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Effect of cold water and inverse lighting on growth performance of broiler chickens under extreme heat stress

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Abstract

The present study was carried out to investigate the effect of provision of extreme heat stress diet (EHD), inverse lighting, cold water on growth performance of broiler chickens exposed to extreme heat stress. The chickens were divided into four treatment groups, (T1, T2, T3, T4) as given below: T1 (EHD 1, 10:00-19:00 dark, 19:00-10:00 light, cool water 9ºC); T2 (EHD 2, 10:00-19:00 dark, 19:00-10:00 light, cool water 9ºC); T3 (EHD 1, 09:00-18:00 dark, 18:00-09:00 light, cool water 14ºC); T4 (EHD 2, 09:00-18:00 dark, 18:00-09:00 light, cool water 14ºC. EHD 1 contained soybean oil, molasses, methionine and lysine; EHD 2 contained the same ingredients as EHD 1 with addition of vitamin C. Groups T1 and T2 were given cooler water than the other two groups, and displayed higher body weight increase and diet intake as compared to T3 and T4 ($p<0.05$). The weights of their liver and gizzard were similar but the weights of the thymus and bursa of fabricius (F) were higher in groups T1 and T2 ($p<0.05$). It was observed that groups T1 and T2 displayed higher concentrations of blood triglyceride, total cholesterol, HDL-cholesterol and blood sugar as compared to T3 and T4; however LDL-cholesterol level was higher in groups T3 and T4 ($p<0.05$). T1 and T2 displayed higher levels of immunity substances such as IgG, IgA and IgM as compared to T3 and T4, but the blood level of corticosterone was lower in groups T1 and T2 ($p<0.05$). T1 and T2 contained higher amount of fecal Lactobacill as compared to T3 and T4; however T3 and T4 contained higher amount of fecal *E. coli*, total aerobic bacteria and coliform bacteria ($p<0.05$). Groups T1 and T2 displayed higher concentrations of cecal total short chain fatty acids, acetic acid and propionic acid but groups T3 and T4 displayed higher concentrations of butyric acid, isobutyric acid, valeric acid and isovaleric acid ($p<0.05$). The present study reports novel results such that the supply of extreme heat stress diet, inverse lighting (10:00-19:00 dark, 19:00-10:00 light) with cold water at 9ºC under extreme heat stress could enhance growth performance of broiler chickens.

Key words

Broiler chicken, Cold water, Heat stress diet, Inverse lighting, SCFA

Introduction

Heat is one of the most important stress factors for poultry production. The optimal temperature for ideal performance of broiler chickens is between 18-22ºC and the typical body temperature for a mature broiler is 41ºC. It is well known that temperature increase in the surrounding environment induces heat stress, which in turn, increase oxidant stress. These stresses lower the growth rate of broiler chickens and results in body weight loss (Lin *et al*., 2006). Broiler chickens lack sweat glands in their skin, and additionally have a layer of insulating feathers covering them. Broiler chickens show a rapid increase in body temperature as the temperature of surrounding environment rises. When they suffer from extreme heat stress, they reduce their dietary intake and have lower metabolic rates. This results in loss of body weight and quick consumption of fat reserves (Quinteiro-Filho *et al.,* 2010).

Extreme heat adds more stress to broiler chickens by lowering the development of immunocytes, blood immunity substance, beneficial microorganisms and short chain fatty acids within the intestinal tract. The damaging reduction of broilers'

growth resulting from the effects of extreme heat stress can spell economic disaster for poultry farms (Yahav, 2000; Abu-Dieyeh, 2006). Some strategies have been suggested on how to mitigate the effects caused by extreme heat stress. Some emphasized genetic and nutritional aspects of broiler chickens, while some strategies emphasized feeding techniques by controlling lighting and restricting diet (Daghir, 1996). The provision of extreme heat stress diet (EHD) containing proper amount of nutrients such as soybean oil, molasses, methionine, lysine and vitamin C enhances growth of broiler chickens (Lin *et al.*, 2006; Park *et al.,* 2013a).

Light does not control body temperature as body temperature is constant and follows a circadian rhytm in birds. Light cycle strongly influences the physical activities of broiler chickens, thus by controlling the light cycle diet utilization can be improved (Sahraei, 2012). Studies have shown that controlled lighting and light itself are important environmental factors which stimulate growth in broiler chickens (Özkan *et al.,* 2012), and inverse lighting under extreme heat stress can likewise enhance their growth (Park *et al.,* 2013a). Diet restriction during extreme heat period is a method used among poultry farms to reduce the metabolic heat of broilers (Sahraei *et al*., 2012), and it was shown that introducing restrictions of night feeding improved growth (Park *et al*., 2013a). Water is not only one of the most important nutrient for animals but it is physiologically necessary to control body temperature of broiler chickens. Water loss under extreme heat stress prevents birds from properly maintaining their body temperature. The effect of cold water provision on broiler chickens under extreme heat were tested and the temperature of their drinking water has been reported to have a positive influence on their growth (Bruno *et al.,* 2011). However, there is still a lack of understanding concerning how diets for extreme heat stress, inverse lighting and cold water interact together. Previous studies have reported that broiler chickens exposed to extreme heat stress had reduced dietary intake and body weight as compared to broiler chickens provided with continuous lighting for 24 hours under normal environmental conditions. They also showed that daytime starvation, inverse lighting and night diet restriction with common drinking water increased body weight (Park *et al*., 2013a; Yoon *et al.*, 2013).

The present study investigated the effect of the provision of extreme heat stress diet, inverse lighting, cool water on growth performance of broiler chickens exposed to extreme heat stress.

Materials and Methods

Experiment design : Three hundred broiler chickens (Abaica) were obtained immediately after hatching from Hanyang Hatchery (Icheon, Gyeonggido, Republic of Korea). They were randomly assigned in 4 treatment groups of 25 chicks each (25 chicks per 1.65m^2) and was done in three replicates. The four treatment groups had different combination of inverse lighting and EHD using results from the previous studies (Park *et al.*, 2013a; Yoon *et al.*, 2013). T1 (EHD 1, 10:00-19:00 dark, 19:00-10:00 light, cool water 9ºC), T2 (EHD 2, 10:00-19:00 dark, 19:00-10:00 light, cool water 9ºC), T3 (EHD 1, 09:00-18:00 dark, 18:00-09:00 light, cool water 14ºC), T4 (EHD 2, 09:00-18:00 dark, 18:00-09:00 light, cool water 14ºC).

Animal experiment followed the regulations suggested by the European Laboratory Animal Handling License Textbook (Scot, 1994) for the Scientific and Ethical Procedure For Animal Experiment, and received approval from the Ethics Committee of Animal Experiments of Kangwon National University, Republic of Korea. The EHD was made to contain the same amount of crude protein and metabolizable energy (Table 1). EHD 1 was composed of the following ingredients: grain ingredients such yellow corn and soybean meal, soybean oil (5%), molasses (2%), methionine (0.45%), lysine (0.45%), and vitamin C (200 ppm). Beef tallow which is an energy source was included in the EHD, was replaced with molasses and soybean oil which offers higher nutrient utilization, palatability and higher amount of essential fatty acids as well. The amount of yellow corn was reduced in order to make room for additional amount of molasses, methionine, lysine and vitamin C. Rice straw litter was stacked 10 cm high from the floor of each pen within the windowless poultry house. The environmental temperature of poultry house was maintained at 33ºC from the experiments initial day to the 3rd and reduced by 2~3ºC each week afterwards. During the broiler's starter phase (Days 1-21), birds were kept in normal environmental conditions, with drinking water, 24 hr continuous lighting and were allowed to feed freely. During broiler's grower phase (Days 22-32), birds were provided with EHD. 6 days prior of being exposed to extreme heat stress (Days 22-27), the birds were provided with continuous lighting, common drinking water and *ad libitum* feeding for EHD under normal environmental conditions. During last 5 days of experiment (Days 28-32), birds were exposed to extreme heat stress, and provided with EHD only during night hours (18:00-09:00). Extreme heat stress lasted for 5 hrs per day (11:00-16:00) and it was set with heat stress of 33±1ºC and 70% humidity. Lighting was restricted and birds were provided with cold water during exposure to extreme heat. There was no ventilation during this period. After 5 hour period poultry house was ventilated and broilers were provided with their typical living environment. Diet intake and body weight of broiler chickens were recorded every 10 days during the experimental period and their growth performance was measured by increase in weight, diet intake and diet efficiency (body weight increase/diet intake). 12 hrs prior to slaughter, the experimental diets were withdrawn and 15 broilers with average body weight were selected from each treatment groups (5 broilers per pen). Blood samples were collected and birds were euthanasized by cervical dislocation according to recommendation of laboratory animal euthanasia to minimize pain and stress for broilers. Liver, gizzard, thymus, spleen, and bursa F of each bird collected tissue

Table 1 : Composition of experimental diets for broiler chickens

Ingredients (% as-fed)	Diets		
	Starter T1-T4 (1-21 days)	GrowerT1, T3 (22-32days)	Grower T2, T4 (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^{-1}	3,100	3,150	3,150
CP, %	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-α-tocopheryl acetate), 45 mg; vitamin K_3 3.0 mg; thiamin 2.5 mg; riboflavin 5mg; vitamin B_6 5mg; vitamin B_{12} 0.02 mg; biotin, 0.18 mg; niacin, 44 mg; pantothenic acid, 17 mg; folic acid, 1.5 mg

fluid was removed from blood by dipping them in saline solution and drying them with filter paper. The weight of each organ was measured and recorded. Blood was collected from each bird by injecting plain tubes (Greine Co Ltd, Australia) into the heart. The blood sample were centrifuged for 20 min at 3000 rpm, at 4ºC in order to separate serum from the blood. Then blood was quickly frozen using liquid nitrogen at -196ºC and stored at -20ºC for further biochemical analysis.

Serum lipid, glucose and immunity substance : The concentration of blood triglyceride, total cholesterol, LDL-C, HDL-C and glucose were measured using enzyme kit (Sigma, USA). Blood IgG, IgA, IgM were quantified using chicken ELISA kit (Bethyl Laboratories, Montgomery, TX, USA). They were processed according to manufacturer's protocols and used precision microplate reader (Molecular Devices Inc, New York, USA) to measure their absorbance at 450nm in order to calculate the amount of antibodies present in them.

Serum Ccorticosterone : The stress hormone within blood corticosterone was quantified using HS EIA kit (Enzyme immunoassay kit, IDS, Ltd., Boldon, UK) according to the manufacturer's manual.

Short chain fatty acids : Bird's caecum was collected by anaerobic method. Due to lack of caecum content from each bird, caecum samples from five birds from each repeat pen were pooled as one sample for 3 repeat groups. Short chain fatty acid (SCFA) was measured by gas chromatographic system (model GC-15A, Shimadzu Corp., Kyoto, Japan) (Zhang *et al.*, 2003). Five grams of caecal content were put into 20 ml screw cap tubes and mixed with 5 ml of distilled water. After homogenization using ultra turrax, it was centrifuged for 10 min under 4ºC at 10,000 rpm. After centrifugation, 1 ml of the supernatant was transfered to an ample bottle and 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 at 10,000 rpm. On GC Glass column (180cm×4mm, Supelco, Inc., Belleponte, PA) charged with 10% SP-1000/1% H_3PO_4 on flame ionization detector and Chromosorb WAW was attached. High purity N_2 (1.8 ml min^{-1}) carrier gas was applied at 100-150ºC with 33 ml min^{-1} flow rate.

Fecal microorganisms : Feces of 15 birds exposed to extreme heat stress from each treatment group (5 broilers per repeat pen) were sampled. The feces were mixed with phosphorus buffered saline (PBS 0.1 M, pH 7.0), diluted 10X (1:9, wt/vol) and series of

dilutions were continued. Cultivation was carried out using sample diluted 10^{-2}~10^{-7}, and they were replanted individually at100 ul on sterilized plates of selective medium for *Lactobacillus* sp. (MRS agar, Oxoid, Basingstoke, UK), *E. coli* sp. (McConkey purple agar, Difco), Coliform bacteria (Violet red bile agar, Difco), Total aerobic bacteria (Nutrient agar, Difco). Sample was aerobically cultured for 24 hr at 37ºC within *E. coli* sp., Coliform and total aerobic bacteria. Sample was stationary cultured within the *Lactobacillus* sp. medium under anaerobic condition for 48 hrs at 37ºC, using sealed anaerobic jars equipped with Anaero Gen sachets. Number of colonies within samples were quantified. All the numbers of microorganism colonies were recorded in germs per gram (Colony-forming unit, CFU g^{-1} of wet of feces .

Statistical analysis : All the data were subjected to ANOVA using GLM procedure of SAS software. Duncan's multiple range test was to list significance p<0.05 level of significance (SAS, 2004).

Results and Discussion

These results discovered the novel facts that supply of extreme heat stress diet, inverse lighting (10:00-19:00dark, 19:00-10:00 light) and cold water at 9ºC to boiler chickens under extreme heat could improve growth performance by reducing energy consumption and increasing immune competence. T1, and T2 displayed higher body weight increase as compared to T3 and T4 (p<0.05) but there was no statistical significance between T1, T2 and T3, T4. Group T2 increased by 106.40% and 105.42% as compared to T3 and T4 respectively. T1 increased by 104.89% and 103.92% as compared to T3 and T4 respectively (p<0.05). The amount of diet intake ranked in descending order from T1, T2, T4 and T3 but there was no statistical significance between T3 and T4. During the entire experimental period, the amount of diet intake for T3 and T4 decreased by 5.35% and 5.19% as compared to T1; and they decreased by 2.39% and 2.22% respectively as compared to T2 (p<0.05). The diet efficiency of entire periods was highest for T2 (p<0.05), but there was no significant difference among T1, T2 and T4 for grower phase. There was no statistical significance among T1, T2 and T4 or T1, T3 and T4 for the entire experimental period in diet efficiency (Table 2). The weights of liver, gizzard, thymus, spleen and bursa F are shown in Table 3. There was no statistical significance observed among treatment groups for liver and gizzard. The weight of thymus and bursa F were significantly higher for T1 and T2 as compared to T3 and T4 (p<0.05). But there was no statistical significance between T1 and T2 or T3 and T4 (Table 3). The body weight increase among T2 was highest of all, but there were no similarities found in body weight increase for pairs of groups T1 and T2 and T3 and T4.

A previous study showed that EHD 2, which contained vitamin C, caused higher body weight increase as compared to EHD 1 (Park *et al.*, 2013a). However, in the present study, there were such differences. It was reported that providing common drinking water and EHD 2 along with inverse lighting (10:00-19:00 dark, 19:00-10:00 light) to fowl exposed extreme heat stress improved growth (Park *et al*., 2013a). T1 and T2 had their lights turned off 1 hour prior being exposed to extreme heat stress and the lights remained off for the next 4 hrs and they were also provided with 9ºC cold water. However, T3 and T4 had their lights turned off 2 hrs prior being exposed to extreme heat and the light remained off for the next 4 hrs and they were also provided with 14ºC cold water. The dark period during daytime and night restricted feeding resulting in reduced activity and metabolic heat generation, which in turn inhibited the increase of body temperature while being exposed to extreme heat stress.

Table 2 : Growth performance of broiler chickens exposed to extreme heat stress

	Groups[1]			
	T1	**T2**	**T3**	**T4**
Days	Body weight gain (g head^{-1} day^{-1})			
0-21	1,070±17.17	1,090±13.21	1,087±15.87	1,078±11.50
22-32	732±12.13^{a}	738±11.67^{a}	631±15.41^{b}	656±17.33^{b}
0-32	1,802±13.07^{a}	1,828±12.12^{a}	1,718±18.29^{b}	1,734±13,88^{b}
	Feed intake (g head^{-1} day^{-1})			
0-21	1,634±8.81	1,637±9.73	1,629±7.32	1,628±8.66
22-32	1,468±5.90^{a}	1,371±8.75^{b}	1,307±10.18^{c}	1,313±7.11^{c}
0-32	3,102±5.92^{a}	3,008±8.44^{b}	2,936±7.03^{c}	2,941±5.24^{c}
	Feed efficiency ratio			
0-21	0.65±0.07	0.67±0.09	0.67±0.03	0.66±0.01
22-32	0.50±0.06^{b}	0.54±0.03^{a}	0.49±0.03^{b}	0.50±0.05^{b}
0-32	0.59±0.03^{ab}	0.61±0.04^{a}	0.58±0.05^{b}	0.59±0.07^{ab}

[1]T1; EHD(extreme heat diet) 1, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T2; EHD 2, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T3; EHD 1, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14!. T4; EHD 2, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. Feed efficiency ratio: body weight gain/feed intake. Different superscripts indicate significant different whereas same superscripts indicate no significant difference (^{a,b,c}p<0.05 among different groups)

Also, providing cold water suppressed body temperature increase and longer dark period (1 hr) allowed sufficient rest. This seemed to have had a significant impact on the growth of birds. Additionally, taking EHD for 6 days prior being exposed to extreme heat stress increased feed intake, nutrient utilization and accumulation of anti-heat stress nutrients, which resulted in reinforcement of heat resistance capability (Park *et al*., 2013a). It was observed that the amount of diet intake was higher for T1 and T2 as compared to T3 and T4. The results agree with the previous study (Park *et al*., 2013a). The body weight increase and diet intake of birds under extreme heat stress is related to body temperature. It was reported that body weight increase and dietary efficiency were lower for birds with body temperature of 32ºC as compared to the birds with body temperature 21ºC (Guo *et al.*, 2003). Soybean oil has lower melting point as compared to beef tallow. Additionally it also contains essential fatty acids and offers high quality energy as well as high energy efficiency. These properties of soybean oil can stimulate diet intake along with molasses (Jacob and Carter, 2008). It is more helpful to increase the intake of essential amino acids such as methionine and lysine rather than increasing intake of protein, because protein generate more metabolic heat when it is disassembled into amino acids (Mendes *et al.,* 1997; Willemsen *et al.,* 2011). It was reported that providing vitamin C to broilers exposed to extreme heat stress would help store energy within the body and maintain a steady metabolic rate which in turn would help to lower blood corticosterone level and improve growth of poultry (Mckee et al., 1997). Vitamin C helps in minimizing heat stress by reinforcing antioxidants, immunity, heart muscle and blood vessels. It also serves as an essential substance for biosynthesis of collagens (Boyera *et al*., 1998). The anti-heat stress nutrients that compose of EHD offer high bioavailability. This stimulates the development of organs within immunity system and this development results in more secretion of IgG, IgA and IgM. It seems that these secretions contribute to lowering the concentration level of corticosterone, which is a stress hormone (Park *et al.,* 2013b). The body weight of T1, T2 was higher compared to T3 and T4, while there was no significant difference between T1 and T2. This is probably somehow related to increased production of acetic acid, propionic acid and total SCFA in caecum. Conclusively, it seems that stimulating the development of *Lactobacillus* (which is good for animal health) and suppressing the development of harmful microorganisms simultaneously helps in maintaining intestinal microbial flora level. Also, the concentration of serum IgG, IgA and IgM has improved immune systems of animals exposed to extreme heat stress and this seems to have improved the overall health of animals suffering with extreme heat stress.

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

Item[2]	Groups[1]			
	T1	T2	T3	T4
Liver	3.19±0.24	3.21±0.11	3.19±0.17	3.20±0.20
Thymus	0.21±0.02[a]	0.20±0.03[a]	0.17±0.03[b]	0.17±0.01[b]
Spleen	0.19±0.02[a]	0.19±0.01[a]	0.17±0.02[b]	0.17±0.02[b]
Bursa of Fabricius	0.20±0.02[a]	0.20±0.02[a]	0.17±0.03[b]	0.18±0.02[b]
Gizzard	1.90±0.13	1.91±0.11	1.88±0.17	1.90±0.15

[1]T1; EHD(extreme heat diet) 1, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T2; EHD 2, 10:00-19:00dark, 19:00-10:00 light with drinking water 9!. T3; EHD 1, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. T4; EHD 2, 09:00-18:00 dark,18:00-09:00 light with drinking water 14ºC. [2]Liver, gizzard is % weight against body weight. Thymus, spleen, bursa of Fabricus is % weight against carcass weight. Different superscripts indicate significant different whereas same superscripts indicate no significant difference ([a,b]$p<0.05$ among different groups)

Table 4 : Serum lipid and glucose levels in broiler chickens exposed to extreme heat stress (mg/dL)

Item	Groups[1]			
	T1	T2	T3	T4
Triglyceride	112.5±3.88[b]	123.8±4.22[a]	96.35±2.17[d]	101.9±2.37[c]
Total cholesterol	121.81±5.07[b]	137.15±5.70[a]	115.81±4.01[c]	107.32±3.85[d]
LDL-C	44.17±3.22[c]	36.56±3.60[d]	72.74±2.98[a]	67.01±2.03[b]
HDL-C	75.68±3.18[b]	85.17±5.35[a]	39.01±5.88[c]	34.77±6.18[c]
Glucose	171.05±5.07[b]	190.13±6.07[a]	146.72±4.87[c]	137.78±2.81[d]

[1]T1; EHD(extreme heat diet) 1, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T2; EHD 2, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T3; EHD 1, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. T4; EHD 2, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. Different superscripts indicate significant different whereas same superscripts indicate no significant difference ([a,b,c,d]$p<0.05$ among different groups)

The triglyceride level were found in the following descending order: T2, T1, T4 and T3. The triglyceride for T3 was lower as compared to T1, T2 and T4 by 14.36%, 22.18% and 8.33%, respectively ($p<0.05$). Total cholesterol concentrations were found in the following descending order: T2, T1, T3 and T4. The total cholesterol for T4 was lower as compared to T1, T2 and T4 by 164.68%, 198.96%, and 108.55% respectively ($p<0.05$). HDL-C concentrations were in the descending order: T2, T1, T3 and T4. HDL-C for T2 was higher as compared to T1, T3 and T4 by 112.54%, 218.33% and 244.95% respectively ($p<0.05$). The blood glucose levels were in the descending order: T2, T1, T3 and T4. The blood glucose of T3 was lower as compared to T1 and T2 by 14.22% and 22.93%. The blood glucose of T4 was also lower as compared to T1, T2 and T3 by 19.45%, 27,53% and 6.09% respectively ($p<0.05$) (Table 4). This study observed that the serum lipid concentrations of T3 and T4 was lowered significantly after being exposed to extreme heat stress. Mumma *et al.,* (2006) claimed that while blood cholesterol, HDL and blood sugar increased due to ACTH, the triglyceride decreased. The results of the present study is partially confirmation.

IgG concentrations were found in the following descending order: T2, T1, T4 and T3. IgG concentration for T2 was higher as compared to T1, T3 and T4 by 115.35%, 154.04% and 134.830% respectively and statistical significance for each treatment group was confirmed ($p<0.05$). IgA levels were found in the following descending order: T1, T2, T3 and T4. IgA of T1 was higher as compared to T2, T3 and T4 by 121.71%, 223.68% and 233.52%, respectively ($p<0.05$). IgM levels were found in the following order: T2>T1>T3 and T4 showing some significance ($p<0.05$) but there was no statistical significance between T1 and T2. IgM level of T1 was higher as compared to T3 and T4 by 241.69% and 305.86%. IgM level of T2 was also higher as compared to T3 and T4 by 244.28% and 309.13% respectively. The corticosterone level was in the following order: T3>T4>T1 and T2 and statistical significance for each treatment group was confirmed ($p<0.05$). Corticosterone level for T2 was lowest and it was lower by 24.54%, 24.26% and 70.90% as compared to T1, T3 and T4 (Table 5). T1 and T2 displayed higher development rate of immunocytes and higher concentration of blood immunity substances, IgG, IgA, IgM compared to T3 and T4. It can be concluded that as compared to T3 and T4 the groups T1 and T2 benefited from lower corticosterone. This was possible due to inverse lighting (10:00-19:00 dark, 19:00-10:00 light) and cold water that was provided to broiler chickens. The provision of inverse lighting and cold water reduced sensitivity of physiological stress resulting from light stimulus and body temperature elevation thus improved immunity of birds. Additionally, the fact

Table 5 : Serum immunoglobulin and corticosterone levels in broiler chickens exposed to extreme heat stress

Item	Groups[1]			
	T1	T2	T3	T4
IgG ($\mu g\ ml^{-1}$)	200.7±3.01[b]	231.5±2.55[a]	150.3±3.71[d]	171.7±2.84[c]
IgA ($\mu g\ ml^{-1}$)	67.44±2.80[a]	55.41±3.17[b]	30.15±3.01[c]	28.88±2.91[d]
IgM ($\mu g\ ml^{-1}$)	97.11±3.32[a]	98.15±4.18[a]	40.18±2.82[b]	31.75±2.77[c]
Corticosterone ($ng\ ml^{-1}$)	44.41±3.94[c]	33.51±4.01[d]	130.17±3.80[a]	115.17±4.21[b]

[1]T1; EHD(extreme heat diet) 1, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T2; EHD 2, 10:00-19:00dark, 19:00-10:00 light with drinking water 9!. T3; EHD 1, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. T4; EHD 2, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. Different superscripts indicate significant different whereas same superscripts indicate no significant difference ([a,b,c,d]$p<0.05$ among different groups)

Table 6 : Changes in the fecal microflora of broiler chickens exposed to extreme heat stress

(log_{10}cfu g^{-1} feces)

Item	Groups[1]			
	T1	T2	T3	T4
Total aerobic bacteria	4.44±0.33[b]	3.97±0.18[c]	6.06±0.27[a]	6.13±0.21[a]
Lactobacillus	8.77±0.11[b]	9.01±0.25[a]	7.12±0.16[c]	7.03±0.10[c]
E. coli	4.01±0.17[b]	3.51±0.22[c]	4.80±0.28[a]	4.77±0.15[a]
Salmonella	3.77±0.11	3.73±0.21	3.80±0.51	3.69±0.13
Coliform bacteria	5.02±0.13[b]	5.10±0.31[b]	5.80±0.18[a]	5.78±0.25[a]

[1]T1; EHD(extreme heat diet) 1, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T2; EHD 2, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T3; EHD 1, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. T4; EHD 2, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. Different superscripts indicate significant different whereas same superscripts indicate no significant difference ([a,b,c]$p<0.05$ among different groups)

that T1 and T2 were provided with inverse lighting for an hour longer than the groups T3 and T4 which helped groups T1 and T2 to recover better from extreme heat stress and they were also provided with sufficient nutrients for cell multiplication of immune system (Singh *et al.*, 2006).

The amount of *Lactobacillus* was, in found the following descending order: T2, T1, T3 and T4 ($p<0.05$) but there was no statistical significance noted between T3 and T4. The amount of *Lactobacillus* for T2 increased by 104.56%, 126.54% and 128.16% as compared to T1, T3 and T4 respectively. The total aerobic bacteria counts were noted in the following descending order: T4, T3, T1 and T2 ($p<0.05$), but there was no statistical significance between T3 and T4. The total aerobic bacteria was lowest in T2 as compared to T1, T3 and T4. It was lower by 10.59%, 34.49% and 35.24%. *E. coli* counts were found in the following order: T3>T4>T1 and T2 ($p<0.05$) but there was no statistical difference noted between T3 and T4. *E. coli* count for T2 was particularly lower than T1, T3 and T4 by 12.47%, 26.88% and 26.42%. The coliform bacteria counts were in the following order: T3>T4>T2 and T4 ($p<0.05$) but there was no statistical difference between T3 and T4. The coliform bacteria of T1 was lower as compared to T2, T3 and T4 by 1.57%, 13.45% and 13.15% (Table 6).

With the exception that propionic acid between T1 and T2 was similar, acetic acid, total SCFAs levels were in the following descending order: T2, T1, T3 and T4 ($p<0.05$). The other SCFAs levels were significant by different among treatment groups ($p<0.05$). Acetic acid of T2 was higher as compared to T1, T3 and T4 by 109.90%, 135.93% and 139.51%. The overall SCFA of T2 was higher as compared to T1, T3 and T4 by 106.59%, 131.32% and 136.61%. Propionic acid for T2 was higher as compared to T3 and T4 by 162.31% and 179.42%. Butyric acid level was, in the descending order: T4, T3, T2 and T1. Butyric acid of T1 was lower by 22.81%, 49,18% and 56.37% as compared to T2, T3 and T4 ($p<0.05$). Isobutyric acid level was in the following order: T1>T3> T2 and T1 ($p<0.05$) but there was no statistical significance noted between T3 and T4. Isobutyric acid level for T1 was lower by 25.35%, 54.99% and 57.55% as compared to T2, T3 and T4. Valeric acid levels was in descending order: T4, T3, T2 and T1 ($p<0.05$), but there was no statistical significance between T3 and T4. Valeric acid level for T1 was lower by 30.21%, 71.22% and 73.57% as compared to T2, T3 and T4. Isovaleric acid levels were in descending order: T3, T4, T1 and T2 but there was no significance between T3 and T4. Isovaleric acid for T2 was lower by 38.28%, 63.39% and 62.76% as compared to T1, T3, and T4 (Table 7). It could be seen that increase *Lactobacillus* for groups T1 and T2 had stimulated immunity of birds and this resulted in an increase of IgG, IgA and IgM. The reason for decrease in IgG, IgA and IgM, which was observed in T3, and T4, was because humoral immunity function was suppressed by extreme heat exposure. *Lactobacillus* sp. and bifidobacteria are widely regarded as beneficial germs; these germs ferment the undigested nutrients which flow in from small intestine. This is known to improve energy provision, lipid metabolism and stimulate immunity as well (Schley and Sodhi, 2002). This study did not investigate the phases of changes that bifidobacteria undergoes. The biological characteristics of IgG, IgA and IgM of the broilers are similar to the biological characteristics of immunity of mammals (Park *et al.,* 2013b). Thymus and spleen are considered as important organs for antibody production in animals and bursa F are especially important for antibody production in birds. These immunity organs are essential for either converting IgM to IgG or vitalizing the effects of IgA (Park, 2011). Therefore, reduction of stress hormone, corticosterone, which results due to increase in concentration levels of IgG, IgA and IgM within blood can also be seen in regression of lymph organs discovered under exposure to extreme heat. The immune organs develops immune system and bursa F is used for advanced studies of development and functions of B lymphocytes (Tizard, 2002).

Table 7 : Concentrations of short chain fatty acid (SCFA) in the cecal contents of broiler chickens exposed to extreme heat stress

(µol g^{-1} of cecum content)

SCFA	Groups[1]			
	T1	T2	T3	T4
Acetic acid	125.3±0.21^{b}	137.7±0.23^{a}	101.3±0.22^{c}	98.7±0.12^{d}
Propionic acid	96.27±0.27^{a}	95.88±0.33^{a}	59.07±0.21^{b}	53.44±0.30^{c}
Butyric acid	8.73±0.23^{d}	11.31±0.21^{c}	17.18±0.32^{b}	20.01±0.21^{a}
Isobutyric acid	3.74±0.10^{c}	5.01±0.22^{b}	8.31±0.10^{a}	8.81±0.33^{a}
Valeric acid	0.97±0.12^{c}	1.39±0.12^{b}	3.37±0.28^{a}	3.67±0.10^{a}
Isovaleric acid	1.75±0.11^{b}	1.08±0.10^{c}	2.95±0.10^{a}	2.90±0.18^{a}
Total SCFA	236.8±0.20^{b}	252.4±0.17^{a}	192.2±0.28^{c}	187.5±0.21^{d}

[1]T1; EHD(extreme heat diet) 1, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T2; EHD 2, 10:00-19:00dark, 19:00-10:00 light with drinking water 9ºC. T3; EHD 1, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. T4; EHD 2, 09:00-18:00 dark, 18:00-09:00 light with drinking water 14ºC. Different superscripts indicate significant different whereas same superscripts indicate no significant difference ($^{a,b,c,d}p<0.05$ among different groups)

Reduction of *E. coli*, coliform, total aerobic bacteria observed in groups T1 and T2 seem to have tendency of improving SCFA and growth rate of broiler chickens. This hypothesis can be tested by comparing the ratios between a control group and experimental group; however there is no defined statistical standard for such comparison. Groups T1 and T2 which were provided with EHD, inverse lighting and cold water benefited from increased amount of SCFAs, such as acetic acid and propionic acid are known to be beneficial to host animals. The increase in *Lactobacillus*, which is known to vitalize animal intestines' functions, seems to be related with the reduction of *E. coli*, coliform and total aerobic bacteria (Devaraj, 2002). *Lactobacillus* secretes bacteriocin which suppresses the development of *E. coli* and other harmful germs. It also generates SCFAs which make the environment within the intestines more favourable for beneficial germs. Therefore, most of the organic acids, lactic acids, acetic acids and propionic acids generated due to fermentation of *Lactobacillus* can suppress colonization of harmful germs within the intestines (Gong *et al*., 2002; Xu *et al.,* 2002). It seems that these mechanisms of *Lactobacillus* are part of the reason for significant reduction of *E. coli*, coliform, total aerobic bacteria within the appendix which was observed in groups T1 and T2. Microorganisms within digestive tract of animals are important because they biosynthesize fermentation products and provide necessary energy for growth of intestinal epithelial cells. They also stimulate the immune system of digestive tract, synthesis of vitamin K and fight against exogenous pathogen colonization (Shakibaie *et al*., 2009). The present study has suggest that the growth performance of broiler chicken can be enhanced by modulating diet and lighting with cold water.

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