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Original Research Article

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

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A B S T R A C T

The current study aimed to compare the effects of increasing concentrations of dietary threonine (Thr), tryptophan (Trp), and glycine (Gly) on growth performance, stress biomarkers, and intestinal function in broiler chickens under multiple stress conditions. Five hundred sixty broiler chickens at 21 d old were randomly allotted to 5 treatments with 8 replicates. Birds in a positive control (PC) treatment were raised under low stock density (16.9 birds/m^2 per cage) with recommended environmental conditions, whereas birds in 4 treatments were subjected to multiple stress conditions: a cyclic heat stress of 30 ± 0.3 °C for 10 h and 23 ± 0.2 °C for 14 h per day with high stock density (25.3 birds/m^2 per cage). A basal diet was assigned to both PC and negative control (NC) treatments. Three additional diets were individually formulated to contain double concentrations of digestible Thr, Trp, or Gly + Ser compared with their concentrations in the basal diet. The experiment lasted for 14 d. Results showed that NC treatment had less growth performance ($P < 0.001$), jejunal goblet cell counts ($P = 0.018$), and trans-epithelial electrical resistance (TEER; $P < 0.001$), but greater ($P = 0.026$) feather corticosterone (CORT) concentrations than PC treatment. Thr treatment showed the least ($P < 0.001$) feed conversion ratio (FCR) among treatments under multiple stress conditions. Thr, Trp, and Gly treatments had less ($P = 0.026$) feather CORT concentrations, but had greater ($P < 0.001$) TEER than NC treatment. In conclusion, increasing concentrations of dietary Thr, Trp, or Gly improve the growth performance and intestinal health in broiler chickens with decreasing stress response under multiple stress conditions.

1. Introduction

Stress is appreciated various physiological reactions in animals exposed to an unfavorable and harmful environment (Stott, 1981). Currently, heat stress is considered the most critical stressor in the poultry because global temperature is steadily increasing (Ratnakaran et al., 2017). Poultry is highly sensitive to heat stress and exhibits various abnormal physiological and behavior responses, which lead to an impairment in productive performance and health (Farag and Alagawany, 2018; Kim et al., 2023). High stocking density is also a critical stressor decreasing productive performance and health in poultry (Goo et al., 2019a). In a practical condition, however, poultry is typically raised under multiple stress conditions derived concurrently from both heat stress and high stocking density. However, research regarding the development of a possible solution to ameliorate stress responses and health problems in poultry exposed to the conventional multiple stress conditions is largely limited.

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Threonine (Thr) is generally considered the third limiting amino acid (AA) for broiler chickens (Kidd et al., 1999). In addition to its primary role in protein synthesis, the other major functions of Thr include maintenance of intestinal integrity, mucin synthesis, and stimulation of immune responses in poultry (Bassareh et al., 2023; Gum, 1992). Previous studies have reported that additional supplementation of dietary Thr over its requirement ameliorates stress responses and improves productive performance and health in broiler chickens (Azzam and El-Gogary, 2015; Eftekhari et al., 2015; Kolbadinejad and Rezaeipour, 2020).

Tryptophan (Trp) is also considered a limiting AA for broiler chickens because of the relatively low concentrations of Trp in the practical corn and soybean meal based-diet (Barua et al., 2021). Recently, Trp supplementation in poultry diets has gained increasing attention because Trp is a precursor AA for serotonin and melatonin synthesis, which is associated with regulation of blood pressure, body temperature, feed intake (FI), growth, and repair of tissues (Le Floc'h et al., 2011; Newberry and Blair, 1993). It has been documented that additional supplementation of Trp in diets decreases stress responses and improves growth performance and health in broiler chickens (Bello et al., 2018; Wang et al., 2014).

Glycine (Gly) is the nonessential AA, but is often considered conditionally essential AA for poultry (Corzo et al., 2004) because its requirement is increased by particular internal and external factors such as feeding low protein diets and exposure to stressful environment (Awad et al., 2015). In particular, the requirements of dietary Gly are possibly increased by increasing metabolic needs of Gly as a precursor AA for the synthesis of purine, glutathione, bile salt, uric acid, and creatine in poultry (Kim et al., 2022; Wyss and Kaddurah-Daouk, 2000). Our previous experiment reported that additional supplementation of dietary Gly improved performance and health with reducing stress responses in broiler chickens (Won et al., 2023).

As a consequence, increasing concentrations of dietary Thr, Trp, and Gly can be expected to improve production and health in broiler chickens exposed to stress conditions. However, no studies have been conducted to compare functional effects of these 3 individual AA in broiler chickens under stress conditions. Furthermore, although the commercial broiler chickens are typically raised under multiple stress conditions, most of previous studies regarding development of dietary treatments for stress managements were performed under a single stress condition of either heat stress or high stocking density, thereby leading to a limitation of the practical application of heat stress-reducing functional nutrients for broiler chickens. Therefore, the present study aimed to compare the effects of increasing concentrations of dietary Thr, Trp, and Gly on growth performance, meat quality, stress biomarkers, and intestinal barrier function in broiler chickens under multiple stress conditions with heat stress and high stocking density.

2. Materials and methods

2.1. Animal ethics

The protocol for the present experiment was approved by the Institutional Animal Care and the Use Committee (IACUC) at Chung-Ang University (IACUC approval no. A2021041). The experiment complied with the ARRIVE guidelines.

2.2. Animals and husbandry

All birds used in this experiment were raised according to the Ross 308 broiler management guideline and were fed a commercial-type diet satisfying energy and nutrient requirements before the start of the experiment. Five hundred sixty broiler chickens at 21 d old with similar body weight (BW) (initial BW = 965.3 ± 0.11 g) were allotted to 5 treatments with 8 replicates per treatment in a completely randomized design. All birds were placed in battery cages (76 cm × 78 cm × 45 cm = width × length × height for each cage). Birds were raised under normal conditions with recommended temperature and humidity (on average, 23 ± 0.7 °C and 44.3% ± 5.08%) and normal stocking density or multiple stress conditions with heat stress and high stocking density. Birds in multiple stress conditions were raised under cyclic heat stress conditions at 30 ± 0.3 °C for 10 h/d and 23 ± 0.2 °C for the remaining time. The average relative humidity was 43.6 ± 4.62%. In addition, 15 birds (25.3 birds/m^2 per cage) were assigned in multiple stress conditions to increase stocking density, whereas 10 birds (16.9 birds/m^2 per cage) were assigned in normal stocking density conditions. A 24-h lighting schedule was used during the whole experiment.

2.3. Dietary treatments

A basal diet was formulated to meet or exceed the energy and nutrient requirement estimates of the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011). This basal diet was provided to birds under normal conditions as the positive control (PC) or those in one treatment group under multiple stress conditions as the negative control (NC). Three additional diets were formulated to increase the concentrations of digestible Thr, Trp, and Gly to 2-fold higher than their respective concentrations in the basal diet, but the same CP concentrations were maintained in all diets (Table 1). The concentrations of 3 AA in treatment diets were determined based on previous studies reporting the beneficial effects of 3 AA on productive performance and health in broiler chickens under stress conditions (Bello et al., 2018; Ismaiel, 2011; Won et al., 2023). The purified form of supplemental Thr (99%, CJ bio, Seoul, Republic of Korea), Trp (99%, CJ bio, Seoul, Republic of Korea), and Gly (99%, Samin chem, Siheung-si, Republic of Korea) was used to increase AA concentrations in diets. The standardized ileal digestible (SID) AA concentrations of ingredients presented in the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011) were used to calculate AA concentrations in diets. However, no information regarding concentrations of digestible Gly itself was available; therefore, the concentrations of digestible Gly in this study was designed to increase concentrations of digestible Gly + Ser (3.20%) than in the basal diet (1.60%). These dietary treatments with increasing concentrations of dietary Thr, Trp, and Gly were assigned in multiple stress conditions. Birds were freely accessed to the diet and water during 14 d of the overall feeding period. The diets were analyzed for CP using the Kjeldahl method (method 990.03; AOAC, 2007) and for total Thr, Trp, Gly, and Ser using L8900 amino acid analyzer (Hitachi, Tokyo, Japan; method 982.30; AOAC, 2007). Moreover, total calcium and total phosphorus of diets were measured using an inductively coupled plasma spectrometer (method 984.27; AOAC, 2007).

2.4. Performance measurement and sampling

The BW gain (BWG) and FI were recorded at the end of the experiment (i.e., 35 d of age). The feed conversion ratio (FCR; g/g) was calculated after mortality correction for FI (Kim et al., 2017).

At the conclusion of the experiment, 2 birds per replicate were selected based on a BW close to the average BW per replicate. One bird was euthanized by CO_2 asphyxiation to collect tissue samples for further analysis and the other bird was used to analyze intestinal permeability.

Table 1
Composition and nutrient levles of experimental diets (%, as-fed basis).

Item	Dietary treatments[1]			
	CON	Thr	Trp	Gly
Ingredients				
Corn	61.81	61.83	61.97	63.29
Soybean meal, 45% CP	27.80	27.77	27.80	26.66
Corn gluten meal	3.53	2.60	3.20	0.50
Soybean oil	3.13	3.29	3.07	3.85
MDCP	1.14	1.14	1.14	1.14
Limestone	1.06	1.06	1.06	1.06
DL-Met	0.24	0.26	0.25	0.31
L-Lys HCl	0.26	0.26	0.26	0.30
L-Thr	0.08	0.79	0.09	0.14
L-Trp	0.00	0.01	0.20	0.02
Gly	0.00	0.04	0.01	1.78
Salt	0.20	0.20	0.20	0.20
Choline (50%)	0.10	0.10	0.10	0.10
$NaHCO_3$	0.30	0.30	0.30	0.30
Coccidiostats	0.10	0.10	0.10	0.10
Antioxidant	0.05	0.05	0.05	0.05
Vitamin premix[2]	0.10	0.10	0.10	0.10
Mineral premix[3]	0.10	0.10	0.10	0.10
Total	100.00	100.00	100.00	100.00
Calculated energy and nutrient level[4]				
AME_n, kcal/kg	3,100	3,100	3,100	3,100
CP	19.25	19.25	19.25	19.25
Digestible Lys	1.04	1.04	1.04	1.04
Digestible Met + Cys	0.76	0.76	0.76	0.76
Digestible Met	0.49	0.50	0.50	0.52
Digestible Thr	0.68	1.36	0.68	0.68
Digestible Trp	0.19	0.19	0.38	0.19
Digestible Gly + Ser	1.60	1.60	1.60	3.20
Total calcium	0.71	0.71	0.71	0.71
Available phosphorus	0.33	0.33	0.33	0.33
Analyzed nutrient level				
CP,	20.3	20.0	20.1	19.5
Total Thr	0.85	1.32	0.80	0.77
Total Trp	0.19	0.19	0.31	0.17
Total Gly	0.78	0.79	0.77	2.37
Total Gly + Ser	1.75	1.73	1.69	3.10
Total calcium	0.71	0.72	0.75	0.74
Total phosphorus	0.50	0.52	0.55	0.51

MDCP= monodicalcium phosphate; AME_n= nitrogen-corrected apparent metabolizable energy.

[1] CON, diet (basal diet) was formulated to meet or exceed the energy and nutrient requirement estimates of the Brazilian Tables for Poultry and Swine (Rostagno et al., 2011). Thr, Trp, and Gly treatment diets had 2-fold higher digestible concentrations of Thr, Trp, and Gly + Ser than the basal diet, respectively, by supplementing 0.79% L-Thr, 0.20% L-Trp, and 1.78% Gly in the basal diet.

[2] Provided per kilogram of the complete diet: vitamin A (from vitamin A acetate), 13,000 IU; vitamin D_3, 5,000 IU; vitamin E (from $_{DL}$-α-tocopherol acetate), 80 IU; 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: Zn as ZnO, 100 mg; Mn as $MnSO_2 \cdot H_2O$, 120 mg; Fe as $FeSO_4 \cdot 7H_2O$, 60 mg; Cu as $CuSO_4 \cdot 5H_2O$, 16 mg; Co as $CoCO_3$, 1000 μg; I as $Ca(IO_3)_2 \cdot H_2O$, 1.25 mg; Se as Na_2SeO_3, 300 μg.

[4] Values were calculated from energy and nutrient concentrations in individual ingredient presented by Brazilian Tables for Poultry and Swine 3rd edition (Rostagno et al., 2011).

2.5. Meat quality

The breast muscle was used to measure meat quality. The right part of breast muscle was used to analyze the pH at 1 and 24 h postmortem, and meat color of lightness (L*), redness (a*), and yellowness (b*). The left part of breast muscle was used to measure water holding capacity (WHC) at 24 h postmortem and thiobarbituric acid reactive substance (TBARS) value at 7 d of storages in a refrigerator (Kim et al., 2012).

2.6. Stress biomarker

The feather corticosterone (CORT) concentrations were analyzed using the method of Bortolotti et al. (2008) with a minor modification. Briefly, collected feathers were separated to vane and rachis using a scalpel. The vane was divided into pieces of less than 5 mm^2 and approximately 100 mg of vane was finally obtained for the analysis. Feather pieces were arranged in 50 mL tubes containing 10 mL methanol. Afterwards, feather CORT was extracted through sonication and incubation processes. Extracted CORT were analyzed using CORT ELISA kit (Enzo Life Sciences Inc., NY), according to manufacturer's protocol. The detailed methods of CORT analysis were reported by Lee et al. (2022).

Blood was collected by the heart puncture using a 10-mL EDTA (Becton and Dickinson company, Diagnostics, UK) to measure heterophils to lymphocyte ratio (H:L ratio). Blood was stained with the wright stain solution (Muto pure chemicals, Tokyo, Japan) and Giemsa stain solution (Ducksan pure chemicals, Ansan, Republic of Korea). The stained blood was then dried aerially and investigated by a microscope. The H:L ratio was calculated by counting heterophils and lymphocytes to 200 cells per person (Cengiz et al., 2015; Yu et al., 2021). Stress biomarker analyses were performed at the BT research facility center, Chung-Ang University.

2.7. Jejunal morphology and goblet cell count

Jejunal morphology was assessed by the method of Nari et al. (2020). Jejunal fragments measuring 2 to 3 cm in length were collected from the middle part of the small intestine. The fragments were immediately flushed and fixed using the 10% neutral-buffered formalin solution. The villus width (VW), villus height (VH), crypt depth (CD), and VH:CD ratio were measured, and the average of 20 measurements was used for the data analysis (Hair-Bejo, 1990). The number of goblet cells was also determined with 10 villi in a 200-μm area from the central part of the villi (Santos et al., 2015).

2.8. Jejunal permeability

Jejunal permeability was expressed as trans-epithelial electrical resistance (TEER) value determined from a two-channel Ussing chamber system (U2500, Warner Instruments, Hamden, CT). The jejunal fragments between the end of the duodenum loop and Meckel's diverticulum were collected and a strip of jejunal mucosa was scraped evenly using the edge of a microscopic slide. The jejunal mucosa fragments were clamped perpendicularly in the middle of the Ussing chamber containing chilled Kreb-Henseleit buffer solutions (Bio-solution co., Suwon, Republic of Korea) at pH 7.4 and 38 °C. The jejunal mucosa were continuously aerated with 95% O_2 and 5% CO_2 mixture. After the 10-min stabilization procedure, short circuit current (Isc) and trans-epithelial voltage (PD) in jejunal mucosa were measured continuously every 10 min for the total of 100 min running time of the Ussing chamber. Afterwards, TEER values were estimated from measured Isc and PD values and were expressed in $\Omega \cdot cm^2$. The detailed procedure was reported in our previous study (Goo et al., 2019a).

2.9. Gene expression

The expression levels of tight junction-associated and selected genes in the jejunal mucosa were analyzed using the reverse-transcriptase-polymerase chain reaction (RT-PCR) (Shin et al., 2018). Shortly, total RNA from mucosa samples was extracted by

Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol and was used to measure gene expression levels of occludin (*OCLN*), claudin-1 (*CLDN1*), zonula occludens-1 (*ZO-1*), junctional adhesion molecule B 2 (*JAM2*), and mucin 2 (*MUC2*). The primers for RT-PCR were produced from the NCBI/Primer-BLAST (Table 2). The primers of the genes (*OCLN*, *CLDN1*, *ZO-1*, *JAM2*, and *MUC2*) were synthesized using available sequences found in public databases. The distinct characteristics of the primers were established by PCR amplification (Aznar and Alarcón, 2002). The $2^{-\Delta\Delta Ct}$ method after normalization to the expression of glyceraldehyde-3-phosphate dehydrogenase gene was used for the relative quantification of target gene expression (Thomsen et al., 2010).

2.10. Statistical analysis

All data were analyzed using the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The replicate was considered an experimental unit. Possible outliers were checked by a normal probability plot and residuals of the raw data, but no outliers were detected. The LSMEANS procedure was used to calculate treatment means and the PDIFF option of SAS was used to separate the treatment means. The significance for statistical analysis was set at $P < 0.05$.

3. Results

3.1. Growth performance and meat quality

Birds in NC and all treatment groups (i.e., Thr, Trp, and Gly treatments) under multiple stress conditions had less ($P < 0.001$) BW, BWG, and FI but greater ($P < 0.001$) FCR than those in PC treatment during a 14-d feeding trial (Table 3). Moreover, birds in Thr and Gly treatments had less ($P < 0.001$) FI than those in NC and Trp treatments under multiple stress conditions. However, birds in Thr treatment showed the least ($P < 0.001$) FCR among dietary treatments under multiple stress conditions, which was similar to the FCR observed in PC treatment. For the meat quality, pH, WHC, meat color, and TBARS values were not affected by multiple stress conditions and dietary treatments (Table 4).

3.2. Stress biomarker

Birds in NC treatment had greater feather CORT concentrations ($P = 0.026$) and blood H:L ratio ($P < 0.001$) than those in PC treatment (Table 5). Under multiple stress conditions, birds in Thr, Trp, and Gly treatments had less feather CORT concentrations ($P = 0.026$) and blood H:L ratio ($P < 0.001$) than those in NC treatment. Birds in Thr treatment had the least ($P < 0.001$) blood H:L ratio among dietary treatments under multiple stress conditions.

3.3. Jejunal morphology and goblet cell counts

Hematoxylin and eosin staining was performed to observe the effect of increasing concentrations of dietary Thr, Trp, and Gly on the jejunal morphology of broiler chickens raised under multiple stress conditions (Fig. 1). No differences in VW, CD, and VH:CD ratio among treatments were observed (Table 6). However, birds in NC treatment had less VH ($P = 0.010$) and goblet cell counts ($P = 0.018$) than those in PC treatment. Birds in Thr, Trp, and Gly treatments,

Table 2
The sequence of the primers used in real-time quantitative-PCR.

RNA target	Primer sequence	Tm[1], °C	Size for PCR product, bp	Accession no.
GAPDH	F: 5′- TGCTGCCCAGAACATCATCC-3′ R: 5′-ACGGCAGGTCAGGTCAACAA -3′	50–65	142	NM_204305
OCLN	F: 5′-TCGTGCTGTGCATCGCCATC-3′ R 5′-CGCTGGTTCACCCCTCCGTA-3′	60	178	NM_205128
CLDN1	F: 5′-CAGACYCYAGGTTTTGCCTT-3′ R: 5′-AATCTTTCCAGTGGCGTAC-3′	58.3	149	NM_001013611
ZO-1	F: 5′-CACACTGTGACCCCAAAA-3′ R: 5′-AAGGTCCATCTCAGTTTCAC-3′	56.3	151	XM_040680632
JAM2	F: 5′-AATTTACAGTTCCTCCCACT-3′ R: 5′-GTCTTTTCCAGTAAGGCAAC-3′	53.2	151	NM_001006257
MUC2	F: 5′-CAGAAGGAACTTCTTCGTACA-3′ R: 5′-AGAGGATGCTCCTGTAGATT-3′	53.9	137	XM_040673077

PCR = polymerase chain reaction; F = forward; R = Reverse; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *OCLN* = occludin; *CLDN1* = claudin-1; *ZO-1* = zonula occludens-1; *JAM2* = junctional adhesion molecule B; *MUC2* = mucin 2.
[1] Tm, amplification temperature.

Table 3
Effect of increasing concentrations of dietary Thr, Trp, and Gly on growth performance of broiler chickens raised under multiple stress conditions.[1]

Item	Dietary treatments[2]					SEM	*P*-value
	PC	NC	Thr	Trp	Gly		
BW, g	2229[a]	2012[b]	2012[b]	2024[b]	1981[b]	15.6	<0.001
BWG, g	1264[a]	1047[b]	1047[b]	1059[b]	1015[b]	15.7	<0.001
FI, g	2012[a]	1773[b]	1659[c]	1740[b]	1676[c]	17.3	<0.001
FCR, g/g	1.59[c]	1.70[a]	1.59[c]	1.64[b]	1.65[ab]	0.016	<0.001

BW = body weight; BWG =body weight gain; FI = feed intake; FCR = feed conversion ratio.
[a – c] Means within a row with no common superscript differ significantly ($P < 0.05$).
[1] Data are least squares means of 8 observations per treatment.
[2] PC, positive control (basal diet under normal conditions); NC, negative control (basal diet under multiple stress conditions). Thr, Trp, and Gly treatment diets had 2-fold higher digestible concentrations of Thr, Trp, and Gly + Ser than the basal diet, respectively, by supplementing 0.79% L-Thr, 0.20% L-Trp, and 1.78% Gly in the basal diet. Thr, Trp, and Gly treatments were assigned to multiple stress conditions.

Table 4
Effect of increasing concentrations of dietary Thr, Trp, and Gly on meat quality of broiler chickens raised under multiple stress conditions.[1]

Item	Dietary treatments[2]					SEM	*P*-value
	PC	NC	Thr	Trp	Gly		
Post-mortem pH							
1 h	6.0	6.3	6.3	6.2	6.3	0.09	0.054
24 h	5.8	5.8	5.8	5.7	5.7	0.06	0.756
WHC, %	75.1	73.6	74.9	73.7	75.5	1.84	0.928
Meat color (CIE Lab value)							
L*	43.3	43.8	43.8	43.9	46.1	0.72	0.077
a*	6.1	4.5	4.7	5.4	4.3	0.57	0.189
b*	15.2	14.3	14.0	14.8	14.6	4.77	0.456
TBARS[3], mg MDA/kg meat	0.31	0.31	0.32	0.30	0.31	0.015	0.909

WHC=water holding capacity; CIE=Commision Internationale de l'Eclairage; L* = lightness; a* = redness; b* = yellowness; TBARS= thiobarbituric acid reactive substances.
[1] Data are least squares means of 8 observations per treatment.
[2] PC, positive control (basal diet under normal conditions); NC, negative control (basal diet under multiple stress conditions). Thr, Trp, and Gly treatment diets had 2-fold higher digestible concentrations of Thr, Trp, and Gly + Ser than the basal diet, respectively, by supplementing 0.79% L-Thr, 0.20% L-Trp, and 1.78% Gly in the basal diet. Thr, Trp, and Gly treatments were assigned to multiple stress conditions.
[3] TBARS values were quantified as malondialdehyde (MDA) equivalents per gram of meat sample at 7 d of storage at 4 °C.

Table 5
Effect of increasing concentrations of dietary Thr, Trp, and Gly on stress biomarkers of broiler chickens raised under multiple stress conditions.[1]

Item	Dietary treatments[2]					SEM	*P*-value
	PC	NC	Thr	Trp	Gly		
CORT, pg/mg	1.35^{b}	6.47^{a}	2.43^{b}	2.55^{b}	2.54^{b}	1.108	0.026
H:L ratio	0.28^{d}	0.62^{a}	0.32^{c}	0.39^{b}	0.36^{bc}	0.014	<0.001

CORT = corticosterone; H:L ratio = heterophil to lymphocyte ratio.
[a – d] Means within a row with no common superscript differ significantly ($P < 0.05$).
[1] Data are least squares means of 8 observations per treatment.
[2] PC, positive control (basal diet under normal conditions); NC, negative control (basal diet under multiple stress conditions). Thr, Trp, and Gly treatment diets had 2-fold higher digestible concentrations of Thr, Trp, and Gly + Ser than the basal diet, respectively, by supplementing 0.79% L-Thr, 0.20% L-Trp, and 1.78% Gly in the basal diet. Thr, Trp, and Gly treatments were assigned to multiple stress conditions.

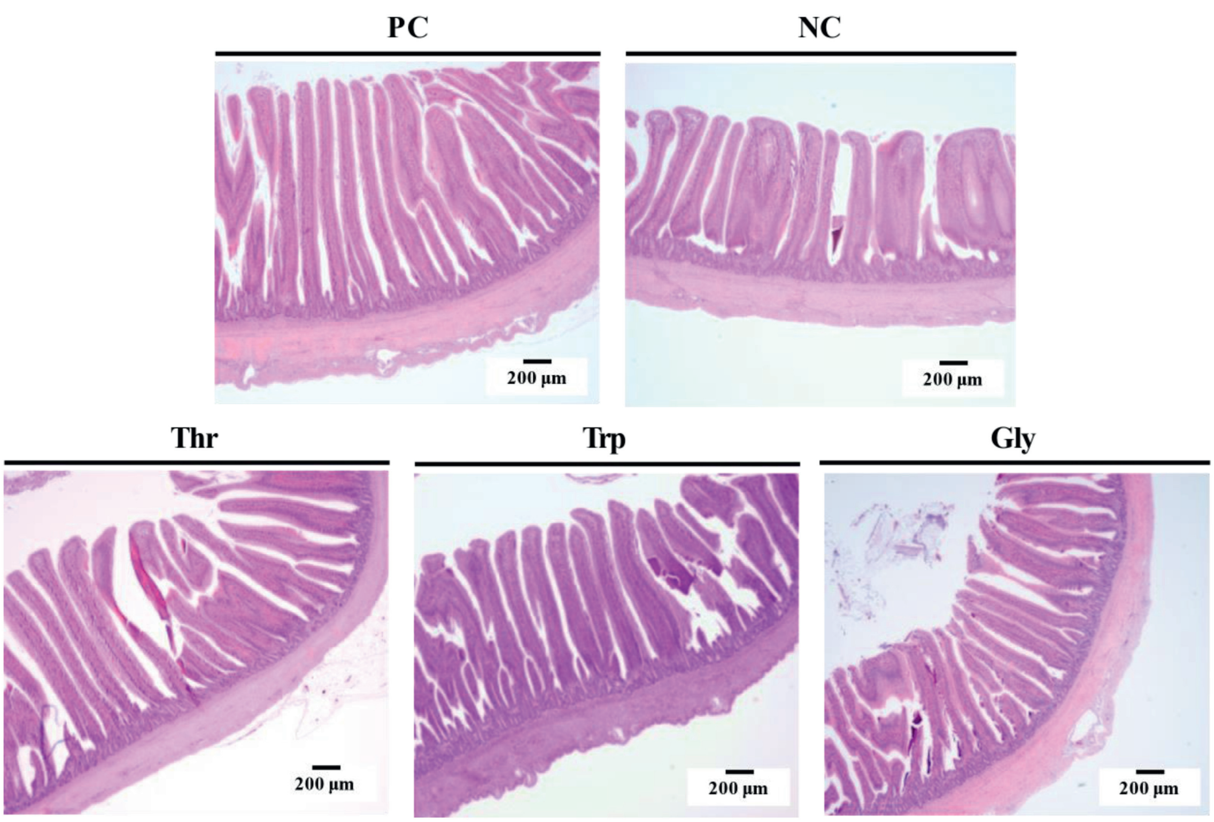

Fig. 1. Hematoxylin and eosin staining of the jejunal section of broiler chickens raised under multiple stress conditions. PC, positive control (basal diet under normal conditions); NC, negative control (basal diet under multiple stress conditions). Thr, Trp, and Gly treatment diets are set to have 2-fold higher digestible concentrations of Thr, Trp, and Gly + Ser than the basal diet.

Table 6
Effect of increasing concentrations of dietary Thr, Trp, and Gly on jejunal morphology and goblet cell counts of broiler chickens raised under multiple stress conditions.[1]

Item	Dietary treatments[2]					SEM	P-value
	PC	NC	Thr	Trp	Gly		
VW, μm	227	215	225	221	236	11.8	0.764
VH, μm	1475[a]	1236[b]	1381[ab]	1334[ab]	1241[b]	51.2	0.010
CD, μm	121	111	106	107	103	8.1	0.600
VH:CD ratio	12.6	11.4	13.2	12.7	12.4	0.65	0.442
Goblet cell[3]	20.8[a]	20.0[b]	20.9[a]	21.2[a]	21.3[a]	0.28	0.018

VW, villus width; VH, villus height; CD, crypt depth.
[a,b] Means within a row with no common superscript differ significantly ($P < 0.05$).
[1] Data are least squares means of 8 observations per treatment.
[2] PC, positive control (basal diet under normal conditions); NC, negative control (basal diet under multiple stress conditions). Thr, Trp, and Gly treatment diets had 2-fold higher digestible concentrations of Thr, Trp, and Gly + Ser than the basal diet, respectively, by supplementing 0.79% L-Thr, 0.20% L-Trp, and 1.78% Gly in the basal diet. Thr, Trp, and Gly treatments were assigned to multiple stress conditions.
[3] Goblet cell counts, cells/200 μm.

which showed similar goblet cell counts to those in PC treatment, had greater ($P = 0.018$) goblet cell counts than those in NC treatment.

3.4. Jejunal permeability and gene expression

Birds in NC treatment had less ($P < 0.001$) TEER values than those in PC treatment (Table 7). Birds in Thr, Trp, and Gly treatments had greater ($P < 0.001$) TEER values than those in NC treatment. The TEER values measured in Thr treatment did not differ from the values measured in PC treatment.

Expression levels of *ZO-1* as tight junction-related genes were decreased ($P = 0.034$) in NC treatment than in PC treatment; however, dietary treatment under multiple stress conditions had no effects on expression levels of *ZO-1* although expression levels measured in Thr treatment did not differ from those measured in PC treatment. Likewise, expression levels of other tight junction-related genes including *OCLN*, *CLDN1*, and *JAM2* were not influenced by multiple stress conditions and dietary treatments. Expression levels of *MUC2* were decreased ($P = 0.003$) in NC treatment than in PC treatment. Birds in Thr and Gly treatments had increased ($P = 0.003$) expression levels of *MUC2* than those in NC treatment.

4. Discussion

The current study revealed that birds in all dietary treatments (i.e., NC, Thr, Trp, and Gly treatments) under multiple stress conditions had less BW, BWG, and FI but greater FCR than those in PC treatment. This result agrees with previous studies reporting that heat stress or high stocking density conditions reduced productive growth performance in broiler chickens compared with normal conditions (Azad et al., 2010; Goo et al., 2019a). Impaired performance in broiler chickens by heat stress has been involved in lower FI, decreased nutrient utilization, increased abnormal behavior, and reduced disease resistance (Dayyani and Bakhtiari, 2013); these impairments has also been observed in broiler chickens raised under high stocking density (Goo et al., 2019a). Thus, it was easily expected that the concurrent stress from heat stress and high stocking density has a synergistically negative effect on broiler performance, although the current study did not investigate the separate effects of heat stress and high stocking density.

Increasing concentrations of dietary Thr, Trp, or Gly had no positive impact on BW and BWG in broiler chickens raised under multiple stress conditions, but showed decreased FCR primarily due to decreased FI. However, increasing concentrations of dietary Gly had no significant effects on FCR. In particular, significant reduction in FI was observed by increasing concentrations of dietary Thr or Gly, but such a reduction in FI was not identified by increasing concentrations of dietary Trp. The reason for reduced FI in broiler chickens by increasing concentrations of dietary Thr and Gly under multiple stress conditions is unclear; however, it may be attributed to increasing heat increment by additional oxidation of supplemented Thr and Gly in the body (Musharaf and Latshaw, 1999). However, increasing concentrations of dietary Trp may enhance the synthesis of serotonin, which in turn stimulate FI in

Table 7
Effect of increasing concentrations of dietary Thr, Trp, and Gly on jejunal permeability and tight junction-related gene expression of broiler chickens raised under multiple stress conditions.[1]

Item	Dietary treatments[2]					SEM	P-value
	PC	NC	Thr	Trp	Gly		
Jejunal permeability							
PD, mV	373[a]	223[c]	327[ab]	284[bc]	333[ab]	26.2	0.003
Isc, μA/cm^2	0.63	0.62	0.60	0.58	0.68	0.038	0.432
TEER, Ω/cm^2	595[a]	362[c]	541[ab]	499[b]	492[b]	28.7	<0.001
Jejunal gene expression							
OCLN	1.23	1.19	1.17	1.09	1.12	0.065	0.560
CLDN1	1.63	1.61	1.62	1.47	1.42	0.089	0.263
ZO-1	1.73[a]	1.36[b]	1.54[ab]	1.40[b]	1.44[b]	0.091	0.034
JAM2	1.16	1.09	1.10	1.02	1.05	0.043	0.162
MUC2	1.54[a]	1.06[c]	1.44[ab]	1.20[bc]	1.36[ab]	0.840	0.003

PD = trans-epithelial voltage; Isc = short circuit current; TEER = trans-epithelial electrical resistance; *OCLN* = occludin; *CLDN1* = claudin-1; *ZO-1*, zonula occludens-1; *JAM2* = junctional adhesion molecule B; *MUC2* = mucin 2.
[a-c] Means within a row with no common superscript differ significantly ($P < 0.05$).
[1] Data are least squares means of 8 observations per treatment.
[2] PC, positive control (basal diet under normal conditions); NC, negative control (basal diet under multiple stress conditions). Thr, Trp, and Gly treatment diets had 2-fold higher digestible concentrations of Thr, Trp, and Gly + Ser than the basal diet, respectively, by supplementing 0.79% L-Thr, 0.20% L-Trp, and 1.78% Gly in the basal diet. Thr, Trp, and Gly treatments were assigned to multiple stress conditions.

animals (Bello et al., 2018), thereby ameliorating the extent of decreased FI. Our results indicated that increasing concentrations of dietary Thr was the most effective in decreasing FCR, the value of which was close to PC treatment. In the current experiment, furthermore, increasing concentrations of dietary Thr was found to improve intestinal health as a measure of intestinal permeability and gene expression, and decreased stress responses as a measure of blood H:L ratio. The extent of the beneficial effects of dietary Thr on intestinal health and stress responses was greater than those of dietary Trp and Gly. This observation may be the possible reason why dietary Thr is more effective in alleviating impairments in broiler performance under multiple stress conditions than dietary Trp and Gly. It is suggested, therefore, that increasing concentrations of dietary Thr by 2-fold higher than its requirement may be a possible solution to ameliorate decreased productivity of broiler chickens under multiple stress conditions.

Decreased meat quality observed in broiler chickens under heat stress (Lu et al., 2007) or high stocking density (Weimer et al., 2022) has been related to maintenance of high temperature and retention of lactic acid in the muscle (Imik et al., 2012; Lu et al., 2007). In previous studies, dietary supplementation of Thr (Dozier et al., 2000), Trp (Bello et al., 2018; Wang et al., 2014), and Gly (Hejdysz et al., 2022) was reported to improve meat quality in broiler chickens under either stress or normal conditions; however, the results have been inconsistent (Petracci et al., 2015). In the current study, increasing concentrations of dietary Thr, Trp, and Gly concentrations under multiple stress conditions had no effect on all meat quality measurements. It was noted, however, that our values pertaining to meat quality measurements were in the normal and typical range of values for breast meat quality reported in previous experiments (Chang et al., 2020), indicating that the current stress conditions and Thr, Trp, or Gly concentrations in diets may have little impact on breast meat quality in broiler chickens.

Feather CORT concentrations and blood H:L ratio were increased under multiple stress conditions as compared to normal conditions in this experiment. These results agree with previous experiments reporting increased stress responses by heat stress or high stocking density (Kridtayopas et al., 2019; Lee et al., 2022). Interestingly, increasing concentrations of dietary Thr, Trp, and Gly decreased feather CORT concentrations and blood H:L ratio under multiple stress conditions, indicating that those 3 AA may have a potential role in alleviating stress responses in broiler chickens under multiple stress conditions. In previous studies, similar results were also observed by increasing concentrations of dietary Thr (Kolbadinejad and Rezaeipour, 2020; Wasman, 2022), Trp (Moneva et al., 2008), or Gly (Won et al., 2023) in broiler chickens. For a functional role of those AA in decreasing stress responses, Baird et al. (2013) demonstrated that Thr can prevent cellular apoptosis and enhance the expression of heat shock proteins, thus stabilizing other proteins under stress conditions. Likewise, Trp is a key precursor AA for serotonin and melatonin synthesis (Le Floc'h et al., 2011), which are associated with the regulation of stress responses. Moreover, Gly decreases oxidative stress by promoting glutathione synthesis (Sekhar et al., 2011), which may lower oxidative stress under stress conditions (Won et al., 2023). Specifically, our results demonstrated that dietary Thr reduced blood H:L ratio to the greatest extent in broiler chickens under multiple stress conditions as compared to dietary Trp and Gly. Similarly, dietary Thr was also the most effective in improving intestinal structure and decreasing intestinal permeability in this study. However, to our knowledge, the reason for this superior effect of dietary Thr remains unknown.

Improvement in intestinal structures is directly associated with increased nutrient utilization and protection from microbial invasion (Teng and Kim, 2018). Stressful conditions are appreciated one significant factor impairing the intestinal structure (Kridtayopas et al., 2019), which confirms our observation. Impaired intestinal structure may be caused by the fact that decreased FI due to stressful conditions reduces nutrient and energy supply for intestinal cells (Porto et al., 2015). Furthermore, oxidative stress caused by various stressors promotes inflammation in intestinal cells and decreases nutrient and oxygen supply to intestinal cells by limiting the blood supply (Lambert, 2009). In the current experiment, increasing concentrations of dietary Thr or Trp increased VH under multiple stress conditions, which is accord with previous experiments indicating that birds fed diets supplemented with Thr or Trp have improved intestinal morphology (Zaefarian et al., 2008). The possible reason may be associated with alleviating stress responses as observed in this study. Moreover, adequate supply of Thr also ensures its utilization by the intestinal mucosa for improvements in mucosal integrity (Gum, 1992). A previous study reported that supplemental Trp improved intestinal structures by stimulating the regeneration of villi and crypt cells in weaned pigs (Koopmans et al., 2006), which may be the reason for our observation that increasing concentrations of dietary Thr and Trp increased VH in broiler chickens under multiple stress conditions. However, increasing concentrations of dietary Gly did not affect VH in broiler chickens raised under multiple stress conditions. Although a previous study showed similar results (Ospina-Rojas et al., 2013), to our knowledge, data pertaining to the effect of increasing concentrations of dietary Gly on intestinal morphology in broiler chickens raised under multiple stress conditions are lacking.

Stress conditions in this study decreased goblet cell counts in broiler chickens, which agrees with previous results (Sandikci et al., 2004). Likewise, the expression of *MUC2* gene, which is required for production of the mucus layer covering the intestinal epithelium (Humam et al., 2021), was also decreased by multiple stress conditions. Therefore, it is suggested that multiple stress conditions decrease goblet cell counts and *MUC2* gene expression, thereby aggravating mucin production and reducing intestinal barrier function in broiler chickens. The present study showed that feeding diets with increasing concentrations of 3 dietary AA to broiler chickens under multiple stress conditions had improved goblet cell counts. In addition, increasing concentrations of dietary Thr and Gly under multiple stress conditions significantly increased the *MUC2* gene expression. These positive effects of dietary Thr and Gly agreed with previous studies investigating goblet cell counts and gene expression of *MUC2* in broiler chickens (Chen et al., 2017; Fan et al., 2019). Dietary Thr plays a role in goblet cell differentiation and is important AA for mucin production (Gum, 1992). Moreover, melatonin synthesized by Trp can downregulate gene expression related to the Notch ligand and receptor, thus increasing goblet cell counts (Li et al., 2017). Furthermore, Gly is required for mucin biosynthesis in intestinal cells because the glycocalyx of the pericellular matrix is present high in transmembrane mucins and tight junctions (Sheng et al., 2013).

The current study showed that broiler chickens raised under multiple stress conditions had less TEER values than those raised under normal conditions, which is similar to the findings of previous studies (Goo et al., 2019a; Song et al., 2014). The TEER value is highly correlated with tight junction-associated gene expression, and this study also showed similar reduction in *ZO-1* gene expression. These results agree with previous studies indicating that heat stress and high stocking density impaired intestinal barrier functions such as increasing intestinal permeability and decreasing tight junction-associated gene expression in broiler chickens (Alhotan et al., 2021). The possible reason may be the intestinal disruption caused by increased inflammatory responses due to various stressors (Song et al., 2014). Moreover, the reduction in blood flow by various stressors can damage tight junctions because decreased blood flow causes intestinal hypoxia, leading to a reduction in cell viability and an increase in intestinal permeability (Lambert et al., 2009). In the current study, increasing concentrations of dietary Thr, Trp, and Gly under multiple stress conditions increased TEER values, with dietary Thr resulting in the greatest TEER values, which were similar to those measured in PC treatment. Therefore, it is likely that increasing concentrations of dietary Thr, Trp, and Gly improve intestinal structure and goblet cell proliferations under multiple stress conditions, which is possible reason for increased TEER values in this study.

The expression levels of *OCLN*, *CLDN*, and *JAM2* were not affected by increasing concentrations of dietary Thr, Trp, and Gly concentrations under multiple stress conditions, although expression levels of *ZO-1* were slightly elevated. Previous studies reported that additional supplementation of dietary Trp (Goo et al., 2019b) or Gly (Won et al., 2023) had no beneficial effects on tight junction-related gene expression in broiler chickens exposed to stressful conditions. The reason for inconsistent results between TEER values and expression of tight junction-related genes in the current study is unclear because intestinal permeability represents integrity of tight junction. Therefore, it is speculated that intestinal permeability is not solely dependent of tight junction integrity (Shin et al., 2018). In addition, the current study focused on gene expression levels rather than protein levels in tight junctions, which limits further speculation regarding our variable results between TEER values and gene expression levels in the tight junction. Therefore, further research is required to verify the association between intestinal permeability and tight junction integrity in broiler chickens raised under stressful conditions.

5. Conclusion

The multiple stress conditions with heat stress and high stocking density impairs productive performance and intestinal health in broiler chickens with increasing stress responses. Increasing concentrations of dietary Thr, Trp, and Gly have a positive effect on growth performance of broiler chickens under multiple stress conditions possibly due to decreased stress responses and improved intestinal health. Among 3 functional AA, increasing concentrations of dietary Thr by 2-fold higher than its recommended concentrations in diets are found to be the most effective in ameliorating the negative outcomes from multiple stress conditions in broiler chickens. Therefore, increasing concentrations of dietary Thr are likely a potential solution to improve growth performance and intestinal health in broiler chickens raised under multiple stress conditions. It should be noted, however, that potential dose effect of supplemented AA may exist because only 2-fold higher concentrations of 3 AA than their recommendations were tested in this study. Further studies are required to compare functional effects of 3 AA at varying concentrations in broiler diets under multiple stress conditions. Furthermore, determination of functional effects of various combination of 3 AA in broiler diets would also be warranted.

Author contributions

All four authors contributed towards the completion of this study and have read and approved this manuscript. **Hyun Woo Kim**: Conceptualization, Methodology, Formal analysis, Writing-Original draft, Investigation. **Jong Hyuk Kim**: Conceptualization, Methodology, Writing-Review and Editing, Investigation, Project administration and Funding acquisition. **Gi Ppeum Han**: Data curation, Methodology, Validation, Writing-Review and Editing. **Dong Yong Kil**: Conceptualization, Methodology, Project administration, Investigation, Writing - Review & Editing, Resources, Supervision.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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