



Effect of Vitamin E Supplementation and High Stocking Density on the Performance and Stress Parameters of Broilers

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■ Keywords

Broiler performance, high stocking density, oxidative stress, vitamin E.

ABSTRACT

The present study was carried out to investigate the effect of dietary supplementation with vitamin E and stocking density on the mortality, body weight gain, feed conversion ratio, European production efficiency factor, heterophil/lymphocyte (H/L) ratio and liver glutathione (GSH) and malondialdehyde (MDA) levels of broilers. In total, 570 one-day-old Vencobb 400 broiler chickens were randomly assigned to three treatments, with six replicates each. G1 served as normal control and birds were provided a normal floor space (0.0465 m²/bird from day 1 to 21 and 0.0929 m²/bird from day 22 to 42) and basal diet containing vitamin E at 70 g/ton feed (n=150). G2 and G3 birds were subjected to high stocking density (0.0279 m²/bird from day 1 to 21 and 0.0557 m²/bird from day 22 to 42). G2 served as the high stocking density (HSD) control, birds were offered a basal diet with no vitamin E (n=210), whereas G3 birds were fed a basal diet containing vitamin E at 70 g/ton feed (n=210). The birds were fed with feed and water *ad libitum* throughout the study period. The supplementation of vitamin E to the birds subjected to HSD had a positive effect on body weight gain, feed conversion ratio (FCR), European Production Efficiency Factor (EPEF), H/L ratio, and liver GSH and MDA levels with those subjected to HSD and not supplemented with vitamin E. The results of the present study indicate that the supplementation of vitamin E at 70g/ton of feed to broilers subjected to HSD could effectively reverse the negative effects of HSD, and improve broiler performance.

INTRODUCTION

In general, broilers are subjected to various stressors, which include heat stress due to high ambient temperature, nutritional stress due to dietary nutritional imbalance, overcrowding stress due to housing of a high number birds per space unit, handling stress due to various procedures, like vaccination, debeaking, etc. High stocking density (HSD), along with high environmental temperature, may induce oxidative stress in birds as their body temperature and metabolic rate are relatively high when compared to mammals. Poultry can only express their full genetic potential when reared under thermoneutral temperatures (Cassuce *et al.*, 2013).

One of the most frequent stressors in broiler farms is HSD, because the farmers tend to reduce housing and equipment costs by overcrowding the birds, which negatively affects broiler performance and health. Moreover, heat stress activates the hypothalamic- pituitary-adrenal (HPA) axis and increases corticosterone levels of chickens, resulting in reduced feed intake, body weight gain, relative immune organ weight and innate immunity (Quinteiro-Filho *et al.*, 2010).



There are several expensive methods available to reduce the negative effects of high temperature and high stocking density, but the dietary manipulation is the easiest approach, and has been supported by several research studies (Souza *et al.* 2011; Khan *et al.* 2012). In this respect, vitamin E supplementation has been investigated because of the reported benefits in laying hens during heat stress, and reduced body vitamin E levels during heat stress (Sahin *et al.*, 2001). Many studies have reported that the inclusion of vitamin E in broiler diets not only reduces oxidative stress, but also improves the overall performance, as demonstrated by the higher body weight gain and reduced FCR of broilers fed vitamin E (Adebiyi *et al.*, 2011).

In general, stress stimulates the HPA axis and elevates blood glucocorticoid levels (Siegel, 1980), thereby accelerating the metabolic rates and increasing the generation of free radicals, in particular reactive oxygen species (ROS). These ROS may react with cell structures and attack proteins, nucleotides, carbohydrates and lipids within cell, and when the levels of natural antioxidants are not sufficient to quench the excessive ROS levels, this state is denoted as oxidative stress (Hidalgo *et al.*, 1988). Oxidative stress leads to the development of many pathological process (Gutteridge, 1993), and causes oxidative damage in the tissues and propagation of lipid peroxidation of the highly unsaturated fatty acids present in the cellular and sub-cellular membrane (Burton and Traber, 1990). This in turn induces a downstream cascade of the formation of reactive oxygen species, resulting in hydroxynonenol (relatively transient ROS) and malondialdehyde free radical. Thus, free radicals cause metabolic disorders, cell death, and growth retardation (Okada, 1996).

Vitamin E (α -tocopherol) is a hydrophobic antioxidant that scavenges the free radicals hydroxyl, alkoxyl, peroxy and superoxide anion through non-enzymatic defense (Amaneh *et al.*, 2015). Vitamin E has been reported to be an excellent biological antioxidant and its administration reduces the physiological response of organisms to stress through enzymatic and non-enzymatic defense mechanisms against free radicals (Dalolio *et al.*, 2015). One of the many approaches proposed to enhance immune response is the supplementation of broiler diets with vitamin E at the level of 65mg/kg Silva *et al.* (2011).

Vitamin E is considered a first line of defense against lipid peroxidation, preventing its chain propagation by quenching free radicals (Bjorneboe *et al.*, 1990). It acts on the early stage of lipid peroxidation (McDowell,

1989), and enhances cell membrane stability (Surai, 2003). Therefore, under stressful conditions, additional dietary vitamin E supplementation is necessary to protect tissues from lipid peroxidation. The performance of pigs was enhanced when the diet was supplemented with vitamin E at the level 100mg/kg of feed (Asghar *et al.*, 1991). The supplementation of vitamin E to Japanese quail diets significantly alleviated heat stress-related performance impairment, suggesting that dietary vitamin E supplementation is necessary under heat stress conditions (Sahin & Kucuk, 2001). The supplementation of vitamin E to broilers is also an important factor for the health of humans consuming chicken meat (Adebiyi *et al.*, 2011) as it is known to improve meat quality by upregulating the expression of antioxidant enzyme genes in broilers (Niu *et al.*, 2017).

The stress level of broilers can be measured by various means, and the heterophil to lymphocyte ratio (H/L ratio) is the most reliable methods (Mashaly *et al.*, 2004). Several studies concluded that H/L ratio is a more reliable indicator of the perceived magnitude of stress than plasma corticosterone values in avian species. Heterophils are important phagocytic cells in avian species. Gross & Siegel (1983) reported that the H/L ratios of 0.2, 0.5 and 0.8 corresponds to low, medium and high degrees of stress in chickens, respectively. Physiological and physical stressors tend to increase the H/L ratio (Maxwell *et al.*, 1998).

Malondialdehyde (MDA) is a three-carbon low-molecular weight aldehyde, and one of the final products of polyunsaturated fatty acids peroxidation in the cells. Blood MDA level is the most frequently used indicators of lipid peroxidation. Jena *et al.* (2013) demonstrated that broiler breeders supplemented with vitamin E had significantly lower malondialdehyde (MDA) levels, increased activities of superoxide dismutase (SOD) and catalase (CAT) enzymes, and ferric reducing antioxidant power (FRAP) in erythrocytes. Serum thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) can also be used as a measure of anti-oxidative status in broilers (Vashan *et al.*, 2012).

No studies had been conducted on the effects of the dietary supplementation of vitamin E at this low dose (70g/ton of feed) of broilers reared at high stocking densities. The aim of the present experiment was to study the effects of dietary vitamin E supplementation (70g/ton of feed) on the mortality rate, body weight gain, feed conversion ratio, European production efficiency factor, H/L ratio, and liver GSH and MDA levels of broilers reared at HSD.



MATERIALS AND METHODS

Ethical Approval

The study was conducted by authorized, qualified and trained veterinarians, scientists, and technicians, in compliance with the guidelines of the Institutional Animal Ethics Committee (IAEC) approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Selvam *et al.*, 2015).

Birds, Experimental Design and Setup

A total of 570 Vencobb 400 broiler chicks were used in this study. The experiment was conducted in poultry research station (PRS) located in Anniyalam, Tamilnadu for a period of 42 days between October and November 2014.

The chicks were housed in semi-closed house divided into pens with floor space of 2.32 square meters. The approximate size of the individual pen was 1.3 x 1.8 x 0.6 m (length x width x height). Each pen was equipped with a brooder, a bell drinker, chick feeder and/or jumbo feeder. The size and floor space of the pen were modified according to number of chicks housed with the help of a PVC sheet. House temperature was maintained at 32 to 34 °C from day 0 to 7, and progressively reduced by 2°C weekly till week 6. House relative humidity (RH) was maintained between 40 to 70% throughout the study period. Lighting was provided 24 hours daily during the first 3 days, 23 hours light and one-hour darkness between day 4 and 7, and 20 hours light and 4 hours of dark till day 42, according to Selvam *et al.* (2015). The chicks

were vaccinated against Marek's disease immediately after hatching.

Upon arrival, chicks were provided with 4% sugar-added water for first four hours to replenish the depleted energy and stimulate feed intake. The chlorinated potable drinking water (Innoclean™, Natural Remedies Pvt Ltd., 1 tablet/500 L) and poultry mash feed manufactured by Biogain Feeds India Pvt Ltd., Bangalore (starter feed with 3,000 kcal metabolizable energy/kg and 20% crude protein from days 1-21, and finisher feed with 3,150 kcal metabolizable energy/kg and 18% crude protein from days 22-42) were provided *ad libitum* (NRC, 1994). All birds were vaccinated against Newcastle disease (ND; LaSota strain) and infectious bursal disease (IBD) by eye drop on days 5 and 14, respectively (Selvam *et al.*, 2015).

Upon arrival, chicks were individually weighed, tagged with a wing band bearing an identification number, and randomly assigned to the treatments G1, G2 and G3, with six replicates each, according to randomized complete block design (RCBD). Treatment G1 served as the control treatment: birds were provided 0.046 m² (days 1-21) and 0.093m² (days 22-42) floor space per bird and a basal diet with the inclusion of vitamin E at 70g/ton feed (n=150). Birds in treatments G2 were housed at HSD (0.027m² from day 1 to 21 and 0.055m² per chick from day 22 to 42) and fed the basal diet with no vitamin E inclusion (n=210). Birds in treatment G3 were housed at HSD and fed the basal diet with the inclusion of vitamin E at 70g/ton feed (n=210).

The optimal dose of vitamin E (70g/ton of feed) was selected based on vitamin E dose-optimization study conducted in our lab (unpublished data). The feed ingredients and the nutritional composition of starter and finisher diets are detailed in Table 1.

Experimental Design and Feeding levels

Group	Overcrowding Stress	No. of birds per replicate	No. of replicates per treatment	No. of chicks/ treatment	Trial duration
G1 – Control (Basal diet with 70 g Vitamin E/ton feed)	No	25	6	150	42 days
G2-HSD Control (HSD + basal diet without Vitamin E)	Yes	35	6	210	
G3-Test Group (HSD + Basal diet with 70 g Vitamin E/ton feed)	Yes	35	6	210	



Table 1 – Basal Diet Composition

Ingredients	Starter Feed (g)	Finisher Feed (g)
Maize	645.2	658.0
Soya	255.0	228.1
Mustard Oil Cake	30.0	40.0
Meat and Bone Meal	32.89	10.0
Oil	16.31	36.2
Lime Stone Powder	3.4	9.52
Salt	1.95	2.23
Sodium Bicarbonate	0.75	0.75
Methionine Hydroxy Analog	3.34	2.91
Lysine	3.38	3.21
Threonine	1.05	1.0
Vitamin Premix	0.5	0.5
Trace Minerals	1.0	1.0
Curatox-FS®	1.0	1.0
Choline Chloride	1.5	1.5
Biotronic®	1.0	1.0
Zigbir®	0.5	0.5
Coxistac® 12 %	0.6	0.6
R Sone® 20%	0.25	0.25
Ronozyme® WX	0.1	0.1
Ronozyme® NP	0.1	0.1
Lincomix® 20	0.08	0.08
Oxifin®	0.1	0.1
Dicalcium Phosphate	-	1.35
Total	1000	1000
500 g of Vitamin Premix contains		
Vitamin A		12.5 MIU
Vitamin D ₃		2.5 MIU
Vitamin E		As per experimental design
Vitamin K		1.5 g
Vitamin B ₁		1.5 g
Vitamin B ₂		5 g
Vitamin B ₆		2 g
Vitamin B ₁₂		0.015 g
Niacin		15 g
Cal. D Pantothenate		10 g
Folic Acid		0.5 g
Organic Nutritive Carrier		0.5

Performance parameters

Birds in individual pens were observed for mortality, three times daily throughout the experimental period. Birds were individually weighed on days 1, 21, and 42 and their body weights were recorded. Feed intake was calculated by subtracting the amount of feed residues from the total amount of feed offered per replicate, and it was measured on days 21 and 42 (Selvam *et al.*, 2015). Feed conversion ratio (FCR) was calculated as feed intake divided by body weight gain. The European Production Efficiency Factor (EPEF) was calculated according to the equation: body weight (kg) x livability (%) x 100/feed conversion ratio x age (d) (Selvam *et al.*, 2015).

Sample collection

Whole blood samples were collected from 12 birds per group (2 birds/replicate) on day 42 in the heparinized centrifuge tubes for H/L ratio analysis. The blood smears were prepared using wedge slide technique, air dried, fixed in methanol, and stained with May-Grunwald-Giemsa stain. One hundred leukocyte including granular (heterophil, eosinophil and basophil) and non-granular (lymphocyte and monocyte) cells were counted on duplicates slide per bird, and the H/L ratio was calculated using the average counts of these cell types (Onbaşilar *et al.* 2007).

Birds were sacrificed by cervical exsanguination method after blood collection. Birds were not fasted before the sacrifice. Liver samples were collected from 12 birds per group (2 birds/replicate) on day 42, and stored at -20 °C until the analysis of glutathione (GSH) and malondialdehyde (MDA) concentrations. Liver samples were homogenized in an ice-cold Tris-HCl buffer (pH 7.4), and centrifuged at 10,000 g for 30 minutes at 4 °C. Reduced GSH level was estimated in the liver homogenate by reaction with Ellman's reagent {5, 5'- dithiobis-(2-nitrobenzoic acid)}, as described by Moron *et al.* (1979), and its final concentration was expressed in µM/g tissue. Lipid peroxidation levels were evaluated by estimating the amount of MDA produced during peroxidation, according to Ohkawa *et al.* (1979), and its final concentration was expressed as µM of MDA produced/g of tissue.

Statistical Analysis

Upon completion of the trial, the raw data were compiled, processed, and expressed as mean ± SEM. Because a randomized complete block experimental design (RCBD) was applied, the data thus generated was analyzed by one-way analysis of variance (ANOVA) according to Latham *et al.* (2015), using replicate as a blocking factor to determine if blocking was effective at reducing the random error. In case of significant differences among treatment ($p < 0.05$), means were subjected to Dunnett's multiple comparison test (IBM SPSS Statistics Version 21.0; SPSS Inc., Chicago, IL, USA), according to Latham *et al.* (2015). Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Mortality

The cumulative mortality percentages observed up to day 42 (at slaughter) were 0.00%, 0.95%, and 0.00% in G1, G2, and G3, respectively (Table 2).



Table 2 – Effect of HSD and vitamin E on Performance, Blood and Biochemical Parameters

Group	G1 - Normal Control (Basal diet with Vitamin E)	G2 - HSD Control (Basal diet without Vitamin E + HSD)	G3 - Test Group (Basal diet with Vitamin E 70g/ton feed + HSD)
Mortality	Day 1-21	0	1
	Day 22-42	0	1
	Total Mortality	0	2
	Percentage	0.00	0.95
Body weight	Day 1	45.94 ± 0.24	45.15 ± 0.28
	Day 21	709.31 ± 6.18	715.14 ± 5.46
	Day 42	2294.40 ± 24.10	***2142.94 ± 21.50
Body weight gain (day 42)	2248.46 ± 24.10	***2097.82 ± 21.50	\$2188.80 ± 20.97
FCR	Day 21	1.50 ± 0.02	1.45 ± 0.02
	Day 42	1.78 ± 0.02	1.82 ± 0.02
EPEF		306.77	278.19
Blood H/L ratio		0.10 ± 0.01	***0.20 ± 0.01
Liver GSH Levels (µM/g liver tissue)		2.53 ± 0.08	***1.85 ± 0.03
Liver MDA Levels (µM/g liver tissue)		6.13 ± 0.12	**6.74 ± 0.12

Values are expressed as Mean ± S.E.M; n=150-210

p<0.01 & *p<0.001 as compared to G1 and \$p<0.05, \$\$ p<0.01 & \$\$\$ p<0.001 as compared to G2 based on one-way ANOVA followed by Dunnett's Multiple Comparison Test using SPSS

Growth performance

The growth performance results are shown in the Table 2.

The broilers reared at HSD and fed vitamin E (G3) presented higher BWG on day 42 compared with G2 birds, which were reared at HSD, but were not supplemented with vitamin E. This finding agrees with the authors Hosseini-Mansoub *et al.* (2010) and Ismail *et al.* (2014) who found that dietary enrichment with vitamin E resulted in the better performance when compared to the birds fed with standard diet without vitamin E. Similarly, Sahin & Kucuk (2001) reported that inclusion of vitamin E in the diet had increased the performance in Japanese quails reared under heat stress (34°C). Khattak *et al.* (2012) reported that Vitamin E supplementation at 300mg/kg produced a better performance in broilers under heat stress. However, our results differ with the author Coetzee & Hoffman (2001) and Nobakht (2012), who reported that there was no difference in body weight gain and FCR between the different dietary levels of vitamin E supplementation. In the present study, HSD reduced the feed consumption of birds that resulted in improvement of FCR without affecting the body weight on day 21. These results are in line with the findings of the study of Dozier *et al.* (2006), who observed a similar improvement in growth responses during high stocking density at early stage. The above result could be attributable to augmentation of metabolic heat production process as non-evaporative heat production at HSD, hence the energy required for heat production had diminished; moreover, the chicks

at neonatal period (approximately the first two weeks) did not achieve the homeothermic condition and were poorly insulated Whittow (1999). Hence chicks were relatively less active and stayed close to each other leading to energy conservation that might result in better FCR on day 21. The fact observed in the present study that the group reared under normal stocking density having achieved the best growth rate but with the expense of consuming more feed and returned poorer FCR, is due to more liberal space allowance, therefore birds were flightier and active and likely to have expended more energy on unproductive movements Ruan *et al.* (1990).

On day 42, G2 was found to have gained 151 g less body weight but with high consumption of feed than G1 group. Similarly, other studies also reported that there was a significant reduction in body weight attributable to stressors such as increased stocking density or feed restriction in birds Sorensen *et al.* (2000); Moreira *et al.* (2004); Thomas *et al.* (2004); Jang *et al.* (2009). The suppression of growth performance in HSD could be due to the metabolic heat dissipation, high production of nitrogen and moisture in the litter with limited air circulation, and also high ammonia emissions Yadgari *et al.* (2006).

However, the inclusion of a diet enriched with vitamin E (70g/ton of feed) resulted in a 91 g higher body weight in broilers reared under HSD indicating the overcrowding stress was ameliorated yet the best performance in terms of body weight was detected in those raised under standard conditions. On the other hand, broiler chicks received the vitamin E



supplementation diets consumed 50 g lesser amount of feed compared with G1 that received the basal diet. The above result was consistent with the results of Seier & Bragg (1993) Niu *et al.* (2009) and Coetzee & Hoffman (2001) who reported that dietary vitamin E could improve broiler growth performance. Similarly, McIlroy *et al.* (1993) recommended that inclusion of vitamin E in the diet may be the most beneficial in situation where there is a challenge to the defense system of the host, and could significantly enhance the performance more certainly under such conditions. Correspondingly Kennedy *et al.* (1992) explored the productivity of 168 broiler chickens fed with vitamin E supplemented diets either 50 mg/kg or 180 mg/kg. The authors reported that at the higher level of vitamin E supplement, productivity was 8.4% more as a result of improved FCR and body weight gain. Similarly, Sahin & Kucuk (2001) found that diet with vitamin E produced a higher performance in Japanese quails reared under heat stress (34°C). The vitamin E supplementation could reduce the effect of heat stress that would have resulted from the high stocking density.

This result suggests that the vitamin E- inclusion in the diet of broilers subjected to high stocking density could effectively reverse the undesirable effects of overcrowding stress after three weeks of age.

EPEF

Higher European Production Efficiency Factor (EPEF) was observed in G1 (306.77) and in G3 (302.48) compared with G2 (278.19), as shown in Table 2.

This result indicates the livability of birds was influenced by stocking density. High stocking density seemed to have stressed the birds, impairing their immunity, and consequently, causing higher mortality. However, mortality rates were not significantly different among treatment groups, as shown in previous reports by Feddes *et al.* (2002), Thomas *et al.* (2004), Dozier *et al.* (2006), Ravindran (2006), who evaluated the effect of HSD on broiler performance. In addition, the lower EPEF observed in G2 are consistent with the findings of Boltol *et al.* (1972) and Proudfoot *et al.* (1979). On the other hand, Roussan *et al.* (2008) and Samar *et al.* (2014) reported that the dietary inclusion of vitamin E increased the livability and the body weight of broiler chickens submitted to heat stress.

Physiological stress

H/L ratio

In our experiment, the result clearly suggests that the vitamin E supplementation had reduced the H/L

(0.2 to 0.1) ratio equal to the G1 when compared to G2 (Table 2). This finding corroborates with Altan *et al.* (2000) and Minka & Ayo (2008), who found an increase in H/L ratios when the birds were subjected to heat and transportation stress. Likewise, Nworgu *et al.* (2007) and Minka & Ayo (2008) observed that the values of TLC and H/L ratio were stable when the broilers chickens were treated with anti-oxidant vitamins.

Oxidative stress

GSH

Glutathione is a reliable indicator of oxidative stress, and the important function is to protect the cells from the oxidative damage. Rama Rao *et al.* (2011) reported that supplementing α -tocopherol in the diet of broiler chicken had increased the activity of glutathione peroxidase activity in the plasma. Vitamin E supplementation might indirectly increase the GSH concentration by controlling the level of oxygenated radical scavenging systems such as GSH-Px, and depress oxidant enzyme activities through homeostatic compensation Kuan *et al.* (1990) which agrees with the result of the present study where vitamin E supplementation significantly improves the liver glutathione concentration in liver of birds under HSD (G3) supplemented with vitamin E when compared to G2 (Table 2). Similarly, the supplementation with vitamin E (200mg/kg) in birds during summer months was known to reduce the lipid peroxidation and increase the activity of glutathione when compared to the normal diet Maini *et al.* (2007).

MDA

MDA is one of the major final products of lipid peroxidation and based on MDA levels oxidative damage can be determined in the cells. In the present study, the liver MDA concentration in G2 was found to be significantly increased indicating an oxidative stress induced lipid peroxidation. However, vitamin E supplementation ameliorated MDA levels to normal in G3 when compared to G2 which is shown in the Table 2. These results are corroborating with the previous research of Tsai *et al.* (2008) who reported that maternal vitamin E supplementation with low doses (40 mg/kg) in hens could supersede the effect of oxidative stress (MDA and ROS) in the neonatal chick brains. Similarly, Sahin *et al.* (2003) reported that supplementation with vitamin E had decreased the serum and liver MDA concentration linearly in Japanese quails. Puthongsiriporn *et al.* (2001) stated



that the hens reared under heat stress (32 °C) was found to have increased level of MDA whereas the birds supplemented with vitamin E (65 IU) had the lowest MDA values.

CONCLUSION

The findings of the present study suggest that the supplementation of vitamin E at 70g/ton of feed can overcome the undesirable effects of overcrowding stress and has a positive impact on productive performance, immune responses and anti-oxidant status of broiler chickens.

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