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Effects of Dietary Threonine Levels and Stocking Density on the Performance, Metabolic Status and Immunity of Broiler Chickens

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ABSTRACT

A total of 240 Cobb 500 broiler chicks, 18 days old, were allocated to 10 treatments groups, each of which included 4 replicates. Experimental treatments consisted of a 5×2 factorial arrangement with 5 levels of L-threonine (Thr) supplementation and 2 levels of stocking density (11.90 birds m^{-2} as the normal stocking density or 16.66 birds m^{-2} as the high stocking density). L-Thr was added to a basal diet at 0.0 (control group), 0.25, 0.50, 0.75 and 1.00 g kg^{-1} diet. Dietary Thr values were 0.69, 0.71 (deficient), 0.74 (adequate; 100% NRC), 0.76 and 0. 79% (excess) of the current NRC recommendations. At 42 days of age, dietary Thr levels had no significant effect on performance, immunity, plasma total protein and glucose (GLU). However, plasma cholesterol (CHO) levels decreased significantly ($p<0.05$) with L-Thr supplementation and the lowest value occurred at 0.74% total dietary Thr. In addition, plasma triiodothyronine (T3) and thyroxine (T4) levels were higher ($p<0.05$) at 0.76% total dietary Thr compared with the control group. The normal stocking density resulted in better performance ($p<0.05$) compared with the high stocking density. However, stocking density did not affect plasma total protein, total Ig, IgG, IgM, total lipids, GLU, CHO, T3 and T4 levels. Interactions between Thr level and stocking density were observed for plasma levels of total lipids and cholesterol. In conclusion, total dietary Thr level at 0.69% (93% of NRC recommendations) is sufficient to improve broiler performance or immunity under high stocking density. However, 0.74% total dietary Thr level has a positive effect on decreasing plasma total lipids and cholesterol levels during growing-finishing phase.

Key words: Broiler performance, cholesterol, immunity, dietary threonine, stocking density

INTRODUCTION

The stocking densities differ between different strains, countries and husbandry systems. However, many producers around the world need to increase stocking densities to maximize profitability. A high stocking density affects performance, welfare, immunity and gut health negatively (Shane, 2000; Heckert *et al*., 2002; Thaxton *et al*., 2006; Estevez, 2007; EL-Gogary and Azzam, 2014).

Birds cannot synthesize threonine therefore it is one of essential amino acids. Threonine participates in protein synthesis and its catabolism produces many products important in metabolism. Threonine is considered as the third limiting amino acid after Met and Lys for broiler chicks (Kidd *et al*., 1999; Ayasan and Okan, 2006; Baylan *et al*., 2006; Ayasan *et al*., 2009). Kidd (2000) reported that L-Thr deficiency resulted in decreasing utilization of TSAA and Lys. In addition, because threonine occurs at a high concentration in γ-globulin, it affects the immune function (Smith and Greene, 1947; Crumpton and Wilkinson, 1963; Tenenhouse and Deutsch, 1966; Azzam *et al*., 2011a, b).

Recently, Houshmand *et al*. (2012) observed significant interactions between protein level and stocking density for BW gain and final BW. Therefore, the aim of our study was to investigate the effects of dietary threonine levels on performance, immune function and metabolic status of broilers chickens during growing-finishing phase at different stocking densities.

MATERIALS AND METHODS

This experiment was conducted at Poultry Unit of the Department of Poultry Production, Teaching and Research Farm, Faculty of Agriculture, Mansoura University, Mansoura, Egypt.

Birds and treatments: Cobb 500 broiler chicks (n = 240), 18 days old, were divided into 10 treatments groups, each of which included 4 replicates (cages). Experimental treatments consisted of a 5×2 factorial arrangement with 5 levels of dietary L-Thr and 2 levels of stocking density. Experimental diet was formulated to meet or exceed the NRC (1994) nutrition recommendations from 21-42 days of age for all nutrients except Thr (Table 1). Crystalline L-Thr (98.5% Thr, PT. Cheil Jedang, Indonesia) was added to the basal diet at 0.0, 0.25, 0.50, 0.75 and 1.00 g kg^{-1} diet to obtain the dietary Thr level at 0.69, 0.71 (deficient), 0.74 (adequate), 0.76 and 0. 79% (excess) of the NRC (1994) recommended concentration. Digestible Thr values are 0.58, 0.60, 0.62, 0.64 and 0.66%. Digestible dietary Thr: Lys ratios are 70, 73, 76, 78 and 80%.

Birds were reared in battery cages and the length, width and height of each cage were 70, 60 and 40 cm, respectively. Thus, the cage floor area was 0.42 m^{-2} (70×60 cm). The numbers of birds located in each cage varied, depending on the stocking density. For the normal and high stocking densities, 5 and 7 birds, respectively, were placed in each cage. The stocking density was 11.90 birds m^{-2} as the normal density and 16.66 birds m^{-2} as the high density. The present study was carried out during March to April. The average daily temperature inside the farm ranged from 19.5-23.3°C. The photo period was 23 L: 1 D throughout the experiment. Chickens were reared 42 days of age and fed a starter ration from one to 17 days of age (3100 kcal of ME kg^{-1} of diet and 22% CP) and a grower ration from 18-42 days of age (Table 1). Feed in mash form and water (via nipple drinkers) were provided freely.

Table 1: Composition and nutrient content of the diets

Compositions	Values (g kg^{-1})	Nutrient level[2]	Values
Corn	696	ME (kcal kg^{-1})	3071.55
Soybean meal, 44%	165	Crude protein (%)	19.58
Corn gluten meal	100	Lysine (%)	1.03 (0.82)
L-Lys. HCL	3	Methionine (%)	0.43 (0.35)
Dl-Meth	0.5	Threonine (%)	0.69 (0.58)
L-Thr	0.0	Dig. Threonine to lysine ratio	0.70
Dicalcium phosphate	16.5	Tryptophan (%)	0.18
Limestone	13	Valine (%)	0.77
Nacl	3	Isoleucine (%)	0.90
Premix[1]	3	Calcium (%)	0.92
Total	1000	Available phosphorus (%)	0.42

[1]Premix provided the following per kilogram of diet, Vitamin A (retinyl acetate): 2654 μg, Vitamin D3 (cholecarciferol): 125 μg, Vitamin E (dl-α-tocopheryl acetate): 9.9 mg, Vitamin K3 (menadione dimethyl pyrimidinol): 1.7 mg, Vitamin B1 (thiamin mononitrate): 1.6 mg, Vitamin B12 (cyanocobalamin): 16.7 μg, Riboflavin: 5.3 mg, Niacin (niacinamide): 36 mg, Calcium pantothenate: 13 mg, Folic acid: 0.8 mg, d-biotin: 0.1 mg, Choline chloride: 270 mg, BHT: 5.8 mg, Fe (iron sulphate monohydrate): 50 mg, Cu (copper sulphatepentahydrate): 12 mg, I (calcium iodate): 0.9 mg, Zn (zinc oxide): 50 mg, Mn (manganous oxide): 60 mg, Se (sodium selenite): 0.2 mg, Co (cobalt sulphate): 0.2 mg. [2]Calculated from data provided by NRC (1994), digestible AAs were calculated from data provided by ileal digestible amino acid values in feedstuffs for poultry RIRDC (2009). The values in parentheses indicate the digestible AAs

Broiler performance parameters: Live Body Weight (BW) and Feed Intake (FI) were measured every week based on a replicate. Body Weight Gain (BWG) was calculated on weekly basis throughout the experimental period. The consumed amounts of feeds were recorded every week and corrected Feed Conversion Ratio (FCR) was then calculated. Mortalities and health status were visually observed and recorded daily throughout the entire experimental period.

Blood sampling and laboratory analyses: Four birds of each treatments slaughtered and blood samples were collected at the end of experiment. The spleen, bursa, gizzard, livers, pancreas and intestine of each bird were collected and weighed. Blood samples were obtained via vena cava puncture and fresh blood was collected in heparinized tubes and centrifuged at 3000×g for 15 min. Plasma obtained was stored at -20°C until analysis. Plasma samples were tested colorimetrically using commercial kits according to the procedures outlined by the manufactures, for determination of total protein (Faulkner and Meites, 1982), cholesterol (Allain *et al*., 1974), glucose (Trinder, 1969) and total lipids (Gordon and Gordon, 1977). Plasma T3 and T4 were determined by RIA technique using Gamma-Coat 125I RIA Kits, Clinical Assay, Cambridge, Medical Diagnostics, Boston, MA, as reported by Akiba *et al*. (1982).

Immunity parameters: The spleen and bursa of each bird were collected and weighed. The immune organ index was calculated expressed as the weight of the organ as a percentage of total BW. Plasma total Ig and IgG were analyzed according to (Meurman *et al*., 1982). IgM was calculated by the difference between total Ig and IgG.

Statistical analyses: All statistical analyses were performed using SPSS Version 16.0 for Windows (SPSS Inc., Chicago, IL) and data was subjected to 2-way ANOVA. Values were considered statistically different at $p<0.05$. When significant differences were found ($p<0.05$), Turkey post hoc tests were performed. The main effects of Thr levels were further tested for linear, quadratic and cubic responses using orthogonal contrasts.

RESULTS AND DISCUSSION

Broiler performance: The effects of stocking density and Thr Levels on the weight gain, feed intake and FCR of broiler chickens at 42 days of age are presented in Table 2. Increasing L-Thr level in the diet did not affect LBW, BWG, feed intake and FCR ($p>0.05$). Our findings suggest that the control diet (0.0% L-Thr and 0.69% total dietary Thr), corresponding to 0.58% digestible of dietary digestible threonine maximized broiler performance. Webel *et al*. (1996) also reported that the NRC (1994) estimate is too high during 3-6 weeks old of broiler chickens The NRC (1994) estimates of Thr requirement for broilers (mixed-sex feeding) is 0.74%. This means that using a 93% of dietary Thr recommendation by NRC (1994) is sufficient to achieve the performance of Cobb broiler chickens during growing-finishing phase with normal stocking density or high stocking density. Our result is an agreement with Penz *et al*. (1991), who recommended 0.68% Thr for optimal feed efficiency from 21-42 days of age. Also, Penz *et al*. (1997) concluded that the total Thr requirement was no greater than 0.70% of diet. In addition, Li (2000) reported that the requirement of the total Thr was 0.66% from 3-6 weeks of age. In general, previous studies suggested that the dietary Thr requirement of broilers chickens may actually be below the current NRC (1994) recommendation (Thomas *et al*., 1987; Kidd *et al*., 2001). However, Ojano-Dirain and

Table 2: Effects of Thr levels and stocking density on growth performance of broilers at 42 days of age[1]

Items	Initial BW (g)	Final BW (g)	BWG (g)	Feed intake/bird (g)	FCR
Total dietary Thr level (%)					
0.69	482.49	2055.18	1572.67	2857.87	1.82
0.71	484.05	2073.69	1589.64	2911.43	1.83
0.74	487.60	2010.93	1523.32	2780.34	1.83
0.76	486.25	1996.83	1510.58	2749.23	1.82
0.79	482.75	1998.24	1515.48	2898.07	1.91
SEM	2.85	42.71	43.22	77.89	0.08
Contrast p-value					
p-value	0.65	0.61	0.59	0.50	0.65
Thr	0.65	0.61	0.59	0.50	0. 64
Linear	0.76	0.16	0.16	0.74	0.26
Quadratic	0.17	0.93	0.85	0.32	0.37
Cubic	0.65	0.48	0.46	0.15	0.61
Stoking density					
Normal (11.90 birds m^{-2})	497.30	2155.72	1658.42	2913.42	1.76
High (16.66 birds m^{-2})	471.96	1898.22	1426.26	2765.36	1.95
SEM	1.80	27.01	27.34	49.26	0.05
p-value	0.00	0.00	0.00	0.04	0.01
Interaction, p-value	0.70	0.27	0.26	0.62	0.29

[1]Data is means of 4 replications, BW: body weight, BWG: Body weight gain, FCR: Feed conversion ratio

Waldroup (2002) reported that Thr requirement of the growing broiler is greater than those recommended by NRC (1994). Ciftci and Ceylan (2004) estimated the digestible Thr was 6.9 g kg^{-1} for growing phases. Our results may be related to optimal hygienic conditions. Recently, Taghinejad-Roudbaneh *et al.* (2013) reported that Thr requirement under stress and non-hygienic conditions was 0.81% for support growth performance. Also, Corzo *et al.* (2007) concluded that the Thr need to maximize growth performance was 0.71-74% on new litter and for used litter growth performance was maximized at 0.73-0.78%.

Birds at normal stocking density (11.90 birds m^{-2}) resulted in better performance ($p<0.05$) compared with the high stocking density (16.66 birds m^{-2}). This indicates to a greater degree of stress on the performance. As shown in Table 2, broiler LBW, BWG and feed intake were affected negatively by high stocking density. Similarly, other researchers (Elwinger, 1995; Thomas *et al.*, 2004; Muniz *et al.*, 2006; EL-Gogary and Azzam, 2014) indicate that increasing the No. of birds per unit depresses growth rate and feed intake. In contrast, Buijs *et al.* (2009) reported that at 39 days of age LBW was not significantly different between birds reared at different stocking densities (6, 15, 23, 33, 35, 41, 47 and 56 kg m^{-2}). Also, FCR was affected adversely by high stocking density. Similarly, Houshmand *et al.* (2012) reported that during the grower phase (22-42 days) broilers raised at a high density had an inferior FCR compared with birds housed at a normal density. However, the FCR found in this study are in disagreement with the findings of other researchers have been concluded that there was no significant effect of stocking density on FCR of broilers (Feddes *et al.*, 2002; El-Deek and Al-Harthi, 2004; Galobart and Moran Jr, 2005; Turkyilmaz, 2008). The difference in management and hygiene may have been responsible for the observed discrepancy between the different studies. The reduction in production performance due to high stocking density could be related to lots of reasons. A reduction in the airflow at the bird level which occurred at the high stocking density, could decrease the dissipation of body heat to the air. Moreover, a reduction in access to water and feed, enhancement ammonia and an unfavorable air quality because of insufficient air exchange (Feddes *et al.*, 2002). In addition, Dozier *et al.* (2005) reported that the negative effect of a high stocking density on broiler growth rate is double as the chicks progressed in BW. Under stressors, the behavioral patterns of birds will be changed and consequently, their energy consumption will increase (Zulkifli and Azah, 2004). Our study found that stocking density did not affect mortality.

No interaction was noted between L-Thr supplementation and stocking density in broiler performance. Our findings are in agreement with others related to lysine and ME. Zuowei *et al*. (2011a) reported that lysine requirement of broilers is not altered by stocking density. In addition, Zuowei *et al*. (2011b) reported that no significant interaction between the diet ME level and the stocking density, suggesting that the ME requirement was not changed by the stocking density.

Mortality rate ranged between 1.66-2% during whole experiment and was not related to any treatment. It was not statistically different among groups. Previous studies reported the same findings ((Sekeroglu *et al*., 2011; Turkyilmaz, 2008; Ravindran *et al*., 2006; Buijs *et al*., 2009; Skomorucha *et al*., 2009; El-Gogary and Azzam, 2014).

Liver, gizzard and pancreas weights: Liver, gizzard, pancreas and intestine weights are presented in Table 3. When treatments affect organ weights, this may be used as an indication that organ function also is affected. The increase in relative weight of gizzard may enhance the digestive capacity of broilers. Also, increased liver weight is always regarded as one indicator of stress conditions. The weights of the liver, gizzard and pancreas remained unaltered by the dietary inclusion of L-Thr or stocking density. Similarly, other reported that there were no significant difference in liver and gizzard weight (p>0.05) due to stocking density (El-Deek and Al-Harthi, 2004; Sekeroglu *et al*., 2011; EL-Gogary and Azzam, 2014). No interaction was noted between L-Thr supplementation and stocking density in organ weight.

Immunity: In our study, the immune organ indexes and the concentrations of immune globulins in plasma were assayed to investigate the effects on the immune system. Immune organs, including the spleen and bursa of Fabricus, have a very important role in the immune response of chicks, especially the spleen (Mast and Goddeeris, 1999). Bursa and spleen weights are presented in

Table 3: Effects of Thr levels and stocking density on weight of digestive organs[1]

	Weight of digestive organs (g)			
Items	Liver	Gizzard	Pancreas	Intestine
Total dietary Thr level (%)				
0.69	46.21	34.92	3.32	93.73
0.71	53.31	35.86	3.49	93.05
0.74	49.54	35.65	3.41	80.93
0.76	48.70	38.83	4.00	92.09
0.79	49.96	39.96	3.52	86.68
SEM	3.10	2.53	0.52	5.06
Contrast p-value				
Thr	0.61	0.57	0.88	0.37
Linear	0.77	0.12	0.56	0.36
Quadratic	0.47	0.70	0.71	0.47
Cubic	0.19	0.91	0.57	0.76
Stocking density				
Normal (11.90 birds m^{-2})	50.91	36.38	3.72	88.41
High (16.66 birds m^{-2})	48.18	37.70	3.39	90.18
SEM	2.00	1.60	0.31	3.36
p-value	0.34	0.57	0.46	0.70
Interaction, p-value	0.14	0.21	0.95	0.84

[1]Data are means of 4 replications

Table 4: Effects of Thr level and stocking density on immune function[1]

Items	Immune function				
	Spleen index (g kg^{-1})	Bursa index (g kg^{-1})	Total Ig,log^2	IgG, Ig,log^2	IgM Ig,log^2
Total dietary Thr level (%)					
0.69	2.54	1.17	6.10	4.22	1.88
0.71	2.41	1.23	6.00	4.13	1.87
0.74	2.33	1.09	5.30	3.58	1.72
0.76	2.25	0.93	5.85	4.05	1.80
0.79	2.43	1.46	5.80	3.98	1.85
SEM	0.24	0.21	0.31	0.21	0.13
Contrast p-value					
Thr	0.97	0.46	0.60	0.44	0.92
Linear	0.66	0.68	0.46	0.31	0.98
Quadratic	0.27	0.24	0.37	0.27	0.88
Cubic	0.82	0.18	0.73	0.49	0.76
Stocking density					
Normal (11.90 birds m^{-2})	2.61	1.27	5.91	4.01	1.87
High (16.66 birds m^{-2})	2.17	1.08	5.71	3.93	1.77
SEM	0.17	0.13	0.22	0.15	0.07
p-value	0.10	0.30	0.50	0.60	0.41
Interaction, p-value	0.24	0.07	0.09	0.12	0.10

[1]n = 4 broilers/group

Table 4. The weights of the bursa and spleen remained unaltered by the dietary inclusion of L-Thr or stocking density. Results are in agreement with those presented by others (Kidd *et al*., 2001; Taghinejad-Roudbaneh *et al*., 2013). Also, Rao *et al*. (2011) found that lymphoid organ (bursa, spleen and thymus) weights and immune responses were not affected by supplementation of L-Thr. The lymphoid organ weights are an indicator of immunity in poultry. Ravindran *et al*. (2006) reported that relative weights of spleen and bursa decreased as density increased (16, 20 and 24 birds m^{-2}). Heckert *et al*. (2002) showed that bursa weight/BW ratios decreased significantly at high stocking density but no effect on the relative weight of the spleen. In this study no significant differences ($p>0.05$) were observed in bursa or spleen weight of the birds due to stocking density. Others are in agreement with our findings (Buijs *et al*., 2009; Tong *et al*., 2012; Houshmand *et al*., 2012). The effects of stocking density and Thr Levels on immune globulins are presented in Table 4. Adding L-Thr has no affect on total Ig, IgG or IgM. This means that the control diet (0.0% L-Thr; 0.69% total dietary Thr), corresponding to 0.58% digestible of dietary digestible threonine is enough to boost the immune function of broiler chickens. In the process of protein anabolism and proteolysis, the serum protein level is always an indicator of the protein metabolism and immunity function situation *in vivo*. Avian total protein contains albumin and α, β and γ-globulin (Lumeij, 1997) thus, high concentrations of total protein are associated with significant increases in levels of serum albumin and globulin (Hunt and Hunsaker, 1965). Tyler *et al*. (1996) suggested that the IgG concentration increases as the total protein concentration increases. In current study, L-Thr did not affect levels of plasma total protein (Table 5). Takahashi *et al*. (1994) found that dietary threonine levels had no effect on the antibody titres to sheep red blood cells or *Brucella abortus* in male broilers. However, Kadam *et al*. (2008) observed that injections of 10, 20 or 30 mg threonine into yolk sac increased the humoral response to sheep red blood cells significantly in broilers at 21 days post-hatch. Recently, in laying hens, Azzam *et al*. (2011b) found that serum total protein and IgG increased significantly at 3 g Thr kg^{-1} diet. In current study, stocking density had no affect on levels of plasma immune globulins (Table 4). Tong *et al*. (2012) showed that no significant difference was noted in the immunological parameters due to different stocking density. However,

Table 5: Effects of Thr level and stocking density on plasma indices of broilers[1]

Items	Total protein ($g\ dL^{-1}$)	Glucose ($mg\ dL^{-1}$)	Total lipids ($mg\ dL^{-1}$)	Cholesterol ($mg\ dL^{-1}$)	T3 ($ng\ mL^{-1}$)	T4 ($ng\ mL^{-1}$)
Total dietary Thr level (%)						
0.69	4.75	222.92	988.96^{a}	233.26^{a}	2.85^{b}	15.12^{b}
0.71	4.70	225.00	921.36^{ab}	204.25^{b}	3.25^{ab}	17.78^{ab}
0.74	4.90	220.41	856.73^{b}	196.75^{b}	3.53^{ab}	19.02^{ab}
0.76	4.87	212.50	874.72^{b}	200.95^{b}	3.67^{a}	20.17^{a}
0.79	4.70	223.75	957.40^{a}	215.30^{ab}	3.45^{ab}	18.45^{ab}
SEM	0.4	11.94	22.83	5.1	0.17	1.3
Contrast p-value						
Thr	0.99	0.95	0.008	0.002	0.03	0.13
Linear	0.41	0.78	0.17	0.03	0.008	0.04
Quadratic	0.77	0.75	0.001	0.00	0.04	0.10
Cubic	0.76	0.52	0.42	0.52	0.66	0.63
Stocking density						
Normal (11.90 birds m^{-2})	4.72	222.16	931.02	213.12	3.30	17.78
High (16.66 birds m^{-2})	4.84	219.66	908.48	206.99	3.39	18.43
SEM	0.26	7.70	14.72	3.3	0.11	0.80
p-value	0.75	0.82	0.31	0.21	0.59	0.57
Interaction, p-value	0.95	0.49	0.04	0.03	0.75	0.81

[1]n = 4 broilers/group

Houshmand *et al*. (2012) concluded that the normal stocking density resulted in higher antibody titer against Newcastle disease compared with the high stocking density. These data suggest the stocking density 16.66 birds m^{-2} did not suppress immune system but suppress broiler performance compared with 11.9 birds m^{-2}. No interaction was noted between L-Thr supplementation and stocking density in immunity.

Blood parameters: The effects of stocking density and Thr Levels on the blood chemicals are presented in Table 5. No significant effects of L-Thr on glucose. However, plasma cholesterol (CHO) and total lipids levels decreased quadratically ($p<0.05$) response to L-Thr supplementation and the lowest levels occurred at 0.74% (100% NRC., 1994). Taghinejad-Roudbaneh *et al*. (2013) also reported that the lowest percentage of fat pad was in diet containing 0.74% Thr. There was interaction between L-Thr supplementation and stocking density in plasma cholesterol (CHO) and total lipids. No significant effects of stocking density on plasma total protein. (Abudabos *et al*., 2013; EL-Gogary and Azzam, 2014) observed that serum total protein did not change by stocking density. However, other disagreements with current result (Sekeroglu *et al*., 2011; Tong *et al*., 2012) reported that level of blood total protein increased by increasing stocking density.

Plasma triiodothyronine (T3) and thyroxine (T4) levels were higher ($p<0.05$) at 0.75 g L-Thr/kg diet (Table 5). In rats, Alfonso *et al*. (2001) also reported that L-Glu increased serum T3 andT4 and they suggested that the importance of EAAs in the regulation of hormone secretion from the pituitary-thyroid axis.

No significant effects of stocking density on stress indicators (cholesterol or glucose). These results are in agreement with other studies who reported no evidence of physiological stress resulting from a high stocking density (Dozier *et al*., 2006; Thaxton *et al*., 2006; Buijs *et al*., 2009; Houshmand *et al*., 2012). Also, stocking density did not affect plasma levels triiodothyronine (T3) and thyroxine (T4). Similarly (Tong *et al*., 2012; EL-Gogary and Azzam, 2014) reported that no effects were found on T3 or T4 due to stocking density.

CONCLUSION

Dietary total Thr level at 0.69% (93% of NRC recommendations) is sufficient to improve broiler performance or immunity under high stocking density. However, 0.74% total dietary Thr level has a positive effect on decreasing plasma total lipids and cholesterol levels.

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