

## Effects of Dietary Brown Rice on the Growth Performance, Systemic Oxidative Status, and Splenic Inflammatory Responses of Broiler Chickens under Chronic Heat Stress

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The aim of this study was to evaluate the effects of dietary brown rice on the growth performance, systemic oxidative status, and splenic inflammatory responses of broiler chickens under both thermo-neutral and chronic heat stress conditions. Forty 12-day-old male broiler chickens (ROSS 308) were randomly assigned to two groups and fed either a control diet (corn-based) or a brown rice-based diet. After seven days (19 days old), both groups were randomly divided into two sub-groups ( $n=10$ ), one of which was exposed to heat stress (33°C for 14 days), while the other was maintained at 24°C. Heat exposure reduced the body weight gain and feed intake ( $p<0.01$ ) of both groups. In terms of oxidative plasma states, heat exposure reduced the glutathione peroxidase activity and increased the ceruloplasmin content, while the 2-thiobarbituric acid reactive substance and reduced glutathione levels were not affected adversely. Heat exposure activated the immune responses, as evidenced by increased plasma immunoglobulin levels, and altered splenic immune-related gene expressions including heat shock proteins, toll-like receptor 4, and interleukin-12. Under both thermo-neutral and heat stress conditions, dietary brown rice improved the growth performance, decreased the immunoglobulin levels, and down-regulated the expression of splenic immune-related genes of broilers, although their systemic oxidative status was not affected. Dietary brown rice should be considered as a valuable component of broiler chicken feeds subjected to both thermo-neutral and heat stress conditions. The positive effects of brown rice on bird performance may be associated with the modulation of the immune responses, as reflected by the decreased production of immunoglobulins and altered splenic immune-related gene expression.

**Key words:** broiler chicken, brown rice, immune responses, oxidative stress

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### Introduction

The global corn demand for use in the production of agricultural feeds and fuel is increasing rapidly (Popp *et al.*, 2016). In Japan, almost 100% of the corn used for animal feeds is imported (Statistics Department, Ministry of Agri-

culture, Forestry and Fisheries, 2019), resulting in a very low level of feedstuff self-sufficiency nationwide. To address this potentially serious problem, several new paddy rice cultivars developed exclusively for animal feeds have been proposed as candidates to substitute corn in poultry feeds and have been assessed in terms of their value. To date, only a few published studies have examined the effect of feeding rice to broiler chickens (Honda *et al.*, 2011; Sittiya *et al.*, 2011; Nanto *et al.*, 2012). The results of these reports have suggested that paddy rice, including brown rice, could be a dietary constituent compatible with corn feed for broiler chickens under normal (non-stressful) conditions.

Heat stress is a major issue in the poultry industry as it may cause physiological changes (Koelkebeck and Odom, 1995; Maak *et al.*, 2003), abnormal amino acid metabolism and neuropeptide expression (Chowdhury, 2019), damage to

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the intestinal morphology (Nanto-Hara *et al.*, 2020), oxidative damage (Mujahid *et al.*, 2005, 2007, 2009; Azad *et al.*, 2010a, 2010b), and immunosuppression (Thaxton *et al.*, 1968; Mashaly *et al.*, 2004; Mahmoud and Yaseen, 2005), ultimately resulting in decreased growth rates and increased mortality. We previously reported that, under heat stress conditions (i.e., during the summer season), Hakata Ichiban-dori chickens (meat chickens) that were fed a diet containing 30% brown rice, had better growth than chickens that were fed a corn-based diet (Hirakawa *et al.*, 2016). Brown rice is a good dietary energy source and is rich in bioactive phytochemicals, such as  $\gamma$ -oryzanol and tocotrienols, both of which have antioxidant and anti-inflammatory properties (Moongngarm *et al.*, 2012). These phytochemicals could reduce oxidative damage and excess inflammatory reactions in heat-stressed chickens, thereby potentially improving their growth rate.

The aim of this study was to elucidate whether a brown rice-based diet would improve the growth performance of chickens subjected to chronic heat stress, with particular reference to the involvement of oxidative status and immune responses.

## Materials and Methods

### Animals, Diet, and Experimental Design

The experimental protocol was approved by the Animal

Care Committee of the Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization (NARO), Japan (Approval number: 1811C087) and the Law (No. 105) and Notification (No. 6) of the Government. Newly hatched (day 0) male broiler chicks (ROSS 308) were purchased from a local hatchery. The chicks were housed in electrically heated battery cages and were given free access to water and a commercial broiler starter diet. At 12 days old, forty chicks of similar body weight were chosen and divided into two groups of 20 birds. The chicks in each group were moved to individual cages and provided with water *ad libitum* and one of the two experimental diets (corn-based or rice-based) for seven days. The experimental feed compositions, including  $\gamma$ -oryzanol and tocotrienol concentrations, are shown in Table 1. Thereafter, the birds in both groups were randomly divided into two sub-groups ( $n=10$ ); one of the two sub-groups of each main group was subjected to heat stress (33°C for 14 days) and the other was maintained at 24°C. The body weight and feed intake of the birds were recorded twice weekly (every three or four days) and on the final day of the experimental period. All birds were euthanized and tissue samples were removed and weighed as quickly as possible. The spleens were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for the cytokine mRNA expression measurements.

Table 1. Composition (%) of experimental diets

Ingredient (%)	Corn-based	Rice-based
Corn	57.65	—
Brown rice	—	57.65
Soybean meal	34.58	34.58
Vegetable oil	4.03	4.03
Calcium Carbonate	1.08	1.08
Dibasic calcium phosphate hydrate	1.59	1.59
Sodium Chloride	0.47	0.47
L-Lysine HCl	0.02	0.02
DL-Methionine	0.27	0.27
Selenium	0.01	0.01
Vitamin Mixture <sup>1</sup>	0.20	0.20
Mineral Mixture <sup>2</sup>	0.10	0.10
Calculated value		
Crude protein (%)	20.1	20.6
Metabolizable energy (kcal/g)	3.1	3.2
$\alpha$ -tocotrienol ( $\mu\text{g/g}$ )	0.08	0.12
$\gamma$ -tocotrienol ( $\mu\text{g/g}$ )	0.01	0.43
$\gamma$ -oryzanol ( $\mu\text{g/g}$ )	—	58.0

<sup>1</sup> The vitamin mixture provided the following (per kilogram of diet): vitamin A (from retinyl acetate) 4,000 IU; cholecalciferol, 600 IU; vitamin E (from DL- $\alpha$ -tocopheryl acetate), 15 IU; vitamin K (menadione sodium bisulfate), 1.5 mg; riboflavin, 10 mg; d-calcium pantothenate, 20 mg; nicotinic acid, 50 mg; choline chloride, 500 mg; pyridoxine hydrochloride, 3 mg; folic acid, 2 mg; thiamine mononitrate, 3 mg; d-biotin, 0.3 mg; vitamin B12 (cyanocobalamin), 20  $\mu\text{g}$ .

<sup>2</sup> The mineral mixture provided the following (per kilogram of diet): iron ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ), 80 mg; manganese ( $\text{MnCO}_3 \cdot n\text{H}_2\text{O}$ ), 60 mg; zinc (ZnO), 40 mg; copper ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ), 8 mg; iodine (calcium iodate), 0.5 mg.

### **Determination of the $\gamma$ -oryzanol and Tocotrienol Contents**

One hundred milligrams of each milled sample were soaked in 1 mL of a methanol/acetonitrile/acetic acid (52/45/3 v/v/v) solution and stirred gently for 2 h. The extracted  $\gamma$ -oryzanol solution was collected through a No. 2 filter paper (Wako, Tokyo, Japan). Then, it was filtered again using DISMIC 03JP050AN filters (Advantec Toyo, Tokyo, Japan). The  $\gamma$ -oryzanol content of the extracts was determined by reverse-phase high-performance liquid chromatography (HPLC) (Yoshie *et al.*, 2009). This system consisted of an intelligent HPLC pump (Shimazu, Kyoto, Japan) equipped with a shim-pack C18 FC-ODS column (150 mm  $\times$  4.6 mm; Shimazu), a CTO-10ASvp column oven (Shimazu), and a UV detector (Shimazu). The conditions were as follows: flow rate, 0.8 mL/min; oven temperature, 30°C; injection volume, 25  $\mu$ L; detection, UV at 320 nm; mobile phase, methanol/acetonitrile/acetic acid (52/45/3 v/v/v). The data were collected in triplicate and with reference to a peak area-concentration calibration curve from authentic  $\gamma$ -oryzanol standards (Tokyo Chemical Industry, Tokyo, Japan).

### **Determination of the 2-thiobarbituric Acid Reactive Substance (TBAR) Content**

The plasma lipid peroxidation was measured by TBARs assay (Yagi, 1976). In brief, 2.0 mL H<sub>2</sub>SO<sub>4</sub> (1/12 N) and 0.25 mL phosphotungstic acid (10% w/v) were added to 10  $\mu$ L plasma sample and incubated for 5 min at room temperature. The samples were centrifuged and the supernatant was removed. Then, the pellets were dissolved in 2.0 mL H<sub>2</sub>O and 0.5 mL TBA reagent (0.67% w/v 4,6-dihydroxy-2-mercaptopyrimidine in 50% v/v acetic acid) and incubated at 95°C for 60 min. Next, the samples were cooled and mixed with 2.5 mL n-butanol. After centrifugation, the n-butanol phase was collected, and fluorescence was measured at a wavelength of 553 nm after excitation at 515 nm (Hitachi F-4500, Tokyo, Japan).

### **Determination of Plasma Reduced Glutathione (GSH) Levels and Glutathione Peroxidase (GPx) Activities as Well as IgY and IgM Immunoglobulins**

The plasma GSH concentration was measured by the method of Ellman (1959). Briefly, the reaction of GSH with Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] gives rise to a yellow product that can be quantified spectrophotometrically at 412 nm. The GPx activity in the blood hemolysates was determined using a commercial kit (Randox Laboratories, Crumlin, UK) that follows a process based on the method of Paglia and Valentine (1967). The IgY and IgM concentrations in the plasma were determined using commercial kits (Immunology Consultants Laboratory, Inc., Portland, OR, USA) according to the manufacturer's guidelines.

### **Determination of Plasma Ceruloplasmin (Cp) Concentration**

The plasma was separated and stored at  $-80^{\circ}\text{C}$  until assayed. The plasma Cp concentration was determined following the procedure of Ravin (1961) using *p*-phenylenediamine. Acetate buffer (2 mL, 0.1 M; pH 5.4) was added to 0.1 mL of plasma sample and pre-incubated for 5 min at 37°C.

Then, 1 mL of 27 mM *p*-phenylenediamine was added as the substrate for the reaction. The reaction was stopped by adding 50  $\mu$ L of 1.5 M sodium azide exactly 30 min later. The resulting color change was spectrophotometrically measured at 530 nm (Shimazu UV-1800; Shimazu). The absorbance of the blank was measured immediately after adding 50  $\mu$ L of 1.5 M sodium azide to a mixture of 1 mL of 27 mM *p*-phenylenediamine and 0.1 mL of plasma. The Cp concentration was calculated using the following equation:

$$\text{Cp concentration (mg/L)} = 875 \times (\text{absorbance of sample} - \text{absorbance of blank}),$$

where 875 is the molar absorbance coefficient.

### **Total RNA Isolation, cDNA Synthesis, and Real-time Polymerase Chain Reaction (PCR)**

Total RNA was extracted from the spleen samples using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 1  $\mu$ g of total RNA using a random primer (TOYOBO, Osaka, Japan) and ReverTra Ace (TOYOBO). Real-time PCR was performed to measure the mRNA expression levels using a QuantStudio 5 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the THUNDERBIRD SYBR qPCR Master Mix (TOYOBO). PCR primers for chicken heat shock protein (HSP) 70, HSP90, toll-like receptor (TLR)4, TLR5, TLR15, interleukin (IL)-4, IL-6, IL-12, and B cell-activating factor (BAFF) were purchased from Qiagen. The expression of 18SrRNA was measured as an internal control and primers (5'-TCAGATACCGTCGTAGTTCC-3', 5'-TTCCGTCAATTCCTTTAAGTT-3') for chicken 18SrRNA were designed based on previously published methods (Li *et al.*, 2005).

### **Statistical Analysis**

A computer-generated SAS (SAS Institute, 1988) application package was used for statistical calculations. The data were analyzed using a two-way ANOVA and the means were compared using Tukey's multiple comparison test. The level of significance used in all studies was  $p < 0.05$ .

## **Results**

### **Growth Performance**

The effects of a brown rice-based diet on the growth performance and spleen weight of broiler chickens that were either subjected or not to heat stress are presented in Table 2. Heat exposure reduced the body weight gain and feed intake of both groups ( $p < 0.01$ ), while it did not affect the feed efficiency and spleen weight per g/kg body weight. Compared with the control (corn-based) diet, the rice-based diet resulted in body weight gains and feed efficiency improvements and spleen weight decreases. No significant interaction ( $p < 0.05$ ) was observed between temperature and diet in terms of body weight gain, feed intake, feed efficiency, or spleen weight during the experimental period.

### **Blood Antioxidant Status**

The effects of dietary brown rice on the plasma oxidative stress status are shown in Table 3. No temperature  $\times$  diet interaction was observed for blood antioxidant levels. Heat exposure affected the TBAR ( $p < 0.01$ ), GPx ( $p < 0.01$ ), and

**Table 2. Effects of dietary brown rice on the growth performance and spleen weight of broiler chickens either subjected or not to heat stress**

	TN		HS		TN	HS	Corn	Rice	Pooled SEM	Significance		
	Corn	Rice	Corn	Rice						T	D	T×D
Body weight gain (g)	1,069 <sup>b</sup>	1,349 <sup>a</sup>	894 <sup>c</sup>	1,064 <sup>b</sup>	1,209	979	981	1,206	41.0	<0.01	<0.01	0.19
Feed intake (g)	1,650 <sup>ab</sup>	1,843 <sup>a</sup>	1,390 <sup>c</sup>	1,492 <sup>bc</sup>	1,746	1,441	1,520	1,668	54.6	<0.01	0.07	0.41
Feed efficiency (g:g)	0.65 <sup>b</sup>	0.73 <sup>a</sup>	0.64 <sup>b</sup>	0.72 <sup>a</sup>	0.69	0.68	0.64	0.72	0.01	0.55	<0.01	0.84
Spleen weight (g/ kgBW)	1.23	1.04	1.21	0.94	1.14	1.08	1.22	0.99	0.09	0.50	0.02	0.71

The values are means of 10 birds per treatment and their pooled SEM.

<sup>a,b,c</sup>  $p < 0.05$  for each treatment; values with different superscript letters are significantly different.

Abbreviations: BW, body weight; TN, thermo-neutral; HS, heat stress; T, temperature; D, diet.

**Table 3. Effects of dietary brown rice on the plasma oxidative status of broiler chickens either subjected or not to heat stress**

	TN		HS		TN	HS	Corn	Rice	Pooled SEM	Significance		
	Corn	Rice	Corn	Rice						T	D	T×D
TBARs (nmol/mL)	3.32 <sup>a</sup>	3.30 <sup>a</sup>	2.71 <sup>ab</sup>	1.94 <sup>b</sup>	3.31	2.33	3.02	2.62	0.34	0.01	0.25	0.28
GSH ( $\mu$ M)	31.1	34.9	42.9	39.5	33.0	41.2	37.0	37.2	7.31	0.27	0.97	0.63
GPx (U/mL)	15.9	15.8	13.4	13.3	15.8	13.3	14.6	14.5	0.70	<0.01	0.86	0.98
Cp (mg/dL)	0.51 <sup>ab</sup>	0.47 <sup>b</sup>	0.62 <sup>a</sup>	0.53 <sup>ab</sup>	0.49	0.57	0.57	0.50	0.34	0.04	0.06	0.40

The values are means of 10 birds per treatment and their pooled SEM.

<sup>a,b</sup>  $p < 0.05$  for each treatment; values with different superscript letters are significantly different.

Abbreviations: TN, thermo-neutral; HS, heat stress; T, temperature; D, diet; TBARs, 2-thiobarbituric acid reactive substances; GSH, reduced glutathione; GPx, glutathione peroxidase; Cp, ceruloplasmin.

**Table 4. Effects of dietary brown rice on the plasma immunoglobulin concentrations of broiler chickens either subjected or not to heat stress**

	TN		HS		TN	HS	Corn	Rice	Pooled SEM	Significance		
	Corn	Rice	Corn	Rice						T	D	T×D
IgY ( $\mu$ g/mL)	2,162	2,055	3,409	2,859	2,108	3,134	2,786	2,457	381	0.01	0.39	0.56
IgM ( $\mu$ g/mL)	225 <sup>bc</sup>	175 <sup>c</sup>	352 <sup>a</sup>	311 <sup>ab</sup>	200	332	288	243	25	<0.01	0.07	0.86

The values are means of 10 birds per treatment and their pooled SEM.

<sup>a,b,c</sup>  $p < 0.05$  for each treatment; values with different superscript letters are significantly different.

Abbreviations: TN, thermo-neutral; HS, heat stress; T, temperature; D, diet; Ig, immunoglobulin.

Cp ( $p < 0.05$ ) levels in the blood of chickens fed both the corn- and rice-based diets. Compared with the corn-based diet, the rice-based diet tended to decrease ( $p = 0.06$ ) the plasma Cp levels. Neither diet nor temperature had a significant effect on the GSH levels.

#### Plasma IgY and IgM Concentrations

Heat stress caused increases in the plasma IgY ( $p < 0.05$ ) and IgM ( $p < 0.01$ ) concentrations (Table 4). Compared to the corn-based diet, the rice-based diet tended to decrease the plasma IgM ( $p = 0.07$ ); however, it did not affect the plasma IgY concentrations. There was no significant effect of the interaction between the environmental temperature and diet on either the IgY or IgM concentrations.

#### Expression of Immune-related Genes in the Spleen

The effects of heat stress and diet on HSPs, TLRs, and cytokine mRNA expression levels in the spleen are shown in Table 5. Heat stress up-regulated the HSP70, TLR4, and IL-

12 mRNA expression and down-regulated the HSP90, IL-4, and IL-6 mRNA expression. Moreover, the rice-based diet decreased the expression of HSP70, HSP90, TLR15, and BAFF in the spleen. The interactions between temperature and diet had significant effects on the TLR4 mRNA expression ( $p < 0.05$ ). Heat exposure increased the TLR4 expression in spleens (corn-based diet in thermo-neutral vs. heat stress), and the brown rice diet inhibited significantly this increment.

## Discussion

Brown rice (unpolished rice), in which the bran layer and germ are retained, has the potential to replace corn as poultry feed under thermo-neutral conditions (Honda *et al.*, 2011; Sittiya *et al.*, 2011; Nanto *et al.*, 2012). However, the efficacy of dietary brown rice as a poultry feed ingredient for birds under stress has not been explored. Therefore, the

**Table 5. Effect of dietary brown rice on the expression of immune-related genes in the spleen of broiler chickens either subjected or not to heat stress**

Gene	TN		HS		TN	HS	Corn	Rice	Pooled SEM	Significance		
	Corn	Rice	Corn	Rice						T	D	T×D
HSP70	1.0 <sup>ab</sup>	0.5 <sup>b</sup>	1.7 <sup>a</sup>	1.0 <sup>ab</sup>	0.7	1.4	1.4	0.7	0.19	<0.01	<0.01	0.75
HSP90	1.0 <sup>a</sup>	0.6 <sup>b</sup>	0.6 <sup>b</sup>	0.4 <sup>b</sup>	0.8	0.5	0.8	0.5	0.10	<0.01	<0.01	0.37
TLR4	1.0 <sup>b</sup>	0.5 <sup>b</sup>	1.9 <sup>a</sup>	0.7 <sup>b</sup>	—	—	—	—	0.17	<0.01	<0.01	0.04
TLR5	1.0	0.8	0.6	0.6	0.9	0.6	0.8	0.7	0.13	0.05	0.55	0.30
TLR15	1.0 <sup>a</sup>	0.6 <sup>b</sup>	0.9 <sup>a</sup>	0.5 <sup>b</sup>	0.8	0.7	1.0	0.6	0.09	0.37	<0.01	0.99
IL-4	1.0 <sup>a</sup>	0.7 <sup>ab</sup>	0.3 <sup>b</sup>	0.5 <sup>ab</sup>	0.8	0.4	0.7	0.6	0.16	0.01	0.58	0.14
IL-6	1.0 <sup>a</sup>	0.7 <sup>ab</sup>	0.5 <sup>b</sup>	0.6 <sup>ab</sup>	0.8	0.5	0.7	0.6	0.12	0.01	0.30	0.14
IL-12	1.0 <sup>b</sup>	0.9 <sup>b</sup>	2.0 <sup>a</sup>	1.4 <sup>ab</sup>	0.9	1.7	1.5	1.1	0.23	<0.01	0.12	0.32
chBAFF	1.0 <sup>a</sup>	0.5 <sup>b</sup>	1.0 <sup>a</sup>	0.2 <sup>b</sup>	0.7	0.6	1.0	0.4	0.14	0.56	<0.01	0.11

The values are means of 10 birds per treatment and their pooled SEM.

<sup>a,b</sup>  $p < 0.05$  for each treatment; values with different letters are statistically different.

Abbreviations: TN, thermo-neutral; HS, heat stress; T, temperature; D, diet; HSP, heat shock protein; TLR, toll-like receptor; IL, interleukin; BAFF, B cell-activating factor.

main objective of this study was to determine the effect of dietary brown rice on the growth performance of chickens subjected to chronic heat stress. Compared to the control (corn-based) diet, the brown rice-based diet increased the body weight gain and feed efficiency of broiler chickens subjected to both thermo-neutral and heat stress conditions; these results indicate that brown rice may offer an alternative nutritional strategy for improving the growth performance of broiler chickens under both thermo-neutral and heat stress conditions.

The results of the present study corroborate those of previous studies (Nanto *et al.*, 2012), as they showed that including 40.7% brown rice in the diet of broiler chickens subjected to thermo-neutral conditions improved their growth performance. Brown rice has more starch, less fiber, and lower moisture content than corn; additionally, rice starch has a smaller granule size (3–8  $\mu\text{m}$ ), lower amylose content, and lower non-starch polysaccharide content than corn starch (Tester *et al.*, 2006). Chicks could utilize this nutrient composition better than the nutrient composition of corn. Further, Honda *et al.* (2011) reported that the true amino acid digestibility in a rice-based diet is significantly higher than that in a corn-based diet. Based on these findings, brown rice nutrients may be also utilized better than corn nutrients under both heat and thermo-neutral conditions, and result in growth performance improvements.

Brown rice contains high concentrations of bioactive secondary metabolites, such as  $\gamma$ -oryzanol and tocotrienols (Moongnarm *et al.*, 2012), and these can reduce oxidative damage and tone down the immune response (Henderson *et al.*, 2012; Islam *et al.*, 2014; Kang and Kim, 2016). Exposing birds to high environmental temperature can affect their growth performance negatively and diminish their antioxidant status and immunological function (Luo *et al.*, 2018; He *et al.*, 2018, 2019). Therefore, the growth performance improvement resulting from dietary brown rice may not only depend on the better utilization of its nutrients

but also on the resulting modulation of oxidative status and immune functions. For this purpose, the effects of a brown rice diet on the systemic oxidative status and immune functions under heat conditions were also investigated in this study.

The level of serum TBARs (major byproducts of lipid peroxidation) indirectly indicates the degree of cellular lipid peroxidation and the accumulation of oxidative damage (Yang *et al.*, 2010); TBAR level increases have been reported owing to heat stress (Mujahid *et al.*, 2007; Azad *et al.*, 2010a). However, contrary to previous findings, in the present study, heat stress caused a decrease in plasma TBAR levels; this could be attributed to the plasma lipid concentration reduction owing to lower feed intake during heat exposure (Rajman *et al.*, 2006). In animals, GSH is the most abundant non-protein thiol compound and functions as the principal non-enzymatic antioxidant in the antioxidant defense system. Enzymes in the GPx family act in the GSH cycle, directly catalyzing GSH oxidation and resulting in the reduction of  $\text{H}_2\text{O}_2$  or peroxides and the generation of oxidized GSH (Dunning *et al.*, 2013). Although the GSH levels were not affected by heat stress in the present study, heat exposure caused a decrease in plasma GPx activities. In addition, the dietary brown rice did not affect the TBAR and GSH levels or the GPx activities in chickens subjected to either the thermo-neutral or heat stress treatments. Cp, an acute-phase protein, is a copper-containing ferroxidase that protects tissues from iron-mediated free radical injury by oxidizing toxic ferrous iron to its non-toxic ferric form (Patel *et al.*, 2002). Conversely, plasma Cp levels in chickens, increase under the effect of several stressors, including corticosterone administration (Lin *et al.*, 2004), lipopolysaccharide (LPS)-challenge (Song *et al.*, 2009), and overcrowding stress (Shakeri *et al.*, 2014), while Cp has also been shown to have antioxidative effects. In our study, heat stress resulted in plasma Cp ( $p < 0.05$ ) concentration increases; additionally, compared to the corn-based diet, the brown rice-based diet



tended to decrease plasma Cp levels ( $p=0.06$ ), thereby suggesting that brown rice may reduce the stress levels caused by heat exposure to some extent.

The immune system of broiler chickens is affected in a considerably negative manner by heat stress. For example, both the macrophage activity in lymphoid organs, such as the spleen, thymus, and bursa of Fabricius (Bartlett and Smith, 2003) and the serum immunoglobulin levels are significantly reduced owing to heat exposure (Sangoh *et al.*, 2013). Contrary to the results of previous reports, the plasma IgG and IgM levels in our study increased by heat stress, while only the latter levels decreased slightly ( $p=0.07$ ) owing to dietary brown rice. Recently, Honda *et al.* (2015) reported that, following multiple environmental stresses, the immunoglobulin levels continue to increase for at least 24 h after the stress stimuli have been terminated. The authors of the study showed that the plasma IgG and IgM levels increase in chickens subjected to heat for seven days. Furthermore, the elevation of plasma immunoglobulin levels by stressors such as cold stress (Zhao *et al.*, 2013) and overcrowding stress (Gomes *et al.*, 2014) in broilers has also been reported. In the present study, heat stress induced the production of immunoglobulins and increased their levels in the circulation; additionally, the brown rice diet resulted in a decrease in the IgM levels, thereby suggesting that brown rice may tone down some of the excess immune reactions induced by heat.

In poultry, the spleen, the largest peripheral immune organ, has been cited for its role in heat stress. In accordance with previous findings (Quinteiro-Filho *et al.*, 2010; Ohtsu *et al.*, 2015), the spleen weight (g/kg body weight) was not affected by heat stress. Feeding a brown rice-based diet decreased the spleen weight, thereby indicating that the spleen immune function may be modulated by dietary brown rice. Liu *et al.* (2014) reported that heat exposure up-regulated the expression of the HSP genes in chicken spleens; these genes are constitutively expressed and play an essential protective role in maintaining the metabolic and structural integrity of the organ. In our study, the HSP70 and HSP90 levels in the spleen increased and decreased, respectively, owing to heat stress. These results demonstrate that different HSPs react differently to a single stress type. In this study, chickens fed the brown rice-based diet had lower HSP70 and HSP90 expressions than the controls, irrespective of the temperature. Recently, the excess expression of HSPs has been regarded as serving as a danger signal to the innate immune system and as promoting receptor-mediated apoptosis (Millar *et al.*, 2003; Davies *et al.*, 2006; Luo *et al.*, 2008). Therefore, the inhibitory effect of dietary brown rice on the HSP transcription levels recorded in our study indicates its potential as an anti-inflammatory therapy in broilers subjected to both thermo-neutral and heat stress.

TLRs play an essential role in initiating the innate and adaptive immune responses. The innate immune system recognizes pathogens by discriminating among certain conserved pathogen structures. Namely, TLR4 senses lipopolysaccharides from gram-negative bacteria (O'Neill *et al.*, 2013), TLR5 recognizes bacterial flagellins (Hayashi *et al.*,

2001), and TLR15 is activated by yeast-derived agonists (Boyd *et al.*, 2012). While TLR5 and TLR15 were not affected by heat stress in our study, the expression of TLR4 was up-regulated by heat exposure and the brown rice diet resulted in a significant decrease in the TLR4 and TLR15 expressions under heat conditions. Pedregosa *et al.* (2011) reported that ischemia and reperfusion injury cause a significant increase in the TLR expression levels in spleen cells. An up-regulated TLR expression in the spleen may induce damage to its immune function. Based on these findings, it could be suggested that brown rice has the potential to prevent immune damage in the spleen. The elevated TLR expression induced by heat stress could stimulate the downstream signaling pathway, thereby inducing inflammatory cytokine production. In this study, the IL-4 (Th2-type cytokines) and IL-6 (pro-inflammatory cytokine) expressions decreased owing to heat exposure, while the IL-12 (Th1-type cytokines) expressions increased. This corroborates the results of our previous study (Ohtsu *et al.*, 2015) that showed that heat stress up-regulates and down-regulates the splenic IL-12 and IL-4 expressions, respectively. Meanwhile, the IL-6 expression decreased owing to heat exposure, which was consistent with the findings of Ohtsu *et al.* (2015). These cytokines were unresponsive to dietary brown rice. These results indicate that feeding a brown rice-based diet did not alter the downstream inflammatory responses despite its inhibitory effects on TLRs.

The chicken homolog of mammalian BAFF (chBAFF), a member of the tumor necrosis factor family of cytokines, plays an important role in the survival and proliferation of chicken B cells (Schneider *et al.*, 2004). Additionally, chBAFF has a strong influence on antibody production (Kothlow *et al.*, 2010). In humans, the deregulated BAFF production is associated with multiple autoimmune disorders, including systemic lupus erythematosus, rheumatoid arthritis, and Sjögren's syndrome (Cheema *et al.*, 2001; Groom *et al.*, 2002; Stohl *et al.*, 2003), thereby suggesting that BAFF may boost excess immune reactions. In our study, the chBAFF mRNA expression in the spleens of brown rice-fed chickens that were subjected to both thermo-neutral and heat stress conditions, was significantly lower than that in controls, thereby indicating that the BAFF transcription and BAFF-induced immune responses were suppressed by dietary brown rice. These results suggest that a brown rice-based diet may prevent excess immune responses in broilers subjected to both thermo-neutral and heat stress conditions.

The results of the present study demonstrated that chickens fed a brown rice-based diet and subjected to both thermo-neutral and heat stress conditions displayed toned down immune reactions, as reflected by the decreased production of immunoglobulins and altered splenic immune-related gene expression. Although the manner through which dietary brown rice modulates the immune system remains unknown, there is a possible mechanism that may account for the role of brown rice in immune modulation. Both rice bran and rice bran extracts exhibit potent antioxidative and anti-inflammatory effects in cell assays (Islam *et al.*, 2014) and in mice ex-

periments (Henderson *et al.*, 2012). Further, dietary supplementation with rice bran has been reported to improve the body weight gain and immune function of broilers (Kang and Kim, 2016). Rice bran contains substantial amounts of hydroxycinnamic acid derivatives, including cycloartenyl ferulate, a natural product of rice bran oil-derived  $\gamma$ -oryzanol. These hydroxycinnamic acid derivatives inhibit NF- $\kappa$ B activity, a transcriptional factor in inflammation (Singh and Aggarwal, 1995; Natarajan *et al.*, 1996). NF- $\kappa$ B is activated by both TLR4 stimulation and BAFF-mediated signaling in spleen cells (Moon *et al.*, 2006). Additionally, NF- $\kappa$ B can act as a transcription factor that plays a positive regulatory role in the expression of HSP70 and HSP90 in the spleen of yellow-feather broilers (He *et al.*, 2019). Further, the down-regulation of NF- $\kappa$ B signaling in peripheral blood mononuclear cells decreases IgM production (Kikuchi *et al.*, 2009). Our results show that dietary brown rice reduced the plasma IgM concentration and down-regulated the expression of HSPs, TLR4, and BAFF in the spleen, thereby indicating that NF- $\kappa$ B activation may be suppressed. This evidence indicates that the immune regulatory effects of dietary brown rice may result from its high  $\gamma$ -oryzanol concentrations; however, further evaluation is required to verify this suggestion.

In conclusion, our results showed that brown rice improved the growth performance of broilers subjected to both thermo-neutral and heat stress conditions. The positive effects of brown rice on bird performance may be associated with the modulation of immune responses, as reflected by the decreased production of immunoglobulins and altered splenic immune-related gene expressions. Further research is required to assess any potential negative impacts of immune suppression, such as an increase in the infection risk under stress, arising from the inclusion of brown rice in the diet.

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### Conflicts of Interest

The authors declare no conflict of interest.

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