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Effects of Wheat-Soybean Meal Based Diet Supplementation with Vitamin A, Vitamin E and Zinc on Blood Cells, Organ Weights and Humoral Immune Response in Broiler Chickens

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Abstract: This experiment was conducted to evaluate the effects of additional supplementation of the diet with vitamin A, vitamin E and zinc, on blood cells, organ weights and humoral immune response of broiler chickens fed a wheat-soybean meal based diet. In a completely randomized design with 2×2×2 factorial arrangement, 224 day-old male broiler chicks were assigned to 32 groups. Factors and their levels were as follows: vitamin A (basal diet, basal diet supplemented with 10,000 IU kg^{-1} retinol acetate); vitamin E (basal diet, basal diet supplemented with 50 IU kg^{-1} α-tocopherol acetate) and Zinc (basal diet, basal diet supplemented with 60 mg kg^{-1} Zn using zinc oxide). Sampling for blood and organ weights were done at 21 day of age. Humoral immune response were evaluated by intramuscular injection of Sheep Red Blood Cells (SRBC) at 21 day of age followed by bleeding at 7 and 14 day post injection. Supplementation of the diet with vitamin A, vitamin E, or zinc significantly ($p = 0.001$) increased the number of White Blood Cells (WBC); but had no effect on Red Blood Cell (RBC) counts, Hematocrit (Ht) and Haemoglobin (Hb). Vitamin A supplementation significantly ($p = 0.016$) decreased the proportion of monocytes in total number of counted monocytes, lymphocytes and heterophils. Supplementation with zinc significantly decreased ($p = 0.003$) the ratio of heterophils to lymphocytes. A significant vitamin A × vitamin E × zinc interaction was found for WBC counts and for the proportions of lymphocytes and heterophils ($p = 0.001$). Supplementing the diet with either vitamin A or vitamin E had no significant effect on relative weights of liver, bursa and spleen. However, addition of zinc to the diet significantly ($p = 0.019$) increased relative weight of spleen. Supplementation of the diet with vitamin E or zinc (but not with vitamin A), significantly increased total antibody titres against SRBC. The results suggest that additional supplementation of the diet with vitamin A, vitamin E, or zinc can be effective in general immune responses by affecting blood cell proportions and also indicated that zinc can be considered as an anti-stress nutrient, regarding the heterophil to lymphocyte ratio index. It also, indicates that vitamin E is more important than vitamin A or zinc in persistency of an immune response.

Key words: Vitamin A, vitamin E, zinc, blood cells, organ weights, antibody response, broiler

INTRODUCTION

Application of antimicrobial growth promoters in poultry nutrition is a strategy which can improve performance and help better protection against diseases and environmental challenges. However, use of antimicrobials in this manner has increased the possibility for pathogenic microorganisms to develop resistance against antibiotics. In search for appropriate alternatives to in-feed antibiotics, vitamin and trace minerals as potential enhancers of immune function against pathogenic and environmental challenges are receiving increasing interest. On the other hand, intensive selection toward rapid growth in broiler lines has negatively affected some of other criteria such as immune responses and stress tolerance in recent commercial broilers (Qureshi and Havenstein, 1994; Cahaner *et al*., 1998). Rapid growth seen in recent commercial broilers might partially have changed nutrient requirements of these lines, especially for micronutrients.

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Vitamins and trace minerals can modulate immune responses due to their extensive involvement in structural components and molecular mechanisms. Vitamin A has a significant role in maintenance of mucosal surfaces, generation of antibody responses, haematopoiesis and function of T and B lymphocytes (Semba, 1999). Feeding a vitamin A-deficient diet to chicks has negatively affected mucosal defence barrier (Uni *et al*., 1998) and decreased bursa and thymus weight (Davis and Sell, 1983). Vitamin A deficiency has been associated with immunosuppression and reduced resistance to infection (Bowman *et al*., 1990; Friedman *et al*., 1991). Vitamin A has a critical role in turnover of microvilli in the intestine and keeping gut integrity (Semba, 2002). Vitamin E as an antioxidant nutrient is involved in maintaining function of the immune cells (Meydani and Beharka, 1998). Providing chicks with a vitamin E-deficient diet reduced lymphocyte numbers in the bursa and thymus (Marsh *et al.,* 1986). Vitamin E has enhanced immunity of chickens to several diseases such as Escherichia coli infection, coccidiosis, infectious bursal diseases and Newcastle diseases (Erf *et al*., 1998). It seems that vitamin E has some effects on the absorption and utilization of vitamin A and protects vitamin A from oxidative breakdown (Gallo-Torres, 1980). Zinc also, has a role in chicken immune function and resistance to diseases (Kidd *et al*., 1996). Zinc contributes in the differentiation of many cell types including immune cells by regulation of DNA transcription, in the form of zinc finger proteins (Luscombe *et al*., 2000). Birds given a diet marginal in zinc showed a decrease in humoral immune response (Burns, 1983).

In spite of considerable researches in the area of vitamins and trace minerals in nutrition, still it is not clear which dose is optimal and what kind of combinations will be beneficial for an immune response. There are many positive and negative interactions among vitamins and trace minerals which might give a completely different result when they are added individually or in combination, into the diet. This study was conducted to evaluate effects of supplementation of the diet with vitamin A, vitamin E and Zinc, singularly or in combination on the haematology, organ weights and antibody response to Sheep Red Blood Cells (SRBC) challenge in broiler chickens.

MATERIALS AND METHODS

Two hundred and twenty four day-old broiler chicks were divided to 32 groups, 7 chicks each and were housed in battery cages and fed a wheat-soybean meal based diet (Table 1). A completely randomized design with a 2×2×2 factorial arrangement was used.. Factors and their levels were as follows: Vitamin A (basal diet, basal diet supplemented with 10,000 IU kg^{-1} retinol acetate); vitamin E (basal diet, basal diet supplemented with 50 IU kg^{-1} α-tocopherol acetate) and zinc (basal diet, basal diet supplemented with 60 mg kg^{-1} Zn using zinc oxide). Vitamin A and vitamin E used in this study were products of BASF, Germany; and zinc oxide was purchased from a local retailer. Chicks had free access to feed and water during the entire period of the experiment.

Table 1: Composition of the basal diets

Ingredients (g 100 g^{-1})	Starter (0-28 day of age)	Grower (28-42 day of age)
Wheat	64.45	71.86
Soybean meal, 44%	26.00	23.34
Fish meal	4.00	-
Dicalcium phosphate	0.88	0.90
Limestone	1.19	1.33
Vitamin premix[1]	0.25	0.25
Mineral premix[2]	0.25	0.25
Salt	0.28	0.24
Sunflower oil[3]	2.50	1.64
DL-Methionine	0.14	0.08
Lysine	0.01	0.06
Enzyme*	0.05	0.05
Total	100	100
Calculated value		
Metabolic energy (Kcal kg^{-1})	2900	2900
Crude protein (%)	20.84	18.20
Calcium (%)	0.91	0.82
Available phosphorus (%)	0.41	0.32
Sodium (%)	0.18	0.18
Zinc (mg kg^{-1})	102	99
Vitamin A (IU)[4]	9000	9000
Vitamin E (IU)	33.30	28.60
Arginine (%)	1.23	1.03
Lysine (%)	1.12	0.91
Methionine+ cystine (%)	0.82	0.61

*Endofeed W, GNC, Biofern Inc., Canada: [1]Provided per kg of diet: Vitamin A, 9000 IU: Vitamin D_3, 2000 IU: Vitamin E, 11 IU: Vitamin K_3, 2 mg: Thiamine, 1.775mg: Riboflavin, 6.6mg: Vitamin B_3, 9.8mg: Vitamin B_5, 29.7 mg; vitamin B_6, 1.176 mg: Vitamin B_9, 1 mg: Vitamin B_{12}, 0.015 mg: Vitamin H_2, 0.1 mg: Choline chloride, 500 mg: [2]Provided per kg of diet: Mn, 76 mg; Zn, 66 mg; Fe, 40 mg; Cu, 4 mg; I, 0.64 mg; Se, 0.2 mg; [3]Containes 608 mg vitamin E per kg (Dorrel, 1978) [4]This amount comes only from vitamin premix

At 21 days of age, one chick from each replicate of treatments was bled and blood samples were collected in EDTA-treated tubes for enumeration of blood cells in haematology lab. Blood films were stained with Giemsa-Wright's stain after drying at room temperature. Differential WBC counts were done using standard avian guidelines of Ritchie *et al*. (1994). At the same day, one chick from each replicate was euthanized by cervical

dislocation, abdominal cavity was opened and liver, spleen and bursa of fabricius were excised and weighed.

In order to investigate the immune response to SRBC, at day 21, all chicks were injected intramuscularly with 0.2 mL of 5% SRBC solution in 9 g L^{-1} sodium chloride solution. Blood was collected from a young male sheep in EDTA-treated tubes and centrifuged at 2000 rpm for 15 min. The supernatant was discarded and cells were washed 3 times with sodium chloride solution. Five percent SRBC suspension was made using collected cells and sodium chloride solution. SRBC solution needed for antibody titre measurement was prepared using blood collected from the same sheep and according to the above procedure. After 7 and 14 days of SRBC injection (21 and 35 days of age, respectively), one chick from each replicate was bled via brachial vein and serum was used for measurement of total antibody response to SRBC using a direct hemagglutination assay described by Haghighi *et al.* (2005). The highest dilution of serum which was able to visibly agglutinate an equal volume of 1% SRBC suspension, was recorded as anti-SRBC titre and expressed as $\log_2$ of the reciprocal of the dilution.

Collected data were analysed using the General Linear Models procedure of the SAS (SAS, 1990). The main effects of vitamin A, vitamin E and zinc together with their interactions were considered in the statistical model used for analysis. Significance of differences among means were tested using new Duncan multiple range test (SAS, 1990).

RESULTS AND DISCUSSION

Haematology: The results of the haematology are shown in Table 2. Supplementation of diet with vitamin A, vitamin E, or zinc significantly ($p = 0.001$) increased the number of White Blood Cells (WBC) in the taken blood samples. Vitamin A, vitamin E and zinc supplementation had no effect on Red Blood Cell (RBC) counts and the amounts of Hematocrit (Ht) and Haemoglobin (Hb). However, a significant vitamin A × vitamin E interaction was seen on RBC, Ht and Hb ($p = 0.013$, $p = 0.014$ and $p = 0.025$, respectively) which means when vitamin A has been added to the diet together with vitamin E, the tendency for reduction of RBC, Ht and Hb, has been moderated. Vitamin A supplementation significantly ($p = 0.016$) decreased the percentage of monocytes in total number of counted monocytes, lymphocytes and heterophils. Vitamin A and vitamin E had no significant effect on the percentage of lymphocytes and heterophils, but supplementation with zinc significantly increased ($p = 0.004$) the percentage of lymphocyte, decreased ($p = 0.002$) the percentage of heterophils and decreased ($p = 0.003$) the ratio of heterophils to lymphocytes. Also, a significant vitamin A × vitamin E interaction was found for the percentage of lymphocytes and heterophils ($p = 0.024$ and $p = 0.049$, respectively). A significant vitamin A × vitamin E × zinc interaction was observed for WBC counts and the percentages of lymphocytes and heterophils ($p = 0.001$). These interactions show that when adding in combination, vitamin A, vitamin E and zinc have complementary or correcting effect on the blood parameters.

In this study, vitamin A and vitamin E significantly increased total number of WBCs. On the other hand, vitamin A significantly decreased the proportion of monocytes among counted WBCs. Monocytes are mononuclear cells which can leave the blood and act as macrophages in tissues. Supplementation of diet with vitamin A had no effect on the proportion of heterophils that is another important member of phygocytic system of the innate immunity. Coodley *et al.* (1993) reported that β-carotene increased the total number of WBCs in HIV infected humans. In a trial on blue tit, supplementation of females' diet with carotenoids resulted in higher leukocyte concentration in blood of progeny (Biard *et al.*, 2005).

Supplementation of the diet with 60 mg kg^{-1} zinc significantly increased the proportion of lymphocytes and decreased the proportion of heterophils in total counted WBCs (Table 2). Reduction in the number of lymphocytes in both central and peripheral lymphoid tissues has been reported in human and animals consuming zinc-deficient diets (Walsh *et al.*, 1994). It has been shown that zinc can perform as a mitogen for lymphocytes (Ruhl *et al.*, 1971; Ruhl and Kirchner, 1978). Rapid increase in intracellular Zn has been observed in mitogen-treated lymphocytes (Shankar and Prasad, 1998; Prasad, 2000) which indicates special requirement for zinc in activated lymphocytes. In the current study, dietary supplementation with zinc significantly decreased the heterophil to lymphocyte ratio. The heterophil to lymphocyte ratio has been indicated as a reliable stress index in poultry (Gross and Siegel, 1983). It has been demonstrated that different stressors such as frustration, fasting, limited access to water and crowding considerably increase the heterophil to lymphocyte ratio (Gross and Chickering, 1987; Cravener *et al.*, 1992; Hocking *et al.*, 1993). Although, we did not have any special stressor during this study, the observed effect of zinc on reducing the heterophil to lymphocyte ratio shows that zinc may act as a potential moderator of stress in poultry. This result is in agreement with findings of Sahin *et al.* (2005) who reported that supplementation of diet with zinc from organic or inorganic sources can reduce adverse effects of heat stress in quail. The combination of vitamin A and vitamin E was also effective

Table 2: Effects of supplementing the diet with vitamin A, vitamin E and Zinc, on blood cell counts of broiler chickens at 21 day of age

Treatment				Hematology							
Vit. A (IU)	Vit. E (IU)	Zn (mg)	n	WBC[1] (10^3 µL^{-1})	RBC (10^6µL^{-1})	Ht (%)	Hb (g dL^{-1})	M (%)	L (%)	H (%)	H L^{-1}
0	0	0	4	17.95	2.52	33.70	12.07	5.75	63.5	30.50	0.48
0	0	60	4	25.35	2.43	32.55	11.57	7.25	74.75	18.00	0.25
0	50	0	4	25.55	2.22	30.35	10.82	4.50	77.75	17.75	0.23
0	50	60	4	22.40	2.18	29.65	10.72	5.50	71.50	23.00	0.32
10000	0	0	4	21.60	2.22	30.67	11.00	4.75	73.25	22.00	0.30
10000	0	60	4	26.62	2.35	31.77	11.42	3.50	76.50	20.00	0.26
10000	50	0	4	24.67	2.28	31.17	11.40	4.00	67.75	28.25	0.42
10000	50	60	4	27.92	2.46	32.87	11.75	4.50	78.50	18.00	0.23
SEM				0.810	0.095	1.057	0.42	0.885	2.143	2.05	0.038
Source of variation				**Probability**							
Vit. A				<0.001	0.912	0.934	0.755	0.016	0.173	0.864	0.536
Vit. E				<0.001	0.181	0.133	0.259	0.266	0.228	0.552	0.424
Zn				<0.001	0.521	0.753	0.884	0.476	0.004	0.002	0.003
Vit. A*Vit. E				0.905	0.013	0.014	0.025	0.198	0.024	0.049	0.020
Vit. A*Zn				0.091	0.110	0.133	0.259	0.198	0.150	0.397	0.374
Vit. E*Zn				<0.001	0.699	0.728	0.787	0.610	0.112	0.114	0.144
Vit. A*Vit. E*Zn				<0.001	0.985	0.960	0.693	0.361	<0.001	<0.001	<0.001
Main effect means											
Vit. A											
0			16	22.81^{b}	2.34	31.56	11.30	5.75^{a}	71.87	22.31	0.32
10000			16	25.21^{a}	2.33	31.62	11.39	4.18^{b}	74.00	22.06	0.30
Vit. E											
0			16	22.88^{b}	2.38	32.17	11.52	5.31	72.00	22.62	0.32
50			16	25.14^{a}	2.28	31.01	11.17	4.62	73.07	21.75	0.30
Zn											
0			16	22.44^{b}	2.31	31.47	11.32	4.75	70.56^{b}	24.62^{a}	0.36^{a}
60			16	25.57^{a}	2.36	31.71	11.36	5.18	75.31^{a}	19.75^{b}	0.27^{b}

[1] WBC, White Blood Cell; RBC, Red Blood Cell; Ht, Hematocrit; Hb, Haemoglobin; M, Monocytes; L, Lymphocytes; H, Heterophils; H/L, Heterophil to Lymphocyte ratio; Vit, Vitamin, Zn, Zinc: [a,b] For each variable, means within a column with no common superscript differ significantly ($p<0.05$)

in reducing the heterophil to lymphocyte ratio which is indicated by significant vitamin A × vitamin E interaction ($p = 0.02$).

Organ weights: The obtained results for organ weights are shown in Table 3. Supplementation of diet with either vitamin A or vitamin E had no significant effect on relative weights of liver, bursa and spleen. Addition of zinc into the diet also had no significant effect on relative weights of liver and bursa, but significantly ($p = 0.019$) increased relative weight of spleen, comparing to other groups. Supplementation of the diet with vitamin E numerically increased relative weight of liver ($p = 0.084$). Singh *et al.* (2006) reported that addition of 200 mg vitamin E kg^{-1} of diet together with 0.2 mg selenium kg^{-1} of diet significantly increased bursal and splenic weights. It has been reported that excessive dietary vitamin E significantly increased liver relative weight (Nockels *et al.*, 1976) which is consistent with the results of this study. A tendency for vitamin E to increase liver relative weight was observed (Table 3). The first role for vitamin E in the liver as well as all the body is acting as an important naturally occurring antioxidant. This role is important in cellular respiration, heme synthesis and metabolism of fatty acids (especially polyunsaturated fatty acids) and lipoproteins (Devlin, 2006).

Spleen is a secondary lymphoid organ which reacts to antigens in bloodstream (Picker and Siegelman, 1999). Spleen is important in both antibody-mediated and cell-mediated immunity (Cyster, 2005). The observed enhancement in spleen relative weight due to supplementation of the diet with zinc can be due to proliferation of lymphocytes in this organ which is also indicated by a larger lymphocyte proportion in the blood in zinc supplemented group (Table 2). Mansour *et al.* (1983) reported that zinc supplementation via multiple intraperitoneal injection in *Schistosoma mansoni* infected hamsters, increased spleen weight and zinc uptake by splenocytes. These authors concluded that spleen may need more zinc to perform its multilateral zinc-dependent immunological functions. Hosea *et al.* (2007) reported a lower proportion of splenic CD90^{+} T-cells in zinc deficient rats. It has been reported that zinc deficiency can cause a significant reduction in weight and growth index of the bursa, thymus and spleen in chicken (Cui *et al.*, 2004).

Antibody response to SRBC: Supplementation of the diet with vitamin E significantly increased total antibody titres against SRBC (Table 4) measured at 7 and 14 days after injection ($p = 0.001$ and $p = 0.040$, respectively). Abdukalikova and Ruiz-Feria (2006) added vitamin E at 3 levels of 40, 80 and 400 IU kg^{-1} to a basal diet and

Table 3: Effects of supplementing the diet with vitamin A, vitamin E and zinc on relative weights (% of body weight) of liver, bursa of fabricius and spleen of broiler chickens at 21 days of age

Treatment				Relative organ weight		
Vit. A (IU)	Vit. E (IU)	Zn(mg)	n	Liver	Bursa	Spleen
0	0	0	4	3.03	0.19	0.08
0	0	60	4	3.34	0.27	0.11
0	50	0	4	3.27	0.26	0.09
0	50	60	4	4.19	0.21	0.14
10000	0	0	4	3.82	0.18	0.11
10000	0	60	4	3.41	0.21	0.15
10000	50	0	4	3.65	0.19	0.12
10000	50	60	4	4.47	0.24	0.16
SEM				0.390	0.031	0.022
Source of variation				**Probability**		
Vit. A				0.180	0.248	0.117
Vit. E				0.084	0.550	0.300
Zn				0.149	0.206	0.019
Vit. A*Vit. E				0.854	0.669	0.630
Vit. A*Zn				0.461	0.669	1.000
Vit. E*Zn				0.107	0.187	0.687
Vit. A*Vit. E*Zn				0.582	0.101	0.747
Main effect means						
Vit. A						
0			16	3.46	0.23	0.108
10000			16	3.84	0.21	0.133
Vit. E						
0			16	3.40	0.21	0.112
50			16	3.90	0.23	0.129
Zn						
0			16	3.45	0.21	0.10^{b}
60			16	3.86	0.23	0.14^{a}

[a,b] For each variable, means within a column with no common superscript differ significantly: ($p<0.05$). Vit, Vitamin; Zn, Zinc

Table 4: Effects of supplementing the diet with vitamin A, vitamin E and zinc on antibody response to SRBC in broiler chickens at 7 and 14 days after injection

Treatment				Antibody Response to SRBC	
Vit. A (IU)	Vit. E (IU)	Zn (mg)	n	7 day post injection	14 day post injection
0	0	0	4	2.500	2.00
0	0	60	4	3.25	2.25
0	50	0	4	4.500	3.50
0	50	60	4	4.75	2.50
10000	0	0	4	3.500	2.25
10000	0	60	4	3.75	2.00
10000	50	0	4	3.500	2.50
10000	50	60	4	5.00	2.75
SEM				0.411	0.448
Source of variation				**Probability**	
Vit. A				0.525	0.559
Vit. E				<0.001	0.040
Zn				0.026	0.559
Vit. A*				0.065	0.559
Vit. E					
Vit. A*Zn				0.525	0.559
Vit. E*Zn				0.525	0.559
Vit. A*				0.145	0.179
Vit. E*Zn					
Main effect means					
Vit. A					
0			16	3.75	2.56
10000			16	3.94	2.37
Vit. E					
0			16	3.25^{b}	2.12^{b}
50			16	4.43^{a}	2.81^{a}
Zn					
0			16	3.50^{b}	2.56
60			16	4.19^{a}	2.37

[a,b] For each variable, means within a column with no common superscript differ significantly: ($p<0.05$). SRBC, Ship Red Blood Cell; Vit, Vitamin; Zn, Zinc

evaluated antibody response of broiler chickens to SRBC. They showed that addition of 80 IU kg^{-1} of the diet significantly increased antibody response to SRBC measured at 4, 8 and 16 days after injection. In another experiment, Leshchinsky and Klasing (2001) reported that supplementation of diet with 50 IU, but not with zero or 200 IU vitamin E kg^{-1}, resulted a higher antibody response to SRBC in broiler chickens. Also it has been reported that injection of 10 IU vitamin E into the amnion of chicken embryos increased antibody response against SRBC in the hatched chicks (Gore and Qureshi, 1997). These results together with our findings in current experiment indicate that vitamin E (at levels around 50 to 100 IU kg^{-1} of the diet) improves humoral immune response and might be important in maintaining a proper immune response over time.

Supplementing the diet with zinc also had a significant effect on enhancement of antibody response to sheep erythrocytes at 7 days after injection ($p = 0.026$). However, this effect was not significant at 14 days after injection. Effect of zinc supplementation on humoral immune function has been shown in several studies. Bartlett and Smith (2003) reported that supplementation of basal diet (containing 34 mg kg^{-1} zing) with 147 mg kg^{-1} zinc from an organic source, significantly improved primary and secondary antibody response to SRBC challenge in broiler chickens. Supplementation of broiler breeder diet with zinc from organic and inorganic sources

(Zn-methionine and ZnO), significantly increased progeny response to SRBC (Kidd *et al*., 1993, 1992). SRBC is a T-cell dependent antigen (Nakae *et al*., 2001) which means after entering the body, it needs cooperation of T-helper cells for activation of B-cells to produce antibody. It has been shown that both vitamin E (Erf *et al*., 1998) and zinc (Hambidge *et al*., 1986) also have immunomodulatory effects on cell-mediated immunity or T-cell function. In this study, vitamin A supplementation had no significant effect on the antibody response against SRBC. There was no significant interaction on antibody response to SRBC too. From the literatures, it can be concluded that humoral immunity is less affected by vitamin A deficiency (Kidd, 2004). On the other hand, it has been demonstrated that both high and low levels of dietary vitamin A can affect lymphocyte proliferation responses to mitogens (Lessard *et al*., 1997). Nevertheless, although SRBC is a T-cell dependent antigen, no effect for vitamin A supplementation on antibody response to this antigen was seen.

CONCLUSION

Under the conditions of this study, it was concluded that additional supplementation of diet with vitamin A, vitamin E and zinc may influence the immune function by altering the proportion of different WBCs in the blood. Vitamin E, in particular, is important in persistency of immune response to SRBC challenge over time. Since vitamin A has a critical role in turnover of microvilli in the intestine and keeping gut integrity, it is possible that inclusion of viscose grains such as barley and wheat into broiler diets can increase their vitamin A requirements for a better maintenance of absorptive surface of the intestine. Results on heterophils to lymphocyte ratio also indicate that additional supplementation of the diet with zinc might be effective in alleviating the stress in broilers.

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