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Effects of anti-heat diet and inverse lighting on growth performance, immune organ, microorganism and short chain fatty acids of broiler chickens under heat stress

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Abstract

The present study investigated the effects of night restricted feeding of extreme heat diet (EHD) containing heat stress resistance nutrients, with inverse lighting program, on their growth performance in broiler chickens exposed to extreme heat stress (33±2ºC). EHD 1 contained soy oil, molasses, methionine and lysine. EHD 2 contained all nutrients of EHD 1 and vitamin C additionally. Three hundred broiler chickens (Ross 308) were randomized into four dietary treatment groups according to a randomized block design on the day they were hatched. The treatment groups were: T1 (EHD 1, 10:00-19:00 dark, 19:00-10:00 light), T2 (EHD 2, 10:00-19:00 dark, 19:00-10:00 light), T3 (EHD 1, 09:00-18:00 dark, 18:00-09:00 light) and T4 (EHD 2, 09:00-18:00 dark, 18:00-09:00 light). The body weight gain of the broilers increased most significantly in T2, followed by T1, T4 and T3 ($p<0.05$). Weights of the immune system, thymus and bursa of Fabricius recorded higher in T1 and T2 than in T3 and T4. The spleen was higher in T1, T2 and T3 than in T4 ($p<0.05$). Blood triglyceride, total cholesterol and blood sugar were higher in T1 and T2 than in T3 and T4 ($p<0.05$). LDL-C recorded high in the order of T4, T3, T2 and T1, but HDL-C showed the inverse order ($p<0.05$). IgG, IgA and IgM were higher in T1 and T2 than in T3 and T4, however, the corticosterone concentration showed the inverse order ($p<0.05$). *Lactobacillus* in feces was higher in T1 and T2 than in T3 and T4, but total aerobic bacteria, *E. coli,* Coliform bacteria was higher in T4 and T3 than in T2 and T1 ($p<0.05$). Contents of acetic acid, propionic acid and total Short chain fatty acid were significantly higher in the order of T2, T1, T3 and T4. Butyric acid, isobutyric acid, valeric acid and isovaleric acid were higher in T4 and T3 than in T1 and T2 ($p<0.05$).

Key words

Corticosterone, Extreme heat diet, Inverse lighting, Immunoglobulin, Short chain fatty acids

Introduction

It is well-known that heat stress arouses oxidative stress and leads to reduced growth performance in poultry production system. Extreme heat stress in summer causes increase in chickens' body temperature. At the same time, the amount of feed intake decreases and water intake increases, affecting economic return of the farm (Quinteiro *et al*., 2011). The ideal environmental temperature for growth of broilers is about 21ºC, and the body temperature of mature chickens should be 41-42ºC. Chickens do not have sweat glands and are covered with feathers, therefore when they are exposed to high-temperature they breathe by opening their mouth to regulate their bodies temperature and pant (Han *et al.,* 2010).

The body temperature of chickens show rapid

increase when environmental temperature rises and under extreme heat stress, feed intake and metabolic rate are lowered. Weight decreases and death rate increases (Quinteiro-Filho *et al.,* 2011; Park *et al.,* 2013a). When environmental temperature increases up to 32ºC, heat stress does not affect the death rate but it does at 37ºC (Niu *et al*., 2009). It was reported that there exists negative phenotype correlation between the growing broilers' body temperature and the resilience to heat stress (Lin *et al*., 2006). Extreme heat oppresses chickens' specific immune reaction and the broilers exposed to 32ºC show a decrease of 24% feed intake (Niu *et al.,* 2009). Extreme heat worsens the development of immune cells, takes away blood immune substances, beneficial enteric microscopic organism, lowers short chain fatty acids (SCFA) and increase harmful microscopic organism, each of which creates more stress (Park *et al.,* 2013a).

Several important nutritional strategies were proposed as environmental factors to alleviate the adverse effect on extreme heat stress with light programme control (Lin *et al.,* 2006). Lighting programme control and light are critical environmental factors which stimulate growth performance of broilers. It is known that supplying of soy oil, molasses, methionine, lysine and vitamin C relieve heat stress (Park *et al.,* 2013b; Yoon *et al.,* 2013). However, there is lack of understanding of the improvement effect of growth performance by correlation between heat stress diet containing such nutrients and lighting programme control. Authors in previous studies have reported that broilers under extreme heat stress showed decrease in body weight and feed intake as compared to chickens under normal environmental conditions over 24 hour consecutive lighting (Park *et al.,* 2013ab). Also under extreme heat stress, with daytime feed restriction and an inverse lighting programme (08:00-20:00 dark, 20:00-08:00 light) and night feed restriction of extreme heat stress diet at night is regarded to improve the body weight gain increase.

In light of the above, the present study was carried out to investigate interaction effect between inverse lighting (presented to experimental design) and extreme heat stress diet (EHD, diet containing nutrients resilient to extreme heat stress) on growth performance of heat stress-exposed broiler chickens in a closed poultry house.

Materials and Methods

Experimental design : Three hundred day-old Ross 308 broiler chicks were arranged in a completely random design of 4 treatment groups on the day of hatching. Each group had 3 repetitions, and each repetition contained 25 broilers. Birds were divided into four groups, based on the previous study results, (Park *et al.,* 2013) : T1 (EHD 1, 10:00-19:00 dark, 19:00-10:00 light), T2 (EHD 2, 10:00-19:00 dark, 19:00-10:00 light), T3 (EHD 1, 09:00-18:00 dark, 18:00-09:00 light), T4 (EHD 2, 09:00-18:00 dark, 18:00-09:00 light).

Animal feeding and management : The experimental diets were set to have equal crude protein and metabolic energy using cereal ingredients such as yellow corn and soybean meal (Table 1). EHD 1 contained cereal ingredients+soy oil 5%+ molasses 2%+ methionine 0.45%+ lysine 0.45%. EHD 2 contained all nutrients of EHD 1 and Vitamin C 200 ppm additionally. Beef tallow, added to EHD as energy source was substituted by soy oil, which contains a high utilization rate, preference and essential fatty acid. Molasses which contain high utilization rate also added preference.

Under extreme heat stress, increase in essential amino acids such as methionine and lysine, rather than total protein level is helpful, because increased total protein level leads to increase in heat generation through metabolism (Leeson and Summers, 1991; Yoon *et al*., 2013). It has been reported that supply of vitamin C influences the storage of nutrients, and improve growth performance by maintaining metabolic rate (McKee *et al*., 1997; Park *et al*., 2013ab). Chickens were allowed *ad libitum* feeding under environmental temperature of 22-25ºC for starter phase (1-21 days), with consecutive lighting and general drinking water. 10 cm of chaff as a straw litter was put on the floor of every pen and the temperature of breeding room was set at 33ºC from the hatching day to three days after. Later the temperature was lowered by 2-3ºC per week. From the 22nd day to the 32nd day of the experiment, EHD was provided. Until the 27th day from the 22nd day the chickens were in consecutive lighting under the general environmental temperature, and EHD was *ad libitum* feeding. For the last five days (from 28 to 32 days) extreme heat environment was maintained and the EHD fed night feed restriction for 15 hr (18:00-09:00). The condition of extreme heat stress was daily five hours (11:00-16:00) of heat stress (33ºC) with the 70% relative humidity and lightening control. During the time ventilation was not carried out. After the daily experiment of extreme heat the air was ventilated and the general environmental temperature was maintained.

Scientific and ethnic procedures for animal experiments were observed, following the manuals from the textbook of European animal experiment license, and it was approved by the Ethics Committee of Animal Experiments in Kangwon National University. During the experiment the amount of feed intake and the body weights were gauged and recorded by ten days. Increase of body weight and feed intake including efficiency (increase of weight/increase of feed intake) are shown.

Blood and tissue collection: Experimental feed was withdrawn 12 hr before slaughter. Fifteen broilers, five from

Table 1 : Composition of experimental diets for broiler chickens

Ingredients (% as-fed)	Diets		
	Starter (1-21 days)	EHD I (Grower 22-32days)	EHD 2 (Grower 22-32days)
Yellow corn	52.00	47.70	47.70
Soybean meal, 44% CP	34.00	25.00	25.00
Corn gluten meal	4.70	5.70	5.70
Wheat meal	-	10.00	10.00
Tallow	5.00	-	-
Soy oil	-	5.00	5.00
Molasses	-	2.00	2.00
Limestone	1.25	1.25	1.25
Dicalcium phosphate	1.70	1.70	1.70
Sodium chloride	0.25	0.25	0.25
DL-Met, 50%	0.30	0.45	0.45
L-Lys HCl, 78%	0.30	0.45	0.45
Trace mineral premix[1)]	0.34	0.34	0.34
Vitamin premix[2)]	0.16	0.16	0.16
Vit. C	-	-	0.02
Total	100	100	100
Chemical composition			
ME, kcal/kg	3,100	3,150	3,150
Crude Proteim, %	22.00	20.00	20.00
Lys, %	1.32	1.15	1.15
Met, %	0.52	0.50	0.50
Met+Cys, %	0.78	0.73	0.73
Ca, %	1.00	0.90	0.90
Available P, %	0.45	0.40	0.40

[1)] Supplied per kilogram 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 kilogram of diet: vitamin A (retinyl acetate), 10,500 IU; vitamin D_3, 4,100 IU; vitamin E (DL-á-tocopheryl acetate), 45 mg; vitamin K_3, 3.0 mg; thiamin, 2.5 mg; riboflavin, 5mg; 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

each pen, were selected, and their blood was collected. Euthanasia was administered by dislocation of cervical vertebral, so as to prevent stress. Liver, gizzard, spleen, thymus and Bursa of Fabricius were collected and put in physiological saline solution. Weights were gauged and recorded after getting rid of water with filter papers. By puncture heart with plain tubes (Greine Co Ltd, Australia), 3 ml of blood from each chicken was obtained. Through centrifugation with 3,000 rpm for 20 min under 4ºC, serum was separated, and was quickly frozen. in liquid nitrogen at -196ºC and kept under -20ºC till biochemical analysis.

Blood lipids and immune substances : Blood lipids, trigriceride, total cholesterol, LDL-C, HDL-C and glucose were measured by enzyme kit (Sigma, USA). Concentrations of immune substances, IgG, IgA, IgM were set by chicken ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). A precision microplate reader (Molecular Devices Inc, New York, USA) was used according to the manufacturer's protocol to measure the absorbance value at 450 nm, and calculate the amount of antibody (Park *et al*., 2013b).

Blood corticosterone : The quantities of stress hormones and corticosterone in blood were determined using HS EIA kit (Enzyme immunoassay kit, IDS, Ltd., Boldon, UK) according to manufacturers protocol (Park *et al*., 2009).

Short chain fatty acid (SCFA) : Caecum from sacrificed chicken was taken in an anaerobic way. Since we had small quantity of sample, was obtained one sample pooled by five samples per repeat pen to get a tri-repeat sample per group was made. SCFA was measured using gas chromatographic system (model GC-15A, Shimadzu Corp., Kyoto, Japan). Five gram of cecal content was put into 20 ml screw cap tubes and were mixed with 5 ml of distilled water. After homogenization, it was centrifuged for 10min under 4ºC, 10,000 rpm. After centrifugation 1 ml of supernatant was to an ample bottle and was acidized with 0.2 ml of 25% H_3PO_4. After homogenization of sample, the bottle was placed on ice for more than 30 min.

Before analysis of GC it was centrifuged for 10 min at10,000 rpm. On GC Glass column (180cm×4mm, Supelco, Inc., Belleponte, PA) charged by 10% SP-1000/1% H_3PO_4 on the flame ionization detector and Chromosorb WAW was attached. Carrier gas was applied at 100-150ºC with high

purity N_2 (1.8 ml min^{-1}). The flow rate was 33 ml min^{-1} (Park *et al*., 2013a).

Microorganisms in feces : Fresh feces from 15 broilers exposed to extreme heat (five per pen) were collected. They were mixed with sterilized physiological saline (phosphorus buffered saline; PBS 0.1 M, pH 7.0) and were then diluted ten times (1:9, wt/vol). Cultivation was carried out using sample diluted 10^{-2}~10^{-7},and they were replanted individually at 100 ul on sterilized plates of selective medium for *Lactobacillus* sp. (MRS agar, Oxoid, Basingstoke, UK), *E. coli* sp. (Mc Conkey purple agar, Difco), coliform bacteria (Violet red bile agar, Difco), total aerobic bacteria (Nutrient agar, Difco). *E. coli* sp., coliform, total aerobic bacteria was aerobically cultivated for 24 hr under 37ºC and *Lactobacillus* sp. was cultivated under anaerobic state under 37ºC, standing cultivation with Anaero Gen sachets using sealed anaerobic jars for 48 hr. The number of colonies as microorganic counters was counted on the respective plates. All numbers of microorganic groups were shown as a form of common logarithm using colony-forming unit (Cfu) g^{-1} of wet of feces content (Park *et al*., 2013b).

Statistical analysis: Analysis of variance on all materials was conducted using GLM procedure by SAS software (2004). After carrying out Duncan's multiple range test with $p<0.05$, statistical significance was tested.

Results and Discussion

During the whole experimental period, T2 showed most significant increase in body weight, followed by T1, T4 and T3. Statistical significance among the groups was approved ($p<0.05$). T1 and T2 appeared to be similar and T1 and T4 also did. T3 was the lowest. Body weights of T2 as compared with those of T1, T3 and T4 increased by 103.70%, 110.58% and 105.59% respectively. Feed intake showed similar results among the groups of T1, T2 and T4. T3 significantly had lowest as compared to the rest ($p<0.05$). T3 feed intake was 7.61% lower than T2. The feed efficiency was highest among all the groups (significantly) in T2 ($p<0.05$), but there was no significant difference between T1, T3 or T4 (Table 2).

There was no significant difference among the groups for liver or gizzard. Thymus and Bursa of Fabricius were higher in T1 and T2 than in T3 and T4. Spleen was significantly higher in T1, T2 and T3 than in T4 ($p<0.05$). T1, T2 and T3 showed similar spleen weights. Bursa of Fabricius and thymus showed no statistical significance between T1 and T2 or between T3 and T4 (Table 3).

Triglycerides were higher by 123.34% in T2 and T1 than in T3 and T4. Total cholesterol was higher in T2 and T1 than in T3 and T4 by 107.71%. LDL-C was high in the order of T4, T3, T2 and T1 (Table 4), but HDL-C showed reverse tendency ($p<0.05$). Glucose was similar in T1 and T2 and was significantly higher by 140.56% than in T3 and T4 ($p<0.05$).

The concentration of IgG was high in the order of T2, T1, T4 and T3. T2 and was found to be higher than T1, T3 and T4 by 9.14%, 50.09% and 74.70%, respectively. Statistical significance exists ($p<0.05$). Concentrations of IgA, IgM were high in the order of T2, T1, T3 and T4. Excluding the similarity between T1 and T2, they also had statistical significance ($p<0.05$) the concentration of IgA in T2 was higher by 5.19%, 111.59% and197.31%compared to T1, T3 and T4, respectively. The concentration of IgM were also found to be 1.20%, 124.20% and 257.88% higher in T2. The concentration of corticosterone was in the decreasing order of T3, T4, T1 and T2, and statistical significance was verified between each of the values. T2 showed lowest value for corticosterone with 13.88%, 75.23% and 71.53% less than groups of T1, T3 and T4, respectively (Table 4).

Lactobacillus was found to be high in the order of T2, T1, T3 and T4. There was statistical significance among the groups ($p<0.05$). Especially, T2 was highest and was increased by 102.96%, 115.61% and 112.80% as compared to T1, T3 and T4, respectively. Total aerobic bacteria was high in the order of T4, T3, T2 and T1 ($p<0.05$). There was no statistically significant difference between T3 and T4. T1 was lowest as compared to T4, T3 and T2, respectively, showing 25.78%, 25.00% and 15.23% lower. *E. coli* was high in the order of T4, T3, T1 and T2. Excluding similarity between T3 and T4, statistical significance was seen ($p<0.05$). T2 appeared lowest, recording 35.28%, 35.77% and 27.64% lower as compared to T4, T3 and T1.Coliform bacteria was high in the order of T4, T3, T2 and T1 ($p<0.05$), not showing any significance between T3 and T4. T1 was lowest and was 30.69%, 20 27% and 8.35% lower than T4, T3 and T2 (Table 5).

Total SCFA, including acetic acid and propionic acid was high in the order of T2, T1, T3 and T4 ($p<0.05$). T2 showed 101.08%, 131.09% and 143.36% more acetic acid than T1, T3 and T4, and 121.13%, 144.73% and 166.75% more propionic acid, whereas total SCFA showed 107.90%, 125.60% and 138.26% more. Butyric acid was in the order of T4, T3, T2 and T1. T1 as compared to T2, T3 and T4, recorded 27.23%, 48.02% and 60.17% less. Isobutyric acid was in the order of T3, T4, T1 and T2 ($p<0.05$) but there was no statistical significance between T1 and T2. T2 when compared with T1, T3 and T4 decreased by 10.26%, 66.78% and 39.16%. Valeric acid was in the order of T4, T3, T1 and T2 ($p<0.05$) and there was no significance between T1 and T2. T2 was lowest compared with T1, T3 and T4 9.66%, 41.01% and 54.50%. Isovaleric acid was in the order of T4, T3, T1 and T2 ($p<0.05$) with no significance between T3 and

Table 2 : Growth performance of broiler chickens exposed to extreme heat stress (g head^{-1})

Groups[1]	T1	T2	T3	T4
Days		Body weight gain		
0-21	785±14.87[2]	790±15.23	768±13.81	737±16.52
22-32	727±15.10[a]	778±14.62[a]	633±18.43[b]	708±14.50[a]
0-32	1,512±12.88[ab]	1,568±17.29[a]	1,418±18.68[c]	1,484±17.01[b]
		Feed intake		
0-21	1,525±5.65[a]	1,527±6.73[a]	1,477±7.32[b]	1,485±8.66[b]
22-32	1,578.7±7.95[a]	1,562±8.51[a]	1,377±5.80[b]	1,513±7.90[a]
0-32	3,102±5.92[a]	3,089±8.45[a]	2,854±4.07[b]	2,998±6.24[a]
		Feed efficiency ratio		
0-21	0.51±0.01	0.52±0.02	0.51±0.04	0.50±0.01
22-32	0.46±0.03	0.50±0.03	0.46±0.03	0.47±0.02
0-32	0.49±0.02[b]	0.51±0.001[a]	0.48±0.01[b]	0.48±0.01[b]

[1]T1 : Extreme heat diet (EHD)1+(10:00-19:00 dark, 19:00-10:00 light); T2 : EHD 2+(10:00-19:00 dark, 19:00-10:00 light); T3 : EHD 1+(09:00-18:00 dark, 18:00-09:00 light); T4 : EHD 2+(09:00-18:00 dark, 18:00-09:00 light). [2]Means±SD. [a,b,c] $p<0.05$.

Table 3 : Weight of lymphoid organs in broiler chickens exposed to extreme heat stress

Item[2]	Groups[1]			
	T1	T2	T3	T4
Liver	3.39±0.51[2]	3.15±0.30	3.09±0.23	3.11±0.25
Thymus	0.20±0.01[a]	0.21±0.02[a]	0.17±0.01[b]	0.17±0.01[b]
Spleen	0.19±0.02[a]	0.18±0.05[a]	0.18±0.05[a]	0.15±0.04[b]
Bursa of Fabricius	0.22±0.07[a]	0.22±0.03[a]	0.17±0.02[b]	0.18±0.05[b]
Gizzard	1.89±0.47	1.93±0.28	1.82±0.10	1.87±0.20

[1]T1 : Extreme heat diet (EHD)1+(10:00-19:00 dark, 19:00-10:00 light); T2 : EHD 2+(10:00-19:00 dark, 19:00-10:00 light); T3 : EHD 1+(09:00-18:00 dark, 18:00-09:00 light); T4 : EHD 2+(09:00-18:00 dark, 18:00-09:00 light). [2]Liver, gizzard is % weight against body weight. Thymus, spleen, bursa of Fabricus is % weight against carcass weight. [a,b] $p<0.05$.

T4. T2 was lowest when compared with T1, T3 and T4 decreased by 43.23%, 70.17% and 71.70%, respectively (Table 6).

A new discovery from these results is that growth performance can be improved for broilers in extreme heat stress if EHD 2 and inverse lightening is provided (10:00-19:00 dark, 19:00-10:00 light). It is considered that night feed restriction of EHD, which led to absorption of resilient-to-heat nutrients and increase in accumulation, made this possible. Besides T1 and T2, especially T2 showed significant increase in body weights. T1 and T2 was given feed supply with restriction at night during the period of extreme heat stress and inverse lightening was carried out. Lights went out in T2 one hour before extreme heat stress to three hours after and in T1, T3 and T4, the rooms were darkened for two hours before the heat to two hours after heat. Allowing enough rest for chickens in T2 helped them to improve growth performance. Less food consumption under extreme heat prevented increase in body temperature by restraining increase of metabolic heat, which could be caused by feed intake and the chickens' resting with no lights for one more hour through inverse lightening increased intake and usage rate of anti-heat nutrients. Inverse lightening is desirable since 24-hour-lightening under extreme heat causes increase in body temperature and an increase of bio-metabolic heat also causes a rapid increase of the body temperature (Park *et al.,* 2013a; Yoon *et al.,* 2013]. T2 showed the highest growth performance with intake of EHD 2 was due to the effect of adding vitamin C. Among the nutrients in EHD, beef tallow contains more saturated fatty acid as compared to vegetable oil. Saturated fatty acid has high melting point and its metabolic heat rate is also relatively high. Soy oil is highly preferred and has low melting point. It also contains essential fatty acid and has high energy usage rate. It can stimulate intake and usage rate in same manner as molasses (Sahraei 2012; Yoon *et al.,* 2013). High level of protein under heat stress brings more metabolic heat when it dissolved in amino acid during bio-metabolic process, it helps in increasing essential amino acids such as methionine and lysine (Leeson and Summers, 1991). The effect of methionine and lysine on body weight gain of the broilers bred at high temperature was reported (Sahraei 2012; Yoon *et al.,* 2013). The supply of vitamin C to the broilers exposed to

Table 4 : Serum lipid, glucose, immunoglobulin and corticosterone levels in broiler chickens exposed to extreme heat stress

Item	Groups			
	T1	T2	T3	T4
Triglyceride (mg dl^{-1})	129.5±4.38^{a,2}	132.1±5.07^{a}	112.8±4.73^{b}	107.1±5.31^{b}
Total cholesterol (mg dl^{-1})	138.12±1.81^{b}	145.54±2.02^{a}	135.12±3.86^{b}	137.27±4.55^{b}
LDL-C (mg dl^{-1})	38.76±2.81^{d}	43.50±1.63^{c}	55.44±4.08^{b}	61.11±4.37^{a}
HDL-C (mg dl^{-1})	97.18±3.81^{a}	88.77±3.51^{b}	69.18±4.08^{c}	64.87±4.15^{d}
Glucose (mg dl^{-1})	193.72±4.73^{a}	187.33±4.07^{a}	150.72±5.74^{b}	137.82±3.81^{c}
IgG (mg ml^{-1})	226.5±2.09^{b,2}	247.2±3.27^{a}	141.5±4.73^{d}	164.7±3.80^{c}
IgA (mg ml^{-1})	50.51±3.11^{a}	53.13±4.22^{a}	25.11±3.56^{b}	17.87±2.37^{c}
IgM (mg ml^{-1})	77.16±3.31^{a}	78.09±1.82^{a}	34.83±4.20^{b}	21.85±1.73^{c}
Corticosterone (mg ml^{-1})	31.42±1.94^{c}	27.06±2.55^{d}	109.26±2.18^{a}	95.03±3.26^{b}

$^{a,b,c,d}p<0.05$.

Table 5 : Changes in the fecal microflora of broiler chickens exposed to extreme heat stres (log_{10} cfu g^{-1})

Item	Groups			
	T1	T2	T3	T4
Lactobacillus	6.76±0.03^{b,2}	6.96±0.12^{a}	6.02±0.06^{c}	6.17±0.09^{d}
Total aerobic bacteria	5.01±0.19^{c}	5.91±0.27^{b}	6.68±0.25^{a}	6.75±0.44^{a}
E. coli	4.74±0.07^{b}	3.43±0.72^{c}	5.34±0.34^{a}	5.30±0.28^{a}
Coliform bacteria	4.72±0.03^{c}	5.15±0.53^{b}	5.92±0.15^{a}	6.81±0.35^{a}

$^{a,b,c,d}p<0.05$.

Table 6 : Concentrations of short chain fatty acid (SCFA) in the cecal contents in broiler chickens exposed to extreme heat stress (mmol g^{-1} of cecum content)

SCFA	Groups			
	T1	T2	T3	T4
Acetic acid	148.5±0.14^{b,2}	150.1±0.32^{a}	114.5±0.17^{c}	104.7±0.27^{d}
Propionic acid	78.51±0.17^{b}	95.10±0.21^{a}	65.71±0.25^{c}	57.03±0.35^{d}
Butyric acid	5.13±0.38^{d}	7.05±0.18^{c}	9.87±0.25^{b}	12.88±0.17^{a}
Isobutyric acid	3.41±0.19^{c}	3.06±0.31^{c}	9.21±0.30^{a}	5.03±0.17^{b}
Valeric acid	2.07±0.12^{c}	1.87±0.25^{c}	3.17±0.23^{b}	4.11±0.15^{a}
Isovaleric acid	1.55±0.12^{b}	0.88±0.05^{c}	2.95±0.17^{a}	3.11±0.08^{a}
Total SCFA	239.1±0.19^{b}	258.0±0.28^{a}	205.41±0.25^{c}	186.6±0.21^{d}

$^{a,b,c,d}p<0.05$.

the high temperature lowers corticosterone and improves the results by maintaining the metabolic rate affecting nutrients, especially saving energy (Park *et al.,* 2013a). Vitamin C has an antioxidant effect, strengthens heart and blood vessels, and at the same time it contributes to minimization of heat stress as an important component for collagen biosynthesis (Boyera *et al.,* 1998). It is deemed that such nutrients in EHD are bio-metabolically used with a high rate, causes less metabolic heat and increase secretion amount of IgG, IgA and IgM in blood by stimulating immune cells to developed. At the same time it lessens the concentration of stress hormones and corticosterone and stimulated broilers' growth ability (Niu *et al.,* 2009). Under heat stress, broilers' weight gain, amount of feed intake and its usage rate are related to their body temperature. When they were exposed to the temperature of 32ºC, weight gain and efficiency of diet drops due to rapid increase of the chickens' body temperature (Geraert *et al.,* 1996; Park *et al.,* 2013ab). Authors reported that when exposed to consecutive lightening and extreme heat, the broilers which were fed EHD with a high ratio of anti-heat nutrients showed significant decrease growth performance (Park *et al.,* 2013a; Yoon *et al.,* 2013). On the other hand, the reason why T2 had the highest body weights, which was similar to T1 but significantly higher than T3 and T4, seems to

be related to the increase of total SCFA, including acetic acid and propionic acid, as can be seen in Table 6. As a result, it maintain the level of intestinal microflora (Table 5) by restraining the growth of harmful microorganism and stimulating the growth of *Lactobacillus,* which is beneficial. Animals exposed to extreme heat are given a high level of immunity by increased concentrations of serum IgG, IgA and IgM (Table 4).

As a result of the broilers' exposure to extreme heat, their blood lipid and glucose significantly decreased. This corresponded to the previous report and it also showed a decrease of bio-lipids which are quickly used as an energy source based on the decrease of feed intake due to heat stress (Park *et al.,* 2013a; Yoon *et al.,* 2013). Development of immune cells and immunity substances in blood showed similar levels in T1 and T2 and T2 was significantly high on blood IgC and this ultimately made it possible to lower stress hormones and corticosterone compared to T3 and T4 when Compared with T3 and T4, T2 enjoyed one additional hour of lights-out and the chickens in T2 were fed sufficient nutrients for cellular multiplication in the immune system with interaction of heat-resilient nutrients (Singh *et al.,* 2006). An increase of IgG, IgA and IgM in T1 and T2 is seen as stimulation of the level of immunity and decreases of IgG, IgA and IgM in T3 and T4 means that the ability of humoral immunity was oppressed by extreme heat. *Lactobacillus* sp. And Bifidobacteria are widely known as beneficial germs. These germs ferment undegraded nutrients and stimulate energy intake and the level of immunity and the improve of metabolism (Schley and Field. 2002; Park and Park, 2009). This study did not investigate the change of Bifidobacteria. Immunoprotein is created in B-cells from bone-marrow and IgG occupies more than 90% of blood. For broilers, IgG, IgA and IgM share similar biological characteristics with mammals' immunoprotein (Higins, 1975; Park and Park, 2009). Thymus and spleen are critical organs for antibody production inanimals, and especially for birds, the bursa of Fabricius plays a role as an immune organ. Broilers' immune system is essential for activating IgA or transforming IgM into IgG (Bienenstock *et al.,* 1973). Therefore, a decrease of stress hormones and corticosterone based on risen concentrations of blood IgG, IgA and IgM is also regarded as the regression result of lymphoid organs shown under extreme heat. Development of the immune system is the basis of its operation and the bursa of Fabricius is used for the development of B lymphocyte and the study of maturity of the operation (Tizard, 2002).

This study considered that decrease of *E. coli*, coliform, total aerobic bacteria in T1 and T2 improved broilers' growth and advance the formation of SCFA. The results of the measures on the scientific procedure experiments can be interpreted comparing the relative ratio with the control group and the absolute standard for related statistics is unknown. In T1 and T2 where inverse lightening and EHD were given, SCFA such as acetic acid and propionic acid, which are beneficial for host animals, increased. Also, *Lactobacillus,* which is beneficial for intestinal functions, increased. This is seen with related to the decrease of *E. coli*, coliforms, and total aerobic bacteria (Yoon *et al.,* 2013). *Lactobacillus* secretes bacteriocin that represses growth of harmful germs, such as *E. coli,* and creates SCFA which improves the intestinal environment for the inhabitation of beneficial germs. Therefore, with most of the organic acid and lactic acid formed by the fermentation of *Lactobacillus*, acetic acid and propionic acid are able to oppress the formation of colonies by harmful germs (Gong *et al.,* 2002; Yoon *et al.,* 2013). The reason why caecum *E. coli*, coliform, total aerobic bacteria in T1 and T2 significantly decreased is regarded to be due to this mechanism. In the digestive canal of animals, microorganisms are critical considering that they provides necessary energy for the development of epithelial cells by biosynthesis of the fermentation products, stimulation of immune system of the digestive canal, synthesis of Vitamin K, and that they provide resilience to the colony formation of exogenous agents of disease (Gong *et al.,* 2002; Xu *et al.,* 2002).

In conclusion, the study results showed inverse lightening (10:00-19:00 dark, 19:00-10:00 light) with night feed restriction of extreme heat stress diet containing soy oil, molasses, methionine, lysine and vitamin C can improve the growth performance of broilers exposed to extreme heat stress. It is considered that the causes of the growth performance are - supply of nutrients which add resilience to extreme heat, stimulating feed consumption by inverse lightening control, intensification of immune system, maintaining intestinal microflora, decreasing consumption of blood triglyceride and glucose as energy sources, decreasing metabolic heat and restraining temperature increase owing to the reduced exposure to the environment of extreme heat stress.

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