Research Square

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Supplementation of Postbiotic RI11 Improves Antioxidant Enzymes Activity, Upregulated Gut Barrier Genes and Reduced Cytokines, Acute Phase Proteins and HSP70 Gene Expression Levels in Heat-Stressed Broilers

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Abstract

Background

To alleviate the adverse impacts of stressful environmental conditions on poultry and promoting the animal's health and growth performance, antibiotics have been added to poultry diets as growth promoters. Nevertheless, improper and overuse of antibiotics as feed additives have resulted 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. Postbiotics produced from probiotic *Lactobacillus plantarum* have been the recent research of interest as dietary additives for livestock and potential alternatives to antibiotics. However, there is very scarce of study has considered the effect of postbiotics on broilers under heat stress. The aim of this work was to evaluate the impacts of feeding different levels of postbiotic RI11 on antioxidant enzyme activity, physiological stress indicators, cytokines and gut barrier genes expression in broilers under heat stress.

Materials and Methods

A total of 252 male Cobb 500 were fed with 1 of 7 diets: NC (negative control, 0.0% RI11) basal diet; OTC (positive control) NC + 0.02% (w/w) oxytetracycline; AA (antioxidant control) NC + 0.02% (w/w) ascorbic acid. Four further groups were NC + 0.2, 0.4, 0.6 and 0.8% postbiotic RI11 (v/w) of the respective levels.

Results

Supplementation of different levels of postbiotic RI11 increased plasma glutathione peroxidase, catalase and glutathione enzymes activity. Postbiotic RI11 groups upregulated the mRNA expression of interleukin 10 and downregulated of interleukin 8, tumor necrosis factor-alpha, heat shock protein 70 and alpha 1-acid glycoprotein levels compared to the NC and OTC groups. Feeding various doges of postbiotic RI11 improved the integrity of the intestinal barrier by the upregulation of zonula occludens-1 and mucin2 mRNA expressions. However, no difference was observed in claudin1, ceruloplasmin, interleukin 6, interleukin 2 and interferon expression, but downregulation for occludin expression as compared with the NC group. Supplementation of postbiotic RI11 in different levels quadratically increased the plasma glutathione peroxidase, catalase and glutathione, interleukin 10, mucin2 and zonula occludens-1 mRNA expression, and reduced plasma interleukin 8, tumor necrosis factor-alpha, alpha 1- acid glycoprotein and heat shock protein 70 mRNA expression. Supplementation of postbiotic RI11 at level 0.6% was sufficient to achieve the improvement in health of broiler chickens under heat stress as compared to other levels.

Conclusions

The results suggested that postbiotic produced from *L. plantarum* RI11 particularly at level 0.6% (v/w) could be used as an alternative to antibiotics and natural sources of antioxidants in the poultry industry.

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, behaviour, health and productive performance causing tremendous economic losses [1–5]. They can adversely affect the biological macromolecules such as proteins, lipids, carbohydrates and DNA by the generation and accumulation of reactive oxygen species (ROS) and free radicals in the cells while performing their normal metabolic functions [6–8], resulting in cell damage and the appearance of pathological symptoms [9, 10]. To lighten the adverse impacts of stressful environmental conditions on poultry and promoting the animal's health and growth performance, antibiotics at sub-therapeutic doses have been added to poultry diets as growth promoters [11–13]. However, the 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 [14, 15].Thus, the use of antibiotics in livestock farming has been prohibited in European Union [16, 17].

Ascorbic acid is a natural antioxidant and health-promoting agent that has the potential to replace antibiotics growth promoters in fighting bacterial infections [18, 19], and have been thought to be beneficial for heat-stressed broiler chickens. Dietary supplementation of ascorbic acid had the advantage of compensating for incompetent biosynthesis of ascorbic acid and had the potential to ameliorate the harmful effects of hot climate in broiler chickens [20]. Data from several studies suggest that ascorbic acid supplementation may compensate for the reduction in growth rate and feed intake [21, 22], improve overall growth performance and antioxidant status, and reduce serum concentrations of corticosterone, acute phase proteins, cholesterol and lipid oxidation [23, 24], decreased heat shock protein70 gene expression [2].

Using postbiotics as dietary additives for livestock and potential alternatives to antibiotics, postbiotics produced from probiotic *Lactobacillus plantarum* have been the subject of several recent researches. 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 the postbiotics but not the living cells [25]. Postbiotics contain several antimicrobial components including bacteriocins and organic acids, which can minimise the pH of the gut and prevent the growth of pathogens in both the feed and animal gut [26]. Recent evidence suggests that the postbiotics produced by *L. plantarum* strains have an inhibitory effect on several gut pathogens such as *L. monocytogenes*, VRE, *S. typhimurium* and *Escherichia coli* [27–30]. It has recently been observed that the dietary supplementation of postbiotics promoted the health and growth performance in broilers [31–33], layers [34, 35] and piglets [36, 37]. More recently, postbiotics have been reported enhancing the growth performance, rumen fermentation, immune status, antioxidant defence system, and gut health in small ruminants [38–40]. Under normal environmental temperature,

dietary supplementation of postbiotics improved health and growth performance of broilers by promoting their immune status, growth genes expression and gut health as their supplementation significantly improved the intestinal villus, decreased the population of Enterobacteriaceae and faecal pH, and increased the population of lactic acid bacteria [25, 31–33, 41]. Moreover, improvements in broiler meat quality and reduction in plasma cholesterol were observed with dietary supplementation of postbiotics in broilers [35, 41–43]. Our recent findings from a companion study [44] revealed that dietary supplementation of postbiotics produced from *L. plantarum* increased body weight, body weight gain, FCR, intestinal villus height, immune response, IGF-1 and GHR mRNA expression, caecum non-pathogenic bacteria population, and reduced Enterobacteriaceae and *E. coli* population in heat-stressed broilers.

Aside from developing a healthy gut and promote growth performance, a preliminary study from this laboratory revealed that the postbiotics produced by *L. plantarum* have high antioxidant activities [40]. Similarly, bacterial cultures of *L. plantarum* were reported to exhibit high antioxidative activities [45, 46]. In heat-stressed broilers, probiotics have been demonstrated to up-regulate the hepatic antioxidant capacity [47–49], inhibited the pro-inflammatory cytokines (TNF-α and IL-1β) and increased the anti-inflammatory cytokines (IL-10) [50]. Another studies reported that chickens fed probiotics increased intestinal epithelial integrity by increased the mucin mRNA expression [51, 52], and postbiotics produced from *L. plantarum* probiotic are expected to provide analogous benefits to those from probiotic bacteria. Whilst 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 enzymes activity, and genes expression related to gut barrier function, acute phase proteins, heat shock protein70 and cytokines in broiler chickens under heat stress.

Materials And Methods

Postbiotic Production

The *Lactobacillus 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.[53]. 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 MRS broth, incubated at 30 °C for 48 h and sub-cultured in the same media for another 24 hours. 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 hours at 30 °C, and centrifuged at 10,000 × *g* for 15 min at 4 °C. Cell-free supernatant (CFS) was filtered using a membrane of cellulose acetate (Sartorius Minisart, 0.22 µm, Gottingen, Germany) following the procedure described by Loh, et al. [54]. The harvested CFS (postbiotic RI11) was kept at 4 °C until applied in feed within 48 hours.

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 (ITAFoS), Universiti Putra Malaysia. The study was conducted following the guidelines approved by 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 and fifty-two Cobb 500 male chicks (one-day-old) were supplied by a local hatchery. The chicks were housed in wire-floor cages placed in two identical rooms. The rooms were environmentally controlled with each of the two 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 days of age (starter period). The chickens in the two rooms were maintained at the recommended temperature of 32 ± 1 °C on the first day, a gradually reduced to around 24 ± 1 °C by 21 days of age. During the finisher period (day 22 to day 42), the birds were divided into 7 treatment groups, 6 replicates per group with 6 chicks in each replicate. The birds were offered 1 of 7 diets: (1) A basal diet without any supplementation as negative control (NC) 0.0% RI11; (2) NC + 0.02% (w/w) oxytetracycline as positive control (OTC); (3) NC + 0.02% (w/w) ascorbic acid as antioxidant control (AA); or four further groups were NC + 0.2, 0.4, 0.6 and 0.8 % postbiotic RI11 (v/w) of the respective levels. The basal diets were formulated using FeedLIVE software [44] following the nutrient specifications of the Cobb 500 Nutrition Guide. 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 in our companion recently published [44].

Samples Collection

At 42 days of age, around 2 hours and 30 minutes 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 [55]. Blood samples (exsanguination) were collected into blood tubes (BD Vacutainer®, New Jersey, USA) containing EDTA as an anticoagulant and kept on ice. Upon centrifugation at 3500 × *g* for 15 min at 4 °C, harvested plasma samples were (1.5 mL microcentrifuge tubes) stored at −80 °C for later determination of glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) concentration. A part of the liver and ileum tissue were collected immediately after slaughtering, frozen in liquid nitrogen and stored at −80 °C for gene expression analysis including interleukin 10 (IL-10), interleukin 8 (IL-8), interleukin 6 (IL- 6), interleukin 2 (IL-2), interferon (IFN), tumor necrosis factor-alpha (TNF-α), heat shock protein 70 (HSP70), alpha 1- acid glycoprotein (α1-AGP), ceruloplasmin (CPN), zonula occludens-1 (ZO-1), mucin2 (MUC2), claudin1 (CLDN1) and occludin (OCLN) mRNA expressions.

Plasma Antioxidant Enzymes Biomarkers

Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity was analysed in plasma samples using the EnzyChrom^{TM} Glutathione Peroxidase Assay Kit (EGPx-100, BioAssay Systems, Hayward, USA), which directly measured the consumption of NADPH in the 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 plus 90 µL of working reagent (80 µL assay buffer, 5 µL glutathione, 3 µL NADPH (35 mM), and 2 µL gr enzyme) were loaded into the microplate well and tap the plate to mix. A 100 µL of substrate solution was added to each sample and control wells. The optical density of the samples and standards were measured immediately at time zero (OD0), and again at 4 min (OD4). The absorbance of the GPx activity was recorded at 340 nm using microplate reader (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA). The NADPH standards were used to plot the standard curve. The standard curve was used to calculate the GPx activity in the plasma samples.

Superoxide Dismutase Activity

Superoxide dismutase (SOD) assays were carried out using EnzyChrom™ Superoxide Dismutase Assay Kit (ESOD-100, BioAssay Systems, Hayward, USA) based on protocol provided from 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 coloured 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 colour decreased. The activity of SOD was determined by measuring the colour intensity at 440 nm using a microplate reader (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA). The standard curve was used to calculate the concentration of SOD in the samples.

Catalase Activity

Catalase (CAT) activity was measured from plasma using the EnzyChrom^{TM} catalase assay kit (ECAT-100, BioAssay Systems, Hayward, USA), 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 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 µM) were loaded into micro-plate well, 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 mM H_2O_2 reagent with 440 µL of distilled water in the serial concentration, then 10 µL of the standard solution with 90 µL of assay buffer were placed into standard wells. After incubation, 100 µL of 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 microplate reader (Multiskan GO, Thermo Scientific, Waltham, Massachusetts, USA). The standard curve was used to calculate the CAT activity in the plasma samples

Glutathione Activity

Glutathione (GSH) activity was measured in plasma using QuantiChrom^{TM} Glutathione Assay Kit (DIGT-250, BioAssay Systems, Hayward, USA) in accordance with the manufacturer's protocol. The principle of

the assay depended on the reaction of 5,5'-dithiobis 2-nitrobenzoic acid with reduced glutathione to form a yellow product. Briefly, 120 µL of 20-fold diluted sample was mixed with 120 µL of reagent A into 1.5 mL tube, centrifuged at 14000 rpm for 5 min and 200 µL of supernatant was transferred into the microplate well. A 100 µL of reagent B was added to each well of samples, tapped the plate for mixing, and incubated for 25 min at room temperature. A 400 µL of the calibrator was mixed in serial dilution with distilled water into separate wells as the standard. A 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, USA). The GSH concentration in the plasma was calculated using the standard curve of glutathione.

RNA extraction and RT-PCR of studied genes

The extraction of total RNA from liver and ileum tissue samples were conducted using an RNeasy® Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's recommendations and protocols. A thirty mg of liver and ileum tissue samples were homogenised with 600 µL of buffer RLT and centrifuged at 4 °C, 10000 x g for 2 min to obtain the supernatant. The collected supernatant was mixed with an equal volume of 70% (v/v) undenatured ethanol. Then, RNeasy spins column was used for RNA binding and 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, USA) at 260/280 nm absorbance ratio. The complementary DNA (cDNA) was generated from purified RNA using a Quantitect® reverse transcription kit (Qiagen, Hilden, Germany) for quantitative PCR.

Reverse transcription real-time PCR was performance on a Bio-Rad CFX96 PCR machine (Bio-Rad Laboratories, Hercules, CA, USA). The standardisation of target genes expression was determined by the GAPDH gene as housekeeping gene. The total of 20 µL PCR reaction mixture for every sample was prepared using QuantiNova™ SYBR Green PCR kit (Qiagen, Hilden, Germany) containing 10 µL of 2X SYBR Green Master Mix, 2 µL of sample cDNA, 1 µL each of 14 µM 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).

Table 1 The primer sequences of target genes used for 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] IL-8 = Interleukin 8, IL-6 = Interleukin 6, IL-2 = Interleukin 2, IL-10 = Interleukin 10, 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, and GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

[2] F = Forward, R = Reverse,

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 TNF-α gene, and 60 °C for IL-6, IL-8, IL-2, OCLN, ZO-1, CLDN1, and MUC2 genes for 30 s, and finally extension at 72 °C for 20 s). Melting curve programme was included post PCR amplification cycles to confirm the amplification specificity of the primers. The efficiency of amplification of both target and housekeeping genes was performed based on standard curve of five-fold serial dilution of cDNA on a real-time PCR machine.. The relative gene expression based on housekeeping gene was quantified following the approach recommended by Livak and Schmittgen [56].

Statistical analysis

This study was subjected to a completely randomized design with the data analyses were performed using the Statistical Analysis System (SAS) 9.4 software (SAS Institute, Cary, North Carolina, USA). All data were analysed by General Linear Model (GLM) procedure of SAS and the comparison of means were identified by Duncan's Multiple Range Test. The determination of linear and quadratic effects of increasing in inclusion of postbiotic RI11 was conducted by orthogonal polynomial contrast of SAS. The significance of statistical difference between treatments was considered at *P*-value < 0.05.

Results

Antioxidant enzymes activity

There was significantly higher GPx activity in the groups fed with postbiotic 0.4%, 0.6% and 0.8% RI11 and AA as compared to 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 or between 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 postbiotic 0.6% RI11 had the highest CAT activity ($P < 0.05$) as compared with the NC and OTC. The postbiotic RI11 groups and AA group were not different ($P > 0.05$) from each other for the CAT activity. The CAT activity results were not significant different between 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 as compared with the NC and OTC, whereas OTC recorded lower ($P < 0.05$) GSH

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

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

Table 2 Antioxidant enzymes activities in broiler chickens fed 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^{bc}	1012.6^{a}	921.4^{bc}	1052^{a}	1163^{a}	1021.9^{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^{bc}	5.217^{abc}	5.231^{abc}	5.329^{ab}	5.638^{a}	5.320^{ab}	0.079	0.027	0.006	0.033
GSH (µM)	53.41^{bc}	52.38^{c}	56.17^{ab}	54.34^{abc}	56.92^{a}	57.47^{a}	56.12^{ab}	0.55	0.013	0.014	0.098

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

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

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

Cytokines mRNA expression

The mRNA expression of IL-6, IL-8, IL-10, IL-2, IFN and TNF-α gene in the ileal tissue of broiler chickens fed different levels of postbiotic RI11 under heat stress are shown in (Table 3). Different levels of postbiotic RI11 supplements 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 groups and AA than the NC. No significant difference for the expression of IL-8 between the postbiotic RI11 groups, OTC and AA treatment groups.

The mRNA expression of IL-10 was higher ($P < 0.05$) in the 0.4% and 0.6% RI11 than NC, OTC and 0.2% RI11 treatment groups. The AA group was higher ($P < 0.05$) for IL-10 expression than NC and OTC, but not significantly different with RI11 groups. No difference ($P > 0.05$) for the IL-10 expression between the NC, OTC, 0.2% and 0.8% RI11 groups. The addition of postbiotic RI11 in different levels down-regulated the TNF-α mRNA expression as compared with the NC, whereas no difference ($P > 0.05$) was found among the postbiotic RI11 treatment groups. The 0.6% RI11 showed lower ($P < 0.05$) mRNA expression of TNF-α

than NC, OTC, AA, and 0.2% RI11. However, no difference ($P > 0.05$) was observed between the latter groups for the mRNA expression of TNF-α. Increasing the level of postbiotic RI11 in the diet led to quadratic increased ($P = 0.01$) the mRNA expression of IL-10 and decreased ($P = 0.015$ and 0014) the mRNA expression for IL-8, IL-6, IL-2 and TNF-α in ileal tissue.

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

Parameters	Dietary treatments[1]							SEM	*P*-values	Contrast, *P*-values[2]	
(mRNA fold change)	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^{ab}	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^{ab}	1.191^{bc}	1.838^{a}	1.920^{a}	1.501^{abc}	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^{ab}	0.732^{ab}	0.761^{ab}	0.564^{bc}	0.413^{c}	0.528^{bc}	0.045	0.002	<.0001	0.014

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

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

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

Gut barrier gene expression

Addition of different levels of postbiotic RI11 as a feed additive 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 the highest significantly in the 0.6% RI11 as compared with NC and OTC. No difference ($P > 0.05$) was observed between the 0.2%, 0.4%, 0.8% RI11and AA or between postbiotic RI11 groups (except 0.6%), OTC, AA and NC for ZO-1 expression. The mRNA expression of MUC2 was higher ($P < 0.05$) in 0.6% RI11 than NC, 0.2% RI11 and OTC, but not different ($P > 0.05$) with 0.4%, 0.8% and AA groups. A significant higher MUC2 expression was observed in the 0.4% and 0.8% RI11 as compared with NC. No difference ($P > 0.05$) in expression of the MUC2 gene was found between NC, OTC, AA and 0.2% RI11. The OCLN mRNA expression was downregulated in the dietary treatments as compared with NC. There was no dietary effect ($P > 0.05$) on the expression of CLDN1 gene. Increasing the level of RI11 in broilers diet quadratic

(*P* = 0.005 and 0.049) upregulated the expression of ZO-1 and MUC2 genes and downregulated (*P* = 0.005) the expression of OCLN gene.

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

Parameters	Dietary treatments[1]							SEM	*P*-values	Contrast, *P*-values[2]	
(mRNA fold change)	NC	OTC	AA	0.2%	0.4%	0.6%	0.8%			Linear	Quadratic
ZO-1	1^{b}	1.010^{b}	1.327^{ab}	1.347^{ab}	1.521^{ab}	1.739^{a}	1.275^{ab}	0.068	0.026	0.044	0.005
MUC2	1^{c}	1.428^{bc}	1.506^{abc}	1.397^{bc}	1.664^{ab}	2.075^{a}	1.673^{ab}	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 ($P < 0.05$).

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

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

Acute phase proteins and HSP70 mRNA expression

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

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

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

Parameters	Dietary treatments[1]							SEM	*P*-values	Contrast, *P*-values[2]	
(mRNA fold change)	NC	OTC	AA	0.2%	0.4%	0.6%	0.8%			Linear	Quadratic
α1-AGP	1[a]	0.87[ab]	0.617[bc]	0.661[abc]	0.425[c]	0.305[c]	0.392[c]	0.054	0.001	<.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[ab]	0.63[b]	0.738[ab]	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 ($P < 0.05$).

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

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

Discussion

Antioxidant Enzymes Activities

Oxidative stress causes the production of ROS varieties, including hydroxyl free radical and superoxide anions. Various researches reported that overflowed of ROS could damage the biological macromolecules such as proteins and nucleic acids, consequently leading to the development of diseases [57]. In chickens, the main antioxidant enzymes are glutathione peroxidase, superoxide dismutase, catalase and glutathione. These enzymes are important to transform reactive species into non-radical and non-toxic products [58].

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 0.2% RI11 and OTC. 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 the various treatments on SOD activity, whereas CAT and GSH activities were enhanced significantly following postbiotic supplementation with 0.4%, 0.6% and 0.8% RI11. This finding is consistent with Wang et al. [59], who indicated that the feed supplementation with probiotic enhanced CAT, GPx and SOD activities in broilers at 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 broiler on probiotic *Bacillus subtilis* increased the GPx, GSH and their mRNA expression level [60]. Likewise, two studies reported that broilers under heat challenge had increased activities of CAT, GPx, GSH and SOD [61, 62]. Hence, the 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 antimicrobial and antioxidant that can safely alleviate the stress and improve the health of animals. As postbiotics possess the probiotic characteristics [34, 39, 41, 44, 63, 64], probiotic studies can provide useful information to understand how postbiotics could improve the antioxidant capability and develop the oxidative resistance in the body under heat stress. Several studies reported that the supplementation of probiotics in the poultry diets reduced the adverse effects of

oxidative stress and enhanced the antioxidant enzymes activities [57, 65], which might reduce cell damages by inhibiting the production of ROS and finally improving the health of animals [66, 67]. Consistent with our results, Shen, et al. [68] reported that blood antioxidant capacities were significantly enhanced by the inclusion of probiotic *L. plantarum* in the diets and promoted growth performance 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 promoting broiler health under normal [57] and high-temperature conditions [69].

Vitamin C has been equally reported to improve the activities of antioxidant enzymes including GPx and GSH in layers [70]. Yun et al. [71] showed that mRNA and activity of GPx were improved in heat-stressed broilers supplemented with vitamin C without affecting SOD activity.

Cytokines mRNA expression

Cytokines are small extracellular signaling protein produced by the host with crucial functions in immunity by enabling cell communication midst immunological development and immune response [72]. The pro and anti-inflammatory cytokines are produced by immune cells such as T lymphocytes, B lymphocytes, macrophages and natural killer cells [73]. T lymphocytes are divided into two 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 which increases cellular immunity, whereas IL-6 and IL-10 are known as a Th2 type cytokine that acts in humoral immunity [74-76]. These small molecules proteins are released when the animal is exposed to infection, inflammation and shock as an immune response [77]. Heat stress affects intestinal integrity and increases intestinal permeability to endotoxin and antigens and inflammatory cytokines [78]. Heat stress has been shown to increase the expression of pro-inflammatory cytokines and suppressed anti-inflammatory cytokines in broilers [79]. Heat stress leads to gut damage and induces commensal bacteria to release endotoxin that encourages the production of the pro-inflammatory cytokines [80]. The current study evident that the increase of the levels of pro-inflammatory cytokines in broilers under heat stress such as IL-8 and TNF-α could be alleviated by postbiotic dietary supplementation. Feeding postbiotic RI11 with various levels would modulate the inflammatory processes by restoring cytokine balance in order to reduce the potential inflammation-induced injury that occur following heat stress in broiler chickens.

In the present study, lower expression of IL-8 and TNF-α, and higher expression of IL-10 were found in postbiotic RI11 fed broilers compared to other treatments. The differential expression of IL-8 seen herein could be due to the interaction between the beneficial bacteria which enhanced by postbiotics and intestinal enterocytes and immune cells of the lamina propria [81]. These findings were in line with that of Kareem et al. [31], who reported reduced cytokines expression in broiler chickens supplemented with various combinations of postbiotics and inulin. Wang [82] documented that supplemented probiotic *B. subtilis* in broiler diets under heat stress decreased the expression levels of IL-6 and TNF-α and increased

IL-10 expression level. Inflammatory cytokines, especially TNF-α, IL-2, IL-8 and IL-6 play important roles in the 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 [83]. In this study, the TNF-α expression was down-regulated by supplementation with postbiotic RI11 in broilers diets under heat stress as compared to the negative control. Recently, supplementation with a polysaccharide-based bioflocculant (PBB) extracted from *B. subtilis* F9 inhibited the expression of TNF-α and IL-1, whereas that of IL-10 was significantly increased as the anti-inflammatory potential of PBB [50]. Previous studies have demonstrated the effect of probiotics in reducing pro-inflammatory cytokine production [84, 85]. In piglets, Yang et al. [86] posited that pre-treatment of porcine epithelial cells with *L. reuteri* led lowered the expression of TNF-α and IL-6. Moreover, the effects of feeding commensal bacteria such as LAB have been reported to have both pro-inflammatory and anti-inflammatory actions [87]. The high population of *Lactobacillus* and *Bifidobacterium* could play a role in anti-inflammatory cytokine expression, whereas the opposite reaction may be due to lowered pathogens load [88]. In piglets, increased lactobacilli population was associated with decreased expression of IL-8 [89]. This was exemplified in this study based on the significant increment in *Lactobacillus* and *Bifidobacterium* count, reduced pathogenic load and down-regulation of IL-8. In other studies, involving probiotics, reduction in IL-8 secretion in intestinal epithelial cells (IECs) was suggested to occur through different pathways [79]. The IL-2, IL-6 and IFN were not affected by the inclusion of postbiotic in broiler diets under heat stress. However, IL-6 expression was up-regulated in broiler fed a combination of postbiotics and inulin [41] and lambs [39] fed postbiotic RG14.

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

Gut barrier genes 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 intestinal epithelial cell (IEC) is the first line of defense and they interact effectively with commensal bacteria and antimicrobial substances to protect intestinal barrier [90]. The IEC function is mediated by multiprotein complexes present at the apical end of the IECs and referred to as tight junctions (TJs). TJs play an immense role in regulating intestinal permeability by shutting the spaces between adjacent IECs [91]. Various factors affect TJ and mucosal barrier functions such as cytokines, probiotics, growth factors and pathogens by transcriptional regulation and post-translational modification of tight junction proteins [90-92]. OCLN, CLDN1 and ZO-1 are some of the major functional components of TJs [93, 94]. 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 TJs was investigated following the postbiotic feeding of broilers exposed to heat stress. The expression of ZO-1 and MUC2 were significantly higher in birds fed with postbiotic RI11 as compared with OTC and negative control, while 0.6% RI11 showed the highest effect among the postbiotic groups. The benefits of inclusion postbiotic at level 0.6% may be attributed to the optimal environment provided for better beneficial bacteria growth, then improve the intestinal integrity, nutrient digestibility and increase the growth performance of broiler. These findings showed that supplementation of postbiotics at various levels prevented the reduction in expression of ZO-1, OCLN and induced MUC2 gens by heat stress. These results were in agreement with the finding of Zhang et al. [95] who found that the addition of probiotics mixture in layer feed resulted in upregulation of ZO-1 mRNA expression under heat stress. Supplementation of probiotic *B. subtilis* in broiler diets significantly increased gene expression of intestinal MUC2 mRNA compared to those fed the control diet coincides with our finding [52]. Broilers fed *L. fermentum* 1.2029 strain showed significantly increased goblet cell density in the jejunum and the level of MUC2 mRNA in both the jejunum and ileum [96]. Inclusion of postbiotics did not affect the expression of 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 were reduced in pulmonary cells in mice and porcine IECs respectively following LPS treatment [86, 97]. A similar scenario regarding the reduced expression of ZO1, MUC2 and OCLN were reported in broilers fed with probiotic after LPS challenge [79].

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

A recent study reported that inclusion of postbiotic in post-weaning lamb increased the expression level of TJs genes which is in line with our finding [39]. The improvement of TJs proteins is attributed to the postbiotics, which contain metabolites of probiotic bacteria with ability to affect the regulation of TJs integrity and mucosal barrier function [39]. The interactions of metabolites and bioactive molecules secreted by probiotics with intestinal immune cell receptors modulate epithelial cell function by increasing tight junction integrity and prevent its disruption [92, 98].

Acute phase proteins and HSP70 mRNA expression

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

HSP70 is a useful indicator of cellular insult and predicting the level of thermal stress in chickens [99]. The protein is highly preserved, and they are expressed under stress conditions such as transportation, feed restriction, unpleasant human contact and high temperature [100-102]. During acute heat stress, the

level of HSP70 is increased through the synthesis of HSP70 mRNA either by increased amount of the protein or activity of the heat shock transcription factor [103, 104]. Earlier studies have reported higher levels of HSP70 in various tissues of broilers following exposure to high ambient temperature [104, 105]. In the present study, the down-regulation of HSP70 mRNA in the heat-stressed birds supplemented with postbiotics indicates the amelioration of the effect of the environmental stressor on birds' health status. Moreover, for intestinal barrier to be improved, the expression of heat shock protein is important for the signaling pathways involved in the survival of IECs [106].

The ability of different levels of postbiotic RI11 to induce lower expression of HSP70 mRNA level as observed in this study could be a mechanism to defend the synthesised TJs 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 [107]. HSP70 induction guards against stresses such as hyperthermia, ischemia and inflammation [101, 108].

This study showed that higher levels of RI11 and ascorbic acid supplementation induced similar positive effect in improving the HSP70 levels in heat-stressed broilers. Both postbiotics and ascorbic acid supplementation had greater positive effect compared to OTC treated birds. Previous studies have also shown the effect of ascorbic acid in the expression HSP70 mRNA [71, 105, 106, 109]. Heat stressed rats supplemented with vitamin C had significantly lower hepatic HSP70 mRNA compared to those not given the dietary treatment; nevertheless, the thermic HSP70 was not affected by the treatment [71].

There is no scientific report to date to investigate the effect of postbiotics on gene expression of HSP70 and APPs in heat-stressed broilers. However, the findings are comparable to other studies that assessed the effect of other substances on the above-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 following exogenous administration of corticosterone [99]. 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 [106, 109].

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. [105] posited a direct relationship between HSP70 level and antioxidant enzyme activities such as SOD, 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. [109] reported that improved serum antioxidant enzymes level could explain the lower HSP70 expression 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.

Conclusion

The results of the present study demonstrated that supplementation of postbiotic RI11 in different levels (particularly 0.6%) in the diet of broiler chickens under heat stress increased the plasma concentration of antioxidant enzymes activities (GPx, CAT and GSH) and reduced the heat stress biomarkers such as acute-phase proteins (α1-AGP and CPN) and HSP70 mRNA expression. It can be observed that higher mRNA expression level of IL-10, but lower expression IL-8 and TNF-α at the mucosal of ileum supplemented with different dosages of postbiotic RI11. The higher production and secretion of MUC2 into the intestinal mucosal and regulation of intestinal barrier by tight junction proteins in broilers receiving various levels of postbiotic RI11 as can be observed in the upregulation of ZO-1 and MUC2 further increase the health and integrity of the intestinal mucosa. Dietary postbiotic RI11 at level 0.6% is suggested to provide satisfactory effects and potential as alternative to antibiotic growth promoter and antioxidant additive in broiler diets to mitigate the detrimental effects of heat stress.

Abbreviations

GPx: Glutathione peroxidase; CAT: Catalase; GSH: Glutathione; SOD: superoxide dismutase; cDNA: complementary deoxyribonucleic acid; CLDN-1: Claudin 1; NC: Negative control; OTC: Oxytetracycline; AA: Ascorbic Acid; EDTA: Ethylenediaminetetraacetic acid; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IFN: Interferon; IL: Interleukin; OCLD: Occludin; RNA: Ribonucleic acid; rpm: revolution per minute; TJP: Tight junction protein; TNF-α: Tumour necrosis factor alpha; v/w: volume/weight; w/w: weight/weight; VRE: Vancomycin-resistant enterococci; CFU: Colony forming units; ZO-1: Zonula occludin 1; MUC2: Mucin 2; HSP70: Heat shock protein70; APPs: Acute phase proteins; α1-AGP: Alpha1-acid glycoprotein; CPN: Ceruloplasmin; M: Molar; mg: milligram; min: minute; mL: millilitre; mM: millimolar; ng: nanogram; nm: nanometre; IECs: Intestinal Epithelial Cells; x g: gravitational force; ROS: Reactive oxygen species.

Declarations

Author contributions

H.A.M., L.T.C., F.H.L. and W.I.I. participated in the conceptualization, methodology, project administration, software, formal analysis and investigation; H.A.M. and I.W.I. involved in sampling, data collection, preparation and writing the original draft; L.T.C., F.H.L., S.A.A., Z.I. and M.N.M. contributed to the supervision, resources, review and editing the manuscript; L.T.C. contributed to the funding acquisition.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All procedures with animals received prior approval from the Animal Care and Use Committee of the Universiti Putra Malaysia (protocol no. UPM/ACUC/AUP-R085/2018), which ascertains that the use and care of research animals are ethical and humane.

Consent for publication

Not applicable.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Mahmoud KZ, Edens FW, Eisen EJ, Havenstein GB. Effect of ascorbic acid and acute heat exposure on heat shock protein 70 expression by young white Leghorn chickens. Comp Biochem physiol C Toxicol Pharmacol. 2003;136(4):329–35.
2. Mahmoud KZ, Edens FW, Eisen EJ, Havenstein GB. Ascorbic acid decreases heat shock protein 70 and plasma corticosterone response in broilers (*Gallus gallus*) subjected to cyclic heat stress. Comp Biochem Physiol Part B Biochem Mol Biol. 2004;137(1):35–42.
3. He X, Lu Z, Ma B, Zhang L, Li J, Jiang Y, et al. Effects of chronic heat exposure on growth performance, intestinal epithelial histology, appetite-related hormones and genes expression in broilers. J Sci Food Agric. 2018;98(12):4471–8.
4. Najafi P, Zulkifli I, Jajuli NA, Farjam AS, Ramiah SK, Amir AA, et al. Environmental temperature and stocking density effects on acute phase proteins, heat shock protein 70, circulating corticosterone and performance in broiler chickens. Int J biometeorol. 2015;59(11):1577–83.
5. Lykkesfeldt J, Svendsen O. Oxidants and antioxidants in disease: oxidative stress in farm animals. Vet J. 2007;173(3):502–11.
6. Matés JM, Pérez-Gómez C, De Castro IN. Antioxidant enzymes and human diseases. Clin Biochem. 1999;32(8):595–603.

7. Mager WH, De Kruijff A. Stress-induced transcriptional activation. Microbiol Mol Biol Rev. 1995;59(3):506–31.
8. Iwagami Y. Changes in the ultrastructure of human cells related to certain biological responses under hyperthermic culture conditions. Hum Cell. 1996;9(4):353–66.
9. Halliwell B, Cross CE. Oxygen-derived species: their relation to human disease and environmental stress. Environ Health Perspect. 1994;102(Suppl 10):5.
10. Halliwell B. Role of free radicals in the neurodegenerative diseases. Drug Aging. 2001;18(9):685–716.
11. Rahimi S, Khaksefidi A. A comparison between the effects of a probiotic (Bioplus 2B) and an antibiotic (virginiamycin) on the performance of broiler chickens under heat stress condition. Iran J Vet Res. 2006;7(3):23–8.
12. Zulkifli I, Abdullah N, Azrin NM, Ho Y. Growth performance and immune response of two commercial broiler strains fed diets containing *Lactobacillus* cultures and oxytetracycline under heat stress conditions. Br Poult Sci. 2000;41(5):593–7.
13. Ramiah SK, Zulkifli I, Rahim NAA, Ebrahimi M, Meng GY. Effects of two herbal extracts and virginiamycin supplementation on growth performance, intestinal microflora population and fatty acid composition in broiler chickens. Asian-Australas J Anim Sci. 2014;27(3):375.
14. Odore R, De Marco M, Gasco L, Rotolo L, Meucci V, Palatucci A, et al. Cytotoxic effects of oxytetracycline residues in the bones of broiler chickens following therapeutic oral administration of a water formulation. Poult Sci. 2015;94(8):1979–85.
15. Shazali N, Foo HL, Loh TC, Choe DW, Rahim RA. Prevalence of antibiotic resistance in lactic acid bacteria isolated from the faeces of broiler chicken in Malaysia. Gut Pathog. 2014;6(1):1.
16. Regulation E. No 1831/2003 of the European Parliament and Council of 22 September 2003 on additives for use in animal nutrition. Official J Eur Commun. 2003;268:29–43.
17. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, et al. Global trends in antimicrobial use in food animals. Proc Natl Acad Sci. 2015;112(18):5649–54.
18. Tajkarimi M, Ibrahim SA. Antimicrobial activity of ascorbic acid alone or in combination with lactic acid on *Escherichia coli* O157: H7 in laboratory medium and carrot juice. Food Control. 2011;22(6):801–4.
19. Verghese RJ, Ramya S, Kanungo R. In vitro Antibacterial Activity of Vitamin C and in Combination with Ciprofloxacin against Uropathogenic *Escherichia coli*. J Clin Diagn Res. 2017;11(12).
20. Njoku P. Effect of dietary ascorbic acid (vitamin C) supplementation on the performance of broiler chickens in a tropical environment. Anim Feed Sci Technol. 1986;16(1–2):17–24.
21. Kadim I, Al-Qamshui B, Mahgoub O, Al-Marzooqi W, Johnson E. Effect of seasonal temperatures and ascorbic acid supplementation on performance of broiler chickens maintained in closed and open-sided houses. Int J Poult Sci. 2008;7(7):655–60.

22. Kutlu H, Forbes J. Changes in growth and blood parameters in heat-stressed broiler chicks in response to dietary ascorbic acid. Livest Prod Sci. 1993;36(4):335–50.
23. Sahin K, Sahin N, Kucuk O. Effects of chromium, and ascorbic acid supplementation on growth, carcass traits, serum metabolites, and antioxidant status of broiler chickens reared at a high ambient temperature (32 °C). Nutr Res. 2003b;23(2):225–38.
24. Ferreira I, Matos Junior J, Sgavioli S, Vicentini T, Morita V, Boleli I. Vitamin C prevents the effects of high rearing temperatures on the quality of broiler thigh meat. Poult Sci. 2015;94(5):841–51.
25. Thanh NT, Loh TC, Foo HL, Hair-Bejo M, Azhar BK. Effects of feeding metabolite combinations produced by *Lactobacillus plantarum* on growth performance, faecal microbial population, small intestine villus height and faecal volatile fatty acids in broilers. Br Poult Sci. 2009;50(3):298–306.
26. Aguilar-Toalá J, Garcia-Varela R, Garcia H, Mata-Haro V, González-Córdova A, Vallejo-Cordoba B, et al. Postbiotics: An evolving term within the functional foods field. Trends Food Sci Technol. 2018;75:105–14.
27. Kareem KY, Ling FH, Chwen LT, Foong OM, Asmara SA. Inhibitory activity of postbiotic produced by strains of *Lactobacillus plantarum* using reconstituted media supplemented with inulin. Gut Pathog. 2014;6(1):23.
28. Choe DW, Foo HL, Loh TC, Hair-Bejo M, Awis QS. Inhibitory property of metabolite combinations produced from *Lactobacillus plantarum* strains. Pertanika J Trop Agric Sci. 2013;36(1).
29. Thanh NT, Chwen LT, Foo HL, Hair-Bejo M, Kasim AB. Inhibitory activity of metabolites produced by strains of *Lactobacillus plantarum* isolated from Malaysian fermented food. Int J Probiotics Prebiotics. 2010;5(1):37.
30. Van Thu T, Foo HL, Loh TC, Bejo MH. Inhibitory activity and organic acid concentrations of metabolite combinations produced by various strains of *Lactobacillus plantarum*. Afr J Biotechnol. 2011;10(8):1359–63.
31. Kareem KY, Loh TC, Foo HL, Akit H, Samsudin AA. Effects of dietary postbiotic and inulin on growth performance, IGF1 and GHR mRNA expression, faecal microbiota and volatile fatty acids in broilers. BMC Vet Res. 2016b;12(1):163.
32. Loh TC, Thanh NT, Foo HL, Hair-Bejo M, Azhar BK. Feeding of different levels of metabolite combinations produced by *Lactobacillus plantarum* on growth performance, fecal microflora, volatile fatty acids and villi height in broilers. Anim Sci J. 2010;81(2):205–14.
33. Rosyidah M, Loh T, Foo H, Cheng X, Bejo M. Effect of feeding metabolites and acidifier on growth performance, faecal characteristics and microflora in broiler chickens. J Anim Vet Adv. 2011;10(21):2758–64.
34. Loh TC, Choe DW, Foo HL, Sazili AQ, Bejo MH. Effects of feeding different postbiotic metabolite combinations produced by *Lactobacillus plantarum* strains on egg quality and production performance, faecal parameters and plasma cholesterol in laying hens. BMC Vet Res. 2014;10(1):149.

35. Choe DW, Loh TC, Foo HL, Hair-Bejo M, Awis QS. Egg production, faecal pH and microbial population, small intestine morphology, and plasma and yolk cholesterol in laying hens given liquid metabolites produced by *Lactobacillus plantarum* strains. Br Poult Sci. 2012;53(1):106–15.
36. Loh TC, Thu TV, Foo HL, Bejo MH. Effects of different levels of metabolite combination produced by *Lactobacillus plantarum* on growth performance, diarrhoea, gut environment and digestibility of postweaning piglets. J Appl Anim Res. 2013;41(2):200–7.
37. Thu TV, Loh TC, Foo HL, Yaakub H, Bejo MH. Effects of liquid metabolite combinations produced by *Lactobacillus plantarum* on growth performance, faeces characteristics, intestinal morphology and diarrhoea incidence in postweaning piglets. Trop Anim Health Prod. 2011;43(1):69–75.
38. Izuddin WI, Loh TC, Samsudin AA, Foo HL. In vitro study of postbiotics from *Lactobacillus plantarum* RG14 on rumen fermentation and microbial population. Rev Bras Zootec. 2018;47.
39. Izuddin WI, Loh TC, Foo HL, Samsudin AA, Humam AM. Postbiotic L.. *plantarum* RG14 improves ruminal epithelium growth, immune status and upregulates the intestinal barrier function in post-weaning lambs. Sci Rep. 2019;9(1):9938.
40. Izuddin WI, Humam AM, Loh TC, Foo HL, Samsudin AA. Dietary Postbiotic *Lactobacillus plantarum* Improves Serum and Ruminal Antioxidant Activity and Upregulates Hepatic Antioxidant Enzymes and Ruminal Barrier Function in Post-Weaning Lambs. Antioxidants. 2020;9(3):250.
41. Kareem KY, Loh TC, Foo HL, Asmara SA, Akit H. Influence of postbiotic RG14 and inulin combination on cecal microbiota, organic acid concentration, and cytokine expression in broiler chickens. Poult Sci. 2016a;96(4):966–75.
42. Kareem KY, Loh TC, Foo HL, Asmara SA, Akit H, Abdulla NR, et al. Carcass, meat and bone quality of broiler chickens fed with postbiotic and prebiotic combinations. Int J Probiotics Prebiotics. 2015;10(1):23.
43. Loh T, Thanh N, Foo H, Hair-Bejo M. Effects of feeding metabolite combinations from *Lactobacillus plantarum* on plasma and breast meat lipids in Broiler Chickens. Rev Bras Cienc Avic. 2013;15(4):307–16.
44. Humam AM, Loh TC, Foo HL, Samsudin AA, Mustapha NM, Zulkifli I, et al. Effects of Feeding Different Postbiotics Produced by *Lactobacillus plantarum* on Growth Performance, Carcass Yield, Intestinal Morphology, Gut Microbiota Composition, Immune Status, and Growth Gene Expression in Broilers under Heat Stress. Animals. 2019;9(9):644.
45. He Z, Wang X, Li G, Zhao Y, Zhang J, Niu C, et al. Antioxidant activity of prebiotic ginseng polysaccharides combined with potential probiotic *Lactobacillus plantarum* C88. Int J Food Sci Technol. 2015;50(7):1673–82.
46. Ji K, Jang NY, Kim YT. Isolation of Lactic Acid Bacteria Showing Antioxidative and Probiotic Activities from Kimchi and Infant Feces. J Microbiol Biotechnol. 2015;25(9):1568–77.
47. Li S, Zhao Y, Zhang L, Zhang X, Huang L, Li D, et al. Antioxidant activity of *Lactobacillus plantarum* strains isolated from traditional Chinese fermented foods. Food Chem. 2012;135(3):1914–9.

48. Jahromi MF, Altaher YW, Shokryazdan P, Ebrahimi R, Ebrahimi M, Idrus Z, et al. Dietary supplementation of a mixture of *Lactobacillus* strains enhances performance of broiler chickens raised under heat stress conditions. Int J Biometeorol. 2016;60(7):1099–110.
49. Sugiharto S, Yudiarti T, Isroli I, Widiastuti E, Kusumanti E. Dietary supplementation of probiotics in poultry exposed to heat stress–a review. Annal Anim Sci. 2017;17(3):591–604.
50. Giri SS, Ryu E, Park SC. Characterization of the antioxidant and anti-inflammatory properties of a polysaccharide-based bioflocculant from *Bacillus subtilis* F9. Microb Pathog. 2019;136:103642.
51. Smirnov A, Perez R, Amit-Romach E, Sklan D, Uni Z. Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. J Nutr. 2005;135(2):187–92.
52. Aliakbarpour H, Chamani M, Rahimi G, Sadeghi A, Qujeq D. The *Bacillus subtilis* and lactic acid bacteria probiotics influences intestinal mucin gene expression, histomorphology and growth performance in broilers. Asian-Australas J Anim Sci. 2012;25(9):1285.
53. Foo H, Loh T, Law F, Lim Y, Kuflin C, Rusul G. Effect of feeding *L. plantarum* I-UL4 isolated from Malaysian Tempeh on growth performance, fecla flora and lactic acid bacteria and plasma cholesterol concentrations in post weaning rats. J Food Sci Biotechnol. 2003;12:403–8.
54. Loh T, Chong S, Foo H, Law F. Effects on growth performance, faecal microflora and plasma cholesterol after supplementation of spray-dried metabolite to postweaning rats. Czech J Animal Sci. 2009;54(1):10–6.
55. Malaysia S. Halal food-production, preparation, handling and storage—general guidelines (second revision). Department of Standards, Selangor Darul Ehsan, Malaysia, MS1500. 2009. hppt://www.standardsmalaysia.gov.my. Accessed 8 June 2019.
56. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2 - \Delta\Delta CT$ method. Methods. 2001;25(4):402–8.
57. Bai K, Huang Q, Zhang J, He J, Zhang L, Wang T. Supplemental effects of probiotic *Bacillus subtilis* fmbJ on growth performance, antioxidant capacity, and meat quality of broiler chickens. Poult Sci. 2017;96(1):74–82.
58. Akbarian A, Michiels J, Degroote J, Majdeddin M, Golian A, De Smet S. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. J Anim Sci Biotechnol. 2016;7:37.
59. Wang WC, Yan FF, Hu JY, Amen OA, Cheng HW. Supplementation of *Bacillus subtilis*-based probiotic reduces heat stress-related behaviors and inflammatory response in broiler chickens. J Anim Sci. 2018;96(5):1654–66.
60. Bai K, Huang Q, Zhang J, He J, Zhang L, Wang T. Supplemental effects of probiotic *Bacillus subtilis* fmbJ on growth performance, antioxidant capacity, and meat quality of broiler chickens. Poult Sci. 2016;96(1):74–82.
61. Altan O, Pabuccuoglu A, Altan A, Konyalioglu S, Bayraktar H. Effect of heat stress on oxidative stress, lipid peroxidation and some stress parameters in broilers. Br Poult Sci. 2003;44(4):545–50.

62. Yang PC, Tu YH, Perdue MH, Oluwole C, Struiksma S. Regulatory effect of heat shock protein 70 in stress-induced rat intestinal epithelial barrier dysfunction. N Am J Med Sci. 2009;1(1):9–15.
63. Foo HL, Loh TC, Abdul Mutalib NE, Rahim RA. The myth and therapeutic potentials of postbiotics: Microbiome and Metabolome in Diagnosis, Therapy, and Other Strategic Applications. In: Faintuch J, Faintuch S, editors. John Fedor. UK: Academic Press; 2019. pp. 201–8.
64. Gao J, Li Y, Wan Y, Hu T, Liu L, Yang S, et al. A novel postbiotic from *Lactobacillus rhamnosus* GG with a beneficial effect on intestinal barrier function. Front Microbiol. 2019;10.
65. Deng W, Dong X, Tong J, Zhang Q. The probiotic *Bacillus licheniformis* ameliorates heat stress-induced impairment of egg production, gut morphology, and intestinal mucosal immunity in laying hens. Poult Sci. 2012;91(3):575–82.
66. 10.1007/978-94-011-2364-8$4
Fuller R. Probiotics: the scientific basis. In: Fuller R, editor. Springer Science & Business Media. 1st ed. UK. 1992. p. 405. DOI 10.1007/978-94-011-2364-8$4.
67. Li N, Yu H, Liu H, Wang Y, Zhou J, Ma X, et al. Horizontal transfer of vanA between probiotic *Enterococcus faecium and Enterococcus faecalis* in fermented soybean meal and in digestive tract of growing pigs. J Anim Sci Biotechnol. 2019;10(1):36.
68. Shen X, Yi D, Ni X, Zeng D, Jing B, Lei M, et al. Effects of *Lactobacillus plantarum* on production performance, immune characteristics, antioxidant status, and intestinal microflora of bursin-immunized broilers. Can J Microbiol. 2014;60(4):193–202.
69. Cramer TA, Kim HW, Chao Y, Wang W, Cheng HW, Kim YHB. Effects of probiotic (*Bacillus subtilis*) supplementation on meat quality characteristics of breast muscle from broilers exposed to chronic heat stress. Poult Sci. 2018;97(9):3358–68.
70. Leskovec J, Levart A, Peric L, Dukic Stojcic M, Tomovic V, Pirman T, et al. Antioxidative effects of supplementing linseed oil-enriched diets with alpha-tocopherol, ascorbic acid, selenium, or their combination on carcass and meat quality in broilers. Poult Sci. 2019.
71. Yun SH, Moon YS, Sohn SH, Jang IS. Effects of cyclic heat stress or vitamin C supplementation during cyclic heat stress on HSP70, inflammatory cytokines, and the antioxidant defense system in Sprague Dawley rats. Exp Anim. 2012;61(5):543–53.
72. Saleh KM, Al-Zghoul MB. Effect of Acute Heat Stress on the mRNA Levels of Cytokines in Broiler Chickens Subjected to Embryonic Thermal Manipulation. Animals. 2019;9(8):499.
73. Jeurissen S. Structure and function of the chicken spleen. Res Immunol. 1991;142(4):352–5.
74. Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev. 2003;8(3):223–46.
75. Xie J, Yu Q, Nie S, Fan S, Xiong T, Xie M. Effects of *Lactobacillus plantarum* NCU116 on intestine mucosal immunity in immunosuppressed mice. J Agric Food Chem. 2015;63(51):10914–20.
76. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today. 1996;17(3):138–46.

77. Hakansson A, Molin G. Gut microbiota and inflammation. Nutrients. 2011;3(6):637–82.
78. Alhenaky A, Abdelqader A, Abuajamieh M, Al-Fataftah AR. The effect of heat stress on intestinal integrity and *Salmonella* invasion in broiler birds. J Therm Biol. 2017;70(Pt B):9–14.
79. Gadde UD, Oh S, Lee Y, Davis E, Zimmerman N, Rehberger T, et al. Dietary *Bacillus subtilis*-based direct-fed microbials alleviate LPS-induced intestinal immunological stress and improve intestinal barrier gene expression in commercial broiler chickens. Res Vet Sci. 2017;114:236–43.
80. De Boever S, Beyaert R, Vandemaele F, Baert K, Duchateau L, Goddeeris B, De Backer P, Croubels S. The influence of age and repeated lipopolysaccharide administration on body temperature and the concentration of interleukin-6 and IgM antibodies against lipopolysaccharide in broiler chickens. Avian Pathol. 2008;37(1):39–44.
81. Galdeano CM, Perdigon G. The probiotic bacterium *Lactobacillus casei* induces activation of the gut mucosal immune system through innate immunity. Clin Vaccine Immunol. 2006;13(2):219–26.
82. Wang W. The Effect of Dietary Supplementation of Probiotic, *Bacillus Subtilis*, on Inflammatory Reactions in the Brain of Broiler Chickens under Heat Stress: Master dissertation. Purdue University. West Lafayette, Indiana. US. 2017; p. 167.
83. Chong DL, Sriskandan S. Pro-inflammatory mechanisms in sepsis. Sepsis-Pro-Inflammatory and Anti-Inflammatory Responses. 17: Karger Publishers; 2011. p. 86–107.
84. Bai AP, Ouyang Q, Zhang W, Wang CH, Li SF. Probiotics inhibit TNF-alpha-induced interleukin-8 secretion of HT29 cells. World J Gastroenterol. 2004;10(3):455–7.
85. Carey CM, Kostrzynska M. Lactic acid bacteria and bifidobacteria attenuate the proinflammatory response in intestinal epithelial cells induced by *Salmonella enterica serovar Typhimurium*. Can J Microbiol. 2013;59(1):9–17.
86. Yang F, Wang A, Zeng X, Hou C, Liu H, Qiao S. *Lactobacillus reuteri* I5007 modulates tight junction protein expression in IPEC-J2 cells with LPS stimulation and in newborn piglets under normal conditions. BMC Microbiol. 2015;15:32.
87. Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, Poiret S, et al. Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. World J Gastroenterol. 2007;13(2):236–43.
88. Herfel TM, Jacobi SK, Lin X, Fellner V, Walker DC, Jouni ZE, et al. Polydextrose enrichment of infant formula demonstrates prebiotic characteristics by altering intestinal microbiota, organic acid concentrations, and cytokine expression in suckling piglets. J Nutr. 2011;141(12):2139–45.
89. Mulder IE, Schmidt B, Stokes CR, Lewis M, Bailey M, Aminov RI, et al. Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. BMC Biol. 2009;7:79.
90. Thomas CM, Versalovic J. Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbes. 2010;1(3):148–63.
91. Suzuki T. Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci. 2013;70(4):631–59.

92. Günzel D, Yu AS. Claudins and the modulation of tight junction permeability. Physiol Rev. 2013;93(2):525–69.
93. Tsukita S, Yamazaki Y, Katsuno T, Tamura A. Tight junction-based epithelial microenvironment and cell proliferation. Oncogene. 2008;27(55):6930.
94. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol. 2001;2(4):285.
95. Zhang P, Yan T, Wang X, Kuang S, Xiao Y, Lu W, et al. Probiotic mixture ameliorates heat stress of laying hens by enhancing intestinal barrier function and improving gut microbiota. Ital J Anim Sci. 2017;16(2):292–300.
96. Cao L, Yang X, Sun F, Liu C, Yao J. *Lactobacillus* strain with high adhesion stimulates intestinal mucin expression in broiler. J Poult Sci. 2012:0110146.
97. Xie W, Wang H, Wang L, Yao C, Yuan R, Wu Q. Resolvin D1 reduces deterioration of tight junction proteins by upregulating HO-1 in LPS-induced mice. Lab Invest. 2013;93(9):991–1000.
98. Krishna Rao R, Samak G. Protection and restitution of gut barrier by probiotics: nutritional and clinical implications. Current Nut Food Sci. 2013;9(2):99–107.
99. Zulkifli I, Najafi P, Nurfarahin AJ, Soleimani AF, Kumari S, Aryani AA, et al. Acute phase proteins, interleukin 6, and heat shock protein 70 in broiler chickens administered with corticosterone. Poult Sci. 2014;93(12):3112–8.
100. Al-Aqil A, Zulkifli I. Changes in heat shock protein 70 expression and blood characteristics in transported broiler chickens as affected by housing and early age feed restriction. Poult Sci. 2009;88(7):1358–64.
101. Zulkifli I, Al-Aqil A, Omar AR, Sazili AQ, Rajion MA. Crating and heat stress influence blood parameters and heat shock protein 70 expression in broiler chickens showing short or long tonic immobility reactions. Poult Sci. 2009;88(3):471–6.
102. Soleimani AF, Zulkifli I, Hair-Bejo M, Omar AR, Raha AR. The role of heat shock protein 70 in resistance to *Salmonella enteritidis* in broiler chickens subjected to neonatal feed restriction and thermal stress. Poult Sci. 2012;91(2):340–5.
103. Craig EA, Gross CA. Is hsp70 the cellular thermometer? Trends Biochem Sci. 1991;16:135–40.
104. Hosseini SM, Afshar M, Ahani S, Vakili Azghandi M. Heat shock protein 70 mRNA expression and immune response of heat-stressed finishing broilers fed propolis (bee glue) supplementation. Arch Anim Breed. 2015;58(2):407–13.
105. Gu XH, Hao Y, Wang XL. Overexpression of heat shock protein 70 and its relationship to intestine under acute heat stress in broilers: 2. Intestinal oxidative stress. Poult Sci. 2012;91(4):790–9.
106. Jang I-S, Ko Y-H, Moon Y-S, Sohn S-H. Effects of vitamin C or E on the pro-inflammatory cytokines, heat shock protein 70 and antioxidant status in broiler chicks under summer conditions. Asian-Australas J Anim Sci. 2014;27(5):749.

107. Malago JJ, Koninkx JF, van Dijk JE. The heat shock response and cytoprotection of the intestinal epithelium. Cell Stress Chaperones. 2002;7(2):191–9.
108. Najafi P, Zulkifli I, Soleimani AF, Kashiani P. The effect of different degrees of feed restriction on heat shock protein 70, acute phase proteins, and other blood parameters in female broiler breeders. Poult Sci. 2015;94(10):2322–9.
109. Roushdy EM, Zaglool AW, El-Tarabany MS. Effects of chronic thermal stress on growth performance, carcass traits, antioxidant indices and the expression of HSP70, growth hormone and superoxide dismutase genes in two broiler strains. J Therm Biol. 2018;74:337–43.