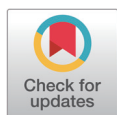


Effects of dietary threonine and arginine on growth performance, stress indicators, and gut health in broiler chickens under multiple stress

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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; $22.6 \pm 0.6^\circ\text{C}$ 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; $29.3 \pm 0.9^\circ\text{C}$ for 10 h, $22.8 \pm 0.8^\circ\text{C}$ 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 *OCN*, *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

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Availability of data and material

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions

Conceptualization: Kim JH.
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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).

Declaration of generative AI

No AI tools were used in this article.

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

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/m² 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/m² and 10 birds/cage). Normal and multiple stress conditions were established in two separate but identical rooms within the same

Table 1. Composition and nutrient levels of basal diet fed to broiler chickens from 1 to 21 d of 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 ₂ SO 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)	3,013.00
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 D₃, 4,000 IU; vitamin E (from DL- α -tocopheryl acetate), 80 mg; vitamin K₃, 4 mg; vitamin B₁, 4 mg; vitamin B₂, 10 mg; vitamin B₆, 6 mg; vitamin B₁₂, 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.

CP, crude protein; MCP, monocalcium phosphate; AMEn, nitrogen-corrected apparent metabolizable energy.

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 d 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 (98.5%, CJ bio) and Arg (99%, CJ bio) 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). 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 CO₂ 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) and a serum separator tube containing a clot activator (Greiner Bio-One). 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). Color characteristics including lightness (CIE L*), redness (CIE a*), and yellowness (CIE b*) were quantified using a spectro colorimeter (Konica Minolta). 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) and centrifuged at 1,610×g for 15 min at 4°C. The WHC was derived from the difference in moisture concentration before and after centrifugation. TBARS

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 ₂ SO 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)	3,100.00	3,100.00	3,100.00
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 D₃, 4,000 IU; vitamin E (from DL- α -tocopheryl acetate), 80 mg; vitamin K₃, 4 mg; vitamin B₁, 4 mg; vitamin B₂, 10 mg; vitamin B₆, 6 mg; vitamin B₁₂, 20 μ g; calcium pantothenate, 20 mg; folic acid, 2 mg; biotin, 200 μ g; 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 μ g; selenium, 200 μ g; iodine, 570 μ g.

CP, crude protein; MCP, monocalcium phosphate; AMEn, nitrogen-corrected apparent metabolizable energy.

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 μ L 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 1,610×g for 10 min. The absorbance of the resulting supernatant was measured at 531 nm using a microplate reader (INNO Microplate Spectrophotometer, LITEK).

Blood parameters

Serum biochemical parameters, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine, were analyzed using a Cobas C720 (Roche Diagnostics). Uric acid concentrations were determined with an automatic analyzer (Labospect 008AS, Hitachi High-Tech). 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).

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) and Giemsa stain solution (Samchun chemicals). After air drying, the stained smears were analyzed microscopically (OS-370DVM, Osun Hitech). 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), following the manufacturer's instructions.

Liver characteristics

Liver color was assessed promptly after euthanasia with a Minolta Chromameter CR-400 (Konica Minolta), recording the values for CIE 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) and EZ-Total Antioxidant Capacity Assay Kit (DG-TAC200, DoGenBio), 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). Reactive oxygen species (ROS) were quantified using the OxiSelect™ In Vitro ROS/RNS Assay Kit (STA-347, Cell Biolabs), and NO levels were determined using the Nitric Oxide Colorimetric Assay Kit (E-BC-K035-M, Elabscience), 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 Hitech). 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). 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% O₂ and 5% CO₂ mixture to sustain tissue function. TEER measurements were derived from recordings of potential difference, 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), as described by Shin et al. [38]. Total RNA was isolated from the jejunal mucosa utilizing TRIzol™ Reagent (15596026, Thermo Fisher Scientific), following the manufacturer's instructions. For cDNA synthesis, DNase I (RNase-free; EN0521, Thermo Fisher Scientific) treatment and the RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Fisher Scientific) were applied. The genes analyzed included occludin (*OCN*), claudin-1 (*CLDN1*), zonula occludens-1 (*ZO-1*), junctional adhesion molecule B (*JAM2*), 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^{-ΔΔC_t} method with glyceraldehyde-3-phosphate dehydrogenase as the reference gene [40].

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

RNA target	Primer sequence	Size for PCR product (bp)	Accession no.
<i>GAPDH</i>	F: 5'-ATGGCATCCAAGGAGTGAGC-3' R: 5'-GGGAACAGAACTGGCCTCTC-3'	130	NM_204305.2
<i>OCN</i>	F: 5'-GTGGAGTCCAGTGATGAGCG-3' R: 5'-TGTCCATCTCAGCACAGAGC-3'	142	NM_205128.1
<i>CLDN1</i>	F: 5'-GCTGACCTGTACTTGAGCTG-3' R: 5'-TGGCACAGGGTTAATGCAAA-3'	171	NM_001013611.2
<i>ZO-1</i>	F: 5'-AGGTGAAGTGTTCGGGTTG-3' R: 5'-AGAAATCCGCTCGATCTCCT-3'	188	XM_015278975.1
<i>JAM2</i>	F: 5'-GTGAATTTACAGTTCCTCCC-3' R: 5'-GTTATGTTGGCTGTTCTAGC-3'	187	NM_001006257.2
<i>HSP70</i>	F: 5'-CCGTGGAGTTCCTCAGATCG-3' R: 5'-CTCTGCCATCTGTTTCGGT-3'	361	NM_001006685.2

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

Statistical analysis

All statistical analyses were performed as a completely randomized design using the PROC MIXED procedure of SAS (SAS Institute). 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.

Table 4. Effects of dietary threonine and arginine on growth performance in broiler chickens raised under multiple stress conditions¹⁾

Items	Treatments ²⁾				SEM	p-value
	PC	NC	Thr	Arg		
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

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.

^{a,b}Means within a variable with no common superscript differ significantly ($p < 0.05$).

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 in broiler chickens raised under multiple stress conditions¹⁾

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	0.09	0.468
	24 h	5.3	5.5	5.4	0.09	0.098
Meat color	CIE L*	48.8	47.2	48.4	0.62	0.166
	CIE a*	3.8	3.5	3.5	0.27	0.818
	CIE b*	9.4	10.2	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.

TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

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 plumage 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 6. Effects of dietary threonine and arginine on relative organ weight in broiler chickens raised under multiple stress conditions¹⁾

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 in broiler chickens raised under multiple stress conditions¹⁾

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)	278.38	297.88	279.25	279.63	16.834	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

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.^{a,b}Means within a variable with no common superscript differ significantly ($p < 0.05$).

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Table 8. Effects of dietary threonine and arginine on blood hematological parameters in broiler chickens raised under multiple stress conditions¹⁾

Items	Treatments ²⁾				SEM	p-value
	PC	NC	Thr	Arg		
RBC ($10^6/\mu\text{L}$)	2.27	2.45	2.39	2.22	0.118	0.524
WBC ($10^6/\mu\text{L}$)	24.54	18.59	18.75	22.29	7.056	0.917
Hb (g/dL)	6.78	7.15	6.59	6.45	0.234	0.192
Hct (%)	30.68	32.23	31.43	29.48	1.381	0.550
Segment (%)	26.44	28.04	17.64	30.48	7.207	0.619
Lymphocyte (%)	72.33	68.93	81.36	68.48	7.083	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 in broiler chickens raised under multiple stress conditions¹⁾

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

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.^{a,b}Means within a variable with no common superscript differ significantly ($p < 0.05$).

H:L ratio, heterophil to lymphocyte ratio.

(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

Table 10. Effects of dietary threonine and arginine on liver characteristics in broiler chickens raised under multiple stress conditions¹⁾

Items		Treatments ²⁾				SEM	p-value
		PC	NC	Thr	Arg		
Liver color	CIE L*	22.2	22.1	23.0	22.0	0.70	0.710
	CIE a*	13.8	13.4	13.6	13.2	0.52	0.854
	CIE 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 (from dark red to light yellowish red) [31].

Table 11. Effects of dietary threonine and arginine on animal welfare score in broiler chickens raised under multiple stress conditions¹⁾

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
Plumage 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 in broiler chickens raised under multiple stress conditions¹⁾

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)	42.01 ^b	53.07 ^a	45.46 ^{ab}	42.74 ^b	2.948	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)	50.63	53.08	56.36	54.88	2.786	0.459
NO (μmol/mg protein)	0.36 ^c	1.02 ^a	0.58 ^b	0.46 ^{bc}	0.060	<0.001

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.

^{a-c}Means within a variable with no common superscript differ significantly ($p < 0.05$).

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

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.

Table 13. Effects of dietary threonine and arginine on jejunal morphology in broiler chickens raised under multiple stress conditions¹⁾

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

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.^{a,b}Means within a variable with no common superscript differ significantly ($p < 0.05$).

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

Jejunal permeability

Birds in PC group had greater ($p < 0.05$) potential difference 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 *OCN*, *CLDN1*, *ZO-1*, *JAM2*, and *HSP70* than those in PC group (Table 15). However, expression levels of *OCN*, *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

Table 14. Effects of dietary threonine and arginine on jejunal permeability in broiler chickens raised under multiple stress conditions¹⁾

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

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.^{a-c}Means within a variable with no common superscript differ significantly ($p < 0.05$).

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 in broiler chickens raised under multiple stress conditions¹⁾

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

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.

^{a-c)}Means within a variable with no common superscript differ significantly ($p < 0.05$).

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

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/m²) showed greater breast meat pH compared to those reared at low SD (12.5 birds/m²) [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 [62,67]. 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 plumage 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 *OCN*, *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 *OCN* 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 *OCN* 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.

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