporn *et al.*, 2001). Vitamin E supplementation of progenitors improves antibody titers in the chicks (Hag *et al.*, 1996) whereas VE supplementation *in ovo* results in enhanced antibody and macrophage response in the chicks (Gore and

Qureshi, 1997). Friedman *et al.* (1998) reported that excessive vitamin E intake has a detrimental effect on antibody production in chickens and turkeys. Accordingly, it is important to establish the optimum efficacy of VE as affected by management practices as well as in combination with other immune enhancing feed ingredients.

The previous results clearly indicate the potential to use ARG and VE as a tool to improve immune response and resistance to infectious challenges in poultry, and potentially as a means to reduce the use of antibiotics in poultry production. However, to date, there are no reports on the effects of the combination and interactions of amino acids and vitamins on cellular and humoral immune response of chickens. We hypothesize that the combination of ARG and VE has a synergistic effect on immune response and health of poultry because each compound acts at different levels of the immune system and with different mechanisms. Then, the objective of this study was to evaluate the effects of ARG and VE supplementation on cell mediated and humoral immune response of broiler chickens.

## Materials and Methods

**Animals and diet:** Two experiments were conducted to evaluate the humoral and cell mediated immune response of birds fed different levels of ARG and VE. One day old commercial broiler male chicks (Cobb 500) were obtained from a local hatchery and wing banded at d 1. The chicks were housed in battery cages inside temperature and light controlled environmental chambers. The birds were brooded following standard temperature regimes, which gradually decreased from 32° to 24°C, and under a 16:8 light:dark cycle throughout the studies.

All the birds were provided with a corn-soybean meal based diet formulated to meet or exceed all of the NRC (1994) requirements, including 22.5% CP and 3150 Kcal/kg of ME for starter feed, 19% of CP and 3250 Kcal/kg of ME for the finisher feed, and 1.2% ARG in both starter and finisher feed. The birds were supplied with water from a low pressure nipple-drinking water system *ad libitum* throughout the study.

In both experiments a 2 x 3 factorial arrangement of treatments was used with 2 levels of ARG: normal ARG (NARG, no further supplementation above the ARG levels in feed) and high ARG (HARG, 0.3% ARG in the drinking water), and three 3 levels of VE: 40, 80, and 400 IU / kg feed of dl- $\alpha$ -tocopheryl acetate (VE40, VE80, and VE400, respectively). Each treatment was replicated six times (three chickens per replicate) for a total of 72 birds in each experiment.

**Evaluation of humoral immune response:** At day 25, four chickens per treatment were injected i.p. with 1 mL of a 10% solution of sheep red blood cells (SRBC<sup>3</sup>) in

PBS, and 2 chickens per treatment were injected with 1 mL of PBS (Experiment 1). Blood samples (2 ml) were collected from the wing vein before and 4, 8, and 16 days after SRBC injection. The samples were centrifuged at 1,500 rpm for 10 min and the serum collected and stored at -20°C until analysis were performed. The hemagglutination assay was performed as described by Wegman and Smithies (1966). The complement was inactivated by heating the serum samples at 56°C for 30 m. The hemagglutination assay was done by adding 0.05 mL of diluent (PBS with 0.05% BSA<sup>2</sup>) to each well of a 96-well microtiter plate. The initial well in each row received 0.05 mL serum, which was then serially and doubly diluted. One drop (0.05 mL) of 2% SRBC solution in PBS was placed in each well. The plates were shaken for 1 min, incubated for 1h, shaken again for 1 min, incubated for 2 h at room temperature and then scored. The antibody titers were expressed as the log<sub>2</sub> of the reciprocal of the highest dilution giving 50% agglutination (Brugh, 1978; Nelson *et al.*, 1995).

**Cell-mediated immune response evaluation:** The cell mediated immune response was assessed using the Cutaneous Basophil Hypersensitivity response to phytohemagglutinin (PHA, lectin from *Phaseolus vulgaris*; PHA-P and PHA-M<sup>3</sup>). Six birds from each treatment were intradermally injected between the 3<sup>rd</sup> and 4<sup>th</sup> digits of the right foot with 100  $\mu$ g of PHA-P in 0.1 mL of sterile PBS solution at d 17 (Experiment 2). The thickness of the skin was measured with a pressure sensitive micrometer before the injection and 24 h after the injection. The same birds were exposed to PHA-M at d 41 following the procedure previously described, but this time the injection was applied in the left foot. The birds exposed to SRBC in Experiment 1 were injected with PHA-M at d 41 and measurements taken as previously described. The CBH response to PHA was evaluated by determining the thickness difference of the interdigital skin before and 24 h after PHA (Corrier and Deloach, 1990).

**Body weights and lymphoid organ weights:** In both experiments chickens were weighed every week and feed consumption calculated on a weekly basis. Mortality was recorded daily. At d 42 all chickens were weighed and euthanized. The bursa of Fabricius, spleen and thymus (all lobes) were immediately removed, carefully stripped of adhering connective tissue, and individually weighed. Relative organ weights were calculated as percentage of live body weight. Body weight, feed consumption and feed conversion, and lymphoid organ weights data from the two experiments were pooled for statistical analysis.

**Statistical analysis:** All data were analyzed as a two way ANOVA with ARG and VE as main effects using the

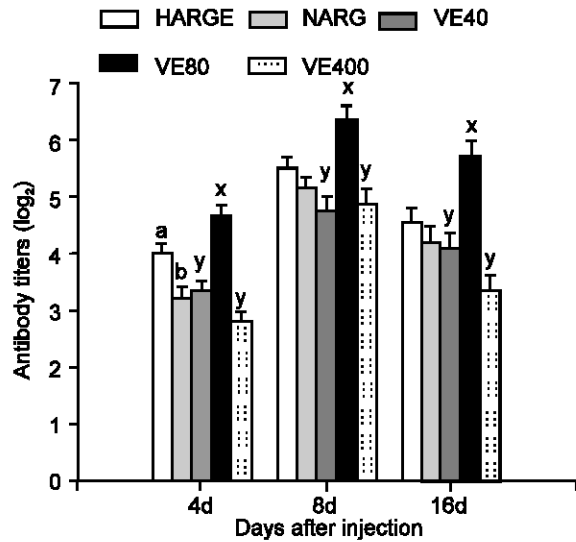
SigmaStat® software. Significance was declared at  $P < 0.05$ , unless otherwise indicated. Means were separated using the Student Newman-Keuls multiple comparison method.

**Results**

**Humoral immune response:** No antibodies against SRBC were detected in blood samples collected before the injection with the SRBC solution, or in the blood of birds injected with PBS. However, 4 d after SRBC injection the antibody titers ( $\log_2$ ) to SRBC were higher in HARG ( $4.00 \pm 0.18$ ) than in NARG birds ( $3.22 \pm 0.18$ ;  $P < 0.01$ ), and in VE80 ( $4.66 \pm 0.22$ ) than in VE40 and VE400 birds ( $3.33$  and  $2.83 \pm 0.23$ , respectively;  $P < 0.001$ ). At 8 and 16 d antibody titers against SRBC were not different between HARG and NARG birds, but the VE80 group maintained higher antibody titers ( $6.37 \pm 0.25$ ) than VE40 and VE80 birds ( $4.75$  and  $4.87 \pm 0.25$ , respectively,  $P < 0.001$ ). Antibody titers between VE40 and VE400 birds were not different at any sampling time. Overall, the antibody titers to SRBC reached their peak at 8 d after SRBC injection (Fig. 1). The interaction between ARG and VE was not significant.

**Cell-mediated immune response:** The results for the Cutaneous Basophil Hypersensitivity test are shown in Fig. 2. We found that birds in the HARG group had a stronger response to the PHA-P injection at d 17 compared with the birds in the NARG group ( $0.641 \pm 0.05$  vs.  $0.463 \pm 0.05$  mm,  $P < 0.05$ ). Birds fed different levels of VE had a similar response against the inoculation with PHA-P at d 17. When the same birds were injected with PHA-M at d 41, birds fed HARG levels had a tendency to have a higher response than birds in the NARG group ( $P = 0.07$ ), whereas birds in the VE40 and VE80 group had a higher response than birds in the VE400 group ( $P < 0.001$ ), and birds in the VE80 group have a tendency to have a higher response than birds in the VE40 group ( $P = 0.06$ ). In the first experiment, when the birds were injected with PHA-M without previous exposure to PHA at d 41, HARG birds had a stronger response ( $1.9 \pm 0.08$ ) than NARG birds ( $1.5 \pm 0.07$ ;  $P < 0.001$ ), whereas VE80 had a higher response than VE400 ( $P < 0.01$ ) but similar to that of VE40. The interaction between ARG and VE was not significant.

**Relative lymphoid organ and body weight:** The relative weights of bursa, spleen, and thymus are shown in Fig. 3. Arginine levels did not affect the relative size of any of the three lymphoid organs evaluated. However, the level of VE affected the relative weight of the bursa and the thymus. The relative size of the bursa was reduced with higher levels of VE (VE400) compared with the bursa of birds fed 40 and 80 IU of VE ( $P < 0.05$ ). The relative weight of the bursa was not different between the VE40 and VE80 birds. On the other hand, the relative size of



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Fig. 1: Antibody titers ( $\log_2$ ) to sheep red blood cells in broilers after 4, 8 and 16 days post-inoculation. The chickens were injected i.p. with 1 mL of a 10% solution of sheep red blood cells at d 25. Broilers (Cobb 500) were fed with 2 levels of arginine: normal (NARG, 1.2% in the feed) and high ARG (HARG, additional 0.3% ARG in the drinking water); and 3 levels of VE (40, 80, 400 IU/kg feed).

<sup>a,b</sup>Different superscripts indicate significant differences between ARG levels.

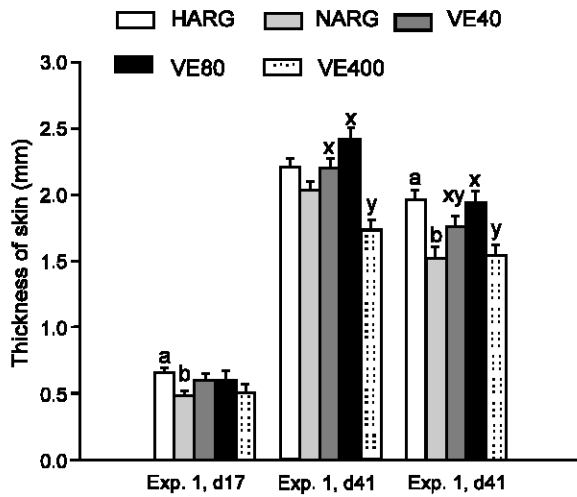
<sup>x, y</sup>Different superscripts indicate significant differences among VE levels

the thymus was enhanced in birds fed the VE80 diet compared with birds in the VE40 and VE400 diets. Neither ARG nor VE had effects on the relative size of the spleen.

Birds in the HARG group were heavier than birds in the NARG group at wks 1 and 2, but afterwards (wk 3 through wk 6) there were not differences in BW between the two groups. Body weight was also higher in the VE40 birds at wks 1 and 2, but afterwards birds fed the VE80 diet were heavier than birds fed the VE40 and VE400 diet (Table 1). There was a significant interaction between the two factors at all weekly BW recorded. In general, it was observed that higher levels of VE reduced BW, but when high levels of VE were combined with HARG levels the BW was improved (Table 1). Feed consumption, feed conversion and mortality were not affected by the dietary treatments (data not shown).

**Discussion**

The SRBC is a T cell-dependent antigen which helps B-cells to produce antibodies (Nelson *et al.*, 1995). It is known that Th2 cells help B cells in producing specific antibody through the production of cytokines which



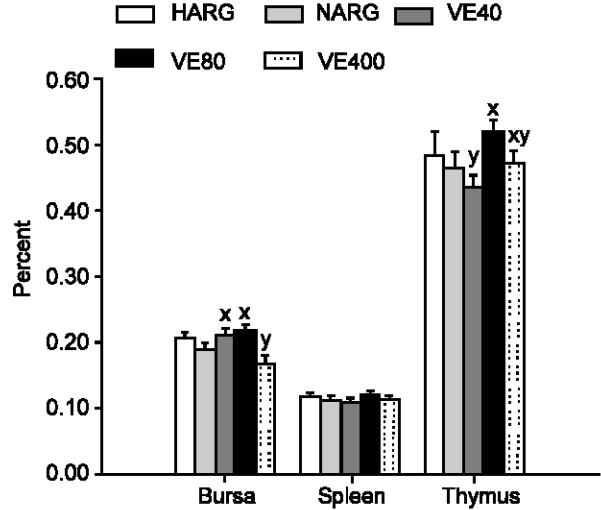
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Fig. 2: Cutaneous Basophil Hypersensitivity (CBH) test responses to PHA-P and PHA-M in broilers (Cobb 500) fed 2 levels of arginine: normal (NARG, 1.2% in the feed) and high (HARG, additional 0.3% ARG in the drinking water); and 3 levels of vitamin E (40, 80, 400 IU/kg feed). The CBH response was determined as the skin thickness difference before and 24 h after an intradermal injection (100  $\mu$ g, PHA-P at d 17 and PHA-M at d 41) between the 3<sup>rd</sup> and 4<sup>th</sup> digits of the right foot.

<sup>a,b</sup>Different superscripts indicate significant differences between ARG levels.

<sup>x,y</sup>Different superscripts indicate significant differences among VE levels

promote activation and growth of B-cells, and, consequently, enhance humoral immunity. The assessment of humoral immune response through the production of hemagglutinin antibodies to SRBC is commonly used because it is rapid and does not involve the use of pathogenic agents. It has been reported that ARG plays an important role as a potent immunological modulator through the production of nitric oxide (Collier and Vallance, 1989), which is involved in modulating T-cell mitogenic responses. Also, VE alters cytokine production and CD4:CD8 T cell ratio (Erf *et al.*, 1998) and it can activate B cell levels of specific antibodies. The results of this experiment showed that ARG supplementation above the NRC (1994) requirements and VE supplementation levels 8 times higher than those recommended by the NRC (1994) greatly improved the humoral immune response of birds challenged with SRBC. We found that high levels of ARG improved the antibody response to SRBC measured 4 d after inoculation; however, although antibody levels in the HARG treatment were numerically higher at 8 and 16 d after SRBC inoculation, they were not significantly



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Fig. 3: Relative lymphoid organ weights of 6 wk - old broilers (Cobb 500) fed 2 levels of arginine: normal (NARG, 1.2% in the feed) and high (HARG, additional 0.3% ARG in the drinking water); and 3 levels of vitamin E (40, 80, 400 IU/kg feed). Relative lymphoid organ weights were calculated as percentages of body weight.

<sup>x,y</sup>Different superscripts indicate significant differences among VE levels.

different from antibody levels in the NARG birds. Supplementation of VE at 80 IU / kg of feed had a positive and significant effect on antibody production at the three sampling periods compared with normal and very high VE levels (40 and 400 IU / kg feed, respectively). Very high levels of VE had a tendency to reduce the antibody response as compared with normal levels of VE, although the differences were not significant. Then, although no interactions were detected between ARG and VE supplementation on antibody response to SRBC, it appears that ARG and VE could play a complementary role on immune response, in which high levels of ARG may enhance the effects of VE, especially in the early stages of antibody production. Kidd *et al.* (2001) did not find significant effects in the primary antibody response against SRBC in diets containing 0.20% ARG supplementation above the NRC (1994) recommendations. The supplementation level used in this experiment (0.3% in the drinking water) is by far higher than the one used by Kidd *et al.* (2001), which may explain the different results. The effects of VE on immune function reported in the literature are highly variable, depending on the dose, the strain and age of the birds, and the immune challenge. Leshchinsky and Klasing (2001) found that dietary levels of 25-50 mg / kg of VE improved immunity while levels of 200 IU / kg of VE

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Table 1: Weekly body weight (g) of broilers fed normal (NARG, 1.2% in the feed) and high (HARG, additional 0.3% in the drinking water) levels of Arginine and different levels of vitamin E (VE, 40, 80 and 400 IU / kg of feed).

Arginine	wk 1	wk 2	wk 3	wk 4	wk 5	wk 6
HARG	201 <sup>a</sup>	578 <sup>a</sup>	1020	1552	2264	2861
NARG	204 <sup>b</sup>	545 <sup>b</sup>	1029	1561	2256	2894
SEM	0.2	6.05	12.8	25.5	26.5	39.3
P- value	<0.001	<0.001	0.604	0.803	0.831	0.558
VE						
VE40	204 <sup>y</sup>	578 <sup>x</sup>	1015 <sup>xy</sup>	1543 <sup>y</sup>	2279 <sup>y</sup>	2919 <sup>x</sup>
VE80	201 <sup>x</sup>	556 <sup>xy</sup>	1058 <sup>x</sup>	1669 <sup>x</sup>	2401 <sup>x</sup>	3053 <sup>x</sup>
VE400	203 <sup>y</sup>	550 <sup>y</sup>	1002 <sup>y</sup>	1458 <sup>y</sup>	2099 <sup>z</sup>	2659 <sup>y</sup>
SEM	0.3	7.8	15.7	31.2	32.4	48.1
P- value	<0.001	<0.025	0.049	<0.001	0.001	<0.001
ARG x VE						
HARG x 40	201.5 <sup>y</sup>	563 <sup>x</sup>	974 <sup>y</sup>	1409 <sup>y</sup>	2118 <sup>y</sup>	2738 <sup>x</sup>
HARG x 80	199 <sup>x</sup>	598 <sup>x</sup>	1095 <sup>x</sup>	1730 <sup>x</sup>	2456 <sup>x</sup>	3105 <sup>y</sup>
HARG x 400	203.5 <sup>z</sup>	574 <sup>x</sup>	991 <sup>y</sup>	1517 <sup>y</sup>	2217 <sup>y</sup>	2739 <sup>x</sup>
NARG x 40	206 <sup>y</sup>	594 <sup>y</sup>	1055 <sup>x</sup>	1677 <sup>x</sup>	2440 <sup>x</sup>	3100 <sup>x</sup>
NARG x 80	202.5 <sup>xz</sup>	515 <sup>x</sup>	1020 <sup>x</sup>	1607 <sup>x</sup>	2345 <sup>xy</sup>	3001 <sup>x</sup>
NARG x 400	203.5 <sup>z</sup>	527 <sup>x</sup>	1013 <sup>x</sup>	1399 <sup>y</sup>	1981 <sup>y</sup>	2579 <sup>y</sup>
SEM	0.5	10.4	22.05	44.1	45.2	68
P- value	<0.001	<0.001	0.006	<0.001	<0.001	<0.01

<sup>a,b,xy,z</sup> Values with different superscripts are statistically different within the same column and the same factor (P<0.05)

have a dampening effect on T helper cell function or T helper cell numbers. Friedman *et al.* (1998) reported that antibody production against *E. coli* and Newcastle disease virus was better when VE was supplemented at 0 to 10 mg / kg of feed, but was depressed with VE levels of 30-150 mg / kg of feed in chickens and turkeys. We found that antibody production against SRBC was higher with the VE80 diet, however, even with the VE400 diet, antibody production was similar than that of the VE40 diet.

We assessed cell-mediated immunity through the hypersensitivity skin test to an intradermal injection of PHA. This approach has been widely used as an *in vivo* screening test due to its practicality and simplicity (Blaese *et al.*, 1973). Goto *et al.* (1978) demonstrated that the PHA skin reaction was a thymus-dependent response using whole and thymectomised chickens. The supplementation of ARG and VE significantly affected the CBH response in the two experiments. High levels of ARG significantly improved the CBH response in naïve birds (experiment 1 at d 17 and experiment 2 at d 41), but in birds that were previously exposed to PHA the effects of ARG were not significant. On the other hand the effects of VE were not significant in naïve birds at d 17, but after a second exposure at d 41 birds fed VE80 diets had a numerically higher response than birds fed VE40 diets (P = 0.06, experiment 1). At d 41 naïve birds in the VE80 diet had a tendency to have a higher response than birds in the VE40 diet. Our results suggest that VE modulation of cell mediated immune response is greater in older than in young birds, and in birds that have been previously exposed to PHA. These

results further suggest the complementarity of ARG and VE in the immune response of broiler chickens, in which high levels of ARG accelerate the immune response in naïve birds while high levels of VE maintain higher immune response when the birds are exposed a second time to the same antigen. Leshchinsky and Klasing (2001) reported that supplemental VE ranging from 0 to 200 IU / kg of feed did not affect CBH response to PHP-A. Boa-Amponsem *et al.* (2000) found that high levels of VE (300 mg / kg) depressed CBH response to PHA-P as compared with VE levels of 10 mg/kg. The previous results agree with our findings in naïve, young chickens; however in older birds and birds sensitized to PHA the VE80 diets tended to have a higher response than birds fed the VE40 diets, while birds consuming the VE400 diets had a reduced response, indicating that the efficacy of VE on cell mediated immune response is affected by age and previous exposure to antigens.

Lymphoid organ size and development has been known to be directly correlated with the health status of animals. In chickens, an arginine deficient diet has been associated with poor development of the thymus and spleen (Kwak *et al.*, 1999), thymus size being a sensitive indicator of health and of acute and chronic stress response (Shelat *et al.*, 1997). In mammals dietary ARG improves thymus weight and function, and enhances lymphocyte mitogenesis (Efron and Barbul, 1998). Konjufca *et al.* (2004) reported that VE at levels of 110 and 220 mg / kg of feed increased the size of the spleen compared with birds fed 16 mg / kg of VE, and this effect was attributed to an increase in the number of lymphocytes. In our experiments we did not find any

effects of ARG or VE on the relative size of the spleen. The relative size of the thymus was higher in birds fed the VE80 diet as compared with birds in the VE40 diet, and birds fed HARG diets had numerically heavier thymus than birds fed the NARG diet. It is interesting to note that the trends in the thymus size (Fig. 3) closely match the trends in antibody titers and CBH response (Fig. 1 and 2), indicating that the size of the thymus is a good indicator of immune function. The relative size of the bursa of Fabricius was not affected by ARG levels, but high levels of VE (VE400) reduced the size of this organ.

High level of ARG did not have significant effects on BW at the end of both experiments, which agrees with the reports of Kidd *et al.* (2001), and Corzo *et al.* (2003), indicating that ARG levels above the NRC requirements do not improve live weight performance. However, Corzo *et al.* (2003) found that high levels of ARG were associated with lower carcass incidence of skin scratch infections and parts defects from processing stresses, suggesting that additional ARG is needed for immunological and connective tissue challenges. On the other hand we found that VE at levels of 80 IU / kg of feed improved BW; however, the effects of VE on BW have been controversial. Villar-Patino *et al.* (2002) reported that diets with VE at 75 mg / kg of feed improved BW of broiler chickens, which agrees with our results; however, Swain *et al.* (2000) found the best response in growth performance using diets with 300 IU of VE / kg of feed, whereas Bartov and Frigg (1992) did not find a significant response in BW using VE levels of 24 and 150 mg / kg of feed. In any case, our results show for the first time that the combination of VE and ARG may have a synergistic effect on growth performance.

In summary, we found that the combination of high levels of ARG and VE play complementary roles in the cellular and humoral immune response of broiler chickens and improves growth performance. The improvement of the immune response can be used as a feeding strategy to reduce the effects of vaccination and other stressors that render the animals susceptible to opportunistic infections such as *E. coli* and Salmonella, and to improve the health status and wellbeing of broiler chickens using naturally occurring feed ingredients. Further research is needed to test the efficacy of ARG and VE on actual viral and bacterial challenges.

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