JAST (Journal of Animal Science and Technology) TITLE PAGE

Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Effects of dietary threonine and arginine on growth performance, stress indicators, and gut health in broiler chickens under multiple stress
Running Title (within 10 words)	Threonine and arginine on multiple-stressed broiler chickens
Author	Yeong Bin Kim, Ju Yeong Park, Ha Neul Lee, Gyu Lim Yeom, Geun Yong Park, Ji Won Shin, Jong Hyuk Kim* * Corresponding author
Affiliation	Department of Animal Science, Chungbuk National University, Cheongju 28644, Republic of Korea
ORCID (for more information, please visit https://orcid.org)	Yeong Bin Kim (https://orcid.org/0009-0007-9151-0135) Ju Yeong Park (https://orcid.org/0009-0008-1938-977X) Ha Neul Lee (https://orcid.org/0009-0007-8352-4182) Gyu Lim Yeom (https://orcid.org/0009-0006-1849-053X) Geun Yong Park (https://orcid.org/0009-0001-3453-6941) Ji Won Shin (https://orcid.org/0009-0000-7167-5425) Jong Hyuk Kim (https://orcid.org/0000-0003-0289-2949)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources **State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available.**	This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2023-00210634).
Acknowledgements	Not applicable.
Availability of data and material	Upon reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions **Please specify the authors' role using this form.**	Conceptualization: Kim JH Data curation: Kim YB, Kim JH

	Formal analysis: Kim YB, Park JY, Lee HN, Yeom GL, Park GY, Shin JW Methodology: Kim YB, Kim JH Validation: Kim JH Investigation: Kim YB, Park JY, Lee HN, Yeom GL, Park GY, Shin JW Writing - original draft: Kim YB, Kim JH Writing review & editing: Kim YB, Park JY, Lee HN, Yeom GL, Park GY, Shin JW, Kim JH
Ethics approval and consent to participate	All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Chungbuk National University (CBNUA-2267-24-01).

CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Jong Hyuk Kim
Email address - this is where your proofs will be sent	jonghyuk@chungbuk.ac.kr
Secondary Email address	mrdgj7@naver.com
Address	Department of Animal Science, Chungbuk National University, Cheongju 28644, Republic of Korea
Cell phone number	82 - 10 - 4935 - 8583
Office phone number	82 - 43 - 261 - 2546
Fax number	82 - 43 - 273 - 2240

Abstract

This study evaluated the effects of dietary threonine (Thr) and arginine (Arg) concentrations on growth performance, meat quality, stress indicators, antioxidant capacity, and gut health in broiler chickens raised under multiple stress conditions. A total of 280 21-d-old broiler chickens were randomly allotted to 4 treatments with 8 replicates. The positive control (PC) group was raised under normal rearing conditions (thermoneutral temperature and low stocking density; 22.6 ± 0.6°C for 24 h and 15.2 birds/m^2), whereas the remaining groups were subjected to multiple stress conditions (cyclic heat stress and high stocking density; 29.3 ± 0.9°C for 10 h, 22.8 ± 0.8°C for 14 h, and 30.3 birds/m^2). Both the PC and negative control (NC) groups received a basal diet, while the other two groups were provided diets containing 1.3-fold higher concentrations of digestible Thr or Arg compared to the basal diet. Experimental diets and water were supplied ad libitum for 14 d. Results indicated that growth performance was decreased ($p < 0.05$) in NC, Thr, and Arg groups compared to PC group. Birds in NC group had greater ($p < 0.05$) heterophil to lymphocyte ratio and feather corticosterone (CORT) concentrations than those in PC group. Under multiple stress conditions, Thr and Arg groups showed less ($p < 0.05$) feather CORT concentrations than NC group. In hepatic tissue, reactive oxygen species levels were increased ($p < 0.05$) in NC and Arg groups compared to PC group. Jejunal nitric oxide levels were increased ($p < 0.05$) in NC group compared to PC, Thr, and Arg groups. Under multiple stress, Thr and Arg treatment groups exhibited greater ($p < 0.05$) transepithelial electrical resistance values than NC group. The expression levels of *OCLN*, *ZO-1*, and *HSP70* were greater ($p < 0.05$) in Thr and Arg groups than in NC group. In conclusion, increasing dietary concentrations of Thr and Arg improves intestinal integrity and mitigates stress response in broiler chickens exposed to multiple stress.

Keywords: Arginine, broiler chicken, heat stress, stocking density, threonine

INTRODUCTION

Stress is defined as an adverse environmental condition that leads to physiological and behavioral disruptions in animals, thereby decreasing productivity [1]. Among the various stressors, heat stress (HS) has become a significant concern in poultry production, particularly in the context of rising global temperatures [2,3]. Broiler chickens are especially vulnerable to HS due to their elevated metabolic heat generation, accelerated growth, and lack of sweat glands [4,5]. As a result, HS negatively affects broiler growth performance, nutrient absorption, and the integrity of the intestinal barrier [5,6]. Furthermore, HS impairs immune function and increases physiological stress, thereby further compromising health and animal welfare [3,7]. While HS is recognized as a major environmental stressor, high stocking density (SD) is also a prevalent stressor in broiler operations due to the birds' rapid growth and short production cycles [8]. Elevated SD exacerbates environmental stress by limiting mobility, intensifying competition for feed and water, and increasing temperatures within housing facilities [9]. These challenges in turn result in decreased feed intake (FI) and performance, heightened welfare issues, and a greater risk of hygiene-related problems [10]. In commercial poultry facilities, HS and high SD frequently coexist, with their combined effects creating a more severe stress environment than each stressor alone [11]. However, most research on nutritional strategies has focused on either HS or high SD separately, and there is limited knowledge regarding effective interventions under simultaneous stress conditions.

Among the essential amino acids, Thr is recognized the third limiting amino acid in broiler diets and is vital for protein synthesis and the maintenance of mucosal health [12,13]. Moreover, Thr is fundamental for the production of mucin glycoproteins, which are integral to forming the protective mucus layer covering the intestinal epithelium [14]. This mucus barrier serves to shield epithelial cells from both mechanical and chemical injury while also reducing the adherence and translocation of pathogenic bacteria [15,16]. Supplementing broiler diets

with Thr has been shown to improve intestinal barrier function and decrease inflammatory responses when chickens are challenged by environmental or pathogenic stressors [17].

Arg is recognized as a conditionally essential amino acid in poultry because birds possess an incomplete urea cycle and are unable to synthesize adequate endogenous quantities [18]. Additionally, Arg acts as a precursor for several metabolites, including nitric oxide (NO), polyamines, proline, creatine, and glutamate, which are involved in vascular regulation, cell proliferation, and energy metabolism [18,19]. In particular, Arg-derived NO is essential for vasodilation, oxygen transport, and modulation of immune responses, linking Arg metabolism with circulatory and immune system [20,21]. Dietary Arg supplementation has been demonstrated to mitigate intestinal inflammation in broiler chickens subjected to lipopolysaccharide or *Clostridium perfringens* challenges [22,23]. Owing to these physiological roles, it is hypothesized that dietary supplementation of Thr and Arg may alleviate physiological stress and enhance health in broiler chickens exposed to HS and high SD. Nevertheless, the effectiveness of these amino acids when both HS and high SD conditions are concurrently present has not been widely studied.

Therefore, this study aimed to evaluate the effects of dietary Thr and Arg supplementation on growth performance, meat quality, stress indicators, antioxidant capacity, and gut health in broiler chickens raised under HS and high SD.

MATERIALS AND METHODS

Animal ethics statement

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Chungbuk National University (CBNUA-2267-24-01).

Animals, diets, and experimental design

Prior to the experiment's commencement, all birds were reared in compliance with the Arbor Acres broiler management guidelines [24], and were fed a grower basal diet formulated to meet the nutrient requirements, which was provided uniformly to all birds until 21 days of age (Table 1). A total of two hundred eighty 21-d-old mixed-sex Arbor Acres broiler chicks were allotted to cages (52 × 61 × 42 cm = width × length × height) with a similar average body weight (BW; 696 ± 6.0 g) among cages. All birds were randomly allotted to 1 of 4 treatments with 8 replicates in a completely randomized design. Birds were raised under normal conditions and multiple stress conditions. Birds in normal conditions were raised under thermoneutral environment (temperature: 22.6 ± 0.6°C; humidity: 75.3 ± 5.7%) with a low SD (SD: 15.2 birds/m^2 and 5 birds/cage), while those under multiple stress conditions were exposed to cyclic HS (temperature: 29.3 ± 0.9°C for 10 h and 22.8 ± 0.8°C for 14 h; humidity: 80.6 ± 8.5% for 10 h and 62.4 ± 7.8% for 14 h) and high SD (SD: 30.3 birds/m^2 and 10 birds/cage). Normal and multiple stress conditions were established in two separate but identical rooms within the same facility, which were physically isolated to prevent environmental interference. All cages and husbandry equipment were identical between rooms to ensure experimental consistency. In addition, sex ratio was intentionally controlled at cage allocation on day 21. Cages in the positive control group contained 3 males and 2 females, whereas cages in the multiple stress groups contained 5 males and 5 females. To ensure consistent SD throughout the study, any birds that died were replaced with individuals of comparable BW. In the event of mortality, replacement birds were selected from a reserve population reared under the same dietary and environmental conditions. They were matched for sex and BW to maintain the original sex ratio and stocking density within each cage. These replacement birds were marked with leg bands and included only in body weight and feed intake calculations, while not included in any sample collections to minimize potential confounding effects. A basal diet was formulated to meet or exceed the energy and nutrient recommendations of the Arbor Acres broiler nutrition

specifications. [24]. Birds raised under normal conditions served as the positive control (PC) group and were only fed the basal diet (Table 2). Conversely, birds exposed to multiple stress conditions were assigned to the negative control (NC) group and were also fed the basal diet. Additionally, two treatment groups under multiple stress conditions received experimental diets formulated to provide either a 1.3-fold increase in digestible Thr (0.94% digestible Thr in diets) or Arg (1.52% digestible Arg in diets) compared to the basal diet (0.72% digestible Thr and 1.17% digestible Arg). Purified supplements of Thr (99%, CJ bio, Seoul, Republic of Korea) and Arg (99%, CJ bio, Seoul, Republic of Korea) were used to achieve the targeted dietary concentrations. All experimental diets were formulated to contain identical concentrations of CP and essential amino acids. The concentrations of Thr and Arg in diets were determined using an amino acid analyzer (L-8900, Hitachi High Tech, Tokyo, Japan). All experimental diets were supplied in mash form, with continuous provision of feed and water ad libitum during the 14-d period from 21 to 35 d of age. A 24-h continuous lighting schedule was applied throughout the experimental period [9]. Mortality was recorded daily on a cage basis, and cumulative mortality was calculated for the experimental period. The BW gain (BWG) and FI were recorded at the conclusion of the experiment. Feed efficiency (FE) was calculated as BWG divided by FI.

Sample collection

At the conclusion of the experiment, two birds from each cage, exhibiting a BW close to the mean BW of their respective cage, were euthanized using CO_2 asphyxiation. Tissue, organ, and feather samples were collected from one bird for subsequent analyses. Blood samples were taken immediately via cardiac puncture and placed into a 10 mL EDTA tube (Greiner Bio-One, Kremsmünster, Austria) and a serum separator tube containing a clot activator (Greiner Bio-One, Kremsmünster, Austria). The second bird was reserved for the assessment of intestinal permeability. The internal organs, namely breast, liver, spleen, kidney, bursa of

Fabricius, and thymus, were dissected and weighed to determine relative organ weights, expressed as percentages of BW.

Breast meat quality

For evaluation of meat quality, the breast muscle served as the primary sample. The pH was measured at 1 h and 24 h postmortem using a pH meter (HANNA instruments, Woonsocket, RI, USA). Color characteristics including lightness (L*), redness (a*), and yellowness (b*) were quantified using a spectro colorimeter (Konica Minolta, Tokyo, Japan). The water holding capacity (WHC) of the breast muscle was assessed 24 h postmortem, and thiobarbituric acid reactive substances (TBARS) were evaluated at 7 d postmortem. The WHC determination followed the method described by Lee et al. [25] with minor modifications. In short, 1.5 g of meat was placed in a 50 mL tube with Whatman filter paper NO.3 (Whatman, Maidstone, UK) and centrifuged at 3,000 rpm for 15 min at 4°C. The WHC was derived from the difference in moisture concentration before and after centrifugation. TBARS values were measured according to the method of Lee et al. [26] with slight modifications. In brief, 5 g of the breast meat sample was transferred to a 50 mL test tube and homogenized with 15 mL of deionized distilled water and 50 μL of butylated hydroxytoluene solution at 15,000 rpm for 20 s. Thereafter, 1 mL of the homogenate was moved into a 15 mL tube, followed by the addition of 2 mL of a mixed solution of thiobarbituric acid and trichloroacetic acid. This mixture was thoroughly vortexed and incubated at 90°C in a water bath for 15 min. The heated sample was cooled on ice, then centrifuged at 3,000 rpm for 10 min. The absorbance of the resulting supernatant was measured at 531 nm using a microplate reader (INNO Microplate Spectrophotometer, LTEK Co., Ltd., Seongnam, Republic of Korea).

Blood parameters

Serum biochemical parameters, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine, were analyzed using a Cobas C720 (Roche Diagnostics, Mannheim, Germany). Uric acid concentrations were determined with an automatic analyzer (Labospect 008AS, Hitachi, Tokyo, Japan). Hematological variables, including red blood cell, white blood cell, hemoglobin, hematocrit, segment, lymphocyte, monocyte, eosinophil, and basophil, were measured by an automated blood cell analyzer (XE2100D, Sysmex, Kobe, Japan).

Stress indicators

The heterophil to lymphocyte ratio (H:L ratio) was determined from blood samples collected in EDTA tubes. Blood smears were prepared using Wright stain solution (Muto pure chemicals, Tokyo, Japan) and Giemsa stain solution (Samchun chemicals, Pyeongtaek, Republic of Korea). After air drying, the stained smears were analyzed microscopically (OS-370DVM, Osun Hithech, Goyang, Republic of Korea). The H:L ratio was obtained by counting heterophils and lymphocytes until a total of 200 cells per stained sample recorded, with independent assessment by each observer [27,28]. Feather corticosterone (CORT) concentrations were determined according to the protocol of Bortolotti et al. [29] with minor adjustments. Briefly, feathers were separated into vane and rachis regions, and approximately 100 mg of vane was finely chopped with scissors. The fragments were placed into 50 mL tubes containing 10 mL of methanol, sonicated, and incubated at 50°C in a water bath for CORT extraction. Quantification of extracted CORT was carried out using a Corticosterone ELISA kit (Enzo Life Sciences Inc., Farmingdale, NY, USA), following the manufacturer's instructions.

Liver characteristics

Liver color was assessed promptly after euthanasia with a Minolta Chromameter CR-400 (Konica Minolta, Tokyo, Japan), recording the values for L*, a*, and b*. After liver color

assessment, samples were photographed for subsequent evaluation of hemorrhagic and fatty liver scores. The hemorrhagic liver score ranged from 0 to 5, where 0 represented a normal liver and 5 denoted extensive and severe hemorrhage [30]. The fatty liver score was evaluated using a scale of 1 to 5, where a score of 1 denoted a normal liver and 5 indicated a dark red to pale yellowish-red discoloration [31]. Liver fat concentration was measured using the Soxhlet method [32]. Liver sample was freeze-dried for 72 h and finely ground for fat extraction.

Animal welfare

At the end of the experiment, 3 birds raised under normal conditions and 6 birds exposed to multiple stress conditions were randomly chosen, representing over 50% of the birds in each cage [33]. All chosen birds were assessed using the Welfare Quality® Assessment protocol for poultry [34]. Four welfare parameters were evaluated: gait score, footpad dermatitis, hock burn, and plumage cleanliness. Each indicator was visually scored by five trained evaluators. Gait score was measured on a 0 to 5 scale, with 0 reflecting normal ambulation and 5 representing severe lameness with an inability to walk. The severity of footpad dermatitis was determined on a scale of 0 to 4, where 0 signified a healthy and undamaged footpad and 4 denoted advanced lesions. Hock burn was graded on a 0 to 4 scale based on the observed degree of dermatitis on the caudal hock. A score of 0 signified no lesions, whereas a score of 4 indicated pronounced dermatitis. Plumage cleanliness was evaluated on a 0 to 3 scale, where 0 represented very clean plumage and 3 signified significant contamination with fecal material.

Antioxidant capacity in the liver and jejunum

Antioxidant capacity was evaluated in both liver and jejunal mucosa tissue. Levels of malondialdehyde (MDA) and total antioxidant capacity (TAC) were measured using the EZ-Lipid Peroxidation (TBARS) Assay Kit (DG-TBA200, DoGenBio Inc., Seoul, Republic of Korea) and EZ-Total Antioxidant Capacity (TAC) Assay Kit (DG-TAC200, DoGenBio Inc.,

Seoul, Republic of Korea), respectively. To standardize MDA and TAC values, protein concentration in liver and jejunal mucosa was quantified using the Pierce BCA Protein Assay Kit (23225, Thermo Fisher Scientific, Waltham, MA, USA). Reactive oxygen species (ROS) were quantified using the OxiSelect™ In Vitro ROS/RNS Assay Kit (STA-347, Cell Biolabs, Inc., San Diego, CA, USA), and NO levels were determined using the Nitric Oxide (NO) Colorimetric Assay Kit (E-BC-K035-M, Elabscience, Houston, TX, USA), respectively. All assessments were performed following the respective manufacturer's instructions.

Jejunal morphology and goblet cell count

Segments of jejunum measuring approximately 3 cm in length were excised, flushed with saline, and placed in 5 mL tubes containing 10% neutral-buffered formalin for fixation. The fixed tissues were then cut into smaller pieces and embedded in paraffin to generate paraffin blocks. Paraffin-embedded sections were prepared by cutting tissues at a thickness of 4 μm and stained with hematoxylin-eosin and periodic acid-Schiff using established protocols [35,36]. The stained sections were observed using a microscope (OS-370DVM, Osun Hithech, Goyang, Republic of Korea). Morphometric parameters including villus height (VH), villus width (VW), crypt depth (CD), and the VH:CD ratio were determined, with the mean value from 10 separate measurements used for subsequent analysis. Goblet cell counts were evaluated in 10 villi within a 200 μm central area of each villus [37].

Jejunal permeability

Jejunal permeability was evaluated by determining transepithelial electrical resistance (TEER) with a 2-channel Ussing chamber system (P2300, Physiologic Instruments Inc., Reno, NV, USA). The mid-portion of the jejunum, positioned near Meckel's diverticulum and adjacent to the duodenal loop, was excised and immediately immersed in Krebs-Henseleit buffer. Once the luminal contents were removed, the intestinal segments were opened longitudinally to isolate

the mucosal layer. These mucosal samples were mounted on chamber inserts prior to placement in the Ussing chamber. The chambers were filled with buffer solution maintained at 40°C via a water bath, and continuously aerated with a 95% O_2 and 5% CO_2 mixture to sustain tissue function. TEER measurements were derived from recordings of potential difference (PD), short-circuit current, and electrical resistance, which were collected every 10 s over a 5 min interval.

Tight junction-related and stress-related gene expression

The expression levels of genes involved in tight junction integrity and cellular stress response in the jejunal mucosa were quantified using a QuantStudio™ 1 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA), as described by Shin et al. [38]. Total RNA was isolated from the jejunal mucosa utilizing TRIzol™ Reagent (15596026, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. For cDNA synthesis, DNase I (RNase-free; EN0521, Thermo Fisher Scientific, Waltham, MA, USA) treatment and the RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific, Waltham, MA, USA) were applied. The genes analyzed included *occludin* (*OCLN*), *claudin-1* (*CLDN1*), *zonula occludens-1* (*ZO-1*), *junctional adhesion molecule B* (*JAM-2*), and *heat shock protein 70* (*HSP70*). Primers for target gene amplification were designed using NCBI Primer-BLAST based on published sequences (Table 3). The specificity of primer pairs was confirmed by conventional PCR analysis [39]. Quantification of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase as the reference gene [40].

Statistical analysis

All statistical analyses were performed as a completely randomized design using the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). Each replicate served as an experimental unit. All data were checked for normal distribution, and outliers were checked

with the UNIVARIATE procedure of SAS [41]. The LSMEANS procedure was conducted to calculate treatment means and the PDIFF option was used to separate means if the difference was significant. Statistical significance and tendency for statistical tests were set at $p < 0.05$ and $0.05 \leq p < 0.10$, respectively.

RESULTS

Growth performance

Birds in NC, Thr, and Arg groups under multiple stress conditions had less ($p < 0.05$) BW, BWG, FI, and FE than those in PC group under normal conditions (Table 4).

Breast meat quality

There was a trend for increased ($p = 0.083$) breast yield in Thr group compared to PC and NC groups (Table 5). Moreover, NC group showed a tendency ($p = 0.098$) toward elevated pH at 24 h postmortem compared to PC group.

Relative organ weight and blood parameters

No significant differences in the relative weights of liver, spleen, kidney, bursa of Fabricius, and thymus were observed among treatment groups (Table 6). Birds in NC and Arg groups had greater ($p < 0.05$) ALT levels in the serum than those in PC group (Table 7). Levels of AST, creatinine, uric acid, and blood hematological parameters did not differ significantly among treatment groups (Table 8).

Stress indicators

Birds in NC, Thr, and Arg treatment groups had greater ($p < 0.05$) H:L ratio than those in PC group (Table 9). Feather CORT concentrations were increased ($p < 0.05$) in NC group than in

PC group. Under multiple stress conditions, birds in Thr and Arg groups had less ($p < 0.05$) feather CORT concentrations than those in NC group.

Liver characteristics and animal welfare score

There were no significant differences in liver color, hemorrhagic score, fatty liver score, and liver fat concentration among treatment groups (Table 10). Similarly, gait score, footpad dermatitis, hock burn, and feather cleanliness did not differ among treatment groups (Table 11).

Antioxidant capacity in the liver and jejunum

Birds in PC and Arg groups had less ($p < 0.05$) ROS levels in the liver than those in NC group (Table 12). Furthermore, birds in Thr and Arg groups had less ($p < 0.05$) NO levels in the jejunum than those in NC group. Notably, birds in Arg group showed the least ($p < 0.05$) NO levels in the jejunum among all treatment groups under multiple stress conditions, approaching the NO levels observed in PC group.

Jejunal morphology

Birds in PC group had greater ($p < 0.05$) VH than those in NC group (Table 13). Birds raised under multiple stress conditions did not differ in VH among treatment groups. Moreover, birds in PC group tended to display increased ($p = 0.096$) VW compared to those in Thr and Arg groups. Birds in NC and Arg groups had less ($p < 0.05$) goblet cell counts than those in PC group.

Jejunal permeability

Birds in PC group had greater ($p < 0.05$) PD and TEER values than those in NC group (Table 14). Birds in Thr and Arg groups had greater ($p < 0.05$) TEER values than those in NC group.

Tight junction-related and stress-related gene expression

Birds in NC group had less ($p < 0.05$) expression levels of *OCLN*, *CLDN1*, *ZO-1*, *JAM2*, and *HSP70* than those in PC group (Table 15). However, expression levels of *OCLN*, *ZO-1*, and *HSP70* were significantly increased ($p < 0.05$) in Thr and Arg groups compared to NC group.

DISCUSSION

In this study, broiler chickens subjected to multiple stress conditions exhibited decreased BW, BWG, FI, and FE compared to those raised under normal conditions. Previous studies have consistently shown that environmental stressors such as HS, increased relative humidity, and high SD adversely affect poultry performance [42-44]. Furthermore, Goo et al. [9] reported that HS or high SD reduced BW, BWG, FI, and FE in broiler chickens. These declines are frequently ascribed to a reduction in FI, which restricts nutrient intake and subsequently lowers broiler productivity [45]. In alignment with these findings, our results indicated that the drop in FI observed in broiler chickens raised under multiple stress conditions may have been a key factor contributing to diminished BW, BWG, and FE. Importantly, growth performance did not differ significantly among treatment groups, signifying that dietary inclusion of 1.3-fold elevated digestible Thr or Arg failed to restore growth performance impaired by HS and high SD. Khalid et al. [46] found that a 1.5-fold increment in dietary digestible Thr had no effect on BWG and FI in broiler chickens exposed to cyclic HS. Similarly, Brugaletta et al. [47] reported that supplementation with 1.37% total Arg (Arg:Lys ratio = 1.20) did not enhance BW and FI in broiler chickens raised under cyclic HS. Several explanations exist as to why Thr and Arg supplementation did not ameliorate growth performance in broiler chickens raised under multiple stress conditions. Broiler chickens under HS and high SD conditions typically consume less FI [48,49], which may have resulted in insufficient intake of Thr and Arg to affect growth performance. In addition, HS has been shown to suppress pancreatic digestive enzyme

activity and increase intestinal permeability [50-52], potentially limiting nutrient digestibility and absorption ability. As a result, while Thr and Arg supplementation improved stress tolerance and supported gut health, these benefits did not compensate for restricted nutrient intake and utilization, resulting in no measurable improvements in growth performance under multiple stress conditions. Importantly, the primary objective of dietary Thr and Arg supplementation in the present study was not to directly stimulate FI or reduce dietary heat increment, but rather to enhance physiological resilience and cellular stress adaptation under conditions of restricted nutrient intake. Therefore, the lack of improvement in growth performance does not contradict the observed benefits on stress biomarkers and gut health, but instead highlights the biological limitation that performance recovery under HS is largely constrained by FI [5,44]. These findings suggest that improvements in stress tolerance and metabolic homeostasis do not necessarily translate into enhanced growth performance when nutrient intake remains insufficient.

Our results indicated that birds in NC group tended to exhibit a greater 24 h postmortem pH compared to those in PC group. The ultimate pH of meat serves as a fundamental quality parameter, influencing WHC, tenderness, color, and shelf life [53]. It is well-established that environmental stressors negatively impact meat quality by affecting muscle metabolism [52]. Typically, acute HS accelerates muscle glycolysis, triggering a rapid decline in meat pH and resulting in pale, soft, and exudative meat [54]. Conversely, chronic stress, such as prolonged HS exposure, elevates pH due to reduced muscle glycogen stores and subsequently diminished lactic acid accumulation [55]. This response can be linked to the metabolic adaptations associated with continuous HS and high SD throughout the 14 d period, which subsequently led to the relatively greater 24 h postmortem pH observed in NC group compared to PC group. Further supporting this, broiler chickens exposed to continuous HS from 35 to 41 d of age exhibited a greater breast meat pH when compared to those maintained at thermoneutral temperatures [55]. Additionally, broiler chickens reared under medium and high SD conditions

(17.5 and 22.5 birds/m^2) showed greater breast meat pH compared to those reared at low SD (12.5 birds/m^2) [56]. Collectively, these findings suggest that chronic HS and high SD may increase breast meat pH in broiler chickens, thereby influencing its physicochemical properties and potentially compromising overall meat quality.

The present study indicated that Thr group exhibited a greater breast yield compared to NC group under multiple stress, as well as PC group under thermoneutral conditions. The elevation in breast yield observed in broiler chickens consuming diets supplemented with Thr during multiple stress exposures may be attributed to several interrelated mechanisms. Dietary Thr plays a direct role in muscle protein accretion by stimulating pathways associated with cellular protein synthesis, notably the mTOR-4E-BP1/S6K and eIF signaling cascades [57-59]. Additionally, Thr acts as a critical precursor for mucins and upregulates the expression of tight junction proteins, which enhances intestinal barrier integrity, decreases epithelial permeability and nutrient leakage, and reduces inflammatory responses [15,60]. In alignment with these findings, our study found that dietary Thr supplementation increased TEER values and tight junction-related gene expression in broiler chickens exposed to multiple stress conditions. Improved intestinal barrier function as a result of Thr administration likely limited the unnecessary allocation of amino acids and energy to immune response and acute phase protein synthesis, ensuring more nutrients remained available for growth [17,61]. Moreover, Thr supplementation is capable of modulating inflammation and oxidative stress, thereby indirectly dampening the overactivation of the hypothalamic-pituitary-adrenal axis and consequently reducing CORT-induced muscle protein catabolism [62,63]. Taken together, these mechanisms indicate that Thr supplementation not only conserves nutrient resources but also fosters a metabolic state ideal for muscle accretion, which ultimately enhances the breast yield of broiler chickens raised under multiple stress conditions.

Among stress biomarkers, the H:L ratio and feather CORT concentrations are widely regarded as reliable indicators of stress in poultry [64]. Specifically, feather CORT is valuable

because it provides cumulative representation of hormonal responses to chronic HS [64]. In the present study, birds in NC group exposed to multiple stress conditions exhibited increased H:L ratios and feather CORT concentrations compared with those in PC group raised under normal conditions. The experience of stress induces sustained activation of the hypothalamic-pituitary-adrenal axis, leading to increased CORT concentrations both in blood and feathers [29,62]. Elevated systemic CORT facilitates the release of heterophils from the bone marrow, suppresses lymphocyte migration, and triggers lymphocyte apoptosis, ultimately resulting in leukocyte redistribution [62,65,66]. Hence, the increase in H:L ratio and feather CORT suggests that HS and high SD provoke a heightened physiological stress response in broiler chickens. Importantly, a 1.3-fold increase in digestible Thr and Arg mitigated feather CORT concentrations in broiler chickens raised under multiple stress conditions. Thr reduces intestinal permeability by promoting mucin synthesis and regulates the release of pro-inflammatory cytokines, helping to ameliorate the inflammatory response [60]. Arg enhances oxygen transport through NO synthesis and modulates the production of inflammatory cytokines, which supports the reduction of inflammation [21]. These mechanisms may help counteract stress in broiler chickens by modulating overactivation of the hypothalamic-pituitary-adrenal axis, subsequently decreasing CORT secretion [67,62]. Thus, dietary supplementation with additional Thr and Arg has the potential to alleviate physiological stress in broiler chickens subjected to stressful environments. In this experiment, we evaluated feather CORT concentrations as a biomarker of stress. Feather CORT is considered a reliable indicator in poultry because it reflects the cumulative incorporation of circulating CORT during feather development [29]. Conventional assessments of stress responses in poultry typically measure the H:L ratio and blood CORT concentrations [65,68]. Nonetheless, blood-derived stress indicators can be influenced by handling during sampling, pathogenic exposures, and fluctuations in the severity or duration of stress, consequently indicating only short-term physiological states [69,70]. Therefore, these measures may not comprehensively represent the

long-term stress burden experienced by the birds. In contrast, CORT becomes integrated into feather keratin through diffusion from the bloodstream as feather grow, creating a retrospective record of hormonal exposure over an extended duration [71]. Consequently, quantifying CORT concentrations in feathers serves as a reliable and non-invasive method for assessing long-term physiological stress responses in poultry [70].

Animal welfare scores, including gait score, footpad dermatitis, hock burn, and feather cleanliness, are utilized to evaluate the physical comfort, mobility, and hygiene status of poultry [72]. These welfare indicators are reported to be affected by factors such as temperature, SD, litter quality, and housing system [72-74]. Our findings for animal welfare scores indicated that multiple stress conditions did not significantly influence the welfare parameters of broiler chickens housed in cages. Previous studies have demonstrated that HS and high SD can increase litter moisture and worsen litter quality, leading to adverse effects on gait score and footpad dermatitis in broiler chickens reared in floor pens [75,76]. Nevertheless, there is a lack of studies examining the impact of environmental stress on animal welfare scores in cage-reared broiler chickens. Since the cage rearing system eliminates the need for litter, welfare parameters in cage-reared birds may be less affected by the deterioration of litter quality, possibly contributing to the absence of significant differences observed in our study. Notably, welfare assessments such as gait score and footpad dermatitis primarily reflect chronic, litter-mediated physical damage rather than acute or subclinical physiological stress responses [77]. Because the cage rearing system eliminates litter-related challenges and the experimental period was relatively short, the applied multiple stress conditions may have been sufficient to induce physiological stress responses without causing overt physical welfare impairments. These findings suggest that the absence of changes in welfare scores does not necessarily indicate a lack of SD stress, but rather reflects differences in the housing system and the nature of the stress response.

Oxidative stress arises when there is an imbalance favoring the production of ROS and reactive nitrogen species (RNS) over the antioxidant defense system, ultimately leading to

cellular injury [78]. Environmental stressors, such as HS and high SD, have been documented to cause oxidative stress in poultry by impairing mitochondrial function, elevating metabolic activity, and increasing the generation of ROS and RNS [79-81]. Significantly, excessive NO formed during stress responses can interact with superoxide, a form of ROS, to produce peroxynitrite, which is a highly reactive oxidant and nitrating species that induces extensive cellular and tissue damage through mechanisms such as cell death [82]. Therefore, the modulation of ROS and RNS under stress is essential for maintaining the physiological functions of broiler chickens. In our study, broiler chickens exposed to multiple stress conditions exhibited elevated hepatic ROS levels, whereas dietary supplementation with 1.3-fold higher digestible Arg significantly decreased liver ROS levels. The positive effects of Arg on hepatic redox homeostasis may be due to its ability to enhance the activities of antioxidant enzymes and support mitochondrial integrity through its metabolites, such as polyamines and creatine [18]. Thus, Arg supplementation exceeding standard recommendations may strengthen hepatic antioxidant capacity in broiler chickens subjected to multiple stress conditions.

Meanwhile, exposure to multiple stress conditions led to elevated jejunal NO levels, which were mitigated by supplementing the diet with additional Thr and Arg. As a critical precursor in the synthesis of mucin glycoproteins, Thr is essential for maintaining a protective barrier on the intestinal epithelium, thus minimizing epithelial injury and reducing the risk of pathogen invasion [14]. The role of Thr in mucin biosynthesis may also suppress the expression of pro-inflammatory cytokines, thereby diminishing intestinal inflammation under stress [83] and consequently decreasing iNOS-driven NO synthesis in the jejunum. Additionally, Arg plays a regulatory role in immune responses by influencing pro-inflammatory cytokine synthesis and downregulating iNOS expression [23], which may help alleviate intestinal inflammation and reduce excess jejunal NO levels. Collectively, these findings imply that dietary supplementation with 1.3-fold increased Thr and Arg effectively limits jejunal NO overproduction in broiler chickens exposed to multiple stress, thereby preserving intestinal barrier function.

Jejunal morphology serves as a crucial indicator of intestinal integrity and nutrient assimilation in poultry [84]. Exposure to various stressors can lead to oxidative stress within the intestine, resulting in damage to epithelial cells and disruption of tight junction structures [85]. In this study, broiler chickens raised under multiple stress conditions showed less VH than those raised under normal conditions, demonstrating that HS and high SD can compromise intestinal architecture. Consistent with our results, previous studies reported reduced jejunal VH in broiler chickens exposed to HS and high SD compared to controls maintained under conditions [86,87]. Therefore, these observations underscore that environmental stressors, such as HS and high SD, have deleterious impacts on the jejunal structure of broiler chickens.

The intestinal mucus layer protects the intestinal epithelium by serving as both a physical and chemical barrier [16]. Goblet cells are critical for sustaining intestinal health; they secrete mucins that entrap pathogens and help regulate immune responses [88,89]. Consequently, the quantification of goblet cells functions as a histological indicator for assessing the structural integrity of the intestinal barrier in poultry [15]. Due to the substantial secretory activity required for mucin glycoproteins, goblet cells are highly susceptible to induction of endoplasmic reticulum (ER) stress [90]. During stress, ER stress can overwhelm the unfolded protein response, which in turn disrupts mucin synthesis and results in goblet cell malfunction [91,92]. In the present study, goblet cell numbers were also diminished in groups exposed to multiple stress conditions in comparison to the normal condition group, aligning with previous studies under HS and high SD [86,93]. Thus, these results suggest that multiple stress conditions impair intestinal health in broiler chickens by decreasing goblet cell populations.

Birds in NC group exposed to multiple stress conditions exhibited decreased expression of *OCLN*, *CLDN1*, *ZO-1*, and *JAM2* compared to those in PC group. These results indicate that multiple stress conditions disrupt the integrity of tight junctions in broiler chickens. These findings accord with previous studies demonstrating that exposure to multiple stress suppresses

tight junction-related gene expression and enhances intestinal permeability [11,48,52]. Nevertheless, supplementing the basal diet with a 1.3-fold increase in digestible Thr and Arg concentrations elevated the expression of *OCLN* and *ZO-1* in broiler chickens under multiple stress. Thr facilitates enhanced mucin synthesis and supports the mucus layer that shields the epithelial surface [14]. An improved mucus barrier limits luminal pathogen access to epithelial cells and suppresses mucosal inflammation, which helps maintain intestinal barrier function [94]. By downregulating inflammatory signaling, the production and arrangement of tight junction proteins and related genes are restored, thus reducing intestinal permeability [95]. Polyamines and NO, produced from Arg metabolism, help sustain epithelial integrity and inhibit pro-inflammatory cytokine expression through immunomodulatory pathways [18,96]. Collectively, these mechanisms enable Arg to limit oxidative stress and inflammation-related impairment of tight junctions, counteracting transcriptional reductions under stress and reducing stress-induced damage to the intestinal barrier.

Heat shock proteins (HSPs) are stress-induced proteins that play a crucial role in maintaining cellular homeostasis during exposure to stress conditions in animals [97]. They are involved in processes such as protein secretion, folding, transport, degradation, and the regulation of transcription factors, which helps prevent apoptosis [98]. Generally, HSPs serve as a major protective mechanism against stress, becoming overexpressed to enhance cellular tolerance and provide cytoprotection under stressful conditions [99]. Among these, HSP70 is regarded as the most temperature-responsive isoform [100]. While HSP70 is normally upregulated in response to stress, there are reports indicating that its expression might not always increase during extended stress exposure [95]. The reduced expression of *HSP70* observed in this study after two weeks of exposure to multiple stress may result from sustained CORT secretion. Continuous elevation of CORT levels is capable of activating glucocorticoid receptors (GRs), which can exert repressive effects on transcription through several pathways [101]. In particular, GRs may directly associate with negative glucocorticoid response elements,

thus hindering the recruitment of RNA polymerase II and decreasing transcriptional initiation [101]. Additionally, GRs are able to transrepress other transcription factors, including nuclear factor kappa-light-chain-enhancer of activated B cells and activator protein-1 or recruit co-repressors that modify chromatin structure, leading to further suppression of gene transcription [101]. These mechanisms may account for the reduction in *HSP70* expression following two weeks of multifaceted stress in broiler chickens. Furthermore, high SD was persistently maintained throughout the experiment, even after the daily 8 h period of HS concluded. As a result, these conditions likely imposed ongoing stress on broiler chickens, contributing to lower *HSP70* expression in this investigation compared to previous study [97]. Under the imposed multiple stress conditions, increasing concentrations of Thr and Arg in diets promoted recovery of *HSP70* expression in broiler chickens. This effect may be linked to the observed reductions in CORT concentrations in Thr and Arg supplemented groups. Collectively, these results indicate that long-term exposure to multiple stress may inhibit tight junction-related gene expression and *HSP70* levels in broiler chickens. Increasing concentrations of Thr and Arg in diets may alleviate this inhibition, supporting improved intestinal integrity and greater cellular resilience to stress.

The resistance measured by passing an electrical current across the epithelium reflects paracellular resistance arising from tight junctions and the lateral intercellular space, a parameter referred to as TEER [102]. TEER serves as a sensitive marker for assessing epithelial barrier integrity [103]. Reduced TEER indicates tight junction disruption and increased passage of luminal antigens or endotoxins into systemic circulation, a process implicated in immune compromise and inflammation [102]. In our investigation, exposure to multiple stress markedly decreased TEER values, corroborating previous findings that report both HS and high SD impair intestinal barrier function [9,11,48]. Notably, supplementation of the basal diet with 1.3-fold greater concentrations of Thr and Arg enhanced TEER in broiler chickens under these stress conditions, indicating that these amino acids help to preserve intestinal barrier integrity

when birds are exposed to multiple stress. These results agree with our gene expression analysis, where elevated dietary Thr and Arg increased the expression of *OCLN* and *ZO-1*, further reinforcing the paracellular barrier. Additionally, Thr and Arg reduced jejunal NO levels, which may signal mitigated intestinal inflammation, since excessive NO generation is associated with epithelial injury and barrier dysfunction [23]. These findings indicate that enhanced dietary Thr and Arg contribute to protection of intestinal barrier integrity under multiple stress conditions by supporting TEER recovery.

In conclusion, exposure to multiple stress conditions combined with HS and high SD impairs growth performance, stress resilience, hepatic antioxidant capacity, and intestinal barrier health in broiler chickens. Under multiple stress conditions, dietary concentrations of 1.3-fold higher digestible Thr and Arg reduce feather CORT concentrations, indicative of reduced physiological stress in broiler chickens. Moreover, increasing concentrations of Thr and Arg in diets enhance intestinal barrier functions and cellular stress adaptation, as reflected by decreased jejunal NO production, restoration of TEER, and upregulation of tight junction and stress-related gene expression in broiler chickens subjected to multiple stress conditions. Nevertheless, supplementation with 1.3-fold higher digestible Thr and Arg does not fully restore growth performance in broiler chickens exposed to multiple stress conditions, which may be attributable to reduced FI and impaired nutrient assimilation.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2023-00210634).

REFERENCES

1. Stott GH. What is animal stress and how is it measured?. J Anim Sci. 1981;52:150-3. https://doi.org/10.2527/jas1981.521150x
2. Lim C, Yoo S, Choi Y, Jeon S, Son Y, Lee W. Assessing climate change impact on forest habitat suitability and diversity in the Korean Peninsula. Forests. 2018;9:259. http://dx.doi.org/10.3390/f9050259
3. Brugaletta G, Teyssier J, Rochell SJ, Dridi S, Sirri F. A review of heat stress in chickens. Part I: Insights into physiology and gut health. Front Physiol. 2022;13:934381. https://doi.org/10.3389/fphys.2022.934381
4. Ensminger ME, Oldfield JE, Heinemann WW. Feeds and Nutrition: Formerly Feeds and Nutrition, Complete. Ensminger Publish; 1990
5. Lara LJ, Rostagno MH. Impact of heat stress on poultry production. Animals. 2013;3:356-69. https://doi.org/10.3390/ani3020356
6. Quinteiro-Filho WM, Ribeiro A, Ferraz-de-Paula V, Pinheiro ML, Sakai M, Sá LRM, et al. Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. Poult Sci. 2010;89:1905-14. https://doi.org/10.3382/ps.2010-00812
7. Kim YB, Yeom GL, Park JY, Lee HN, Park GY, Shin JW, et al. Nutritional strategies to alleviate heat stress in broiler chickens and laying hens: Role of functional nutrients. J Anim Sci Technol. 2025;67:949-81. https://doi.org/10.5187/jast.2500205
8. Hall AL. The effect of stocking density on the welfare and behaviour of broiler chickens reared commercially. Anim Welf. 2001;10:23-40. https://doi.org/10.1017/S096272860002323X
9. Goo D, Kim JH, Park GH, Reyes JBD, Kil DY. Effect of heat stress and stocking density on growth performance, breast meat quality, and intestinal barrier function in broiler chickens. Animals. 2019b;9:107. http://dx.doi.org/10.3390/ani9030107

10. Shynkaruk T, Long K, LeBlanc C, Schwean-Lardner K. Impact of stocking density on the welfare and productivity of broiler chickens reared to 34 d of age. J Appl Poult Res. 2023;32:100344. https://doi.org/10.1016/j.japr.2023.100344
11. Kim HW, Kil DY, Kim JH. Effects of dietary concentration of threonine on growth performance, meat quality, antioxidant capacity, stress biomarker, and intestinal barrier function in broiler chickens raised under multiple stress conditions. Anim Feed Sci Technol. 2025;323:116303. https://doi.org/10.1016/j.anifeedsci.2025.116303
12. Kidd MT, Kerr BJ. Limiting amino acid responses in commercial broilers. J Appl Poult Res. 2000;9:223-33. https://doi.org/10.1093/japr/9.2.223
13. Qaisrani SN, Ahmed I, Azam F, Bibi F, Saima, Pasha TN, et al. Threonine in broiler diets: an updated review. Ann Anim Sci. 2018;18:659-74. https://doi.org/10.2478/aoas-2018-0020
14. Mao X, Zeng X, Qiao S, Wu G, Li D. Specific roles of threonine in intestinal mucosal integrity and barrier function. Front Biosci. 2011;3:1192-200. https://doi.org/10.2741/e322
15. Smirnov A, Sklan D, Uni Z. Mucin dynamics in the chick small intestine are altered by starvation. J Nutr. 2004;134:736-42. https://doi.org/10.1093/jn/134.4.736
16. Kim YS, Ho SB. Intestinal goblet cells and mucins in health and disease: Recent insights and progress. Curr Gastroenterol Rep. 2010;12:319-30. https://doi.org/10.1007/s11894-010-0131-2
17. Bortoluzzi C, Rochell SJ, Applegate TJ. Threonine, arginine, and glutamine: Influences on intestinal physiology, immunology, and microbiology in broilers. Poult Sci. 2018;97:937-45. http://dx.doi.org/10.3382/ps/pex394
18. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Rhoads JM, et al. Arginine metabolism and nutrition in growth, health and disease. Amino acids. 2009;37:153-68. https://doi.org/10.1007/s00726-008-0210-y

19. Stöckler S, Holzbach U, Hanefeld F, Marquardt I, Helms G, Requart M, et al. Creatine deficiency in the brain: a new, treatable inborn error of metabolism. Pediatr Res. 1994;36:409-13. https://doi.org/10.1203/00006450-199409000-00023
20. Ignarro LJ, Buga GM, Wei LH, Bauer PM, Wu G, del Soldato P. Role of the arginine-nitric oxide pathway in the regulation of vascular smooth muscle cell proliferation. Proc Natl Acad Sci USA. 2001;98:4202-8. https://doi.org/10.1073/pnas.071054698
21. Liu G, Kim WK. The functional roles of methionine and arginine in intestinal and bone health of poultry: review. Animals. 2023;13:2949. https://doi.org/10.3390/ani13182949
22. Tan J, Liu S, Guo Y, Applegate TJ, Eicher SD. Dietary L-arginine supplementation attenuates lipopolysaccharide-induced inflammatory response in broiler chickens. Br J Nutr. 2014;111:1394-404. https://doi.org/10.1017/S0007114513003863
23. Zhang B, Gan L, Shahid MS, Lv Z, Fan H, Liu D, et al.. In vivo and in vitro protective effect of arginine against intestinal inflammatory response induced by Clostridium perfringens in broiler chickens. J Anim Sci Biotechnol. 2019;10:73. https://doi.org/10.1186/s40104-019-0371-4
24. Aviagen. Ross Boiler: Nutrition specifications. [Internet]. Aviagen. Inc.; 2022 [cited 2025 Jul 16]. https://aviagen.com/assets/Tech_Center/Ross_Broiler/RossBroilerNutritionSpecifications2022-EN.pdf
25. Lee SJ, Lee SY, Kim GD, Kim GB, Jin SK, Hur SJ. Effects of self-carbon dioxide-generation material for active packaging on pH, water-holding capacity, meat color, lipid oxidation and microbial growth in beef during cold storage. J Sci Food Agric. 2017;97:3642-8. https://doi.org/10.1002/jsfa.8223
26. Lee HJ, Yim DG, Jo C. Effect of plasma-activated organic acids against Salmonella Typhimurium and Escherichia coli O157: H7 inoculated on pork loin and its quality

characteristics. Innov Food Sci Emerg. 2023;88:103455. https://doi.org/10.1016/j.ifset.2023.103455

27. Cengiz Ö, Köksal BH, Tatlı O, Sevim Ö, Ahsan U, Üner AG, et al. Effect of dietary probiotic and high stocking density on the performance, carcass yield, gut microflora, and stress indicators of broilers. Poult Sci. 2015;94:2395-403. https://doi.org/10.3382/ps/pev194
28. Yu DG, Namgung N, Kim JH, Won SY, Choi WJ, Kil DY. Effects of stocking density and dietary vitamin C on performance, meat quality, intestinal permeability, and stress indicators in broiler chickens. J Anim Sci Technol. 2021;63:815-26. https://doi.org/10.5187/jast.2021.e77
29. Bortolotti GR, Marchant TA, Blas J, German T. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. Funct Ecol. 2008;22:494-500. https://doi.org/10.1111/j.1365-2435.2008.01387.x
30. Diaz GJ, Squires EJ, Julian RJ. The use of selected plasma enzyme activities for the diagnosis of fatty liver-hemorrhagic syndrome in laying hens. Avian Dis. 1999;43:768-73. https://doi.org/10.2307/1592746
31. Choi YI, Ahn HJ, Lee BK, Oh ST, An BK, Kang CW. Nutritional and hormonal induction of fatty liver syndrome and effects of dietary lipotropic factors in egg-type male chicks. Asian-Australas J Anim Sci. 2012;25:1145-52. https://doi.org/10.5713/ajas.2011.11418
32. Association of Official Analytical Chemists (AOAC). Official Methods of Analysis. 18th ed. AOAC; 2007.
33. Weimer SL, Mauromoustakos A, Karcher DM, Erasmus MA. Differences in performance, body conformation, and welfare of conventional and slow-growing broiler chickens raised at 2 stocking densities. Poult Sci. 2020;99:4398-407. https://doi.org/10.1016/j.psj.2020.06.009

34. Welfare Quality. Welfare Quality® Assessment protocol for poultry. 1st ed. Welfare Quality® Consortium; 2009.
35. Luna LG. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. 3rd ed. McGraw Hill; 1968.
36. Tarachai P, Yamauchi K. Effects of luminal nutrient absorption, intraluminal physical stimulation, and intravenous parenteral alimentation on the recovery responses of duodenal villus morphology following feed withdrawal in chickens. Poult Sci. 2000;79:1578-85. https://doi.org/10.1093/ps/79.11.1578
37. Santos RR, Awati A, Roubos-van den Hil PJ, Tersteeg-Zijderveld MHG, Koolmees PA, Fink-Gremmels J. Quantitative histo-morphometric analysis of heat-stress-related damage in the small intestines of broiler chickens. Avian Pathol. 2015;44:19-22. https://doi.org/10.1080/03079457.2014.988122
38. Shin JE, Kim JH, Goo D, Han GP, Pitargue FM, Kang HK, et al. Effect of dietary supplementation of betaine on productive performance, egg quality and jejunal tight junction-related gene expression in laying hens raised under hot environmental conditions. Livest Sci. 2018;214:79-82. https://doi.org/10.1016/j.livsci.2018.05.013
39. Aznar R, Alarcón B. On the specificity of PCR detection of listeria monocytogenes in food: a comparison of published primers. Syst Appl Microbiol. 2002;25:109-19. https://doi.org/10.1078/0723-2020-00079
40. Thomsen R, Sølvsten CAE, Linnet TE, Blechingberg J, Nielsen AL. Analysis of qPCR data by converting exponentially related Ct values into linearly related X0 values. J Bioinf Comput Biol. 2010;8:885-900. https://doi.org/10.1142/S0219720010004963
41. Steel RGD, Torrie JH, Dicky DA. Principles and procedures of statistics, a biometrical approach. 3rd ed. McGraw Hill; 1997.
42. Cheng TK, Coon CN, Hamre ML. Effect of environmental stress on the ascorbic acid requirement of laying hens. Poult Sci. 1990;69:774-80. https://doi.org/10.3382/ps.0690774

43. Najafi P, Zulkifli I, Jajuli NA, Farjam AS, Ramiah SK, Amir AA, et al. Environmental temperature and stocking density effects on acute phase proteins, heat shock protein 70, circulating corticosterone and performance in broiler chickens. Int J Biometeorol. 2015;59:1577-83. https://doi.org/10.1007/s00484-015-0964-3
44. Kim HW, Lee SY, Kim YB, Kim JH. Influence of functional nutrients on poultry performance and health by reducing stress response under environmental stress. Worlds Poult Sci J. 2025;81:879-915. https://doi.org/10.1080/00439339.2025.2506399
45. Olgun O, Abdulqader AF, Karabacak A. The importance of nutrition in preventing heat stress at poultry. Worlds Poult Sci J. 2021;77:661-78. https://doi.org/10.1080/00439339.2021.1938340
46. Khalid A, Bashir S, Kalsoom A, Rehman HFU, Rashid MA, Sandhu MA, et al. Effects of high dietary threonine supplementation on growth performance, health biomarkers, and intestinal histology in cyclic heat-stressed broilers. Vet World. 2025;18:646-57. https://doi.org/10.14202/vetworld.2025.646-657
47. Brugaletta G, Laghi L, Zampiga M, Oliveri C, Indio V, Piscitelli R, et al. Metabolic and microbiota response to arginine supplementation and cyclic heat stress in broiler chickens. Front Physiol. 2023;14:1155324. https://doi.org/10.3389/fphys.2023.1155324
48. Goo D, Kim JH, Choi HS, Park GH, Han GP, Kil DY. Effect of stocking density and sex on growth performance, meat quality, and intestinal barrier function in broiler chickens. Poult Sci. 2019;98:1153-60. http://dx.doi.org/10.3382/ps/pey491
49. Andretta I, Kipper M, Schirmann GD, Franceschina CS, Ribeiro AML. Modeling the performance of broilers under heat stress. Poult Sci. 2021;100:101338. https://doi.org/10.1016/j.psj.2021.101338
50. Routman KS, Yoshida L, Frizzas de Lima AC, Macari M, Pizauro Jr JM. Intestinal and pancreas enzyme activity of broilers exposed to thermal stress. Braz J Poult Sci. 2003;5:23-7. https://doi.org/10.1590/S1516-635X2003000100003

51. Al-Zghoul MB, Alliftawi ARS, Saleh KMM, Jaradat ZW. Expression of digestive enzyme and intestinal transporter genes during chronic heat stress in the thermally manipulated broiler chicken. Poult Sci. 2019;98:4113-22. http://dx.doi.org/10.3382/ps/pez249
52. Kim HW, Kim JH, Han GP, Kil DY. Increasing concentrations of dietary threonine, tryptophan, and glycine improve growth performance and intestinal health with decreasing stress responses in broiler chickens raised under multiple stress conditions. Anim Nutr. 2024;18:145-53. https://doi.org/10.1016/j.aninu.2024.03.018
53. Petracci M, Mudalal S, Soglia F, Cavani C. Meat quality in fast-growing broiler chickens. Worlds Poult Sci J. 2015;71:363-74. https://doi.org/10.1017/S0043933915000367
54. Zaboil G, Huang X, Feng X, Ahn DU. How can heat stress affect chicken meat quality? - a review. Poult Sci. 2019;98:1551-6. http://dx.doi.org/10.3382/ps/pey399
55. Mancinelli AC, Baldi G, Soglia F, Mattioli S, Sirri F, Petracci M, et al. Impact of chronic heat stress on behavior, oxidative status and meat quality traits of fast-growing broiler chickens. Front Physiol. 2023;14:1242094. https://doi.org/10.3389/fphys.2023.1242094
56. Tong HB, Lu J, Zou JM, Wang Q, Shi SR. Effects of stocking density on growth performance, carcass yield, and immune status of a local chicken breed. Poult Sci. 2012;91:667-73. https://doi.org/10.3382/ps.2011-01597
57. Kimball SR, Jefferson LS. Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. J Nutr. 2006;136:227S-31S. https://doi.org/10.1093/jn/136.1.227S
58. Tesseraud S, Abbas M, Duchene S, Bigot K, Vaudin P, Dupont J. Mechanisms involved in the nutritional regulation of mRNA translation: features of the avian model. Nutr Res Rev. 2006;19:104-16. https://doi.org/10.1079/NRR2006120
59. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. Cell. 2012;149:274-93. https://doi.org/10.1016/j.cell.2012.03.017

60. Tang Q, Tan P, Ma N, Ma X. Physiological functions of threonine in animals: beyond nutrition metabolism. Nutrients. 2021;13:2592. https://doi.org/10.3390/nu13082592
61. Chen Y, Zhang H, Cheng Y, Li Y, Wen C, Zhou Y. Dietary L-threonine supplementation attenuates lipopolysaccharide-induced inflammatory responses and intestinal barrier damage of broiler chickens at an early age. Br J Nutr. 2018;119:1254-62. https://doi.org/10.1017/S0007114518000740
62. Sapolsky RM, Romero LM, Munck AU. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev. 2000;21:55-89. https://doi.org/10.1210/edrv.21.1.0389
63. Dong H, Lin H, Jiao HC, Song ZG, Zhao JP, Jiang KJ. Altered development and protein metabolism in skeletal muscles of broiler chickens (Gallus gallus domesticus) by corticosterone. Comp Biochem Physiol A Mol Integr Physiol. 2007;147:189-95. http://dx.doi.org/10.1016/j.cbpa.2006.12.034
64. Ataallahi M, Nejad JG, Song J, Kim J, Park K. Effects of feather processing methods on quantity of extracted corticosterone in broiler chickens. J Anim Sci Technol. 2020;62:884-92. https://doi.org/10.5187/jast.2020.62.6.884
65. Gross WB, Siegel HS. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. Avian Dis. 1983;27:972-9. https://doi.org/10.2307/1590198
66. Davis AK, Maney DL, Maerz JC. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol. 2008;22:760-72. https://doi.org/10.1111/j.1365-2435.2008.01467.x
67. Abo-Al-Ela HG, El-Kassas S, El-Naggar K, Abdo SE, Jahejo AR, Al Wakeel RA. Stress and immunity in poultry: light management and nanotechnology as effective immune enhancers to fight stress. Cell Stress Chaperones. 2021;26:457-72. https://doi.org/10.1007/s12192-021-01204-6

68. Post J, Rebel JMJ, ter Huurne AAHM. Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress. Poult Sci. 2003;82:1313-8. https://doi.org/10.1093/ps/82.8.1313
69. Romero LM, Fairhurst GD. Measuring corticosterone in feathers: Strengths, limitations, and suggestions for the future. Comp Biochem Physiol A Mol Integr Physiol. 2016;202:112-22. https://doi.org/10.1016/j.cbpa.2016.05.002
70. Lee C, Kim JH, Kil DY. Comparison of stress biomarkers in laying hens raised under a long-term multiple stress condition. Poult Sci. 2022;101:101868. https://doi.org/10.1016/j.psj.2022.101868
71. Fairhurst GD, Marchant TA, Soos C, Machin KL, Clark RG. Experimental relationships between levels of corticosterone in plasma and feathers in a free-living bird. J Exp Biol. 2013;216:4071-81. https://doi.org/10.1242/jeb.091280
72. Kwon B, Park J, Kim D, Lee K. Assessment of welfare problems in broilers: focus on musculoskeletal problems associated with their rapid growth. Animals. 2024;12:1116. https://doi.org/10.3390/ani14071116
73. Taira K, Nagai T, Obi T, Takase K. Effect of litter moisture on the development of footpad dermatitis in broiler chickens. J Vet Med Sci. 2014;76:583-6. https://doi.org/10.1292/jvms.13-0321
74. Dikmen BY, Gündüz M. Comparison of cage versus floor rearing systems on broiler performance, welfare and behavioral parameters. J Hell Vet Med Soc. 2025;76:8563-74. https://doi.org/10.12681/jhvms.35239
75. Tahamtani FM, Pedersen IJ, Riber AB. Effects of environmental complexity on welfare indicators of fast-growing broiler chickens. Poult Sci. 2020;99:21-9. https://doi.org/10.3382/ps/pez510

76. Kuter E, Cengiz Ö, Köksal BK, Sevim Ö, Tatlı O, Ahsan U, et al. Litter quality and incidence and severity of footpad dermatitis in heat stressed broiler chickens fed supplemental zinc. Livest Sci. 2023;267:105145. https://doi.org/10.1016/j.livsci.2022.105145
77. de Jong IC, Gunnink H, van Harn J. Wet litter not only induces footpad dermatitis but also reduces overall welfare, technical performance, and carcass yield in broiler chickens. 2014;23:51-8. https://doi.org/10.3382/japr.2013-00803
78. Surai PF, Kochish II, Fisinin VI, Kidd MT. Antioxidant defence systems and oxidative stress in poultry biology: an update. Antioxidants. 2019;8:235. https://doi.org/10.3390/antiox8070235
79. Akbarian A, Michiels J, Degroote J, Majdeddin M, Golian A, De Smet S. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. J Anim Sci Biotechnol. 2016;7:37. https://doi.org/10.1186/s40104-016-0097-5
80. Oke OE, Akosile OA, Oni AI, Opowoye IO, Ishola CA, Adebiyi JO, et al. Oxidative stress in poultry production. Poult Sci. 2024;103:104003. https://doi.org/10.1016/j.psj.2024.104003
81. Aryal B, Kwakye J, Ariyo OW, Ghareed AFA, Milfort MC, Fuller AL, et al. Major oxidative and antioxidant mechanisms during heat stress-induced oxidative stress in chickens. Antioxidants. 2025;14:471. https://doi.org/10.3390/antiox14040471
82. Radi R. Peroxynitrite, a stealthy biological oxidant. J Biol Chem. 2013;228:26464-72. https://doi.org/10.1074/jbc.R113.472936
83. He F, Wu C, Li P, Li N, Zhang D, Zhu Q, et al. Functions and signaling pathways of amino acids in intestinal inflammation. Biomed Res Int. 2018;2018:9171905. https://doi.org/10.1155/2018/9171905

84. Liu K, Jia M, Wong EA. Delayed access to feed affects broiler small intestinal morphology and goblet cell ontogeny. Poult Sci. 2020;99:5275-85. https://doi.org/10.1016/j.psj.2020.07.040
85. Mishra B, Jha R. Oxidative stress in the poultry gut: potential challenges and interventions. Front Vet Sci. 2019;6:60. https://doi.org/10.3389/fvets.2019.00060
86. Kridtayopas C, Rakangtong C, Bunchasak C, Loongyai W. Effect of prebiotic and synbiotic supplementation in diet on growth performance, small intestinal morphology, stress, and bacterial population under high stocking density condition of broiler chickens. Poult Sci. 2019;98:4595-605. http://dx.doi.org/10.3382/ps/pez152
87. Mazzoni M, Zampiga M, Clavenzani P, Lattanzio G, Tagliavia C, Sirri F. Effect of chronic heat stress on gastrointestinal histology and expression of feed intake-regulatory hormones in broiler chickens. Animal. 2022;16:100600. https://doi.org/10.1016/j.animal.2022.100600
88. Moal VL, Servin AL. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. Clin Microbiol Rev. 2006;19:315-37. https://doi.org/10.1128/cmr.19.2.315-337.2006
89. Dharmani P, Srivastava V, Kissoon-Singh V, Chadee K. Role of intestinal mucins in innate host defense mechanisms against pathogens. J Innate Immun. 2009;1:123-35. https://doi.org/10.1159/000163037
90. Johansson MEV, Hansson GC. Goblet cells need some stress. J Clin Invest. 2022;132:e162030. https://doi.org/10.1172/JCI162030
91. Luo K, Cao SS. Endoplasmic reticulum stress in intestinal epithelial cell function and inflammatory bowel disease. Gastroenterol. Res Pract. 2015;2015:328791. http://dx.doi.org/10.1155/2015/328791

92. Ma X, Dai Z, Sun K, Zhang Y, Chen J, Yang Y, et al. Intestinal epithelial cell endoplasmic reticulum stress and inflammatory bowel disease pathogenesis: an update review. Front Immunol. 2017;8:1271. https://doi.org/10.3389/fimmu.2017.01271
93. Zhang C, Zhao XH, Yang L, Chen XY, Jiang RS, Jin SH, et al. Resveratrol alleviates heat stress-induced impairment of intestinal morphology, microflora, and barrier integrity in broilers. Poult Sci. 2017;96:4325-32. http://dx.doi.org/10.3382/ps/pex266
94. Duangnumsawang Y, Zentek J, Boroojeni FG. Development and functional properties of intestinal mucus layer in poultry. Front Immunol. 2021;12:745849. https://doi.org/10.3389/fimmu.2021.745849
95. Al-Sadi R, Boivin M, Ma T. Mechanism of cytokine modulation of epithelial tight junction barrier. Front Biosci. 2009;14:2765-78. https://doi.org/10.2741/3413
96. Fathima S, Al Hakeem WG, Selvaraj RK, Shanmugasundaram R. Beyond protein synthesis: the emerging role of arginine in poultry nutrition and host-microbe interactions. Front Physiol. 2024;14:1326809. https://doi.org/10.3389/fphys.2023.1326809
97. Balakrishnan KN, Ramiah SK, Zulkifli I. Heat shock protein response to stress in poultry: A review. Animals. 2023;13:317. https://doi.org/10.3390/ani13020317
98. Kampinga HH, Hageman J, Vos MJ, Kubota H, Tanguay RM, Bruford EA, et al. Guidelines for the nomenclature of the human heat shock proteins. Cell Stress Chaperones. 2009;14:105-11. https://doi.org/10.1007/s12192-008-0068-7
99. Madkour M, Salman FM, El-Wardany I, Abdel-Fattah SA, Alagawany M, Hashem NM, et al. Mitigating the detrimental effects of heat stress in poultry through thermal conditioning and nutritional manipulation. J Therm Biol. 2022;103:103169. https://doi.org/10.1016/j.jtherbio.2021.103169
100. Kregel KC. Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. J Appl Physiol. 2002;92:2177-86. https://doi.org/10.1152/japplphysiol.01267.2001

101. Oakley RH, Cidlowski JA. The biology of the glucocorticoid receptor: New signaling mechanisms in health and disease. J Allergy Clin Immunol. 2013;132:1033-44. https://doi.org/10.1016/j.jaci.2013.09.007

102. Farré R, Fiorani M, Rahiman SA, Matteoli G. Intestinal permeability, inflammation and the role of nutrients. Nutrients. 2020;12:1185. http://dx.doi.org/10.3390/nu12041185

103. Buzza MS, Netzel-Arnett S, Shea-Donohue T, Zhao A, Lin C, List K, et al. Membrane-anchored serine protease matriptase regulates epithelial barrier formation and permeability in the intestine. Proc Natl Acad Sci USA. 2010;107:4200-5. https://doi.org/10.1073/pnas.0903923107

885 **Table 1.** Composition and nutrient levels of basal diet fed to broiler chickens from 1 to 21 d of

886 age (as-fed basis)

Items	Basal diet
Ingredient, %	
Corn	56.67
Soybean meal, 46% CP	30.22
Corn gluten meal	6.39
Soybean oil	2.19
MCP	1.75
Limestone	1.14
Salt	0.30
98.5% DL-Met	0.29
55% L-Lys-H_2SO_4	0.44
98.5% L-Thr	0.02
99% L-Arg	0.04
50% Choline	0.10
$NaHCO_3$	0.15
Vitamin premix[1)]	0.15
Mineral premix[2)]	0.15
Total	100.00
Calculated energy and nutrient content	
AME_n, kcal/kg	3,013
CP, %	22.25
Digestible Lys, %	1.25
Digestible Met + Cys, %	0.96
Digestible Trp, %	0.21

Digestible Thr, %	0.84
Digestible Arg, %	1.34
Total calcium, %	0.85
Available phosphorus, %	0.46

[1)]Provided per kilogram of the complete diet: vitamin A (from vitamin A acetate), 12,000 IU; vitamin D_3, 4,000 IU; vitamin E (from DL-α-tocopheryl acetate), 80 mg; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 10 mg; vitamin B_6, 6 mg; vitamin B_{12}, 20 μg; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 200 μg; niacin, 60 mg.

[2)]Provided per kilogram of the complete diet: iron, 57.14 mg; copper, 16 mg; zinc, 64.29 mg; manganese, 85.71 mg; cobalt, 170 μg; selenium, 200 μg; iodine, 570 μg.

Table 2. Composition and nutrient levels of experimental diets fed to broiler chickens from 21 to 35 d of age (as-fed basis)

Items	Dietary treatments[1)]		
	Basal diet	Thr	Arg
Ingredient, %			
Corn	65.23	65.23	66.10
Soybean meal, 46% CP	22.89	22.94	22.84
Corn gluten meal	6.28	5.94	5.00
Soybean oil	1.74	1.79	1.77
MCP	1.32	1.32	1.32
Limestone	0.86	0.86	0.86
Salt	0.30	0.30	0.30
98.5% DL-Met	0.26	0.26	0.28
55% L-Lys-H_2SO_4	0.49	0.49	0.50
98.5% L-Thr	0.00	0.23	0.02
99% L-Arg	0.08	0.09	0.46
50% Choline	0.10	0.10	0.10
$NaHCO_3$	0.15	0.15	0.15
Vitamin premix[2)]	0.15	0.15	0.15
Mineral premix[3)]	0.15	0.15	0.15
Total	100.00	100.00	100.00
Calculated energy and nutrient content			
AME_n, kcal/kg	3,100	3,100	3,100
CP, %	19.50	19.50	19.50
Digestible Lys, %	1.08	1.08	1.08
Digestible Met + Cys, %	0.86	0.86	0.86

Digestible Trp, %	0.18	0.18	0.18
Digestible Thr, %	0.72	0.94	0.72
Digestible Arg, %	1.17	1.17	1.52
Total calcium, %	0.65	0.65	0.65
Available phosphorus, %	0.36	0.36	0.36
Analyzed nutrient content			
Total Thr, %	0.74	0.96	0.78
Total Arg, %	1.16	1.13	1.48

[1)]Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

[2)]Provided per kilogram of the complete diet: vitamin A (from vitamin A acetate), 12,000 IU; vitamin D_3, 4,000 IU; vitamin E (from DL-α-tocopheryl acetate), 80 mg; vitamin K_3, 4 mg; vitamin B_1, 4 mg; vitamin B_2, 10 mg; vitamin B_6, 6 mg; vitamin B_{12}, 20 µg; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 200 µg; niacin, 60 mg.

[3)]Provided per kilogram of the complete diet: iron, 57.14 mg; copper, 16 mg; zinc, 64.29 mg; manganese, 85.71 mg; cobalt, 170 µg; selenium, 200 µg; iodine, 570 µg.

Table 3. The sequence of the primers used in quantitative real-time PCR

RNA target	Primer sequence	Size for PCR product, bp	Accession no.
GAPDH	F: 5'-ATGGCATCCAAGGAGTGAGC-3' R: 5'-GGGAACAGAACTGGCCTCTC-3'	130	NM_204305.2
OCLN	F: 5'-GTGGAGTCCAGTGATGAGCG-3' R: 5'-TGTCCATCTCAGCACAGAGC-3'	142	NM_205128.1
CLDN1	F: 5'-GCTGACCTGTACTTGAGCTG-3' R: 5'-TGGCACAGGGTTAATGCAAA-3'	171	NM_001013611.2
ZO-1	F: 5'-AGGTGAAGTGTTTCGGGTTG-3' R: 5'-AGAAATCCGCTCGATCTCCT-3'	188	XM_015278975.1
JAM2	F: 5'-GTGAATTTACAGTTCCTCCC-3' R: 5'-GTTATGTTGGCTGTTCTAGC-3'	187	NM_001006257.2
HSP70	F: 5'-CCGTGGAGTTCCTCAGATCG-3' R: 5'-CTCTGCCATCTGGTTTCGGT-3'	361	NM_001006685.2

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *OCLN*, occludin; *CLDN1*, claudin-1; *ZO-1*, zonula occludens-1; *JAM2*, junctional adhesion molecule B; *HSP70*, heat shock protein 70.

907 **Table 4.** Effects of dietary threonine and arginine on growth performance of broiler chickens raised under multiple stress conditions[1)]

	Treatments[2)]					
Items	PC	NC	Thr	Arg	SEM	*p*-value
BW, g	1,808[a]	1,471[b]	1,461[b]	1,477[b]	24.0	<0.001
BWG, g	1,112[a]	776[b]	765[b]	781[b]	24.0	<0.001
FI, g	1,553[a]	1,209[b]	1,173[b]	1,245[b]	27.0	<0.001
FE, g/kg	715[a]	642[b]	652[b]	627[b]	10.9	<0.001
Mortality, %	2.5	1.3	0.0	1.3	1.53	0.723

908 [a,b]Means within a variable with no common superscript differ significantly ($p < 0.05$).

909 [1)]Data are least squares means of 8 observations per treatment.

910 [2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress
911 conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-
912 fold higher digestible arginine.

913 BW, body weight; BWG, body weight gain; FI, feed intake; FE, feed efficiency.

Table 5. Effects of dietary threonine and arginine on breast meat quality of broiler chickens raised under multiple stress conditions[1)]

Items		Treatments[2)]				SEM	*p*-value
		PC	NC	Thr	Arg		
Breast yield, %		20.1	20.0	21.6	21.1	0.50	0.083
pH	1 h	6.1	6.2	6.1	6.1	0.09	0.468
	24 h	5.3	5.5	5.4	5.5	0.09	0.098
Meat color	L*	48.8	47.2	48.4	49.2	0.62	0.166
	a*	3.8	3.5	3.5	3.6	0.27	0.818
	b*	9.4	10.2	10.3	10.3	0.61	0.700
Water holding capacity, %		71.1	67.9	68.9	68.6	2.42	0.811
TBARS, mg MDA/kg meat		0.34	0.31	0.31	0.31	0.015	0.368

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

L*, lightness; a*, redness; b*, yellowness; TBARS, tiobarbituric acid reactive substances; MDA, malondialdehyde.

Table 6. Effects of dietary threonine and arginine on relative organ weight of broiler chickens raised under multiple stress conditions[1)]

Items	Treatments[2)]				SEM	*p*-value
	PC	NC	Thr	Arg		
Liver, %	2.11	2.00	1.92	1.91	0.062	0.113
Spleen, %	0.09	0.09	0.10	0.07	0.008	0.203
Kidney, %	0.54	0.53	0.54	0.53	0.026	0.985
bursa of Fabricius, %	0.24	0.20	0.19	0.19	0.032	0.647
Thymus, %	0.32	0.31	0.29	0.27	0.023	0.427

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

Table 7. Effects of dietary threonine and arginine on serum biochemical parameters of broiler chickens raised under multiple stress conditions[1)]

Items	Treatments[2)]				SEM	*p*-value
	PC	NC	Thr	Arg		
ALT, U/L	3.38[b]	4.00[a]	3.63[ab]	4.13[a]	0.196	0.044
AST, U/L	278	298	279	280	16.8	0.816
Creatinine, mg/dL	0.08	0.10	0.09	0.10	0.013	0.654
Uric acid, mg/dL	5.85	4.21	4.13	3.85	0.637	0.133

[a,b]Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 8. Effects of dietary threonine and arginine on blood hematological parameters of broiler chickens raised under multiple stress conditions[1)]

Items	Treatments[2)]				SEM	*p*-value
	PC	NC	Thr	Arg		
RBC, 10^6/μL	2.27	2.45	2.39	2.22	0.118	0.524
WBC, 10^6/μL	24.5	18.6	18.8	23.3	7.06	0.917
Hb, g/dL	6.78	7.15	6.59	6.45	0.234	0.192
Hct, %	30.7	32.2	31.4	29.5	1.38	0.550
Segment, %	26.4	28.0	17.6	30.5	7.21	0.619
Lymphocyte, %	72.3	68.9	81.4	68.5	7.08	0.557
Monocyte, %	0.63	2.65	0.44	0.53	1.004	0.357
Eosinophil, %	0.00	0.01	0.03	0.00	0.014	0.541
Basophil, %	0.61	0.38	0.54	0.53	0.259	0.930

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

RBC, red blood cell; WBC, white blood cell; Hb, hemoglobin; Hct, hematocrit.

Table 9. Effects of dietary threonine and arginine on stress indicators of broiler chickens raised under multiple stress conditions[1)]

Items	Treatments[2)]				SEM	*p*-value
	PC	NC	Thr	Arg		
Blood H:L ratio	0.20[b]	0.30[a]	0.29[a]	0.28[a]	0.020	0.006
Feather corticosterone, pg/mg	3.43[b]	5.07[a]	2.69[b]	3.11[b]	0.439	0.005

[a,b]Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

H:L ratio, heterophil to lymphocyte ratio.

Table 10. Effects of dietary threonine and arginine on liver characteristics of broiler chickens raised under multiple stress conditions[1)]

Items		Treatments[2)]				SEM	*p*-value
		PC	NC	Thr	Arg		
Liver color	L*	22.2	22.1	23.0	22.0	0.70	0.710
	a*	13.8	13.4	13.6	13.2	0.52	0.854
	b*	2.5	2.4	3.1	2.2	0.50	0.624
Hemorrhagic score[3)]		0.16	0.63	0.34	0.47	0.212	0.465
Fatty liver score[4)]		1.13	1.03	1.09	1.03	0.074	0.753
Fat concentration, %		21.9	21.7	23.1	22.5	1.28	0.852

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

[3)]The hemorrhagic score is graded subjectively on a scale from 0 to 5 with 0 indicating normal liver, 1 up to 10 subcapsular petechial or ecchymotic hemorrhages, 2 for more than 10 subcapsular petechial or ecchymotic hemorrhages, and 3 to 5 for large and massive hemorrhages [30].

[4)]The color scored from 1 (dark red) to 5 (light yellowish red), the color of normal liver was scored 1 and the color score from 2 to 5 (rom dark red to light yellowish red) [31].

L*, lightness; a*, redness; b*, yellowness.

Table 11. Effects of dietary threonine and arginine on animal welfare score of broiler chickens raised under multiple stress conditions[1)]

Items	Treatments[2)]				SEM	*p*-value
	PC	NC	Thr	Arg		
Gait score	0.36	0.59	0.35	0.35	0.123	0.447
Footpad dermatitis	0.04	0.06	0.13	0.06	0.030	0.179
Hock burn	0.18	0.22	0.21	0.13	0.075	0.840
Feather cleanliness	0.20	0.30	0.16	0.21	0.068	0.527

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

Table 12. Effects of dietary threonine and arginine on liver and jejunal antioxidant status of broiler chickens raised under multiple stress conditions[1)]

Items	Treatments[2]				SEM	*p*-value
	PC	NC	Thr	Arg		
Liver						
TAC, mM trolox/mg protein	0.14	0.14	0.14	0.14	0.005	0.655
MDA, μmol/mg protein	0.17	0.18	0.16	0.19	0.015	0.452
ROS, mM	42.0[b]	53.1[a]	45.5[ab]	42.7[b]	2.95	0.040
NO, μmol/mg protein	0.48	0.51	0.43	0.47	0.044	0.601
Jejunum						
TAC, mM trolox/mg protein	0.10	0.11	0.10	0.10	0.006	0.399
MDA, μmol/mg protein	1.08	1.05	1.04	1.04	0.082	0.976
ROS, mM	50.6	53.1	56.4	54.9	2.79	0.459
NO, μmol/mg protein	0.36[c]	1.02[a]	0.58[b]	0.46[bc]	0.060	<0.001

[a-c] Means within a variable with no common superscript differ significantly ($p < 0.05$).

963 [1)]Data are least squares means of 8 observations per treatment.

964 [2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress
965 conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-
966 fold higher digestible arginine.

967 TAC, total antioxidant capacity; MDA, malondialdehyde; ROS, reactive oxygen species; NO, nitric oxide.

Table 13. Effects of dietary threonine and arginine on jejunal morphology of broiler chickens raised under multiple stress conditions[1)]

Items	Treatments[2)]				SEM	*p*-value
	PC	NC	Thr	Arg		
VH, μm	1,119[a]	1,007[b]	1,000[b]	1,007[b]	30.2	0.027
VW, μm	180	164	161	159	6.2	0.096
CD, μm	170	160	148	153	7.8	0.239
VH:CD ratio	6.7	6.4	6.8	6.6	0.27	0.719
Goblet cell, cell/200 μm	29.1[a]	24.1[b]	27.4[ab]	24.0[b]	1.31	0.026

[a,b]Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

VH, villus height; VW, villus width; CD, crypt depth; VH:CD ratio, villus height to crypt depth ratio.

Table 14. Effects of dietary threonine and arginine on jejunal permeability of broiler chickens raised under multiple stress conditions[1)]

Items	Treatments[2)]				SEM	*p*-value
	PC	NC	Thr	Arg		
PD, mV	40[a]	27[c]	32[b]	32[b]	1.4	<0.001
Isc, μA/cm^2	200	197	197	201	5.0	0.913
TEER, Ω/cm^2	206[a]	137[c]	164[b]	165[b]	7.3	<0.001

[a-c]Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

PD, potential difference; Isc, short circuit current; TEER, transepithelial electrical resistance.

Table 15. Effects of dietary threonine and arginine on tight junction-related and stress-related gene expression of broiler chickens raised under multiple stress conditions[1)]

	Treatments[2)]					
Items	PC	NC	Thr	Arg	SEM	*p*-value
OCLN	1.00[a]	0.44[c]	0.67[b]	0.71[b]	0.054	<0.001
CLDN1	1.00[a]	0.47[b]	0.59[b]	0.60[b]	0.059	<0.001
ZO-1	1.00[a]	0.44[b]	0.90[a]	0.94[a]	0.076	<0.001
JAM2	1.00[a]	0.61[b]	1.66[b]	1.63[b]	0.080	0.005
HSP70	1.00[a]	0.52[c]	0.78[b]	0.86[ab]	0.097	<0.001

[a-c]Means within a variable with no common superscript differ significantly ($p < 0.05$).

[1)]Data are least squares means of 8 observations per treatment.

[2)]PC, positive control (thermoneutral conditions and basal diet); NC, negative control (multiple stress conditions and basal diet); Thr, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible threonine; Arg, multiple stress conditions and basal diet supplemented with 1.3-fold higher digestible arginine.

OCLN, occludin; *CLDN1*, claudin-1; *ZO-1*, zonula occludens-1; *JAM2*, junctional adhesion molecule B; *HSP70*, heat shock protein 70.