Supplementation of postbiotic RI11 improves antioxidant enzyme activity, upregulated gut barrier genes, and reduced cytokine, acute phase protein, and heat shock protein 70 gene expression levels in heat-stressed broilers

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ABSTRACT The aim of this work was to evaluate the impacts of feeding different levels of postbiotic RI11 on antioxidant enzyme activity, physiological stress indicators, and cytokine and gut barrier gene expression in broilers under heat stress. A total of 252 male broilers Cobb 500 were allocated in cages in environmentally controlled chambers. All the broilers received the same basal diet from 1 to 21 d. On day 22, the broilers were weighed and grouped into 7 treatment groups and exhibited to cyclic high temperature at 36 ± 1°C for 3 h per day until the end of the experiment. From day 22 to 42, broilers were fed with one of the 7 following diets: negative control, basal diet (0.0% RI11) (NC group); positive control, NC diet + 0.02% (w/w) oxytetracycline (**OTC** group); antioxidant control, NC diet + 0.02% (w/w) ascorbic acid. The other 4 other groups were as follows: NC diet + 0.2% cell-free supernatant (postbiotic RI11) (v/w), NC diet + 0.4% cell-free supernatant (postbiotic RI11) (v/w), NC diet + 0.6% cell-free supernatant (postbiotic RI11) (v/w), and NC diet + 0.8% cell-free supernatant (postbiotic RI11) (v/w). Supplementation of different levels (0.4, 0.6, and 0.8%) of postbiotic RI11 increased plasma glutathione peroxidase, catalase, and glutathione enzyme activity. Postbiotic RI11 groups particularly at levels of 0.4 and 0.6% upregulated the mRNA expression of IL-10 and downregulated the IL-8, tumor necrosis factor alpha, heat shock protein 70, and alpha-1-acid glycoprotein levels compared with the NC and OTC groups. Feeding postbiotic RI11, particularly at the level of 0.6%, upregulated ileum zonula occludens-1 and mucin 2 mRNA expressions. However, no difference was observed in ileum claudin 1, ceruloplasmin, IL-6, IL-2, and interferon expression, but downregulation of occludin expression was observed as compared with the NC group. Supplementation of postbiotic RI11 at different levels quadratically increased plasma glutathione peroxidase, catalase and glutathione, IL-10, mucin 2, and zonula occludens-1 mRNA expression and reduced plasma IL-8, tumor necrosis factor alpha, alpha-1-acid glycoprotein, and heat shock protein 70 mRNA expression. The results suggested that postbiotics produced from *Lactiplantibacillus plantarum* RI11 especially at the level of 0.6% (v/w) could be used as an alternative to antibiotics and natural sources of antioxidants in poultry feeding.

Key words: postbiotic RI11, antioxidant enzyme activity, gut barrier gene expression, heat biomarker gene expression, cytokine gene expression

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INTRODUCTION

Environmental stressors such as disease challenge and heat stress are the major problems faced by global poultry production, having negative influences on animal physiology, behavior, health, and productive performance, thus causing tremendous economic losses

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(Mahmoud et al., 2003, 2004; Lykkesfeldt and Svendsen, 2007; Najafi et al., 2015a; He et al., 2018). They can adversely affect the biological macromolecules such as proteins, lipids, carbohydrates, and DNA by generation and accumulation of reactive oxygen species (**ROS**) and free radicals in the cells while performing their normal metabolic functions (Mager and De Kruijff, 1995; Iwagami, 1996; Matés et al., 1999), resulting in cell damage and appearance of pathological symptoms (Halliwell and Cross, 1994; Halliwell, 2001). To lighten the adverse impacts of stressful environmental conditions on poultry and promote the animal's health and growth performance, antibiotics at subtherapeutic doses have been added to poultry diets as growth promoters (Zulkifli et al., 2000; Rahimi and Khaksefidi, 2006; Ramiah et al., 2014). However, improper and overuse of antibiotics as feed additives have caused the emergence of antibiotic-resistant bacteria and increased the levels of antibiotic residues in animal products, which have disastrous effects on the health of both animals and humans (Shazali et al., 2014; Odore et al., 2015). Thus, the use of antibiotics in livestock farming has been prohibited in the European Union (Regulation, 2003; Van Boeckel et al., 2015).

Ascorbic acid is a natural antioxidant and health-promoting agent that has the potential to replace antibiotic growth promoters in fighting bacterial infections (Tajkarimi and Ibrahim, 2011; Verghese et al., 2017), and it has been thought to be beneficial for heat-stressed broiler chickens. Dietary supplementation of ascorbic acid has the advantage of compensating for incompetent biosynthesis of ascorbic acid and has the potential to ameliorate the harmful effects of hot climate in broiler chickens (Njoku, 1986). Data from several studies suggest that ascorbic acid supplementation may compensate for the reduction in growth rate and feed intake (Kutlu and Forbes, 1993; Kadim et al., 2008), improve overall growth performance and antioxidant status, and reduce serum concentrations of corticosterone, acute phase proteins, and cholesterol and reduce lipid oxidation (Sahin et al., 2003; Ferreira et al., 2015), and decrease heat shock protein (**HSP**) 70 gene expression (Mahmoud et al., 2004).

Using postbiotics as dietary additives for livestock and potential alternatives to antibiotics, postbiotics produced from the probiotic *Lactiplantibacillus plantarum* have been the subject of several recent research studies. The mechanism of action of postbiotics is not different from that of probiotics owing to the fact that the same secondary metabolites from probiotics are presented in postbiotics but not in living cells (Thanh et al., 2009). Postbiotics contain several antimicrobial components including bacteriocins and organic acids, which can minimize the pH of the gut and prevent the growth of pathogens in both the feed and animal gut (Aguilar-Toalá et al., 2018). Recent evidence suggests that postbiotics produced by *L. plantarum* strains have an inhibitory effect on several gut pathogens such as *Listeria monocytogenes*, vancomycin-resistant enterococcus, *Salmonella typhimurium*, and *Escherichia coli* (Thanh et al., 2010; Van Thu et al., 2011; Choe et al., 2013; Kareem et al., 2014). It has recently been observed that dietary supplementation of postbiotics promotes the health and growth performance in broilers (Loh et al., 2010; Rosyidah et al., 2011; Kareem et al., 2016b), layers (Choe et al., 2012; Loh et al., 2014), and piglets (Thu et al., 2011; Loh et al., 2013b). More recently, postbiotics have been reported to enhance growth performance, rumen fermentation, immune status, the antioxidant defense system, and gut health in small ruminants (Izuddin et al., 2018, 2019, 2019b, 2020). Under normal environmental temperature, dietary supplementation of postbiotics improves health and growth performance of broilers by promoting their immune status, growth gene expression, and gut health as their supplementation significantly improves the intestinal villus, decrease the population of Enterobacteriaceae and fecal pH, and increase the population of lactic acid bacteria (Thanh et al., 2009; Loh et al., 2010; Rosyidah et al., 2011; Kareem et al., 2016b). Moreover, improvements in broiler meat quality and reduction in plasma cholesterol levels are observed with dietary supplementation of postbiotics in broilers (Choe et al., 2012; Loh et al., 2013a; Kareem et al., 2015, 2016a). Our recent findings revealed that dietary supplementation of postbiotics produced from *L. plantarum* increased BW, BW gain, feed conversion ratio, intestinal villus height, immune response, IGF-1 and GHR mRNA expression, the nonpathogenic bacteria population in the cecum, and reduced the Enterobacteriaceae and *E. coli* population in heat-stressed broilers (Humam et al., 2019).

Aside from developing a healthy gut and promoting growth performance, a preliminary study from this laboratory revealed that postbiotics produced by *L. plantarum* have high antioxidant activities (Izuddin et al., 2020). Humam et al. (2020) reported that dietary supplementation of different postbiotics mitigates adverse impacts of heat stress by increasing antioxidant enzyme activity, total antioxidant capacity, acute phase protein expression, and HSP70 expression in broilers. Similarly, bacterial cultures of *L. plantarum* were reported to exhibit high antioxidative activities (Ji et al., 2015; He et al., 2015). It has been documented that feeding broilers on the probiotic *Bacillus subtilis* increased the levels of glutathione peroxidase (**GPx**) and glutathione (**GSH**) and their mRNA expression level (Bai et al., 2016). In heat-stressed broilers, probiotics have been demonstrated to upregulate hepatic antioxidant capacity (Li et al., 2012; Jahromi et al., 2016; Sugiharto et al., 2017), inhibit secretion of proinflammatory cytokines (tumor necrosis factor alpha [**TNF-α**] and IL-1β), and increase levels of anti-inflammatory cytokines (IL-10) (Giri et al., 2019). Izuddin et al. (2020) reported that lambs that received postbiotics had higher regulation of rumen barrier function by upregulation of occludin (**OCLN**), claudin-1 (**CLDN1**), and CLDN4 expression. Other studies reported that chickens fed with probiotics showed increase in intestinal epithelial integrity by increase in mucin mRNA expression (Smirnov et al., 2005; Aliakbarpour et al., 2012), and

postbiotics produced from the *L. plantarum* probiotic are expected to provide analogous benefits to those from probiotic bacteria. Although considerable research has investigated the beneficial impacts of postbiotics on broiler chickens under normal temperature, there is still a scarcity of information on their impacts on heat-stressed broilers. Therefore, the purpose of this work was to investigate the impacts of feeding different inclusion levels of postbiotic RI11 on the antioxidant enzyme activity and gene expression related to gut barrier function, acute phase proteins, HSP70, and cytokines in broiler chickens under heat stress.

MATERIALS AND METHODS

Postbiotic RI11 Production

The *L. plantarum* RI11 strain was procured from the Industrial Biotechnology Laboratory, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. The culture was preserved by the revival of culture following the procedure of Foo et al. (2003). The culture was kept at −20°C in de Man, Rogosa and Sharpe (**MRS**) medium (Merck, Darmstadt, Germany) with 20% (v/v) glycerol.

A volume of 100 µL from stock culture was activated in 10 mL of MRS broth, incubated at 30°C for 48 h, and subcultured in the same media for another 24 h. The activated culture was spread onto a plate and incubated at 30°C for 48 h. A single colony was picked from the plate, inoculated twice into MRS broth (10 mL), and incubated at 30°C for 48 h and 24 h. Active cells of *L. plantarum* RI11 was first washed using a 0.85% (w/v) NaCl (Merck, Darmstadt, Germany) sterile solution, then adjusted to 10^9 cfu/mL, and used as an inoculum. For preparing the working culture of the *L. plantarum* RI11 strain, 10% (v/w) 10^9 cfu/mL bacterial cells were inoculated into MRS media, incubated for 10 h at 30°C, and centrifuged at 10,000 × *g* for 15 min at 4°C. The cell-free supernatant was filtered using a membrane of cellulose acetate (0.22 µm; Sartorius Minitart, Gottingen, Germany) following the procedure described by Loh et al. (2009). The harvested cell-free supernatant (postbiotic RI11) was kept at 4°C until applied in feed within 48 h.

Ethical Note, Birds, Diets, Experimental Design, and Housing

The feeding trial was performed at the research facilities of the Institute of Tropical Agriculture and Food Security, Universiti Putra Malaysia. The study was conducted following the guidelines approved by the Animal Ethics Committee of Universiti Putra Malaysia (protocol no. UPM/ACUC/AUP-R085/2018), which ascertains that the use and care of research animals are ethical and humane. Two hundred fifty-two Cobb 500 male chicks (1-day-old) were supplied by a local hatchery. The chicks were housed in wire-floor cages placed in 2 identical rooms. The rooms were environmentally controlled, with each of the 2 measuring 9.1 × 3.8 × 2.3 m (length × width × height), whereas measurement of each cage was 120 (length) × 120 (width) × 45 (height) cm. The birds were reared following the management recommendations of Cobb 500 from 1 to 21 d of age (starter period). The chickens in the 2 rooms were maintained at the recommended temperature of 32 ± 1°C on the first day, and the temperature was gradually reduced to around 24 ± 1°C by 21 d of age. During the finisher period (day 22–day 42), the birds were divided into 7 treatment groups, 6 replicates per group with 6 chicks in each replicate. The birds were offered one of the 7 diets: 1) a basal diet without any supplementation as the negative control (NC group) (0.0% RI11); 2) NC + 0.02% (w/w) oxytetracycline as the positive control (OTC group); 3) NC + 0.02% (w/w) ascorbic acid as the antioxidant control (AA group); or 4) four further groups in which NC diet is supplemented with the following levels of postbiotic, that is, NC + 0.2% postbiotic RI11 (v/w), NC + 0.4% postbiotic RI11 (v/w), NC + 0.6% postbiotic RI11 (v/w), and NC + 0.8% postbiotic RI11 (v/w). The postbiotic RI11 has been chosen based on its role to alleviate the adverse effect of heat stress and enhance the growth performance as compared with other postbiotics from previous studies (Human et al., 2019, 2020). The basal diets were formulated using FeedLIVE software following the nutrient specifications of the Cobb 500 Nutrition Guide (Cobb-Vantress, 2008). From day 22 to day 42, broilers were subjected to 36 ± 1°C for 3 h per day from 11:00 am to 2:00 pm. Approximately, it took 45 min for the temperature to rise from 24 to 36°C. Nonetheless, it took 1 h and 30 min for the temperature to decline from 36 to 24°C. The management and environmental conditions of this current experiment were described by our companion in a recently published article (Humam et al., 2019).

Sample Collection

At 42 d of age, around 2 h and 30 min after the daily heat stress, 2 chickens from each cage (12 chickens per treatment group) were selected in random and slaughtered following the Halal procedure, as recommended in the Malaysian Standard (Malaysia, 2009). Blood samples (exsanguination) were collected in blood tubes (BD Vacutainer, Franklin Lakes, NJ) containing EDTA as an anticoagulant and kept on ice. Upon centrifugation at 3,500 × *g* for 15 min at 4°C, harvested plasma samples were (1.5-mL microcentrifuge tubes) stored at −80°C for later determination of GPx, superoxide dismutase (**SOD**), catalase (**CAT**), and GSH concentration. A part of the liver and ileal tissue was collected immediately after slaughtering, frozen in liquid nitrogen, and stored at −80°C for gene expression analysis including IL-10, IL-8, IL-6, IL-2, interferon (**IFN**), TNF-α, HSP70, alpha-1-acid glycoprotein (**α1-AGP**), ceruloplasmin (**CPN**), zonula

occludens-1 (**ZO-1**), mucin 2 (**MUC2**), CLDN1, and OCLN mRNA expressions.

Plasma Antioxidant Enzyme Biomarkers

Glutathione peroxidase activity was analyzed in plasma samples using the EnzyChrom Glutathione Peroxidase Assay Kit (EGPx-100; BioAssay Systems, Hayward), which directly measured the consumption of NADPH in enzyme-coupled reactions. The assay was carried out as recommended in the manufacturer's protocol. The detection range of the kit was 40–800 U/L GPx. Approximately, 10 μL of the sample and 90 μL of the working reagent (80 μL of assay buffer, 5 μL of GSH, 3 μL of NADPH (35 mmol), and 2 μL of glutathione reductase) were loaded into the microplate well, and the plate was tapped to facilitate mixing. A volume of 100 μL of the substrate solution was added to each sample and control well. The optical density of the samples and standards was measured immediately at time 0 (OD0) and again at 4 min (OD4). The absorbance of the GPx activity was recorded at 340 nm using a microplate reader (Multiskan GO; Thermo Scientific, Waltham, Massachusetts). The NADPH standards were used to plot the standard curve. The standard curve was used to calculate GPx activity in the plasma samples.

Superoxide dismutase activity was analyzed using the EnzyChrom Superoxide Dismutase Assay Kit (ESOD-100; BioAssay Systems, Hayward) based on the protocol provided by the manufacturer. The detection range of the kit was 0.05–3 U/mL SOD. The test depended on the addition of xanthine oxidase to the sample as a source of superoxide, and this superoxide reacted with a specific dye to form a colored product. Based on the activity of SOD in the sample, which acted as a superoxide scavenger, the superoxide was reduced, and then, the intensity of color decreased. The activity of SOD was determined by measuring the color intensity at 440 nm using a microplate reader (Multiskan GO; Thermo Scientific, Waltham, Massachusetts). The standard curve was used to calculate the concentration of SOD in the samples.

Catalase activity was measured from plasma using the EnzyChrom catalase assay kit (ECAT-100; BioAssay Systems, Hayward), according to the manufacturer's protocol. The detection range of the kit was 0.2–5 U/L CAT. The test depended on the degradation of H_2O_2 using the redox dye. After the preparation of the assay, 10 μL of the sample, positive control, and assay buffer as blank plus 90 μL of substrate buffer (50 μmol) were loaded into the microplate well, and then, the plate was shaken and incubated at room temperature for 30 min. During the incubation time, the standard curve was prepared by mixing 40 μL of the 4.8 mmol H_2O_2 reagent with 440 μL of distilled water in serial concentration, and then, 10 μL of the standard solution and 90 μL of assay buffer were placed into standard wells. After incubation, 100 μL of the detection reagent was combined in each well and incubated for 10 min at room temperature. Finally, the optical density of CAT was read at 570 nm using a microplate reader (Multiskan GO; Thermo Scientific, Waltham, Massachusetts). The standard curve was used to calculate CAT activity in the plasma samples.

Glutathione activity was measured in plasma using the QuantiChrom Glutathione Assay Kit (DIGT-250; BioAssay Systems, Hayward) following the manufacturer's protocol. The principle of the assay depended on the reaction of 5,5′-dithiobis-2-nitrobenzoic acid with reduced GSH to form a yellow product. In brief, 120 μL of the 20-fold diluted sample was mixed with 120 μL of reagent A into a 1.5-mL tube and centrifuged at 14,000 rpm for 5 min, and 200 μL of the supernatant was transferred into the microplate well. A volume of 100 μL of reagent B was added to each well of samples, and the plate was tapped for mixing and was incubated for 25 min at room temperature. A volume of 400 μL of the calibrator was mixed in serial dilution with distilled water into separate wells as the standard. A volume of 300 μL of distilled water was pipetted into a separate well as a blank. After incubation, the absorbance was read at 412 nm using a microplate reader (Multiskan GO; Thermo Scientific, Waltham, Massachusetts). The GSH concentration in the plasma was calculated using the standard curve of GSH.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction of the Studied Genes

The extractions of total RNA from liver and ileal tissue samples were conducted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and protocols. Thirty milligrams of liver and ileal tissue samples was homogenized with 600 μL of buffer RLT and centrifuged at 4°C and at 10,000 × *g* for 2 min to obtain the supernatant. The collected supernatant was mixed with an equal volume of 70% (v/v) undenatured ethanol. Then, the RNeasy spin column (Qiagen, Hilden, Germany) was used for RNA binding, and a series of buffer RW1 and buffer RPE were used for RNA purification. RNase-free water was used to elute the purified RNA from the spin column. The purified RNA was confirmed for its concentration and purity using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) at 260/280 nm absorbance ratio. The cDNA was generated from purified RNA using a Quantitect reverse transcription kit (Qiagen, Hilden, Germany) for quantitative PCR.

Reverse transcription real-time PCR was performed on a Bio-Rad CFX96 PCR machine (Bio-Rad Laboratories, Hercules, CA). The standardization of target gene expressions was determined by the GAPDH gene as a housekeeping gene. A total of 20 μL of PCR reaction mixture for every sample was prepared using the QuantiNova SYBR Green PCR kit (Qiagen, Hilden, Germany) containing 10 μL of 2X SYBR Green Master

Table 1. The primer sequences of target genes used for real time-polymerase chain reaction (RT-qPCR).

Target gene[1]	Primer sequence, 5′–3′[2]	Accession no.	Product size (bp)
	R-CTTGTGGATGGCATGATCT		
IL-8	F-GCCCTCCTCCTGGTTTCA G	AJ009800	74
	R-TGGCACCGCAGCTCATT		
IL-6	F-GCTCGCCGGCTTCGA	AJ250838	71
	R-GGTAGGTCTGAAAGGCGAACAG		
IL-2	F-GTGGCTAACTAATCTGCTGTCCA	NM 204153	144
	R-CCGTAGGGCTTACAGAAAGG		
IL-10	F-TAACATCCAACTGCTCAGCTC	NM 001004414	172
	R-TGATGACTGGTGCTGGTCTG		
IFN-γ	F-GAGCCATCACCAAGAAGATGA	NM 205149	214
	R-TAGGTCCACCGTCAGCTACA		
TNF-α	F-GCTGTTCTATGACCGCCCAGTT	NM 204267.1	140
	R-AACAACCAGCTATGCACCCCA		
HSP70	F-AGCGTAACACCACCATTCC	NM_001006685.1	372
	R-TGGCTCCCACCCTATCTC		
α1-AGP	F-TCTGATCTAGACCTGCAGGCTC	AY584568.1	814
	R-ATCCTCGCCATGGGGTTGGTG		
CPN	F-GAGAGTAAGGGTGGGGGTGGG	XM_015291853.1	4134
	R-TATTTCACATTTTCCACAAGG		
MUC2	F-TTCATGATGCCTGCTCTTGTG	XM_421035	93
	R-CCTGAGCCTTGGTACATTCTTGT		
CLDN1	F-CATACTCCTGGGTCTGGTTGGT	NM_001013611.2	100
	R-GACAGCCATCCGCATCTTCT		
OCLN	F-ACGGCAGCACCTACCTCAA	XM_025144248	123
	R-GGGCGAAGAAGCAGATGAG		
ZO-1	F-CTTCAGGTGTTTCTCTTCCTCCTC	XM_015278981	131
	R-CTGTGGTTTCATGGCTGGATC		
GAPDH	F-CTGGCAAAGTCCAAGTGGTG	NM_204305	275
	R-AGCACCACCCTTCAGATGAG		

[1]IFN-γ: interferon gamma; TNF-α: tumor necrosis factor alpha; HSP70: heat shock protein 70; α1-AGP: alpha-1-acid glycoprotein; CPN: ceruloplasmin; MUC2: mucin 2; CLDN1: claudin 1; OCLN: occludin; ZO-1: zonula occludens-1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.
[2]F: forward; R: reverse.

Mix, 2 μL of sample cDNA, 1 μL each of 14 μmol of the respective forward and reverse primers, and 6 μL of RNase-free water. The sequence of forward and reverse primers of target and housekeeping genes is depicted in Table 1.

The real-time PCR machine was programmed as follows: initial denaturation at 95°C for 10 min, following by 40 cycles of denaturation, annealing, and extension (denaturation at 95°C for 15 s; annealing at 57°C for GADPH, HSP70, IL-10, and IFN genes, 59°C for α1-AGP and CPN genes, 55°C for the TNF-α gene, and 60°C for IL-6, IL-8, IL-2, OCLN, ZO-1, CLDN1, and MUC2 genes for 30 s; and a final extension at 72 °C for 20 s). The melting curve program was included after PCR amplification cycles to confirm the amplification specificity of the primers. The efficiency of amplification of both target and housekeeping genes was analyzed based on the standard curve of 5-fold serial dilution of cDNA on a real-time PCR machine. The relative gene expression based on the housekeeping gene was quantified following the approach recommended by Livak and Schmittgen (2001).

Statistical Analysis

This study was subjected to a completely randomized design, with data analyses being performed using Statistical Analysis System (SAS) 9.4 software (SAS Institute, Cary, North Carolina). All data were analyzed using the general linear model procedure of SAS, and the comparisons of means were identified using Duncan's multiple range test. The determination of linear and quadratic effects of increasing levels of postbiotic RI11 was conducted by orthogonal polynomial contrast of SAS. The significance of the statistical difference between treatments was considered at P-value <0.05.

RESULTS

Antioxidant Enzyme Activity

There was significantly higher GPx activity in the 0.4, 0.6, and 0.8% RI11 and AA treatment groups than in the NC, 0.2% RI11, and OTC treatment groups (Table 2). No significant difference in the GPx activity was observed between the NC, 0.2% RI11, and OTC groups or between the 0.4, 0.6, and 0.8% RI11 and AA groups. The SOD results were not different ($P > 0.05$) between the dietary treatment groups in broilers under heat stress. The 0.6% RI11 group had the highest CAT activity ($P < 0.05$) as compared with the NC and OTC groups. The postbiotic RI11 and AA groups were not different ($P > 0.05$) from each other with regard to the CAT activity. The CAT activity results were not significantly different among the NC, 0.2% RI11, OTC, and AA treatment groups. The highest GSH activity ($P < 0.05$) was observed in the 0.4 and 0.6% RI11 groups as compared with the NC and OTC groups, whereas the

Table 2. Antioxidant enzyme activities in broiler chickens fed with different levels of postbiotic RI11 under heat stress.

Parameters	Dietary treatments[1]							SEM	*P*-values	Contrast, *P*-values[2]	
	NC	OTC	AA	0.2%	0.4%	0.6%	0.8%			Linear	Quadratic
GPx (μmol/L)	644^{c}	717.2^{b,c}	1,012.6^{a}	921.4^{b,c}	1052^{a}	1163^{a}	1,021.9^{a}	50.53	0.002	0.001	0.014
SOD (U/mL)	1.971	1.793	2.312	2.127	1.998	2.694	2.027	0.081	0.062	0.26	0.249
CAT (U/L)	4.711^{c}	4.870^{b,c}	5.217^{a,b,c}	5.231^{a,b,c}	5.329^{a,b}	5.638^{a}	5.320^{a,b}	0.079	0.027	0.006	0.033
GSH (μM)	53.41^{b,c}	52.38^{c}	56.17^{a,b}	54.34^{a,b,c}	56.92^{a}	57.47^{a}	56.12^{a,b}	0.55	0.013	0.014	0.098

$^{a\text{-}c}$Means with different superscripts in the same row indicate significant difference ($P < 0.05$), n = 8 samples per group.

Abbreviations: CAT, catalase; GPx, glutathione peroxidase; GSH, glutathione; SOD, superoxide dismutase.

[1]Dietary treatments: NC = negative control (0.0% RI11) basal diet only; OTC = basal diet + 0.02% (w/w) oxytetracycline (positive control); AA = basal diet + 0.02% (w/w) ascorbic acid (antioxidant control); 0.2%, 0.4%, 0.6%, and 0.8% = basal diet + sequential levels of postbiotic RI11 (v/w).

[2]Contrast, *P*-values = comes from polynomial contrast for (0.0% to 0.8%) treatments.

OTC group recorded lower ($P < 0.05$) GSH activity than the 0.8% RI11 group. The activity of GSH was not different ($P > 0.05$) among the postbiotic RI11 groups and AA group or between the OTC, NC, and 0.2% RI11 groups.

Increasing the level of postbiotic RI11 in diet increased quadratically the GPx and CAT activities ($P = 0.014$ and 0.033, respectively).

Cytokine mRNA Expression

The mRNA expression of the IL-6, IL-8, IL-10, IL-2, IFN, and TNF-α gene in the ileal tissue of heat-stressed broiler chickens fed with different levels of postbiotic RI11 is shown in Table 3. Different levels of postbiotic RI11 did not affect ($P > 0.05$) the IL-6, IL-2, and IFN mRNA expression. The IL-8 expression was significantly downregulated in the RI11 and AA groups than in the NC group. No significant difference was observed in the expression of IL-8 between the postbiotic RI11, OTC, and AA treatment groups.

The mRNA expression of IL-10 was higher ($P < 0.05$) in the 0.4 and 0.6% RI11 groups than in the NC, OTC, and 0.2% RI11 treatment groups. The AA group showed higher ($P < 0.05$) IL-10 expression than the NC and OTC groups, but the expression was not significantly different as compared with RI11 groups. No difference ($P > 0.05$) was observed for IL-10 expression between the NC, OTC, 0.2, and 0.8% RI11 groups. The addition of postbiotic RI11 at different levels downregulated TNF-α mRNA expression as compared with the NC group, whereas no difference ($P > 0.05$) was found among the postbiotic RI11 treatment groups. The 0.6% RI11 group showed lower ($P < 0.05$) mRNA expression of TNF-α than the NC, OTC, AA, and 0.2% RI11 groups. However, no difference ($P > 0.05$) was observed between the latter groups for mRNA expression of TNF-α. Increasing the level of postbiotic RI11 in the diet led to quadratic increase ($P = 0.01$) in the mRNA expression of IL-10 and decreased ($P = 0.015$ and 0.014) the mRNA expression of IL-8, IL-6, IL-2, and TNF-α in ileal tissue.

Gut Barrier Gene Expression

The addition of different levels of postbiotic RI11 in the diet of broiler chickens upregulated ($P < 0.05$) the expression of ZO-1 and MUC2 genes (Table 4). The expression of ZO-1 was higher in the 0.6% RI11 group as compared with the NC and OTC groups. No difference ($P > 0.05$) was observed among the 0.2, 0.4, 0.8% RI11 and AA groups or among the postbiotic RI11 (except 0.6%), OTC, AA, and NC groups for ZO-1 expression. The mRNA expression of MUC2 was higher ($P < 0.05$) in the 0.6% RI11 group than in the NC, 0.2% RI11, and OTC groups, but not different ($P > 0.05$) from the 0.4, 0.8, and AA groups. A significant higher MUC2 expression was observed in the 0.4 and 0.8% RI11 groups as compared with the NC group. No difference ($P > 0.05$) in the expression of the MUC2 gene was found among the NC, OTC, AA, and 0.2% RI11 groups. The OCLN mRNA expression was downregulated in the dietary treatment groups as compared with the NC group. There was no dietary effect ($P > 0.05$) on the expression of the CLDN1 gene. Increasing the level of

Table 3. Expression of IL-6, IL-8, IL-10, IL-2, IFN, and TNF-α in the ileal tissue of heat-stressed broiler chickens fed with different levels of postbiotic RI11.

Parameters (mRNA fold change)	Dietary treatments[1]							SEM	*P*-values	Contrast, *P*-values[2]	
	NC	OTC	AA	0.2%	0.4%	0.6%	0.8%			Linear	Quadratic
IL-6	1	0.890	0.720	0.740	0.653	0.5524	0.501	0.062	0.322	0.025	0.505
IL-8	1^{a}	0.719^{a,b}	0.579^{b}	0.572^{b}	0.484^{b}	0.422^{b}	0.478^{b}	0.052	0.039	0.001	0.015
IL-10	1^{c}	0.952^{c}	1.743^{a,b}	1.191^{b,c}	1.838^{a}	1.920^{a}	1.501^{a,b,c}	0.087	0.001	0.003	0.010
IL-2	1	0.680	0.684	0.683	0.655	0.648	0.601	0.051	0.446	0.038	0.225
IFN	1	0.730	0.688	0.728	0.970	0.687	0.717	0.059	0.630	0.195	0.887
TNF-α	1^{a}	0.826^{a,b}	0.732^{a,b}	0.761^{a,b}	0.564^{b,c}	0.413^{c}	0.528^{b,c}	0.045	0.002	<0.0001	0.014

^{a,b,c}Means with different superscripts in the same row indicate significant difference ($P < 0.05$), n = 8 samples per group.

Abbreviations: IFN, interferon; TNF-α, tumor necrosis factor alpha.

[1]Dietary treatments: NC = negative control (0.0% RI11) basal diet only; OTC = basal diet + 0.02% (w/w) oxytetracycline (positive control); AA = basal diet + 0.02% (w/w) ascorbic acid (antioxidant control); 0.2%, 0.4%, 0.6%, and 0.8% = basal diet + sequential levels of postbiotic RI11 (v/w).

[2]Contrast, *P*-values = comes from polynomial contrast for (0.0% to 0.8%) treatments.

Table 4. Changes in ZO-1, MUC2, CLDN1, and OCLN mRNA expression levels in ileal tissue of heat-stressed broiler chickens fed with different levels of postbiotic RI11.

Parameters (mRNA fold change)	Dietary treatments[1]							SEM	*P*-values	Contrast, *P*-values[2]	
	NC	OTC	AA	0.2%	0.4%	0.6%	0.8%			Linear	Quadratic
ZO-1	1^{b}	1.010^{b}	$1.327^{a,b}$	$1.347^{a,b}$	$1.521^{a,b}$	1.739^{a}	$1.275^{a,b}$	0.068	0.026	0.044	0.005
MUC2	1^{c}	$1.428^{b,c}$	$1.506^{a,b,c}$	$1.397^{b,c}$	$1.664^{a,b}$	2.075^{a}	$1.673^{a,b}$	0.080	0.012	0.002	0.049
CLDN1	1	0.936	1.127	1.217	1.396	1.227	1.136	0.061	0.502	0.528	0.073
OCLN	1^{a}	0.449^{b}	0.346^{b}	0.412^{b}	0.446^{b}	0.480^{b}	0.432^{b}	0.048	0.002	0.001	0.005

[a,b,c]Means with different superscripts in the same row indicate significant difference ($P < 0.05$), n = 8 samples per group.

Abbreviations: CLDN1, claudin-1; MUC2, mucin 2; OCLN, occludin; ZO-1, zonula occludens-1.

[1]Dietary treatments: NC = negative control (0.0% RI11) basal diet only; OTC = basal diet + 0.02% (w/w) oxytetracycline (positive control); AA = basal diet + 0.02% (w/w) ascorbic acid (antioxidant control); 0.2%, 0.4%, 0.6%, and 0.8% = basal diet + sequential levels of postbiotic RI11 (v/w).

[2]Contrast, *P*-values = comes from polynomial contrast for (0.0% to 0.8%) treatments.

RI11 in broilers' diet quadratically ($P = 0.005$ and 0.049) upregulated the expression of ZO-1 and MUC2 genes and downregulated ($P = 0.005$) the expression of the OCLN gene.

Acute Phase Protein and HSP70 mRNA Expression

The results of hepatic α1-AGP, CPN, and HSP70 mRNA expression in heat-stressed broiler chickens fed with different levels of postbiotic RI11 are shown in Table 5. There was no diet effect ($P > 0.05$) on the expression of the hepatic CPN gene. The expression of α1-AGP was downregulated ($P < 0.05$) when broilers were fed with 0.4, 0.6, and 0.8% RI11 diets as compared with NC and OTC diets. The α1-AGP expression level in the AA group was lower ($P < 0.05$) than that in the NC group, whereas it was not different ($P > 0.05$) as compared with all postbiotic RI11 groups. No significant difference was found for α1-AGP expression between the NC, OTC, and 0.2% RI11 treatment groups.

The expression of HSP70 mRNA was downregulated ($P < 0.05$) in the postbiotic 0.4, 0.6, and 0.8% RI11 groups as compared with the NC group. There was no significant difference observed for the HSP70 expression level between the NC, OTC, and 0.2% RI11 groups or between the AA and RI11 treatment groups. There was significant downregulation ($P < 0.05$) in the expression of α1-AGP and HSP70 genes by increasing the dietary level of postbiotic RI11.

DISCUSSION

Antioxidant Enzyme Activities

Oxidative stress causes the production of ROS varieties, including hydroxyl free radical and superoxide anions. Various research studies reported that overflow of ROS could damage biological macromolecules such as proteins and nucleic acids, consequently leading to development of diseases (Bai et al., 2017). In chickens, the main antioxidant enzymes are GPx, SOD, CAT, and GSH. These enzymes are important to transform reactive species into nonradical and nontoxic products (Akbarian et al., 2016).

In this study, the heat-stressed chickens supplemented with various levels of RI11 (excluding 0.2%) recorded increased GPx activity, but there was no difference between the 0.2% RI11 and OTC groups. However, the result signified that higher levels of RI11 are required to improve GPx activity in broiler chickens under heat stress. There was no effect of various treatments on SOD activity, whereas CAT and GSH activities were enhanced significantly after postbiotic supplementation with 0.4, 0.6, and 0.8% RI11. This finding is consistent with that of the study by Wang et al. (2018), who indicated that feed supplementation with probiotics enhanced CAT, GPx, and SOD activities in broilers on day 21, which may be one of the mechanisms of its beneficial effects on health and growth performance of broilers. Another study found that feeding broilers on the probiotic *B. subtilis* increased activities of GPx and GSH and their mRNA expression level (Bai et al.,

Table 5. Changes in the hepatic α1-AGP, CPN, and HSP70 gene expression level in heat-stressed broilers fed with different levels of postbiotic RI11.

Parameters (mRNA fold change)	Dietary treatments[1]							SEM	*P*-values	Contrast, *P*-values[2]	
	NC	OTC	AA	0.2%	0.4%	0.6%	0.8%			Linear	Quadratic
α1-AGP	1^{a}	$0.87^{a,b}$	$0.617^{b,c}$	$0.661^{a,b,c}$	0.425^{c}	0.305^{c}	0.392^{c}	0.054	0.001	<0.0001	0.004
CPN	1	0.917	0.837	0.879	0.922	0.733	0.741	0.046	0.685	0.120	0.958
HSP70	1^{a}	$0.778^{a,b}$	0.63^{b}	$0.738^{a,b}$	0.568^{b}	0.559^{b}	0.604^{b}	0.043	0.018	0.001	0.024

[a,b,c]Means with different superscripts in the same row indicate significant difference ($P < 0.05$), n = 8 samples per group.

Abbreviations: α1-AGP, alpha-1-acid glycoprotein; CPN, ceruloplasmin; HSP70, heat shock protein 70.

[1]Dietary treatments: NC = negative control (0.0% RI11) basal diet only; OTC = basal diet + 0.02% (w/w) oxytetracycline (positive control); AA = basal diet + 0.02% (w/w) ascorbic acid (antioxidant control); 0.2%, 0.4%, 0.6%, and 0.8% = basal diet + sequential levels of postbiotic RI11 (v/w).

[2]Contrast, *P*-values = comes from polynomial contrast for (0.0% to 0.8%) treatments.

2016). Likewise, 2 studies reported that broilers under heat challenge had increased activities of CAT, GPx, GSH, and SOD (Altan et al., 2003; Yang et al., 2009). Hence, dietary postbiotic RI11 showed the capacity to improve antioxidant activities (concentrations of GPx, CAT, and GSH) in the plasma of heat-stressed broilers. Postbiotics are a natural source of antimicrobials and antioxidants that can safely alleviate stress and improve the health of animals. As postbiotics possess the probiotic characteristics (Loh et al., 2014; Kareem et al., 2016a; Foo et al., 2019; Gao et al., 2019; Humam et al., 2019; Izuddin et al., 2019), probiotic studies can provide useful information to understand how postbiotics could improve antioxidant capability and develop oxidative resistance in the body under heat stress. Several studies reported that supplementation of probiotics in poultry diets reduced adverse effects of oxidative stress and enhanced antioxidant enzyme activities (Deng et al., 2012; Bai et al., 2017), which might reduce cell damage by inhibiting the production of ROS and finally improving the health of animals (Barrow, 1992; Li et al., 2019). Consistent with our results, Shen et al. (2014) reported that blood antioxidant capacities were significantly enhanced by the inclusion of the probiotic *L. plantarum* in the diets and growth performance was promoted in broilers.

This study is the first attempt to provide data on the effect of different levels of postbiotic RI11 on antioxidant activities in heat-stressed broilers. However, probiotics have been reported for their ROS removal capacity and promotion of broiler health under normal (Bai et al., 2017) and high-temperature conditions (Cramer et al., 2018).

Vitamin C has been equally reported to improve the activities of antioxidant enzymes including GPx and GSH in layers (Leskovec et al., 2019). Yun et al. (2012) showed that mRNA and activity of GPx were improved in heat-stressed broilers supplemented with vitamin C without affecting SOD activity.

Cytokine mRNA Expression

Cytokines are small extracellular signaling proteins produced by the host with crucial functions in immunity by enabling cell communication amid immunological development and immune response (Saleh and Al-Zghoul, 2019). The proinflammatory and anti-inflammatory cytokines are produced by immune cells such as T lymphocytes, B lymphocytes, macrophages, and natural killer cells (Jeurissen, 1991). T lymphocytes are divided into 2 types of cells, which are Th1 and Th2. Generally, IL-2, IL-8, IFN-γ, and TNF-α are known as a Th1-type cytokine that increases cellular immunity, whereas IL-6 and IL-10 are known as a Th2-type cytokine that acts in humoral immunity (Mosmann and Sad, 1996; Kidd, 2003; Xie et al., 2015). These small molecules of proteins are released when the animal is exposed to infection, inflammation, and shock as an immune response (Hakansson and Molin, 2011). Heat stress affects intestinal integrity and increases intestinal permeability to endotoxin, antigens, and inflammatory cytokines (Alhenaky et al., 2017). Heat stress has been shown to increase the expression of proinflammatory cytokines and suppress anti-inflammatory cytokines in broilers (Gadde et al., 2017). Heat stress leads to gut damage and induces commensal bacteria to release endotoxin that encourages the production of the proinflammatory cytokines (De Boever et al., 2008). The present study shows that the increase in the levels of proinflammatory cytokines in broilers under heat stress such as IL-8 and TNF-α could be alleviated by postbiotic dietary supplementation. Feeding postbiotic RI11 at various levels would modulate the inflammatory processes by restoring cytokine balance to reduce the potential inflammation-induced injury that occurs after heat stress in broiler chickens.

In the present study, lower expressions of IL-8 and TNF-α and higher expression of IL-10 were found in the postbiotic RI11–treated groups than in other treatment groups. The differential expression of IL-8 seen herein could be due to the interaction between the beneficial bacteria that is enhanced by postbiotics and intestinal enterocytes and immune cells of the lamina propria (Galdeano and Perdigon, 2006). These findings were in line with those of the study by Kareem et al. (2016b), who reported reduced cytokine expression in broiler chickens supplemented with various combinations of postbiotics and inulin. Wang (2017) documented that the supplemented probiotic *B. subtilis* in diets of broilers under heat stress decreased the expression levels of IL-6 and TNF-α and increased the IL-10 expression level. Inflammatory cytokines, especially TNF-α, IL-2, IL-8, and IL-6, play important roles in inducement and prolongation of inflammation caused by macrophages. The high levels of TNF-α have the capability to increase tissue damage or sepsis and death (Chong and Sriskandan, 2011). In this study, TNF-α expression was downregulated by supplementation of postbiotic RI11 in broilers under heat stress as compared with the NC group. Recently, supplementation with a polysaccharide-based bioflocculant extracted from *B. subtilis* F9 inhibited the expression of TNF-α and IL-1, whereas that of IL-10 was significantly increased owing to the anti-inflammatory potential of the polysaccharide-based bioflocculant (Giri et al., 2019). Previous studies have demonstrated the effect of probiotics in reducing proinflammatory cytokine production (Bai et al., 2004; Carey and Kostrzynska, 2013). In piglets, Yang et al. (2015) posited that pretreatment of porcine epithelial cells with *Lactobacillus reuteri* lowered the expression of TNF-α and IL-6. Moreover, the effects of feeding commensal bacteria such as lactic acid bacteria have been reported to have both proinflammatory and anti-inflammatory actions (Foligne et al., 2007). The high population of *Lactobacillus* and *Bifidobacterium* could play a role in anti-inflammatory cytokine expression, whereas the opposite reaction may be due to the lowered pathogen load (Herfel et al., 2011). In piglets, an increased population of lactobacilli was associated with decreased expression of IL-8 (Mulder et al., 2009). This

was exemplified in this study based on the significant increment in the *Lactobacillus* and *Bifidobacterium* count, the reduced pathogenic load, and downregulation of IL-8. In other studies, involving probiotics, reduction in IL-8 secretion in intestinal epithelial cells (**IEC**) was suggested to occur through different pathways (Gadde et al., 2017). The expressions of IL-2, IL-6, and IFN were not affected by the inclusion of postbiotics in diets of broilers under heat stress. However, IL-6 expression was upregulated in broilers fed with a combination of postbiotics and inulin (Kareem et al., 2016a) and lambs (Izuddin et al., 2019) fed with postbiotic RG14.

The results of the present study allowed us to suggest that postbiotic RI11 influences cytokine expression dynamics of broilers by the modulation of the balance between anti-inflammatory and proinflammatory cytokines under heat stress. Therefore, postbiotics could ameliorate heat tolerance by upregulation of cytokine expression to tissue stability and repairing mechanisms that are working during and after heat stress recovery, which reflected positively on the health and growth performance.

Gut Barrier Gene Expressions

The gut mucosal barrier is mainly formed by the intestinal epithelium and remains an essential part of the immune response in the intestine. Upon entry of foreign bodies such as pathogenic microbes, the IEC is the first line of defense, and they interact effectively with commensal bacteria and antimicrobial substances to protect the intestinal barrier (Thomas and Versalovic, 2010). The IEC function is mediated by multiprotein complexes present at the apical end of the IEC, and they are referred to as tight junctions (**TJ**). Tight junctions play an immense role in regulating intestinal permeability by shutting the spaces between adjacent IEC (Suzuki, 2013). Various factors affect TJ and mucosal barrier functions such as cytokines, probiotics, growth factors, and pathogens by transcriptional regulation and post-translational modification of TJ proteins (Thomas and Versalovic, 2010; Günzel and Yu, 2013; Suzuki, 2013). Occludin, CLDN1, and ZO-1 are some of the major functional components of TJ (Tsukita et al., 2001, 2008). The gene for mucin produced by goblet cells is often illustrated as MUC2, a vital component of the mucous layer covering the intestinal epithelium.

In this study, the expression of these TJ was investigated after the postbiotic feeding of broilers exposed to heat stress. The expression of ZO-1 and MUC2 was significantly higher in birds fed with postbiotic RI11 than in those fed with OTC and NC diets, and the 0.6% RI11 group showed the highest effect among the postbiotic groups. The benefits of inclusion of postbiotics at the level of 0.6% may be attributed to the optimal environment provided for better growth of beneficial bacteria, then improvement of the intestinal integrity and nutrient digestibility, and increase in the growth performance of broilers. These findings showed that supplementation of postbiotics at various levels prevented the reduction in expression of ZO-1 and OCLN and induced MUC2 expression by heat stress. These results were in agreement with the finding of the study by Zhang et al. (2017), who found that addition of probiotic mixture in layer feed resulted in upregulation of ZO-1 mRNA expression under heat stress. Supplementation of the probiotic *B. subtilis* in broiler diets significantly increased gene expression of intestinal MUC2 mRNA compared with that in those fed with the control diet, which coincides with our finding (Aliakbarpour et al., 2012). Broilers fed with the *Lactiplantibacillus fermentum* 1.2029 strain showed significantly increased goblet cell density in the jejunum and level of MUC2 mRNA expression in both the jejunum and ileum (Cao et al., 2012). The inclusion of postbiotics did not affect the expression of the CLDN1 gene in this study. These results corroborate the reports from studies conducted previously in mice and pigs, in which the expression of OCLN and ZO-1 was reduced in pulmonary cells of mice and porcine IEC, respectively, after lipopolysaccharide treatment (Xie et al., 2013; Yang et al., 2015). A similar scenario with regard to the reduced expression of ZO1, MUC2, and OCLN was reported in broilers fed with probiotics after lipopolysaccharide challenge (Gadde et al., 2017).

To the best of our knowledge, there is a paucity of data relating to the expression of TJ-related genes in broilers under heat stress. However, the expression of these TJ components illustrates the potential of RI11 to enhance barrier function and prevent antigen entry. The postbiotics used in this study could have improved barrier function and production of mucin and HSP, thereby modulating signaling pathways and survival of IEC.

A recent study reported that inclusion of postbiotics in postweaning lambs increased the expression level of TJ genes, which is in line with our finding (Izuddin et al., 2019). The improvement of the expression level of TJ proteins is attributed to postbiotics, which contain metabolites of probiotic bacteria with ability to affect the regulation of TJ integrity and mucosal barrier function (Izuddin et al., 2019). The interactions of metabolites and bioactive molecules secreted by probiotics with intestinal immune cell receptors modulate epithelial cell function by increasing TJ integrity and prevent its disruption (Günzel and Yu, 2013; Krishna Rao and Samak, 2013).

Acute Phase Protein and HSP70 mRNA Expression

The results from this study showed lower expression of AGP mRNA and HSP70 in heat-stressed broilers supplemented with postbiotics compared with those in the control group. The parameters were also reduced in birds supplemented with AA diet, but not as observed in the postbiotic-treated groups.

Heat shock protein 70 is a useful indicator of cellular insult and predicting the level of thermal stress in

chickens (Zulkifli et al., 2014). The protein is highly preserved, and it is expressed under stress conditions such as transportation, feed restriction, unpleasant human contact, and high temperature (Al-Aqil and Zulkifli, 2009; Zulkifli et al., 2009; Soleimani et al., 2012). During acute heat stress, the level of HSP70 is increased via the synthesis of HSP70 mRNA either by the increased amount of the protein or activity of the heat shock transcription factor (Craig and Gross, 1991; Hosseini et al., 2015). Earlier studies have reported higher levels of HSP70 in various tissues of broilers after exposure to high ambient temperature (Gu et al., 2012; Hosseini et al., 2015). In the present study, the downregulation of HSP70 mRNA in heat-stressed birds supplemented with postbiotics indicates the amelioration of the effect of the environmental stressor on health status of birds. Moreover, for the intestinal barrier to be improved, the expression of HSP is important for the signaling pathways involved in the survival of IEC (Jang et al., 2014).

The ability of different levels of postbiotic RI11 to induce a lower level of expression of HSP70 mRNA as observed in this study could be a mechanism to defend the synthesized TJ proteins from the negative impact of heat stress. Accordingly, HSP70 acts as a chaperone by interacting with proteins to defend synthesized proteins against additional injury and reduce ROS production (Malago et al., 2002). Heat shock protein 70 induction guards against stresses such as hyperthermia, ischemia, and inflammation (Zulkifli et al., 2009; Najafi et al., 2015b).

This study showed that higher levels of RI11 and ascorbic acid supplementation induced a similar positive effect in improving the HSP70 levels in heat-stressed broilers. Birds in both the postbiotic-treated and AA groups had a greater positive effect than the OTC-treated birds. Previous studies have also shown the effect of ascorbic acid in the expression of HSP70 mRNA (Gu et al., 2012; Yun et al., 2012; Jang et al., 2014; Roushdy et al., 2018). Heat-stressed rats supplemented with vitamin C had significantly lower hepatic HSP70 mRNA expression than those not given the dietary treatment; nevertheless, thermic HSP70 expression was not affected by the treatment (Yun et al., 2012).

There is no scientific report to date to investigate the effect of postbiotics on gene expression of HSP70 and acute phase proteins (**APPs**) in heat-stressed broilers. However, the findings are comparable with other studies that assessed the effect of other substances on the previously listed proteins. There was no effect of the various treatments on the mRNA expression of CPN. This could be related to the time variation and response kinetics of different APPs in avian species. For instance, hepatic α1-AGP was reported to be faster in reaction than CPN after exogenous administration of corticosterone (Zulkifli et al., 2014). Moreover, some authors have shown that variation of broiler breeds and examined organs could influence the response of APPs to heat stress and targeted treatments (Jang et al., 2014; Roushdy et al., 2018).

Another likely mechanism for the positive impact of postbiotics on the HSP70 and α1-AGP mRNA expression level is the interaction with antioxidant activities. Gu et al. (2012) posited a direct relationship between the HSP70 level and antioxidant enzyme activities such as those of SOD and GPx and total antioxidant capacity. In this study, we observed improved antioxidant enzyme activity and reduced HSP70 and APPs mRNA expression in the heat-stressed broilers supplemented with various levels of postbiotic RI11. Roushdy et al. (2018) reported that the improved expression level of serum antioxidant enzymes could explain the lower HSP70 expression level in the different strains of broilers under heat stress. Such interaction with HSP70 mRNA expression could be vital in ameliorating the extent of mucosal oxidative injury. From the current results, it can be suggested that the increase in antioxidant capacity and gut mucosal barrier gene expression in broilers fed with postbiotic RI11 may increase the resistance to heat stress, which leads to reduction in cell damage and decrease in the production of APPs and HSP70 by switching off the related genes and then resulted positively on the general health and performance of broilers. The molecular finding in the present study can be linked to that of previous works of Humam et al. (2019, 2020), who reported that broilers fed with postbiotics showed improvement in health and performance, and it could be attributed to the higher regulation of gene expression related to growth, immune response, antioxidant capacity, and mucosal barrier, leading to better health and higher meat production.

CONCLUSION

The results in this study demonstrated that the supplementation of 0.6% postbiotic RI11 in broiler chickens under heat stress increased the plasma concentration of antioxidant enzyme activities (GPx, CAT, and GSH) and reduced the heat stress biomarkers such as acute phase protein (α1-AGP and CPN) and HSP70 mRNA expression. The higher mRNA expression level of IL-10, but lower expression of IL-8 and TNF-α, was observed in those broiler chickens supplemented with different dosages of postbiotic RI11. In addition, the postbiotic RI11–treated groups showed upregulation in ZO-1 and MUC2 expression, further increasing the health and integrity of the intestinal mucosa. Dietary postbiotic RI11 at the level of 0.6% is suggested to provide satisfactory effects and have potential as an alternative to antibiotic growth promoters and antioxidant additive in broiler diets to mitigate the detrimental effects of heat stress.

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DISCLOSURES

The authors declare no conflicts of interest.

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