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Research Article
Effect of Some Organic Acids on Body Weight, Immunity and Cecal Bacterial Count of Chicken during Heat Stress

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Abstract
Objective: The aim of the experiment was to evaluate the effect of heat stress on body weight, immune response and cecal bacterial count in parent broiler chickens and to determine the ameliorating effects of some organic acids to overcome heat stress. **Materials and Methods:** Day-old, 1920 male broiler parent chicks were used in a 2×2 factorial completely randomized design. The experimental chicks were randomly and equally distributed into 2 major groups (960 chicks each) which further divided into 4 subgroups (480 chicks each) with 8 replicates. Two subgroups were exposed to heat (H) at 35°C from 22-42 days of age and the other two subgroups was lift normal. Four replicates in each subgroup were given organic acids (OA) in drinking water at the first week, days 19-27 and last week of age whereas the other 4 replicates in the same subgroup were given normal drinking water. **Results:** Heat stress caused significant decrease in body weight, coliforms count and serum lysozyme level at 5th week of age. The body weight has been significantly ameliorated by organic acids. Deleterious effects of heat stress on immunity has been confirmed by the significant decrease of bursa/body weight ratio and total leukocyte count (TLC) at 4th week of age. The bacterial count was significantly increased in the group exposed to heat at 5th week of age. **Conclusion:** It is concluded that heat stress has deleterious effects on chickens and organic acids has significantly ameliorated some of these effects.

Key words: Body weight, Broiler parents, cecal bacterial count, heat stress, immunity, lysozyme, organic acids

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

High ambient temperatures can be devastating to commercial broilers; coupled with high humidity they can have an even more harmful effect. Heat stress interferes with the broilers comfort and suppresses productive efficiency. During periods of heat stress the broiler has to make major thermoregulatory adaptations in order to prevent death from heat exhaustion. The result is that the full genetic potential of the broiler is often not achieved[1].

The European Union ban on the prophylactic use of in-feed antibiotics has accelerated the search for alternatives for use within the poultry industry[2]. These alternatives include acidifiers (organic acids), prebiotics, probiotics, enzymes, herbal products, microflora enhancers and immune-stimulants. Most of these alternatives have direct or indirect effects on microflora[3]. Thus, alternatives to antibiotics are of great interest to the poultry industry[4].

Organic acids have strong bacterio-static effects and have been used as Salmonella-control agents in feed and water supplies for poultry[5]. Acidification with various weak organic acids to diets such as formic, fumaric, propionic, lactic and sorbic acid have been reported to decrease colonization of pathogen and production of toxic metabolites, improve digestibility of protein and uptake of Ca, P, Mg and Zn and serve as substrates in the intermediary metabolism[6].

Several studies demonstrated that supplementation of organic acids to broiler diets increased growth performance, enhanced mucosal immunity, reduced diseases and management problems[7-11].

During summer months, the temperature increases to very high degrees which may affect the performance of poultry and need a solution. However, the organic acids might have an ameliorating effects against heat stress. Therefore, the current study was conducted with the following objectives:

- To check if the organic acids have positive effects on the body weight of chickens in heat stress
- To study the effects of organic acids to enhance the immune responses of chickens exposed to heat stress
- To evaluate the impact of organic acids on the cecal bacterial count of chickens exposed to heat stress

MATERIALS AND METHODS

Poultry birds: A total number of 1920 one-day-old male broiler parent chicks were housed from day 1 till day 35 of age at the poultry farm of the Department of Animal Production and Management, College of Agriculture, University of Tripoli. The chickens were provided with feed and water for *ad libitum* consumption.

Organic acids: A commercial mixture of organic acids was used utilizing the following formula: Each 100 mL contains 65 mg citric acid, 57 mg orthophosphoric acid and 13 mg lactic acid.

Experimental design: One-day-old, 1920 male broiler parent chicks were used in a 2×2 factorial completely randomized design. The experimental chicks were randomly and equally distributed into 2 major groups (960 chicks each) which further divided into 4 subgroups (480 chicks each). Each subgroup contained 8 replicates of 60 chicks in each replicate. Two of the 4 subgroups were exposed to heat (H) at 35°C from 22-35 days of age using temperature adjusted heaters while the temperature of the other two subgroups was lift normal. Four replicates in each subgroup were given organic acids (OA) in drinking water at the first week, days 19-27 and continued till the end of the last week of age (5th week) whereas the other 4 replicates in the same subgroup were given normal drinking water. Chickens in all groups were vaccinated via drinking water on days 7, 10 and 14 against Newcastle disease (ND), Infectious bursal disease (IBD) and Infectious bronchitis (IB), respectively. Booster doses were similarly given on days 18 and 21 for IBD and ND, respectively. Blood sampling: Two sets of blood samples were collected on day 28 (4th week) and day 35 (5th weeks) of age from two birds of each replicate for hematology and serology. First set of blood samples from wing veins or heart were collected in heparinized vials (0.2 mL of 1% heparin/5 mL of blood) for leucocytic count. Second set of blood samples were kept in an oblique position at the refrigerator for 15 min then centrifuged at 5000 rpm at cooling centrifuge to separate sera for immunological tests.

Determination of total leukocytic count (TLC): Total leukocytic count were determined with the help of an improved Neubauer counting chamber using method described by Natt and Herrick[12] in which 20 µL of blood was added to 4 mL of Natt and Herrick's solution. The leukocyte stained dark blue. Total leukocyte concentration was obtained by counting all leukocytes in the nine large squares at the ruled area of the counting chamber using the following equation which described by Campbell and Ellis[13]:

$$\text{TLC/mm}^3 = \text{total cells in 9 squares} + 10\% \text{ of total cell} \times 200$$

Determination of differential leucocytic count (DLC): A fresh drop from each blood sample was smeared on clean glass slide and air dried before staining with Wright-Giemsa stain. One hundred white blood cells were counted under oil immersion and results were expressed in percentage.

Assessment of humoral immune response: Assessment of humoral immunity was carried out using haemagglutination and haemagglutination inhibition tests (HA and HI) according to the method described by OIE[14].

Body weight and lymphoid organs weight: Live body weight (LBW) was determined on weekly basis. At 4th and 5th weeks of age, the bursa and spleen weights of two birds from each replicate were determined and the bursa/body weight and spleen/body weight ratios were calculated according to the method of Heckert *et al.*[15] as follows:

$$\text{Bursa/body weight (\%)} = \frac{\text{Bursa weight}}{\text{Body weight}} \times 100$$

$$\text{Spleen/body weight (\%)} = \frac{\text{Spleen weight}}{\text{Body weight}} \times 100$$

Cecal bacterial count: For the intestinal bacterial count, two birds from each replicate were randomly selected and humanely euthanized and dissected. Cecum from each dissected chick was removed using sterile pair of scissors and squeezed down using sterile forceps to collect approximately 1 g of cecal contents into sterile falcon tube.

The cecal bacterial populations were determined at 4th and 5th weeks of age. Approximately 1 g of the cecal contents was mixed with 9 mL of sterile peptone water and homogenized for 3 min. From the initial 10^{-1} dilution, 10-fold serial dilutions were subsequently made in 0.1% peptone for aerobic bacteria. The samples form cecum were diluted to 10^{-5}, 10^{-7} and 10^{-9}. For each dilution 0.1 mL was inoculated in three plates of brain heart infusion for total aerobic bacterial count and MacConkey agar for coliforms bacteria. The total numbers of bacterial colonies were counted at 24 h. The laboratory procedures used to determine the total bacterial count and Coliform counts protocols were adopted from those of Salanitro *et al.*[16], Iannotti *et al.*[17] and Dawsom *et al.*[18].

Serum lysozyme level: Sera collected were tested for lysozyme level using the method of Lie *et al.*[19]. 20 mL of 2% agarose dissolved in phosphate buffer was mixed with 20 mL suspension of 24 h culture of *Micrococcus* lysodeikticus at 67°C. This mixture was poured out in Petri's dish (14 cm diameter). After solidifying at room temperature 32 wells were made (5 mm diameter). Fifty microliters of undiluted sera were poured out in each well. Eight standard dilutions [from 0.025-3.125 (ug mL^{-1})] of lysozyme (Sigma) were used in the same quantity as well. The samples were incubated for 20 h at 37°C and lytic diameters were measured.

Statistical analysis: Data obtained were subjected to statistical analysis with two way ANOVA (univariate analysis) using SPSS 11.5 computer program (SPSS Inc. Chicago, Illion, USA).

RESULTS

The effect of heat and organic acids on body weight, bursa/body weight ratio, spleen/body weight ratio and hemagglutination inhibition (HI) titer at 4th and 5th weeks of age are shown in Table 1. Body weight revealed no significant difference between tested groups at 4th week of age. At the 5th week of age, significant decrease in body weight was observed in the group that exposed to heat as compared to

Table 1: Effect of heat and organic acids on body weight, bursa/body weight ratio, spleen/body weight ratio and Hemagglutination inhibition (HI) titer of broiler parents at 4th and 5th weeks of age (Means±SE)

	Body weight (g)		Bursa/body weight (ratio)		Spleen/body weight (ratio)		HI (antibody titer)	
Variable (n = 16)	4th week	5th week	4th week	5th week	4th week	5th week	4th week	5th week
Temperature								
Normal	1108±27	2602±52	0.214±0.11	0.123±0.019	0.111±0.008	0.079±0.006	3.8±0.5	2.4±0.34
High	1127±27	2454±52*	0.179±0.11*	0.126±0.019	0.092±0.008	0.078±0.006	2.5±0.5	1.7±0.34
p-value	0.614	0.050	0.034	0.907	0.109	0.946	0.105	0.133
Organic acid								
Organic acids	1145±27	2617±52*	0.185±0.11	0.117±0.019	0.102±0.008	0.082±0.006	3.1±0.5	2.1±0.34
No organic acids	1089±27	2439±52	0.208±0.11	0.132±0.019	0.101±0.008	0.076±0.006	3.2±0.5	1.9±0.34
p-value	0.156	0.022	0.144	0.562	0.958	0.0541	0.813	0.610
Temperature×Organic acids (p-value)	0.793	0.393	0.282	0.367	0.873	0.379	0.479	0.610

*Means differ significantly at $p \leq 0.05$

Table 2: Effect of heat and organic acids on hematology of broiler parents at 4th and 5th weeks of age (Means±SE)

	Heterophil (%)		Lymphocyte (%)		H: L		TLC (10^3/mm^3)	
Variable (n = 16)	4th week	5th week	4th week	5th week	4th week	5th week	4th week	5th week
Temperature								
Normal	20±1.7	31±3.3	74.1±1.9	65±3.5	0.291±0.03	0.640±0.15	10.2±0.42	14.16±1.4
High	19±1.7	29±3.3	74.6±1.9	66±3.5	0.259±0.03	0.528±0.15	7.7±0.42**	13.02±1.4
p-value	0.593	0.698	0.856	0.797	0.540	0.614	0.001	0.446
Organic acid								
Organic acids	19±1.7	32±3.3	74.6±1.9	63±3.5	0.261±0.03	0.675±0.15	9.1±0.42	15.47±1.4*
No organic acids	20±1.7	27±3.3	74.0±1.9	66±3.5	0.288±0.03	0.493±0.15	8.6±0.42	11.71±1.4
p-value	0.740	0.306	0.820	0.251	0.605	0.415	0.537	0.017
Temperature×Organic acids (p-value)	0.898	0.061	0.683	0.108	0.782	0.056	0.143	0.124

H:L: Heterophil : lymphocyte ratio, TLC: Total leucocyte count, *Means differ significantly at p≤0.05, **Means differ significantly at p≤0.01

Table 3: Effect of heat and organic acids on total cecal bacterial count, coliforms bacteria and serum lysozyme level of broiler parents at 4th and 5th week of age (Means±SE)

	Total bacterial count		Coliforms bacteria		Serum lysozyme (mg mL^{-1})	
Variable (n = 16)	4th week	5th week	4th week	5th week	4th week	5th week
Temperature						
Normal	163±46	175±44	72±16	201±88	1.63±0.04	2.18±0.03
High	145±46	330±44*	83±16	389±88	1.66±0.04	2.05±0.03**
p-value	0.784	0.021	0.647	0.642	0.628	0.006
Organic acid						
Organic acids	134±46	227±44	70±16	142±88	1.70±0.04	2.14±0.03
No organic acids	175±46	277±44	84±16	447±88	1.60±0.04	2.09±0.03
p-value	0.541	0.440	0.546	0.493	0.107	0.212
Temperature×Organic acids (p-value)	0.105	0.541	0.166	0.024[a]	0.107	0.212

*Means differ significantly at p≤0.05, **Means differ significantly at p≤0.01

normal temperature group and significant increase was observed in the group given organic acids as compared to no organic acids group with no interaction between heat and organic acids.

Bursa/body weight ratio revealed significant decrease in the group which exposed to heat at 4th week of age, while spleen/body weight ratio showed no significant difference. At the 5th week of age Bursa/body weight ratio and spleen/body weight ratio revealed no significant difference between tested groups. Hemagglutination inhibition (HI) titer revealed no significant difference between tested groups at 4thand 5thweek of age.

The effect of heat and organic acids on lymphocyte (%), Heterophiles (%), Total leukocytic count (TLC) and Heterophiles: lymphocyte (ratio) are shown in Table 2. The effect on lymphocyte (%) revealed no significant difference between tested groups at 4th week of age. On 5th week of age, lymphocyte (%) revealed no significant difference. Heterophiles (%) revealed no significant difference between tested group at 4th and 5th week of age. Total leukocytic count (TLC) revealed significant ($p<0.01$) decrease in the group exposed to heat at 4th week of age. On 5th week of age TLC revealed significant increase in organic acid group with no interaction. Heterophiles: lymphocyte (ratio) revealed no significant difference between tested groups at 4th and 5th week of age.

The effect of heat and organic acids on total cecal bacterial count, Coliforms bacteria and Serum lysozyme level at 4th and 5th weeks of age is shown in Table 3. Total cecal bacterial count revealed no significant difference between tested groups at 4th week of age. Total cecal bacterial count revealed significant increase in the group exposed to heat at 5th week of age. Coliforms bacteria revealed no significant difference between tested groups at 4th week of age. Organic acids significantly decreased the number of coliforms bacteria in the cecum at the presence of heat stress (interaction) at 5th week of age. Serum lysozyme level revealed no significant difference between tested groups at 4th week of age. On 5th week of age, serum lysozyme level revealed significant decrease in the group exposed to heat.

DISCUSSION

Heat stress, a potential threat to the fast-growing broiler industry of tropical countries got worse when summer temperature reaches to its extremity since most of the farming is still practiced in conventional manner. High summer temperature and stress can cause under-performance, suppressed immunity and high mortality in meat-type chicken[20]. The negative effects of heat stress on broilers range from reduced growth to decreased poultry quality and safety. However, a major concern should be the negative impact of heat stress on poultry welfare[1]. The supplementation of organic acids in the diet of broilers enhanced nutrient utilization, growth and feed efficiency[21].

Data obtained from this study showed that broiler parents exposed to heat stress (high temperature, 35°C) had significant decrease in body weight (g) at 5th week of age when compared with normal temperature group. Similar results were reported by Lara and Rostagno[1], who stated that broilers maintained in hot environments reduce their feed consumption. This is a part of their physiological adaptation to heat stress. The reduction in feed intake results in a decrease in the daily intake of nutrients responsible for growth.

Our results revealed a significant increase in body weight (g) in broiler parents supplemented with organic acids in drinking water when compared with group given normal drinking water (control group) at 5th week of age. These results are in agreement with Luckstadt and Mellor[22] who stated that the use of organic acids has great role in enhancement of nutrient utilization and growth and feed conversion efficiency and also agree with Owens *et al*.[23] who reported that the supplementation of organic acids in the diet of broiler chicken improved the body weight gain when compared with the un-supplemented group. The improved body weight gain is probably due to the beneficial effect of organic acids on the gut flora. These results disagree with Pirgozliev *et al*.[24] who declared that dietary organic acids did not significantly affect weight gain or feed efficiency.

Regarding bursa/body weight ratio, the results revealed significant decrease between broiler parents exposed to high temperature and normal temperature at 4th week of age. These were in accordance with Bartlett and Smith[25] who observed that broilers subjected to heat stress significantly reduced thymus, bursa, spleen and liver weights. Moreover, Niu *et al*.[26] reported reduced lymphoid organ weights in broilers under heat stress conditions. In the current study, broiler parents exposed to high temperature (heat stress) showed slight reduction in spleen/body weight ratio when compared with birds kept under normal conditions at 4th and 5th weeks of age. Similar results were obtained by Bartlett and Smith[25]. Ghazi *et al*.[27] investigated the effects of heat stress on the immune response in poultry. In general, all studies show an immune-suppressing effect of heat stress on broilers and laying hens. For instance, lower relative weights of thymus and spleen has been found in laying hens subjected to heat stress. The humeral immune response of heat-stressed broiler parents tested by hemagglutination inhibition (HI) titer showed lower titer as compared to birds reared under optimal conditions at 4th and 5th weeks of age. These results are similar to those reported by Bartlett and Smith[25] who observed that broilers subjected to heat stress had lower levels of total circulating antibodies, as well as lower specific IgM and IgG levels, both during primary and secondary humoral responses. Ghazi *et al*.[27] stated that heat stress had an immune-suppressing effect on broilers and laying hens. Bozkurt *et al*.[28] observed reduction in systemic humoral immune response in laying hens fed diets supplemented with mannan-oligosaccharide or an essential oil mixture under hot environmental conditions. Concerning the effect of organic acids supplementation on humoral immune response, the results showed that hemagglutination inhibition (HI) titer was higher in broiler parents supplemented with organic acids in drinking water when compared with group given normal drinking water at 5th week of age. Haque *et al*.[29] have indicated that citric acid supplementation (0.5%) enhanced the non-speci c immunity. Ghazala *et al*.[30] reported that birds fed on organic-acid-supplemented diet had heavier immune organs (bursa of Fabricius and thymus) and also a higher level of globulin in their serum. Concentration of globulin is used as an indicator for measuring immune response. They also suggested that the improvement in bird immunity could be related to the inhibitory effects of organic acids on gut system pathogens. Houshmand *et al*.[31] found that at 21 days of age of the broiler, dietary addition of organic acids resulted in significant increases in antibody titers against Newcastle disease. Similarly, antibody titers against Newcastle disease in laying hens also increased by increasing the levels of formic acid.

The effect of heat stress on hematology of broiler parents is also studied. The results revealed that broilers exposed to high temperature showed slightly high lymphocyte with decreased heterophils and heterophil: lymphocyte ratio. These results are not in harmony with Aengwanich[32] who demonstrated that broilers subjected to heat stress revealed decreased numbers of lymphocytes in the cortex and medulla areas of the bursa. However, recent studies by Prieto and Campo[33] and Felver-Gant *et al*.[34] demonstrated that heat stress can alter levels of circulating cells. It has been shown that heat stress causes an increase in heterophil : lymphocyte ratio, due to reduced numbers of circulating lymphocytes and higher numbers of heterophils. The effect of high temperature on total leukocytic count (TLC) showed a significant decrease in broiler parents reared under heat stress when compared with those maintained under optimal condition at 4th week of age. These findings were in accordance with Imtiaz *et al*.[35] who observed that total leukocyte count (TLC) was significantly reduced in group exposed to heat stress. Lokande *et al*.[36] reported that heat stress reduces significantly TLC in broilers. The effect of organic acids supplementation on total leukocytic count (TLC) revealed a significant increase in number of white blood cells in broiler parents supplemented with organic acids in drinking water when compared with un supplemented group. These results were in agreement with Al-Saad *et al*.[37] who observed a significant increase in number of White Blood Cells (WBC) in blood samples of probiotic and organic acids groups compared to antibiotic group.

In current study, broiler parents exposed to high temperature showed a significant increase in total ceacal bacterial count when compared with birds kept under normal temperature at 5th week of age. These results were in conformity with Park *et al*.[38] who verified that extreme heat stress is not beneficial to broiler's cecum microflora or it stimulate proliferation of harmful pathogens *Escherichia coli*, *Salmonella* and total aerobic bacteria. Also, Abdel-Mohsein *et al*.[39] reported that total aerobic count in fresh fecal samples of broiler chickens subjected to chronic heat stress are significantly higher in count. The effect of organic acids on broiler parents revealed a reduction in both total bacterial count and coliforms bacteria in group supplemented with organic acids when compared with un-supplemented group. These results are in accordance with Adil *et al*.[40] who confirmed that the addition of organic acids to the diets of broiler chicken significantly decrease the cecal viable and coliform counts compared to the un-supplemented group. In this study, broiler parents kept under high temperatures (heat stress) showed high count of coliforms bacteria when compared with those reared under normal temperature at 4th and 5th weeks of age. Organic acids were significantly decreased the number of coliforms bacteria in the cecum at the presence of heat stress (interaction) at 5th week of age. Ali *et al*.[41] stated that inclusion of organic acids into chicken diets altered the populations of intestinal microflora and reduced the total number of coliforms bacteria and microbial total counts.

Serum lysozyme level revealed a significant decrease in broiler parents reared under hot environment (heat stress) when compared with broiler parents kept under normal temperature. Several studies[25-27] have investigated the effects of heat stress on the immune response in recent years. In general, all studies showed an immune-suppressing effect of heat stress on broilers and laying hens, although using different measurements.

CONCLUSION

Heat stress has adverse effect on broiler parents' body weight, immune response and intestinal microbiology. Supplementation of organic acids in drinking water improves broiler parents' body weight, enhances immune response and decreased the effect of heat stress. It is highly recommended that poultry producers should take good care to avoid the negative impacts of heat stress on their poultry populations especially during hot seasons through adding anti-heat stress feed and water additives such as organic acids.

SIGNIFICANCE STATEMENT

This study discovers the beneficial effects of organic acids to ameliorate the deleterious effects of heat stress in broiler parents. This study will help the producers to overcome the heat stress and will help the researchers to uncover critical areas of the effect of heat stress on immune response that many researchers were not able to explore. Thus a new theory on these issues maybe arrived.

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