The Journal of Agricultural Sciences - Sri Lanka
Vol. 19, No 3, September 2024. Pp 517-529
http://doi.org/10.4038/jas.v19i3.10245

Thermotolerance in Broiler Chickens: Evaluating the Influence of Embryonic Thermal Manipulation and *in-ovo* Feeding of Lysine and Cysteine in a Hot Tropical Environment

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Received: 27th April 2023 / Accepted: 04th August 2024

ABSTRACT

Purpose: *The physiological mechanism of action of low or high combinations of lysine and cysteine in ovo feeding combinations needs to be elucidated. This study aimed to determine the influence of high or low combinations of lysine and cysteine on the growth, thermotolerance, haematochemical, and antioxidant parameters of heat-stressed broiler embryos.*

Research Method: *A total of 2000 broiler hatching eggs were exposed to a relative humidity of 55% and temperature of 39.0ºC for 6 hours daily from day 10 until day 18. On day 18, 1500 eggs having living embryos were taken at random into 6 treatments containing 250 eggs per treatment with 5 replicates of 50 eggs viz: eggs without in-ovo injection (UIJ), eggs treated with in-ovo injection of 0.5mL distilled water (DWT), 2mg lysine (LYS), 3.4mg cysteine (CYS), 3.4mg cysteine+2mg lysine (CLHD) or 1.7mg cysteine+1mg lysine (CLLD).*

Findings: *The blood total protein of CLLD birds was higher (P<0.05) compared to the others at the starter phase. The ALT of CLHD and CLLD birds were lower (P<0.05) than other treatments at the finisher phase. The malondialdehyde of DWT and UIJ broilers was higher than the other treatments at the starter and finisher phases.*

Research Limitation: *This study was carried out in a hot season. More studies are needed under different seasons of the year using in ovo amino acids.*

Originality/Value: *It can be concluded that in-ovo injection of lysine-cysteine positively altered the growth rate, and biochemical and antioxidant indices of broilers. The low combination of the amino acids had a more sustained effect at the market age.*

Keywords: *Amino acids, Antioxidant, Growth, Physiology, Post-hatch, Broiler chicken, in ovo feeding.*

INTRODUCTION

There are persistently high temperatures and relative humidity in the tropical environments, and these harsh conditions prevent broiler chickens raised in these regions from performing optimally due to heat stress (Oke, 2018; Kpomasse *et al.*, 2021; Oke *et al.*, 2021a; Oke *et al.*, 2022; Kpomasse *et al.*, 2023). Production of chicks that can withstand various environmental conditions, including thermal challenges, is an indicator of the poultry industry's success. The fast-embryonic development in chickens is linked with excessive free radical production, as a result of oxidative stress (Deeming and Pike, 2013). Therefore, to achieve the full genetic potential of the growth performance, it is vital to optimize embryonic development in chickens. This is necessary because the maternal nutrient reserve of the developing embryos is limited and can be exhausted during hatching.

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Perinatal feeding is important for the development of embryos and can determine the vitality and overall health condition of chickens during post-hatch growth (Geng *et al.*, 2022).

Studies have shown that the chicken embryo's physiological state can be enhanced by *in ovo* injection (Kadam *et al*., 2013; Oke *et al*., 2021b; Oyelola *et al*., 2024). This technique enhances the direct delivery of substances to the embryos before hatching (Kadam *et al*., 2013). *In ovo* feeding additives, including trace elements, sugars, proteins, different amino acids, vitamins, fatty acids, and medications like stimulants and hormones, which are advantageous to bird's embryonic development or post-hatch performance, have been documented (Ohta and Kid, 2001; McGruder *et al*., 2011; Bakyaraj *et al*., 2012; Dong *et al*., 2013; Bilalissi *et al*., 2019).

Amino acids have exceptional antioxidant capability and preserve the equilibrium of free radicals (Bin *et al*., 2017). Soluble amino acids can reduce cell damage occasioned by oxidative stress and eliminate free radicals (Kim *et al*., 2020). Based on the type of reaction formed, sulfur-containing amino acids like cysteine have different ways of eliminating reactive oxygen species (Parcell *et al*., 2002). Various studies (Hansen and Lewis, 1993; Tuitoek *et al*., 1997) have shown that balancing amino acids is crucial in the diets of animals. Lysine plays a major role in the synthesis of protein through the physiological metabolism of the body (Liu *et al*., 2012; Kheiri *et al*., 2017). L-lysine is also a precursor to L-carnitine and other numerous useful compounds (Hullár *et al*., 2008). *In ovo* injection of lysine (separate use) enhanced blood profile, gastrointestinal health, and muscle weight (Ebrahimi *et al*., 2017).

Cysteine functions as a semi-essential amino acid as it can be made from serine and methionine through trans-sulfuration (Rehman *et al*., 2020). Feeding of amino acids can influence the post-hatch growth of chickens (Fisher *et al*., 1998; Seifdavati *et al*., 2008). Our earlier study (Ajayi *et al*., 2022) showed that the use of cysteine alone improved hatching. However, the physiological mechanism of action of the low or high combinations of lysine and cysteine is not yet elucidated. Moreover, there is limited data on the influence of the *in ovo* injection of these amino acids on the growth rate of broilers. Therefore, the present study evaluated the influence of *in ovo* injection of high or low mixtures of cysteine and lysine on the growth rate and physiological responses of broilers at the post-hatch growth phase.

MATERIALS AND METHODS

Experimental Procedure

Eggs collection, incubation, and candling : Two thousand hatching eggs of Ross 308 broilers were purchased from a reputable farm and were incubated under normal incubation conditions from the first day to the 10^{th} day of incubation. Then, the eggs were exposed to an increase in temperature of about 39.6ºC for a total of 6 hours per day, from embryonic day 10 to 17.5. Candling was done on day 17.5 to check for evidence of a living embryo. Eggs with dead embryos were removed from the incubator.

*In Ovo Injection:*On day 18, a total of 1500 eggs were allocated to six treatments, each with five replicates (50 eggs per replicate): eggs without *in ovo* injection (UIJ), eggs treated with *in ovo* injection of 0.5 mL distilled water (DWT), 2 mg/egg lysine (LYS), 3.4 mg cysteine (CYS), 3.4 mg cysteine + 2 mg lysine (CLHD), or 1.7 mg cysteine + 1 mg lysine (CLLD). The solution was prepared as described by Elnesr *et al*. (2019). A 0.5 mL portion of the prepared solution was injected into a hole drilled above the air chamber of the eggs, and the holes were sealed with wax after the procedure. The eggs were then transferred into hatching baskets labelled according to the treatment. The hatcher's relative humidity and temperature settings were 36.7ºC and 65%, respectively. *Post-Hatch Phase:* At the end of the hatch, the chicks were reared from day old according to the six in-ovo treatments, with 200 chicks per treatment and 5 replicates of 40 chicks each. The birds were raised for eight weeks after hatch and were fed *ad libitum* in an open-sided poultry house. A commercially available starter feed (from hatch to 28 days) and finisher feed (from 29 to 56 days) (New Hope Agriculture and Technology, Nigeria Limited) were provided to the chickens. All management conditions were practised according to the Ross manual.

Data Collection

Meteorological observations: The ambient relative humidity and temperature of the environment were monitored during the experiment in the morning (7:00 am), afternoon (1:00 pm), and night (1:00 am) using a thermo-hygrometer. The data obtained are shown in Table 1.

Growth performance: The weights of the birds were determined weekly on a replicate basis during the experiment for eight weeks, using a weighing scale.

Heamatological parameters of broiler birds: Samples of blood were collected from four birds selected at random from each replicate at the starter and

Table 1: Meteorological observations during the experimental period

Morning			Afternoon		Night	
Week	Temperature	Relative humidity	Temperature	Relative humidity	Temperature	Relative humidity
2	28.0	84.40	33.0	60.00	28.50	76.80
3	25.8	80.00	33.8	48.80	27.80	75.40
4	26.6	83.00	33.0	64.60	28.50	81.60
5	28.0	79.40	33.2	63.00	27.40	80.80
6	27.4	83.00	34.0	69.00	28.20	79.75
7	27.8	76.50	32.2	64.75	28.20	83.25
8	27.0	70.00	34.0	50.00	27.00	79.33

finisher phase via the brachial veins into sterile bottles containing anticoagulant and then gently mixed to prevent blood clotting. Hematological tests, which included packed cell volume (PCV), red blood cell (RBC), white blood cell (WBC), hemoglobin, lymphocyte, heterophil, eosinophil, basophil, and monocyte, were done using an automated hematological analyzer to determine the effect of phytochemicals on these parameters. PCV was determined using microhematocrit. A capillary tube was 3/4 filled with well-mixed blood and sealed at one end with heat sealing or a clay pad. The tube was spun in a high-speed micro hematocrit centrifuge for 6 minutes, and the PCV was read using a microhematocrit reader with a sliding cursor. The plasma's appearance (such as normal, icteric, hemolyzed, or lipemic) and buffy coat thickness, which provides a very approximate estimate of total WBC, were both recorded.

Serum chemistry of broiler birds: The total protein, creatinine, alanine transferase, albumin, globulin, glucose, and triglyceride were evaluated at the starter and finisher phases of this experiment. Blood samples were collected into plain bottles having no anticoagulant and were centrifuged at 750g for 15 minutes and stored in a freezer at -20°C until use.

*Superoxide dismutase, malondialdehyde, and catalase:*The blood malondialdehyde concentrations, superoxide dismutase, and catalase activity were measured as described by Zhang *et al*. (2020).

Physiological Response of Broiler Birds: Data were collected twice per week from week 1 to week 8 between 13:00 and 14:00 hours. The number of breaths per minute, or respiratory rate (RR), of the birds was measured. The measurements of RR were obtained for 15 seconds, and the value per minute was calculated by multiplying the result by 4. A digital thermometer with 0.1°C precision was gently inserted into each bird's rectum (colon) until it beeped to determine the rectal temperature (RT) of the birds.

Carcass Evaluation: Two birds per replicate (eight per treatment) were randomly selected on day 56 to evaluate carcass and organ relative weights. Selected birds were deprived of feed overnight. The birds were processed, and the weights of the cut parts, which included breast meat, drumstick, shank, thigh, wing, neck, head, and intestinal organs (heart, liver, gizzard, kidney, lung, pancreas, proventriculus, and lymphoid organs (bursa of Fabricius and spleen)), were recorded and expressed as relative body weights.

Statistical Analysis: Using SAS (2008) in a Completely Randomized Design, an analysis of variance (ANOVA) was conducted on the data collected. The differences among the means were compared using Tukey's HSD test at $P < 0.05$.

RESULTS AND DISCUSSION

Growth Rate

The LYS chick weights were higher compared to UIJ and DWT treatments on day 1 (post-hatch) (Figure 1). On day 7, the chick weights of CYS were higher than the chicks in the other treatments. The weights of LYS chicks were lower than CYS but higher than the weights of the other chicks in the other treatment groups. On day 14, CLLD and CLHD chicks were higher compared to the control birds. On day 21, CLLD chicks were comparable to those of LYS and higher than the other treatments. On day 28, the weights of the birds in the control groups were lower than *in ovo*-treated birds. There was a similarity in the weights of CLLD chicks and LYS whose weights were similar to the other treatments. The weights of CLLD chickens were significantly higher than the other birds on day 47. A similar trend was recorded in week 7. UIJ, DWT, and CLH chickens were comparable but lower than LYS and CYS birds at day 56. CLLD was birds were similar to LYS in their weights.

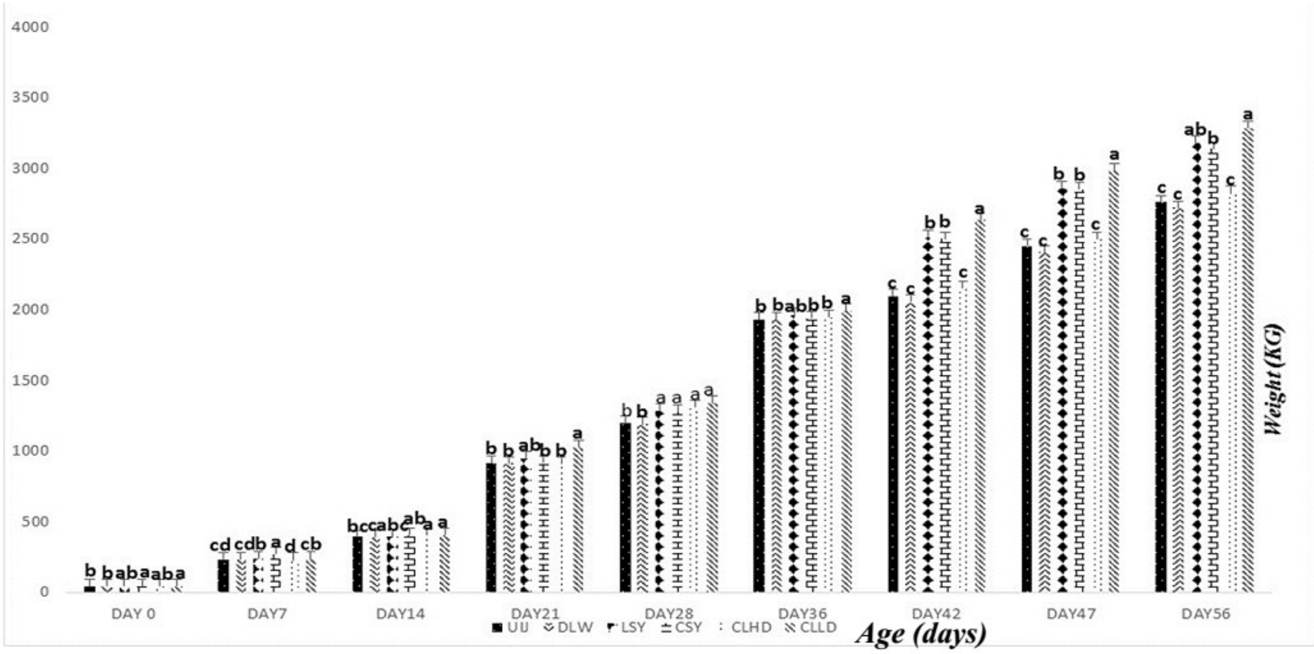

Figure 1: Post-hatch growth rate of broiler birds treated with *in ovo* lysine and cysteine from day 1 to 56
Eggs without an ovo injection (UIJ), eggs treated with an ovo injection of 0.5mL distilled water (DWT), 2mg/egg lysine (LYS), 3.4mg cysteine(CYS), 3.4mg cysteine+2mg lysine (CLHD) or 1.7mg cysteine+1mg lysine(CLLD).

The meteorological data recorded in this study showed that the birds were stressed as the temperature ranged between 25.8 and 35.0°C and the relative humidity ranged between 48.80 and 84.40%. These were above the thermoneutral zone for broiler birds (Dei and Bumbie, 2011; Oke *et al.* 2020; Tona *et al.*, 2022). Although there is a paucity of information on the optimal dose of an ovo combination of lysine and cysteine, it is interesting to note that the chicks treated with the lower combination of cysteine and lysine (CLLD) had a catch-up growth from post-hatch day 14 onward in the present study. This suggests that the growth-promoting effect of the treatment became more pronounced on the birds than those of other treatment groups at this age. Moreover, the influence of in-ovo feeding of cysteine alone on the birds was not consistent from day 21 and waned at post-hatch day 36. The general lower weights recorded in the birds of in-ovo feeding of a higher combination of lysine plus cysteine indicates that the dose was not optimal for the birds. The improved growth of the birds *in ovo*-treated eggs in our trial agrees with the findings in an earlier study (Gaafar *et al.*, 2013), which indicated that a mixture of amino acids could enhance the growth performance of birds. *In ovo* amino acids have been used to enhance the growth of broilers (Ohta *et al.*., 1999; Bhanja and Mandal, 2005; Kadam *et al.*, 2008; Wang *et al.* 2020). The present study reveals the post-hatch growth rate of broiler birds influenced by *in ovo* combinations of cysteine and lysine. The growth pattern from weeks 1 to 8 reflects the effective utilization of the substance injected into the eggs. Different studies have documented the adverse effect of thermal stress on the growth of broiler birds (Geraert *et al.*, 1996; Habibian *et al.*, 2014; Lara and Rostagno, 2013). Beyond hatching, our result on the growth rate of the birds indicates that *in ovo* cysteine and lysine ameliorated the effect of harsh environmental temperature, which characterizes the tropical environment (Meteyake *et al.*, 2020).

Haematochemical Parameters

At the starter and finisher phases (Tables 2 and 3), there was no significant treatment effect (P>0.05) on most of the biochemical parameters. However, total protein, globulin, and cholesterol levels of blood showed a significant effect (P<0.05) on *in ovo* injection of cysteine and lysine of the thermally challenged embryo during incubation. The total protein and globulin of CLLD (10.7 mg/dL) were higher compared to the others (CLHD: 8.33 mg/dL; CYS: 8 mg/dL; LYS: 8.13 mg/dL; DWT: 8.63 mg/dL; UIJ: 8.66 mg/dL). However, at the finisher phase, lower values of ALT were observed in CLHD and CLLD birds (Table 3).

The result of haematochemical parameters can reflect an animal's physiological and nutritional efficiency (Toghyani *et al.*, 2010) and can be used as an indication of the impact of a nutritional factor on the animal's diet (Khan and Zafar, 2005). It has been documented that total protein and globulin levels are the main protein transporters in avian species (Laurie *et al.*, 2003). The lower dose combination of *in ovo* injection of cysteine and lysine showed higher total protein values of blood at the starter phase. The observed increase in the blood total protein suggests that the

Table 2: Effect of *in-ovo* feeding of lysine and cysteine on serum biochemical parameters of broilers at the starter phase (day 21)

Parameters	UIJ	DWT	LYS	CYS	CLHD	CLLD	SEM	P value
Total protein*	8.66[b]	8.63[b]	8.13[b]	8.00[b]	8.33[b]	10.7[a]	0.16	0.0009
Creatinine*	0.80	0.83	0.67	0.76	0.56	0.87	0.55	0.184
ALT (U/l)	43.33	40.33	40.33	38.33	38.33	45.00	2.34	0.170
Albumin*	3.66	3.20	3.20	3.33	3.16	3.80	0.66	0.055
Globulin*	5.00[b]	5.43[ab]	4.99[b]	4.66[b]	5.16[b]	6.90[a]	0.18	0.008
ALB: GLB	0.73	0.59	0.68	0.72	0.61	0.55	0.68	0.218
Glucose*	351.33	350.67	344.67	300.33	351.33	324.00	23.65	0.078
Cholesterol*	198.00[a]	216.00[a]	206.67[ab]	212.66[a]	202.23[ab]	187.67[b]	15.80	0.004
Triglyceride*	96.01	95.67	97.00	96.00	95.34	96.33	8.98	0.997

*Eggs without an ovo injection (UIJ), eggs treated with an ovo injection of 0.5mL distilled water (DWT), 2mg/egg lysine(LYS), 3.4mg cysteine(CYS), 3.4mg cysteine+2mg lysine (CLHD) or 1.7mg cysteine+1mg lysine(CLLD), ALT, aminotransferase. AST, aspartate aminotransferase ALB: GLB, albumin globulin ratio, *mg/dl, [abc] Means within the row with different superscripts differ significantly at P<0.05.*

in ovo injection of the blend of lysine and cysteine was beneficial to support the normal protein level in the blood at this phase. The positive influence of *in ovo* lysine and cysteine on plasma total protein and globulin indicates the beneficial impact of *in ovo* feeding of amino acids on liver function by increasing protein synthesis. The results of the current study are in contrast to the findings obtained by Elwan *et al.* (2019), who reported that *in ovo* methionine-cysteine mixture did not significantly influence serum globulin and total protein in broiler birds. Frdoust *et al.* (2019) also observed that L-methionine *in ovo* injection did not influence the serum protein level in broiler birds.

In this study, the CLLD birds had lower cholesterol concentrations compared to control groups, which might be a result of a sufficient amount of L-carnitine, a plasma lipid-lowering enzyme synthesized from lysine and methionine that decreases cholesterol and plays a role in carbohydrate metabolism (Tanaka *et al.*, 2004). The cholesterol values are, however, generally within the normal range (Sturkie *et al.*, 2000). There is a scarcity of data on the influence of *in ovo* lysine or cysteine on the blood cholesterol of broilers. However, the findings of the present study are consistent with the report of Parsaeimehr *et al.* (2014), who indicated that dietary L-carnitine reduced cholesterol levels in broilers' blood. The positive synergetic effect of the lower dose mixture of lysine and cysteine observed in the serum biochemical parameter suggests that the birds were not adversely influenced by *in ovo* injection of cysteine and lysine. Similarly, Elnesr *et al.* (2019) reported that *in ovo* injection of methionine and cysteine significantly lowered the total cholesterol concentration in broiler chickens. The results of Wandita *et al.* (2018) also showed that dietary supplementation of purified amino acid influenced the total cholesterol concentration of broiler chickens. Additionally, Adejumo *et al.* (2021) reported low cholesterol levels when sulfur-containing amino acids were supplemented in the diets of broiler chickens.

Serum activity of ALT is commonly used as a biomarker for hepatocellular conditions (Hoffmann and Solter, 2008). The lower activities of ALT in CLLD birds may suggest that the higher mixture of the amino acids did not positively influence the liver. This result is at variance with that of Sogunle *et al.* (2019), who observed that the effect of *in ovo* amino acid did not influence the blood profile of FUNAAB alpha chicken. Packed cell volume (PCV) value measures the percentage composition of blood cells in the animal body and other content such as plasma and effective nutrient utilization of feed components in the animal body (Melillo *et al.*, 2007). The present study showed that the values obtained for PCV were in the normal range of 27–33% for healthy broiler chickens (Nse *et al.*, 2014). Malfunctioning of the circulatory system is often detected when the RBC fails to efficiently carry oxygen to tissues of the body for proper cardiovascular function (Talebi *et al.*, 2005). According to Nse *et al.* (2014), a lower value of RBC and hemoglobin (HB) indicates undernutrition, especially dietary vitamins and amino acids. Hemoglobin is responsible for cellular respiration, and a decrease in hemoglobin is an essential biomarker of anemia (Clancy and McVicar, 2007). The HB values of the birds in this study were in the normal range (7–13 g/dL) (Bounous and Stedman, 2000). Physiological indications of stress in poultry include changes in the number of circulating leucocytes, most especially pronounced in heterophilia and lymphocytopenia, which are reliable indicators of stress (Campo *et al.*, 2008). The lower H:L ratio of *in*

Table 3: Effect of *in-ovo* feeding of lysine and cysteine on serum biochemical parameters of broilers at the finisher phase (day 56)

Parameters	UIJ	DWT	LYS	CSY	CLHD	CLLD	SEM	P value
Total protein*	7.47	8.63	8.63	8.50	7.53	7.80	0.33	0.018
Creatinine*	0.63	0.70	0.70	0.70	0.70	0.63	0.78	0.891
ALT#	41.00^{ab}	42.33^{ab}	42.67^{ab}	45.00^{a}	38.33^{bc}	34.33^{c}	2.90	0.002
AST#	189.67	207.00	201.33	211.67	194.67	198.00	25.84	0.158
Albumin*	2.73	3.33	3.00	3.16	2.83	2.43	1.98	0.207
Globulin*	4.73	5.30	5.63	5.33	4.70	5.36	1.22	0.288
ALB: GLB	0.60	0.63	0.54	0.59	0.60	0.45	0.89	0.630
Glucose*	295.00	307.00	331.00	312.33	306.33	292.00	29.44	0.834
Cholesterol*	187.00	192.67	204.00	211.67	205.00	184.67	17.04	0.282
Triglyceride*	89.00	97.00	97.33	88.33	86.33	91.00	66.99	0.286

*Eggs without in ovo injection (UIJ), eggs treated with in ovo injection of 0.5 mL distilled water (DWT), 2 mg/egg lysine (LYS), 3.4 mg cysteine (CYS), 3.4 mg cysteine + 2 mg lysine (CLHD), or 1.7 mg cysteine + 1 mg lysine (CLLD), ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB:GLB, albumin:globulin ratio; *mg/dL, #U/L, ^{ABC} Means within the row with different superscripts differ significantly P<0.05.*

Table 4: Effect of *in-ovo* injection of lysine and cysteine on hematological parameters of broilers at the starter phase

Parameters	UIJ	DWT	LYS	CSY	CLHD	CLLD	SEM	P value
PCV (%)	32.33^{ab}	35.00^{a}	34.00^{a}	30.67^{ab}	28.67b	32.67^{ab}	0.62	0.014
HB (g/dl)	10.53^{ab}	10.73^{a}	10.73a	9.66^{ab}	8.55b	8.80^{ab}	0.26	0.010
RBC (x103/µl)	3.27^{a}	3.32^{a}	3.36^{a}	3.24^{a}	2.40^{b}	3.18^{a}	0.08	0.0003
WBC (x106/L)	16.33	16.41	16.30	16.57	15.800	16.27	0.09	0.305
Platelet (x103/µl)	13.73	15.20	14.36	13.43	13.07	13.50	0.29	0.362
Lymphocyte (%)	55.67b	64.67^{ab}	65.00^{ab}	66.00^{a}	62.00^{ab}	67.33^{b}	1.152	0.016
Heterophil (%)	36.00^{a}	31.33^{ab}	27.33^{b}	22.67^{b}	29.00b	26.66^{ab}	0.905	0.001
H: L	0.64^{a}	0.48^{b}	0.42^{b}	0.40^{b}	0.47^{b}	0.39^{b}	0.400	0.019
Monocyte (%)	3.33	3.67	2.67	2.67	2.67	4.00	0.185	0.107
Eosinophil (%)	4.67^{a}	3.33^{bc}	4.00^{abc}	3.67^{abc}	4.33^{bc}	3.00^{c}	0.185	0.009
Basophil (%)	0.67	0.33	0.67	0.33	0.00	0.00	0.114	0.366

PCV: packed cell volume. HB: hemoglobin RBC: red blood cell WBC: white blood cell H:L: *heterophil/lymphocyte ratio. abc: different superscripts along the row differ significantly P<0.05, eggs without in ovo injection (UIJ), eggs treated with in ovo injection of 0.5mL distilled water (DWT), 2mg/egg lysine(LYS), 3.4mg cysteine(CYS), 3.4mg cysteine+2mg lysine (CLHD) or 1.7mg cysteine+1mg lysine(CLLD).*

ovo cysteine and lysine injected birds, as compared to the control, indicates the birds were not facing stress. Information is scarce on *in ovo* sulfur-containing amino acid on hematological parameters. However, the result of the present study corresponds to that of Tombarkiewicz *et al.* (2020), which indicated that the effect of folic acid and methionine *in ovo* did not influence the hematology of birds.

Table 4 reveals the influence of *in-ovo* lysine and cysteine on the hematological parameters of broilers. The PCV, RBC, HB, and lymphocytes of DW and LYS were not significantly different from other treatment groups except for CLHD birds which had a lower value. However, the heterophil of CLLD was significantly higher than the other treatment group. UIJ had the lowest value for eosinophil in the treatment group. The H:L ratio of LYS, CYS, and CLLD birds was similar to DWT and CLLD birds but lower than UIJ.

At the finisher phase (Table 5), PCV, HB, WBC, and LYM were influenced *P<0.05)*(by *ovo* cysteine and lysine. CLHD had the lowest (P<0.05) PCV.

Oxidative Stress Parameters of Broiler Birds

In the starter phase (Table 6), the MDA of UIJ and DLW were similar but lower than the other treatment groups. The CAT of CSY and CLLD were similar but higher than the other treatments. However, in the finisher phase (Table 7), the MDA of UIJ and DLW were similar but lower than the other treatment groups.

Table 5: Effect of *in-ovo* injection of lysine and cysteine on hematological parameters of broilers at the finisher phase

Parameters	UIJ	DWT	LYS	CSY	CLHD	CLLD	SEM	P value
PCV %	32.00^{a}	30.67^{ab}	28.00^{ab}	34.00^{a}	24.00^{b}	35.00^{a}	1.067	0.005
HB (g/dl)	10.20^{ab}	10.23^{ab}	8.43^{bc}	10.53^{a}	7.03^{c}	10.46^{a}	0.351	0.000
RBC (x103/μl)	3.02^{ab}	3.28^{a}	2.47b	3.27^{a}	1.53^{c}	3.37^{a}	0.166	0.000
WBC (x106/μL)	15.92^{ab}	14.97^{ab}	13.37^{b}	15.97^{ab}	13.36^{b}	16.55^{a}	0.330	0.036
Platelet (103/μL)	12.56	13.57	13.00	13.33	12.26	12.47	25.55	0.700
Lymphocyte,%	61.67^{bcd}	66.67^{abc}	57.67^{cd}	73.67^{cd}	54.33^{abc}	70.67^{a}	4.203	0.007
Heterophil (%)	28.67	24.67	28.67	22.00	28.00	25.33	3.965	0.299
H:L	0.47	0.37	0.50	0.29	0.51	0.35	0.087	0.047
Monocyte (%)	4.33	3.67	2.33	3.67	3.33	2.67	0.268	0.302
Eosinophil (%)	3.00	6.00	4.00	4.00	3.00	3.00	0.339	0.040
Basophil (%)	0.33	0.67	0.00	0.67	0.33	0.00	0.114	0.366

PCV: packed cell volume. HB: hemoglobin RBC: red blood cell WBC: white blood cell H:L: *Heterophil/lymphocyte ratio. abc: different superscripts along the row differ significantly P<0.05, eggs without in ovo injection (UIJ), eggs treated with in ovo injection of 0.5 mL distilled water (DWT), 2 mg/egg lysine (LYS), 3.4 mg cysteine (CYS), 3.4 mg cysteine + 2 mg lysine (CLHD), or 1.7 mg cysteine + 1 mg lysine (CLLD).*

Table 6: Effect of *in-ovo* injection of lysine and cysteine on oxidative stress parameters of broilers at starter phase

Parameters	UIJ	DWT	LYS	CSY	CLHD	CLLD	SEM	P value
MDA mg/dl.	10.039^{a}	6.480^{a}	4.717^{b}	1.656^{c}	4.816^{b}	2.193^{bc}	0.724	0.001
SOD mg/dl.	5.167	6.073	7.042	9.509	11.060	14.835	1.060	0.590
CAT(U/L) mg/dl.	2.777	4.267	6.362	8.068	3.335	8.083	0.760	0.143

MDA, malondialdehyde SOD, superoxide dismutase CAT, catalase, abc: Means within the row with different superscripts differ significantly *P<0.05, eggs without in ovo injection (UIJ), eggs treated with in ovo injection of 0.5mL distilled water (DWT), 2mg/egg lysine (LYS), 3.4mg cysteine (CYS), 3.4mg cysteine+2mg lysine (CLHD) or 1.7mg cysteine+1mg lysine (CLLD).*

Table 7: Effect of *in-ovo* injection of lysine and cysteine on oxidative stress parameters of broilers at finisher phase

Parameters	UIJ	DWT	LYS	CSY	CLHD	CLLD	SEM	P value
MDA mg/dl.	6.302^{a}	6.007^{a}	2.231^{c}	2.318^{c}	3.517^{bc}	1.646^{cd}	0.456	0.002
SOD mg/dl.	3.697	3.551	7.195	8.843	7.123	8.316	0.697	0.088
CAT(U/L) mg/dl.	3.089^{b}	5.590^{b}	4.328^{b}	10.615^{a}	5.928^{b}	10.897^{a}	0.732	0.001

MDA: malondialdehyde, SOD: superoxide dismutase, CAT: catalase, abc: different superscripts differ significantly at P<0.05 along the row,*eggs without in ovo injection (UIJ), eggs treated with in ovo injection of 0.5mL distilled water (DWT), 2mg/egg lysine (LYS), 3.4mg cysteine (CYS), 3.4mg cysteine+2mg lysine (CLHD) or 1.7mg cysteine+1mg lysine (CLLD).*

Table 8: Physiological parameters of *in-ovo* feeding of cysteine and lysine

Parameters	UIJ	DWT	LYS	CSY	CLHD	CLLD	SEM	P value
RT (°C)	41.45^{a}	41.50^{a}	41.57^{a}	41.05^{ab}	40.66^{b}	40.58^{b}	0.493	<0.0001
HR (beats/min)	327.60	333.24	328.24	335.50	334.67	334.73	8.613	0.162
RR (breaths/min)	40.00	42.80	39.60	42.40	41.60	42.00	5.229	0.680

RT, rectal temperature, HR, heart rate, RR, respiratory rate, abc: different superscripts differ significantly at P<0.05, eggs without in ovo injection (UIJ), eggs treated with in ovo injection of 0.5mL distilled water (DWT), 2mg/egg lysine (LYS), 3.4mg cysteine (CYS), 3.4mg cysteine+2mg lysine (CLHD) or 1.7mg cysteine+1mg lysine (CLLD).

Table 9: Effect of *in ovo* injection of cysteine and lysine on carcass parameters broiler chickens

Parameters	UIJ	DWT	LYS	CSY	CLHD	CLLD	SEM	P value
Head (%)	2.50	3.20	2.60	2.84	2.66	2.29	0.106	0.4685
Neck (%)	4.90	3.67	3.09d	3.83b	4.02	5.37	0.214	0.9909
Wing (%)	7.58	7.70	8.25	6.19	9.90	7.26	0.417	0.1773
Breast (%)	34.16	29.00	29.72	33.98	36.19	29.79	1.603	0.8938
Drumstick (%)	18.85	14.90	21.75	19.19	19.86	20.78	0.794	0.2924
Shank (%)	4.11	4.22	4.34	3.82	4.97	4.10	0.193	0.8571
Intestine (%)	4.80	4.74	4.60	4.11	3.53	5.00	0.208	0.3055
Spleen (%)	0.12	0.13	0.12	0.16	0.15	0.12	0.008	0.4253
Bursa (%)	0.08	0.00	0.00	0.00	0.16	0.00	0.035	0.3000
Liver (%)	2.37	2.11	1.87	1.96	2.31	2.60	0.105	0.5976
Kidney (%)	0.56	0.48	0.73	0.51	0.54	0.58	0.029	0.4164
Heart (%)	0.62	0.42	0.58	0.51	0.57	0.45	0.024	0.3536
Gizzard (%)	3.22	2.78	3.25	2.81	2.87	2.54	0.113	0.7268
Proventriculus (%)	0.10	0.11	0.16	0.09	0.15	0.06	0.018	0.0877
Intestine (cm)	226.00	191.00	211.50	202.50	207.50	238.50	6.860	0.3531

[abc] Means within the row with different superscripts differ significantly *P<0.05, eggs without in ovo injection (UIJ), eggs treated with in ovo injection of 0.5mL distilled water (DWT), 2mg/egg lysine (LYS), 3.4mg cysteine (CYS), 3.4mg cysteine+2mg lysine (CLHD) or 1.7mg cysteine+1mg lysine (CLLD).*

MDA concentration is the end product of lipid peroxidation, which is an indication of lipid damage as a result of oxidative stress; the blood MDA increases under stress conditions (Liang *et al*., 2015). Numerous physiological processes, including development, reproduction, and immunity, have been shown to be negatively impacted by free radicals and lipid peroxidation (Gladine *et al*., 2007). The significantly higher level of MDA in the control birds indicated that higher levels of free radicals were generated and overwhelmed the antioxidant status of the birds.

The efficacy of an antioxidant in scavenging radicals depends on the absolute concentration of the antioxidant at the site of stress and the species of radical generated (Surai, 2006). The decrease in high antioxidant concentrations has been linked with lipid peroxidation reduction in chickens (Liang *et al*., 2015). The lower levels of serum MDA in the birds treated with *in ovo* amino acid compared to the control birds suggest that the amino acid enhanced the antioxidant status of the birds, thereby improving the thermotolerance of the birds.

The similarity in the SOD of the chickens across the treatments in this study indicates that *in ovo* amino acid did not upregulate this parameter during the post-hatch growth phase. Our previous study showed that this oxidative stress marker was improved in newly hatched chicks when cysteine and lysine were administered *in ovo* (Ajayi *et al*., 2022). Similarly, Elnesr *et al*. (2019) also reported that *in ovo* injection of methionine and cysteine decreased the concentration of MDA in chicks. The increase in activity of these antioxidant enzymes (catalase) at the later phase of growth may be ascribed to the birds' age and susceptibility to environmental variables. Different reports have documented that a decrease in the activity of catalase in the blood is caused by environmental stress to which birds are exposed during their development (Kant *et al*., 2011; Roushdy *et al*., 2020).

Physiological Parameters of Broiler Birds

Heart rate and respiratory rate values were not significantly different across the treatment groups, except for rectal temperature (RT), which had a lower value in CLHD and CLLD birds (Table 8).The rectal temperature (RT) can reflect the core body temperature. RT increases when the body fails to maintain homeostasis (He *et al*., 2019; Berihulay *et al*., 2019). The results of the current study indicate that *in ovo* cysteine and lysine reduced rectal temperature ($P < 0.05$) compared to the control groups. In line with the results of this study, *in ovo* administration of L-Leucine also caused a lower rectal temperature in chickens (Han *et al*., 2020).

Carcass Characteristics of Broiler Chicken at Slaughter Age

As shown in Table 9, the carcass traits of the birds were similar *(P>0.05)* across the different treatments. This observation suggests that the relative weight of the body parts was not impacted by the treatments.

CONCLUSION

In conclusion, the combinations of lysine and cysteine *in ovo* injection altered the growth rate, thermotolerance, and some biochemical indices in broiler chickens during the post-hatch growth. Overall, the lower combination of the amino acids had a more lasting effect on the growth rate, particularly. The antioxidant indices of the chickens treated with the lower combination of the amino acids were superior during the post-hatch growth.

Animal Welfare Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Data Availability Statement

The datasets generated and analyzed during the present study are available from the corresponding author upon reasonable request.

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