Journal of Animal and Veterinary Advances 12 (4): 464-471, 2013
ISSN: 1680-5593

Diet with Soy Oil, Molasses, Methionine, Lysine and Vitamin C Improves the Growth Performance of Broilers Exposed to Extreme Heat Stress

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Abstract: This study investigated the effects of feeding the broilers that were exposed to Extreme Heat Stress (EHS, 33±2°C) with Extreme Heat Stress Diet (EHSD) containing an adequate amount of soy oil and other nutrients on their growth performance. Five hundred broiler chickens (Ross 308) were randomized into five dietary treatment groups according to a randomized block design on the day they were hatched. Each group was further divided into four repeat pens with of 25 chickens. Five dietary treatment groups were: T1 (Normal ambient condition + Concentrated basal Diet (CD), T2 (Extreme Heat Stress (EHS) + CD), T3 (EHS + Extreme Heat Stress Diet (EHSD) prepared from CD with tallow replaced with soy oil 5% and containing molasses 2%), T4 (EHS + EHSD prepared from CD with tallow replaced with soy oil and containing molasses 2% and methionine and lysine of 1.5 times greater quantities than in CD) and T5 (EHS + EHSD prepared from CD with tallow replaced with soy oil and containing molasses 2%, methionine and lysine of 1.5 times greater quantities than in CD and vitamin C 200 ppm). The body weight gain of the broilers increased significantly in T4 and T5 compared with that in T1 and T2. Weights of the lymphoid organ, bursa of Fabricius, thymus and spleen were similar between all groups. Serum concentrations of IgG, IgG and IgM were higher in T4 and T5 than inT1 and T2 but the corticosterone concentration decreased significantly in them. In T4 and T5, Lactobacillus in the ceca increased but *Escherichia coli* form and total aerobic bacteria decreased significantly ($p<0.05$), compared with those in T1 and T2. Contents of acetic acid, propionic acid and total SCFA were significantly higher in T4 and T5 than in T1 and T2.

Key words: Extreme heat stress, lymphoid organ, immunoglobulin, microflora, SCFA

INTRODUCTION

It is a well-known fact that extreme heat stress among broilers is the cause of economic damage in poultry production since it results in a considerable decrease of feed intake and body weight gain and a high mortality (Mendes *et al*., 1997; May *et al*., 1998; Quinteiro-Filho *et al*., 2011). The ambient temperature must be maintained at approximately 21°C for optimal feed utilization and body weight gain of broiler chickens (Reece and Daton, 1971). It has been known that the normal body temperature of a fully grown boiler is 41-42°C and its body temperature rise as the ambient temperature increases and that its feed intake and body weight gain decrease under the high ambient temperature. It has been observed that when the ambient temperature rose up to 32°C, the extreme heat stress began to occur with no impact on the mortality but the mortality increased when the temperature rose to 37°C (Donkoh, 1989; Cooper and Washburn, 1998; Han *et al*., 2010). It has been reported that there is a negative genotype correlation between body weight and resistance to heat stress in growing broilers. The resistance to heat stress of the strains selected for growth was less than that of a slow growing random bred control strain. Restriction of feed consumption of the growth selected strains so that body weights were reduced resulted in a increase in resistance to heat stress (Washburn *et al*., 1980). The extreme heat represses the specific immune reaction in chickens and feed intake decreases by 24% in broilers exposed to 32°C temperature (Niu *et al*., 2009). A number of nutritional strategies to mitigate the negative effect of extreme heat stress have been suggested. Under the extreme heat stress an increase in amino acids, rather than total protein level is helpful because an increased total protein level leads to an increase in heat generation through metabolism. Addition of high energy components that are palatable to broilers such as soy oil and molasses to the

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feed stimulates them to have more feed intake and addition of vitamin C helps minimize heat stress (Leeson and Summers, 1991; Temim *et al*., 2000). Thereported effects of methionine and lysine in the feed on the body weight gain of broilers bred under high temperatures were varied depending on the method used by the researchers (Knight *et al*., 1994; Mendes *et al*., 1997; Gonzalez-Esuerra and Lesson, 2006). Soy oil is an ingredient of high energy utilization in broilers and it is also used with molasses as an excellent energy source (Jacob and Carter, 2008; Sharifi *et al*., 2012). It has been reported that the supply of vitamin C influences the storage of nutrients and improve growth performance by maintaining metabolic rate (McKee *et al*., 1997). However, there is no full understanding of the relation between the extreme heat stress diet which contains soy oil instead of tallow and is reinforced with adequate amounts of molasses, methionine, lysine and vitamin C and on the growth rate of broilers.

The objective of this study was to investigate the effect of night restricted feeding of an extreme heat stress diet containing soy oil and reinforced nutrient ingredients on immunoglobulin in blood, lymphoid organ, microorganisms in the cecum, short chain fatty acid and growth performance in broilers exposed to night lightings and extreme heat stress. Material and method is not complete. For example researchers can't see any subjects about the role of experimental matters on performance and blood parameters of broilers. Therefore, need more attention.

MATERIALS AND METHODS

Experimental design: On the day of hatching, 500, 1 day old Ross 308 male broiler chickens were randomized into 5 dietary treatment groups according to a randomized block design. Each group comprised four repeats of a pen of 25 chickens. The five dietary treatment groups were: T1 (Normal ambient condition+Concentrated basal Diet (CD), T2 (Extreme Heat Stress (EHS)+CD), T3 (EHS+Extreme Heat Stress Diet (EHSD) prepared from CD with tallow replaced with soy oil 5% and containing molasses 2%), T4 (EHS+EHSD prepared from CD with tallow replaced with soy oil 5% and containing molasses 2% and methionine and lysine of 1.5 times greater quantities than in CD) and T5 (EHS+EHSD prepared from CD with tallow replaced with soy oil 5% and containing molasses 2%, methionine and lysine of 1.5 times greater quantities than in CD and vitamin C 200 ppm). CD and EHSD were prepared by mixing corn and soybean meal as main ingredients according to the NRC (1994) nutrient requirement. The metabolic energy of tallow and soy oil was adjusted as the same with NRC. For EHSD in T3, T3 and T5, tallow in CD was replaced with more soy oil and molasses having a high energy utilization and broilers' liking. Methionine, lysine and vitamin C were adjusted at a higher level than in CD and the actual component ratio of the feed depending upon addition of nutrient sources was accomplished by mixing corn of which the amount was decreased. Crude protein and ME were adjusted to the same level in CD and EHSD and they were mixed to meet or exceed the NRC nutrient requirement slightly (Table 1). The pen floor was covered with 10 cm of rice husks and the temperature in the pen was maintained at 33°C for the 1st 3 days and then gradually lowered by 2-3°C per week. Until the age of 27 days when the study commenced, each group was fed *ad libitum* with CD or EHSD formula for each group and plain drinking water (25-28°C) under continuous lighting in the normal ambient condition. In all groups except T1 for 5 days from the age of 28 days to the age of 32 days, plain drinking water was supplied during the day and the lightings were put out during the day and the experimental feed were not supplied and at the time of EHS, the lightings were put on during the night and EHSD were restrictedly supplied. After 2 h from the beginning of EHS, restricted feeding of 120 g of experimental feed per chicken and plain drinking water were supplied (60 g per chicken manually during the time of 18:00 to 24:00 and 60 g per chicken automatically during the time of 24:00-08:00). Chickens were subjected to 5 h of EHS

Table 1: Composition of Concentrated basal Diet (CD) for broiler chickens

	Growing stage	
Ingredients (as-fed%)	Starter (0-21 days)	Grower (22-32 days)
Yellow corn	52.00	50.00
Soybean meal	34.00	25.00
Corn gluten meal	4.70	5.70
Wheat meal	-	10.00
Tallow	5.00	5.00
Limestone	1.25	1.25
Dicalcium phosphate	1.70	1.70
Sodium chloride	0.25	0.25
DL-methionine (50%)	0.30	0.30
L-lysine HCl (78%)	0.30	0.30
Trace mineral premix[1]	0.34	0.34
Vitamin premix[2]	0.16	0.16
Total	100.00	100.00
Chemical composition		
ME (kcal kg^{-1})	3,100.00	3,150.00
Crude protein (%)	22.00	20.00
Lysine (%)	1.32	1.15
Methionine (%)	0.52	0.50
Methionine + cystine (%)	0.78	0.73
Calcium (%)	1.00	0.90
Available phosphorus (%)	0.45	0.40

[1]Supplied per kg of diet: Fe, 80 mg; Zn, 80 mg; Mn, 70 mg; Cu, 7 mg; I, 1.20 mg; Se, 0.30 mg; Co, 0.70 mg. [2]Supplied per kg of diet: vitamin A (retinyl acetate); 10,500 IU; vitamin D_3, 4,100 IU; vitamin E (DL-α-tocopheryl acetate); 45 mg; vitamin K_3, 3.0 mg; Thiamin, 2.5 mg; Riboflavin, 5 mg; vitamin; B_6, 5 mg; vitamin B_{12}, 0.02 mg; Biotin, 0.18 mg; Niacin, 44 mg; Pantothenicacid, 17 mg; Folic acid, 1.5 mg

(33±1°C) a day (11:00-16:00) at the relative humidity of 70% and 12 h of the light dark cycle (dark during the time of 08:00-20:00, light during the time of 20:00-08:00). After each daily EHS, the ambient temperature was maintained at 25°C. The growth performance including the feed intake, body weight gain and feed efficiency of the broiler chickens were determined at 21, 27 and 32 days, respectively. Feed efficiency was calculated by dividing body weight gain during 32 days by feed intake. Scientific and ethical procedures for the animal experiment complied with the regulations set forth in the European Laboratory Animal Handling License textbook (Scot, 1994) which was approved by the International Animal Care and Use Committee, IACUC, Kangwon National University, Republic of Korea.

Slaughtering and blood collection: Experimental feed was withdrawn 12 h before slaughtering. At the end of the experimental period, 20 chickens which were most close to the average body weight of the chickens were selected from the repeat pens of each group (5 male chickens per pen). After collecting blood samples from them, they were peacefully sacrificed by cervical dislocation in accordance with the laboratory animal euthanasia guideline (Close *et al*., 1997). About 3 mL of blood were collected from the heart via a plain tube (Greine Co., Ltd. Australia). Each blood sample was subjected to centrifugation at 3,000 rpm, 4°C for 20 min before separation of serum which was then frozen rapidly in liquid nitrogen at -196°C and stored at -20°C until the subsequent biochemical analysis thereof.

Determination of serum immunoglobulin: Serum concentrations of IgG, IgA and IgM were quantified by using the chicken ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). After processing the serum according to the manufacturer's protocol, the quantity of antibody was calculated by measuring the absorbance at 450 nm by using a precision microplate reader (Molecular Devices Inc., New York, USA).

Determination of serum corticosterone: Serum corticosterone was determined from the separated serum by using Corticosterone HS EIA kit (Enzymeimmunoassay kit, IDS, Ltd. Boldon, UK).

Determination of cecal microflora: In order to examine the intestinal microorganisms immediately after slaughtering, the cecum was excised by using an anaerobic method and kept on ice then stored under anaerobic condition in sealed anaerobic jars (Oxoid Basingstoke, UL) having AnaeroGen sachets (Oxoid, Hampshire, UK). The contents of the cecum were initially diluted 10 times (1:9, wt./vol.) by blending them with the sterilized anaerobic physiological saline (phosphorus buffered saline; PBS 0.1 M, pH 7.0) and then a series of dilution was continuously done. All these procedures were performed in the anaerobic condition in an anaerobic chamber (hydrogen 5%, CO_2 5%, balanced nitrogen). From each diluted sample (10^{-2}~10^{-7}), 100 μL therof was taken and cultured at the sterilized plate selective medium, i.e., *Lactobacillus* sp. (MRS agar, Oxoid, Basingstoke, UK), *Escherichia* sp. (McConkey purple agar), coliform bacteria (Violet red bile agar, Difco), total aerobic bacteria (Nutrient agar, Difco). *Escherichia coli* sp., coliform and total aerobic bacteria were aerobically cultured at 37°C for 24 h and *Lactobacillus* sp. was cultured in the stationary anaerobic condition by using sealed anaerobic jars with AnaeroGen sachets at 37°C for 48 h. Then, the number of colonies in each plate medium was counted by a microorganism counter. The numbers of all microorganism colonies as the Colony-Forming Unit (CFU)/g of the cecum contents were represented in common logarithm.

Determination of short chain fatty acids: Because the volume of each cecum collected from the sacrificed broiler was small, seven ceca per pen of the treatment group were gathered into one sample which was used to determine Short Chain Fatty Acid (SCFA) by using a gas chromatographic system (model GC-15A, Shimadzu Corp., Kyoto, Japan) (Zhang *et al*., 2003). The 5 g of the cecum content was put into a 20 mL screw cap tube and blended with 5 mL of distilled water. Then, the mixture was homogenized by using an ultra turrax and subjected to centrifugation at 3,300×g and 4°C for 10 min. After centrifugation, 1 mL of the supernatant was transferred to an ampule and acidified by adding 0.2 mL of 25% H_3PO_4 solution thereto. The homogenized sample was kept on ice for 30 min or more. Then, it was subjected to centrifugation at 10,000 rpm for 10 min before performing Gas Chromatographic (GC) analysis thereof. The GC system had a flame ionization detector and a glass column (180 cm×4 mm, Supelco, Inc., Belleponte, PA) charged with 10% SP-1000 1%/H_3PO_4 in Chromosorb WAW as attached and the column was operated together with N_2 (1.8 mL min^{-1}) of high purity as carrier gas at 100-150°C. The oven, the detector and injection had the temperature of 135, 190-200 and 190-200°C, respectively and its flow rate was 33 mL min^{-1}.

Statistical analysis: Data analysis was done by ANOVA using the GLM procedure of SAS Software. The statistically significant difference in all data was tested at $p<0.05$ according to Duncan's multiple range test (SAS, 2004).

Table 2: Growth performance of broiler chickens fed experimental diets for 32 days (g/head)

Items	Groups T1	T2	T3	T4	T5	Pooled SEM
Body Weight Gain (BWG, 0-21 days)	1,064	1,059	1,058	1,060	1,066	20.215
Daily BWG (22-27 days)	64.83^{d}	63.00^{d}	82.00^{a}	79.66^{b}	68.01^{c}	0.8902
Daily BWG (28-32 days)	41.37^{b}	37.44^{c}	23.25^{e}	34.02^{d}	50.41^{a}	1.5071
BWG (0-32 days)	1,658^{b}	1,624^{b}	1,666^{b}	1,708^{a}	1,726^{a}	22.081
Feed Intake (FI, 0-21 days)	1,624	1,620	1,638	1,631	1,624	27.025
Daily FI (22-27 days)	93.33^{b}	96.83^{b}	127.2^{a}	127.7^{a}	97.71^{b}	18.155
Daily FI (28-32 days)	105.4^{a}	96.04^{b}	76.32^{c}	75.80^{c}	110.5^{a}	3.7711
FI (0-32 days)	2,707^{a}	2,681^{b}	2,781^{a}	2,772^{a}	2,760^{a}	21.098
Feed efficiency (0-32 days)	0.61^{b}	0.61^{b}	0.60^{b}	0.62^{ab}	0.63^{a}	0.0052
Mortality (%)	1	0	0	0	0	-

T1: Normal ambient condition + Concentration basal Diet, CD; T2: Extreme Heat Stress, EHS+CD; T3: (EHS+Extreme Heat Stress Diet (EHSD) prepared from CD with tallow replaced with soy oil and containing molasses 2%); T4: (EHS+EHSD prepared from CD with tallow replaced with soy oil and containing molasses 2% and methionine and lysine of 1.5 times greater quantities than in CD); T5: (EHS+EHSD prepared from CD with tallow replaced with soy oil and containing molasses 2%, methionine and lysine of 1.5 times greater quantities than in CD and vitamin C 200 ppm); $^{a-d}$ $p<0.05$

RESULTS

Growth performance: The growth performance of the broilers exposed to EHS and fed EHSD for the entire experiment period is summarized in Table 2. The body weight gain of the broilers increased throughout the period particularly significantly in T4 and T5, compared with T1 and T2. Although, the weight gain in T3 was relatively higher than in T1 and T2 and broilers in group T2 which were fed with CD under EHS showed less weight gain, inter-group differences between these groups were not statistically significant. After supplying normal drinking water and CD *ad libitum* under continuous lightings during days 0-21, all treatment groups showed similar levels of the body weight gain. The body weight gain during days 22-27 was higher in groups T3, T4 and T5 in that order than in groups T1 and T2 with statistical significance of difference between the groups. During days 28-32, the body weight gain was highest in group T5 followed by T1, T2, T4 and T3 in the order named which also showed significant gains. The feed intake was significantly lowest in group T2. T3, T4 and T5 showed a tendency of some higher feed intake than group T1 but there were no significant inter-group differences. The feed intake was similar in all groups during days 0-21. Although, it was significantly higher in group T3 and T4 than in group T1 and T2 during days 22-27, it was similar among group T1, T2 and T5 during the same period. During days 28-32, the feed intake was higher in group T5 than in group T1 but without any significant difference and the feed intake in these two groups was significantly higher than in group T2, T3 and T4. T2 showed the significantly higher feed intake than group T3 and T4 but the difference between these groups was not statistically significant. The feed efficiency was higher in groups T4 and T5, compared with group T1 and T2 but the difference between groups T4 and T5 and the difference between group T1, T2, T3 and T4 were not statistically significant. Such results indicate that EHSD formula for group T4 and T5 could improve the growth performance of the EHS exposed broilers.

Table 3: Lymphoid organ weight of broiler chickens fed experimental diets for 32 days organ weight/body weight (%)

Items	Groups T1	T2	T3	T4	T5	Pooled SEM
Bursa of Fabricius	0.23^{a}	0.22^{a}	0.18^{b}	0.21^{a}	0.24^{a}	0.0057
Spleen	0.17^{ab}	0.18^{a}	0.15^{b}	0.18^{a}	0.19^{a}	0.0032
Thymus	0.20^{a}	0.21^{a}	0.17^{b}	0.18^{b}	0.22^{a}	0.0069

Lymphoid organ development: The weights of the lymphoid organ, bursa of Fabricius, thymus and spleen of the broilers that were exposed to EHS and fed with EHSD are summarized in Table 3. T1, T2, T4 and T5 showed similar weight gains of the bursa of Fabricius while group T3 showed a weight loss and there was a statistically significant difference between these groups. The weight of the spleen decreased significantly in group T3 but there was no significant difference between group T1 and T3 and between group T2, T4 and T5. The weight of thymus tended to have increased slightly with no significant difference in group T5, compared with that in Group T1 and T2 while it decreased significantly in group T3 and T4. Consequently, it was shown that the weight of the lymphoid organ in broilers exposed to EHS did not change significantly after the intake of EHSD.

Serum concentrations of IgG, IgA, IgM and corticosterone: The serum concentrations of immunoglobulin and corticosterone determined after feeding the ERHS exposed broilers with EHSD are summarized in Table 4. Serum IgG concentration was higher in T3, T4 and T5 than in groups T1 and T2 and there was a statistically significant difference between these groups, respectively. Particularly, T5 showed the highest level among all treatment groups which was

Table 4: Serum levels of immunoglobulins and corticosterone of broiler chickens fed experimental diets for 32 days (μg mL^{-1})

	Groups					
Items	T1	T2	T3	T4	T5	Pooled SEM
IgG	141.7^{c}	97.02^{e}	101.5^{d}	222.1^{b}	269.7^{a}	1.9085
IgA	30.13^{d}	30.38^{d}	34.07^{c}	64.74^{a}	50.10^{b}	0.3702
IgM	40.61^{e}	47.95^{d}	64.85^{b}	61.88^{c}	70.13^{a}	0.6683
Corticosterone	90.04^{a}	67.19^{c}	75.61^{b}	65.01^{d}	57.33^{e}	0.7541

Table 5: Cecum microflora in broiler chickens fed experimental diets for 32 days (log_{10} cfu g^{-1})

	Groups					
Items	T1	T2	T3	T4	T5	Pooled SEM
Lactobacillus	4.72^{e}	4.15^{d}	5.83^{c}	7.51^{b}	7.38^{a}	0.1815
Escherichia	2.83^{e}	3.13^{c}	3.73^{b}	4.16^{a}	3.09^{d}	0.1377
Coliform bacteria	7.03^{d}	7.60^{c}	8.57^{a}	8.07^{b}	6.73^{e}	0.1215
Total aerobic bacteria	7.81^{d}	8.12^{c}	9.06^{a}	8.68^{b}	7.37^{e}	0.0831

higher by 190.33 and 277.98%, respectively than groups T1 and T2. Serum IgA concentration was higher in groups T3, T4 and T5 than in groups T1 and T2 and there was a statistically significant difference between these groups, respectively. Particularly, T4 showed the highest level among all treatment groups which was higher by 166.77 and 164.91%, respectively than T1 and T2. Serum IgM concentration was higher in groups T3, T4 and T5 than in groups T1 and T2 and there was a statistically significant difference between these groups, respectively. Particularly, T5 showed the highest level among all treatment groups which was higher by 172.69 and 146.25%, respectively than group T1 and T2. These results indicate that feeding the broilers exposed to EHS with EHSD formula in groups T4 and T5 could increase further the serum concentrations of IgG, IgA and IgM. The serum concentration of corticosterone was lower in group T3, T4 and T5 than in group T1 and T2 and there was a statistically significant difference between these groups, respectively. Particularly, T5 showed the lowest level among all treatment groups which was lower by 36.33 and 14.67%, respectively than group T1 and T2. Such results indicate that feeding the broilers exposed to EHS with EHSD formula in groups T4 and T5 increased the concentration of immunoglobulin and lowered the concentration of corticosterone in serum.

Cecal microflora: The changes in cecal microflora determined after feeding the EHS exposed broilers with EHSD are summarized in Table 5. The count of *Lactobacillus* sp. was higher in groups T3, T4 and T5 than in groups T1 and T2 and there was a statistically significant difference between these groups, respectively. Particularly, T5 showed the highest count among all treatment groups which was higher by 156 and 178%, respectively than groups T1 and T2. The count of total aerobic bacteria and the count of coliform bacteria were higher in groups T3 and T4 than in groups T1 and T2 but they were significantly lower in groups T5. Particularly, groups T5 showed a tendency that they were lower by 5.63 and 9.24%, respectively than groups T1 and lower by 4.27 and 11.45%, respectively than T2. The count of *Escherichia* sp. was the lowest in groups T1 followed by T5, T2, T3 and T4 in the order named and there was a statistically significant difference between these groups, respectively. Such results indicate that feeding the broilers exposed to EHS with EHSD formula in group T5 improved maintenance of cecum microflora.

Table 6: Cecum Short Chain Fatty Acid (SCFA) in broiler chickens fed experimental diets for 32 days (μmol/g of cecal content)

	Groups					
SCFA	T1	T2	T3	T4	T5	Pooled SEM
Acetic acid	100.77^{b}	87.01^{c}	86.43^{c}	128.19^{a}	127.03^{a}	8.1760
Propionic acid	33.09^{b}	24.34^{c}	22.03^{c}	36.51^{a}	35.81^{a}	0.5380
Butyric acid	10.51^{b}	13.74^{a}	15.87^{a}	5.12^{c}	4.89^{c}	0.7489
Isobutyric acid	6.50^{b}	8.09^{a}	7.88^{a}	4.67^{c}	4.71^{c}	0.5801
Valeric acid	5.52^{a}	5.66^{a}	5.93^{a}	3.09^{b}	1.55^{c}	0.1255
Isovaleric acid	3.07^{a}	3.26^{a}	3.70^{a}	1.17^{b}	1.25^{b}	0.3821
Total SCFA	159.40^{b}	142.10^{c}	141.84^{c}	178.70^{a}	175.20^{a}	7.1885

Cecal SCFA: The changes in the content of cecum SCFA determined after feeding the EHS exposed broilers with EHSD are summarized in Table 6. The contents of acetic acid, propionic acid and total SCFA were significantly higher in groups T4 and T5 than in groups T1 and T2. They were significantly lower in groups T3 than T1 but there was no statistically significant difference between groups T2 and T3. The contents of butyric acid, isobutyric acid, valeric acid and isovaleric acid were significantly lower in groups T4 and T5 than in groups T1 and T2. Meanwhile, the contents of butyric acid and isobutyric acid were significantly higher in groups T2 and T3 than in T1 but there was no significant difference between groups T2 and T3. The contents of valeric acid and isovaleric acid were similar between groups T1, T2 and T3.

DISCUSSION

The current study showed that when EHS exposed broilers were restrictively fed with EHSD their body weight gain was improved. Results showed that the EHS exposed broilers in T4 and T5 which were restrictively fed with EHSD during the night under lightings for 12 h showed a significant body weight gain, compared with those in T1 and T2 which were fed with CD in the normal ambient condition under continuous lightings and those in T3 which were fed with EHSD

formulated by replacing tallow with soy oil and containing molasses 2%. Such difference can be considered as the result of the synergic action of a proper level of nutrients including soy oil, methionine, lysine and vitamin C which were contained in the feed for T4 and T5. It can be assumed that these nutrients which were supplied from 1 week prior to the exposure of broilers to EHS had a higher bioavailability and was sufficient in quantity and provide good resistance against EHS in broilers. This interpretation is supported by the fact that feed intake was similar among T1, T3, T4 and T5. Since, an increased intake of protein in EHS exposed broilers would have led to increased generation of metabolic heat, an increase in the contents of essential amino acids such as methionine and lysine in the feed would be helpful to decrease EHS. Also, supplying soy oil and molasses which are highly palatable to broilers may stimulate feed intake. In addition, supplying vitamin C may help minimize EHS (Leeson and Summers, 1991). Since, these nutrients have a high bioavailability in broilers, they are thought to have contributed to an increase in outputs of IgG, IgA and IgM in serum and at the same time, a decrease in stress hormone and corticosterone levels by stimulating development of lymphoid organ (Table 4) (Han *et al*., 2010). The body weight gain, feed intake and feed utilization in EHS exposed broilers are affected by environmental temperatures. The extreme heat stress lower body weight gain and feed efficiency (Austic, 1985; Geraert *et al*., 1996; Cooper and Washburn, 1998; Guo *et al*., 2003). In the earlier study, researchers reported that when EHS exposed broilers were *ad libitum* fed with EHSD under continuous lightings while normal drinking water was supplied, their growth performance was significantly decreased. Researchers fed EHS exposed broilers *ad libitum* under continuous lightings with EHSD. However, while the other ingredients than molasses and vitamin C had the same level in the earlier study as in this study, the levels of molasses (5%) and vitamin C (300 ppm) were higher than that in this study (Park *et al*., 2012). Meanwhile, it is thought that the reason why the body weight was significantly higher in T4 and T5 than in T1, T2 and T3 was probably associated with an increase in production of SCFA such as acetic acid and propionic acid in the ceca (Table 6). Consequently, it can be argued that the intestinal microflora was maintained by stimulating the growth of *Lactobacillus* sp. beneficial to health and inhibiting the growth of harmful microorganisms at the same time (Table 3). This could meant that the health of EHS exposed broilers was better as their immun competence was stronger due to the higher level of IgG, IgA and IgM in serum (Table 4).

The growth of the lymphoid organs was similar among most treatment groups except T3 where it was significantly lower. However, in T4 and T5, serum IgG, IgA and IgM concentrations were higher and the levels of stress hormone and corticosterone lower, compared with T1, T2 and T3. This result showed that the feed intake was decreased by EHS and thus a sufficient amount of nutrients required for cell proliferation of the lymphoid organ secreting immunity substances needed to be supplied (Bartlett and Smith, 2003; Singh *et al*., 2006). The increase of IgG, IgA and IgM in T4 and T5 resulted from the stimulation of the immunologic competence by an increase in *Lactobacillus* sp. and the decrease IgG, IgA, and IgM in T1, T2 and T3 means that humoral immunity was compromised by EHS. It has been reported that an increase of beneficial Lactobacillus and bifidobacteria in the cecum stimulates immune system (Suk and Washburn, 1995; Park and Park, 2009). The immune proteins are produced in B-cells of bone marrow and IgG which is a humoral immunity indicator. Biological characteristics of IgG, IgA and IgM in broiler are similar to those of immune protein in mammals (Higgins, 1975). The thymus and the spleen are important organs for antibody production in animals and particularly, the lymphoid organ in birds includes the bursa of Fabricius. This lymphoid organ in broilers is essential to convert IgM to IgG or activate IgA action (Bienenstock *et al*., 1973). It can be, therefore, speculated that the decease in stress hormone and corticosterone concentrations due to a lower level of of IgG, IgA and IgM in serum would result from the regression of the lymphoid organ found in EHS exposed broilers. The growth of the lymphoid organ is the basis for the functionality of the immune system and the bursa of Fabricius is used in the study as an indicator for on the development and functional maturity of B lymphocytes (Wang *et al*., 2000; Tizard, 2002).

In general, a higher level of short chain fatty acid such as acetic acid and propionic acid is beneficial to the host animal (Table 6) and the increase in Lactobacillus may have helped activate intestinal functions in EHSD-fed broilers in T4 and T5 which was associated with a decreased count of harmful Escherichia, coliform and total aerobic bacteria (Table 5) (Devaraj *et al*., 2002). Lactobacillus secretes bacteriocin which inhibits the growth of harmful bacteria such as Escherichia and produces short chain fatty acid which improve the intestinal environment so that number of beneficial bacteria may increase in the git. Therefore, acetic acid and propionic acid together with most organic acids and lactate that are produced from fermentation of Lactobacillus can inhibit the proliferation of harmful bacteria in the intestine (Gibson and Wang, 1994;

Gibson *et al*., 1995; Devaraj *et al*., 2002; Gong *et al*., 2002; Xu *et al*., 2002). This may lend explanation to the reason as to why the count of Escherichia, coliform and total aerobic bacteria was significantly lowered in the ceca of birds in T4 and T5. Microorganisms in the alimentary tract of an animal is very important from the viewpoint that they produce fermentation products to supply intestinal epithelial cells with the energy required for their growth, stimulate the alimentary tract immune system, synthesize vitamin K and resist against the proliferation of pathogenic organisms (Shakibaie *et al*., 2009).

CONCLUSION

The results of this study suggest that restricted feeding of diet containing 5% soy oil, 2% molasses, 0.45% methionine, lysine 0.45% and 200 ppm vitamin C under night lightings can improve growth performance of broilers exposed to extreme heat stress. This is believed that such a diet stimulates immune system, encourages the production of short chain fatty acids in ceca and increases beneficial gut microflora such as Lactobacillus in the gut.

ACKNOWLEDGEMENT

This study was supported by Rural Development Administration (Project No: Agenda), Republic of Korea, 2011.

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