Effect of synbiotics on thyroid hormones, intestinal histomorphology, and heat shock protein 70 expression in broiler chickens reared under cyclic heat stress

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ABSTRACT This study examined effect of a dietary synbiotic supplement on the concentrations of plasma thyroid hormones, expressions of heat shock protein 70 (HSP70), and intestinal histomorphology in broiler chickens exposed to cyclic heat stress (HS). Three hundred and sixty day old male Ross 708 broiler chicks were randomly distributed among 3 dietary treatments containing a synbiotic (PoultryStar me^{US}) at 0 (control), 0.5 (0.5×), and 1.0 (1.0×) g/kg. Each treatment contained 8 replicates of 15 birds each housed in floor pens in a temperature and lighting controlled room. Heat stimulation was established from days 15 to 42 at 32°C for 9 h daily. The results indicated that under the HS condition, both synbiotic fed groups had lower liver and hypothalamus HSP70 levels ($P < 0.001$) compared to control group; however, HSP70 mRNA expression was not different among treatments ($P > 0.05$). There were no treatment effects on the levels of triiodothyronine (T_3) and thyroxine (T_4) as well as T_3/T_4 ratio ($P > 0.05$). Compared to controls, 1.0× HS broilers had greater villus height in the duodenum ($P < 0.01$), and greater villus height and villus height:crypt depth ratios in the ileum ($P < 0.01$). There were no differences among treatments on the measured intestinal parameters in the jejunum ($P > 0.05$). The results suggest that the synbiotic may ameliorate the negative effects of HS on chicken health as indicated by the changes in the intestinal architecture and the levels of HSP70. Dietary synbiotic supplement could be a feasible nutritive strategy for the poultry industry to improve the health and welfare of chickens when exposed to hot environmental temperature.

Key words: broiler chicken, heat stress, synbiotic, intestinal histomorphology, heat shock protein 70

2020 Poultry Science 99:142–150
http://dx.doi.org/10.3382/ps/pez571

INTRODUCTION

Heat stress (**HS**) is a severe problem in the poultry industry; it negatively affects production, increases morbidity and mortality, and is consequently responsible for heavy economic loss, particularly during hot seasons and in the tropical locations (Lara and Rostagno, 2013; Mignon-Grasteau et al., 2015). Stressful conditions, such as those experienced during elevated temperatures, alter the activity of the neuroendocrine system which stimulates the hypothalamic-pituitary-adrenal (**HPA**) axis to increase plasma corticosterone (**CORT**) (Quinteiro-Filho et al., 2010) and reduce circulating thyroid hormones such as triiodothyronine ($\mathbf{T_3}$) and thyroxine ($\mathbf{T_4}$) (Sohail et al., 2010). Combined, these effects result in impaired metabolism function (Bahrami et al., 2012; Farag and Alagawany, 2018) and, ultimately, reduced BW gain. Further, HS negatively affects the immune system (Padgett and Glaser, 2003), and causes dysfunction of the intestinal barrier (Lambert, 2009), which further affects production and leads to poor health and welfare in broiler chickens (Song et al., 2014).

Exposure to HS increases the synthesis of heat shock proteins (**HSPs**) which are produced in all cells and tissues in response to stress. These specialized proteins function to facilitate the synthesis, conformation, and turn-over of other proteins (Yahav et al., 1997). One type of HSPs, heat shock protein 70 (**HSP70**), is vital to stress recovery and is responsible for repairing damaged cells (Ming et al., 2010), regulating the refolding of damaged proteins (Maloyan et al., 1999), inhibiting oxidation and apoptosis (Xing et al., 2015), and controlling protein assembly, disassembly, and translocation (Ryan and Pfanner, 2001). In all, these processes may

Received April 21, 2019.
Accepted September 15, 2019.
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stabilize the internal environment and improve tolerance to stress (Kamboh et al., 2013).

Recent studies have demonstrated that the stress response and overall health of poultry share a reciprocal relationship with the structure of the intestine and the microbial population in the intestine (Sohail et al., 2010; Deng et al., 2012; Ashraf et al., 2013; Song et al., 2014). Stress, including HS, alters the microbial composition in the gut of poultry (Yu et al., 2012), which subsequently influences the turnover of intestinal epithelial cells and tight junctions, causing damage to the integrity and morphology of the intestine (Song et al., 2014), leading to "leaky gut" (Pearce et al., 2013). In addition, HS leads to hypoxia and ischemia in the intestinal epithelia due to the redistribution of the systemic blood flow circulation from internal to peripheral body surface to dissipate heat (Al-Fataftah and Abdelqader, 2014; Song et al., 2014), leading to weakened absorption, digestion, and increased permeability to toxins and luminal antigens. Conversely, an impaired microbial population increases response to stressors (Sudo et al., 2004) and has a negative impact on the health and welfare of poultry (Stanley et al., 2014), suggesting these systems share a mutual relationship.

Numerous studies have demonstrated an improvement in the stress response and overall health of poultry following dietary supplementation with probiotics and synbiotics (Ghareeb et al., 2008; Sohail et al., 2010; 2011; Ashraf et al., 2013). Probiotics are live microorganisms that benefit the host by maintaining or restoring the balance of the intestinal bacterial populations (Fuller, 1989). Synbiotics refer to a combination of probiotics and prebiotics, the latter of which improves the survival and implantation of favorable microbes in the gut (Awad et al., 2009), and together these compounds have been shown to improve or protect intestinal morphology (Deng et al., 2012; Lei et al., 2013). Further, dietary supplements of probiotics increase serum concentrations of T_3 and T_4 (Tollba et al., 2004; Sohail et al., 2010), and influence HSP70 expression in the heart of heat-stressed broiler chickens (Khan et al., 2016) and in the liver, kidney, and spleen of heat-stressed pigs (Gan et al., 2013). These results suggest that, similar to the functions of probiotics, synbiotics may protect the health of animals including broilers chickens experiencing HS.

A small body of conflicting literature, however, exists regarding the effects of synbiotics on heat-stressed poultry. Several authors report no effect (Sohail et al., 2015), while others report promising results (Ashraf et al., 2013; Sohail et al., 2013; Song et al., 2014). These contrasting results may be partly due to variations in synbiotic composition (i.e., bacterial strains and prebiotic types) as well as different levels of the synbiotic. Considering the potential for improvement to gut microbiota, intestinal histomorphology, thyroid hormone expression, HSPs, and overall bird health, we aimed to build upon the existing literature by investigating the effectiveness of a combination of bacterial strains derived from the 4 gastrointestinal sections of poultry at 2 concentrations. Further, previous researches have not reported changes in HSPs following dietary supplementation with synbiotics. Therefore, the objective of this study was to observe the influence of the synbiotic on intestinal histomorphology, thyroid hormones, and HSP70 expression in broiler chickens reared under HS. We hypothesized that dietary supplementation of the synbiotic (PoultryStar me^{US}) will alleviate the negative effects of HS via the regulation of HSP70 expression and thyroid hormonal secretion and improve the intestinal architecture in broiler chickens under HS.

MATERIALS AND METHODS

All procedures in this experiment were approved by the Animal Care and Use Committee of Purdue University in West Lafayette, Indiana, USA.

Birds and Housing

Three hundred and sixty day old male broiler chicks (Ross 708 strain) were obtained from a commercial hatchery (Pine Manor/Miller Poultry, Goshen, IN). Chicks were weighed in 15 bird-group and assigned to 1 of 24 floor pens (110 cm × 110 cm per pen) with equal weight distribution among the pens. All pens were in a temperature and lighting controlled room at the Poultry Research Farm of Purdue University.

The management procedure followed the guidelines of Aviagen (2014). Heat stress was established at 32°C daily for 9 h (08:00 am to 17:00 pm) from days 15 to 42 (Mohammed et al., 2018). Data loggers (HOBO, Onset Computer Corporation, Bourne, MA) were fixed 30 cm above the litter surface to monitor the room temperature and humidity.

Dietary Treatments

The synbiotic product, PoultryStar me^{US} (BIOMIN America Inc., San Antonio, TX), was used in this study. It included a prebiotic (fructo-oligosaccharides) and a probiotic mixture of 4 microbial strains selected from the 4 different sections of poultry gastrointestinal tract: *Pediococcus acidilactici* isolated from the cecum, *Bifidobacterium animalis* from the ileum, *Enterococcus faecium* from the jejunum, and *Lactobacillus reuteri* from the crop. The prebiotic was chosen to further improve the growth and activity of beneficial microflora, whereas the probiotic bacteria were selected for their ability to stabilize a healthy gut microbial community and regulate the pathogenic bacteria. The synbiotic activity and survivability have been examined in previous reports (Murugesan and Persia, 2015; Yan et al., 2015).

Birds were fed 1 of 3 diets: (1) a regular diet (control), (2) the regular diet mixed with 0.5 (0.5×, 1×10^6 cfu/g) or (3) 1.0 (1.0×, 2×10^6cfu/g) g·kg^{-1} synbiotic.

The PoultryStar diet was supplemented from days 1 to 42 and made by the step-up procedure (Mahmoud et al., 2015). In brief, a small amount of the regular diet was mixed with the respective amount of PoultryStar then gradually integrated with a larger amount of the regular diet until the total amount of each of the diets was homogeneously mixed. The birds were fed the starter diet from days 1 to 14, the grower diet from days 15 to 28, and the finisher diet from days 29 to 42. The diet formulation was reported previously (Mohammed et al., 2018). Water and feed were supplied ad libitum.

Sample Collection

On day 42, 2 broiler chickens were randomly selected from each pen (16 broiler chickens/treatment) and sedated with sodium pentobarbital (30 mg/mL) within 2 min of the birds removal from its home pen. A 5 mL of blood samples was collected via cardiac puncture in a plasma separator tube with EDTA and then centrifuged at 3,000× *g* for 15 min to collect the plasma. Birds were euthanized immediately following blood collection, and samples of the liver, hypothalamus, and intestine (2 cm at the midpoint of the duodenum, jejunum, and ileum based on its anatomic markers) were collected (Akbarian et al., 2013). All samples were stored at –80°C until analyses were performed except for the intestinal samples. Intestinal samples were gently flushed with 0.9% saline to remove the intestinal contents and fixed in 10% formalin until analysis.

Thyroid Hormone Analysis

Analysis of plasma concentrations of total T_3 and T_4 were performed by using the commercial chicken ELISA kits (MyBioSource, Inc., San Diego, CA). The T_3/T_4 ratio was calculated by dividing the value of T_3 by the value of T_4. The detection range was 0.5 to 8 ng/mL for T3 and 20 to 320 ng/mL for T4.

Intestinal Histomorphology

The intestinal samples were processed by followed the previously published protocol (Thompson and Applegate, 2006). Briefly, the intestinal samples were dehydrated with increasing concentrations of ethanol, cleared with xylene (Surgipath Medical Industries, Richmond, IL), and embedded with paraffin wax (Thermo fisher scientific, Kalamazoo, MC). Cross sections (5 μm) were stained with hematoxylin and eosin (GeneCopoeia, Rockville, MD). The stained sections were dehydrate with ethanol, clean with xylene, and mounted with DPX mountant (Grounds, 2014). The software of ImageJ (National Institutes of Health, USA) was used to determine the morphometric measurements of villus height and crypt depth of the duodenum, jejunum, and ileum by using an Olympus BX40 F-3 microscope (Olympus Cooperation, Tokyo, Japan) attached to a digital video camera (Q- imaging, 01-MBF-200R-CLR-12, SN: Q32316, Canada) as described in Samuel et al. (2017).

Western Blot Analysis

Approximately 1 cm^3 of each of liver and hypothalamus sample was homogenized in 1× RIPA buffer solution (Sigma-Aldrich, St. Louis, MO), then centrifuged at 15,000× *g* for 5 min at 4°C and, the supernatant was collected. Protein concentration of each sample was identified by using BioTek Microplate reader at 280 nm wavelength (Bio Tek Epoch, Vermont). The concentration of 5 mg/mL of each sample was prepared by adding RIPA lysis buffer, and then stored at –20°C until further analyses.

Western blot analysis of HSP70 was conducted based on the protocol descripted previously (Felver-Gant et al., 2012). Briefly, 30 μg of total protein per sample was separated with a 10% SDS-PAGE. After electrophoreses, the proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat dry milk in TBST solution for 1 h at room temperature to block nonspecific binding, then incubated with a primary antibody (anti-mouse HSP70 IgG; Thermo fisher scientific, Rockford, MA) in a dilution of 1:5,000 at 4°C overnight, followed by a secondary antibody (horse anti-mouse horseradish peroxidase conjugated IgG) at a 1:5,000 dilution for 1.5 h. Visualization of the site of antigen-antibody complex was carried out with chemiluminescence solution (Immobilon Western Chemiluminescent HRP Substrate, Millipore, Billerica, MA). Immunoreactive bands were detected using the gel-imaging system (UVP, LLC, The ChemiDoc-It2 Imager, Upland, CA) with the Image Analysis Software (UVP, LLC, VisionWorksLS Image Acquisition and Analysis Software, Upland, CA).

mRNA Expression Analysis

Real-time PCR analysis for HSP70 mRNA expressions in the liver and hypothalamus were conducted following the protocol previously reported by Felver-Gant et al. (2012). The primers and probes were: Forward primer (5′-CACCATCACTGGCCTTA ACGT-3′), Reverse primer (5′-TTATCCAAGCCA TAGGCAATAGC-3′), and Taqman probe (5′-ATGC GTATTATCAATGAGCCCA-3′). Each tissue sample was homogenized by using tissue homogenizer, RNA was extracted by using RNeasy Mini Kit (Qiagen, Valencia, CA), and total RNA was determined by the optic density at 260 nm (NanoDrop-1000, Thermo Fisher Scientific, Waltham, MA). RNase-free water (Ambion Inc.) was added to make an equal amount of RNA (100 ng/μL) across all samples. 1 μL of 20× Enzyme mix (includes MuLV Reverse Transcriptase and RNase inhibitor protein) was added to 9 μL of the sample followed by 10 μL of 2× Reverse Transcriptase Buffer

Mix (includes dNTPs, random octamers, and oligo dT-16) (High Capacity RNA-to-cDNA Kit, Applied Biosystems, Foster City, CA). The sample mixtures were loaded to the Hybaid PCR Express thermo cycler (Midwest Scientific, St. Louis, MO), and amplified using the following cycling conditions: 37°C for 60 min followed by 95°C for 5 min with a final holding temperature of 4°C. The tubes were then stored at −20°C until PCR analysis.

The PCR analysis condition was set up at 25 μL of PCR Master mix (Applied Biosystems), 7.75 μL RNase-free water (Ambion Inc.), 3.5 μL of TaqMan probe, 4.5 μL of genespecific TaqMan forward and reverses primers each, and 5 μL of sample cDNA. The cycling condition for real-time PCR was: 50°C for 2 min, followed 95°C for 10 min, and finally 40 cycles of 95°C for 20 s and 60°C for 1 min, and then analyzed with 7500 Real-Time PCR software (Applied Biosystems). 2-ΔΔCt method was used to calculate the average gene expression relative to the glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**) which was used as an endogenous control for each sample (Livak and Schmittgen, 2001). The average ΔCt value from the negative control group was the calibrator for the gene of each particular tissue. To assure accuracy and consistency, all samples were measured in duplicates and standards in triplicates with a standard deviation of less than 2.0 and a coefficient of variation less than 2.0%.

Statistical Analysis

The experimental design was conducted in a completely randomized design. Pen was considered as the experimental unit. Means of the data were analyzed by using the JMP software (SAS Institute, Cary, NC). The normality of the data was analyzed by the Shapiro-Wilk test. The overall effects of the dietary synbiotic supplementation were analyzed statistically by one-way analysis of variance (ANOVA). Means were compared by Tukey- Kramer test when a significant difference was detected. Statistical significance was declared when the coefficients were at a probability of α equal to or less than 0.05. Least square means and SEM are presented.

RESULTS

Plasma Thyroid Hormone Measurements

The synbiotic had no effect on plasma T_3, T_4, and the T_3/T_4 ratio in HS broiler (Table 1, $P = 0.080$, 0.766, and 0.303, respectively).

Table 1. Effect of dietary supplementation of the synbiotic in 2 concentrations on the thyroid hormones of heat-stressed broiler chickens at 42 D of age.

Treatment[1]	Control	0.5×	1.0×	SEM	*P*-value
T3 (ng · mL^{-1})	1.81	1.26	1.30	0.26	0.080
T4 (ng · mL^{-1})	175.43	164.26	160.52	21.13	0.766
T3/T4 ratio	0.012	0.008	0.008	0.002	0.303

[1]Basal dietary supplemented with 0 (Control), 0.5 (Low), and 1 (High) g kg^{-1} synbiotic (PoultryStar me^{US}). n = 8 (as each pen was considered as an experimental unit, each pen included 15, each treatment included 8 pens).

T3 = triiodothyronine and T4 = thyroxine.

Histomorphological Measurements in the Intestine

The synbiotic effects on the villus height, crypts depth, and the ratio of villus height and crypts depth in the duodenum, jejunum, and ileum in HS broiler are presented in Table 2 and Figure 1. In the duodenum, the villus height ($P = 0.009$) was increased with a tended increase of villus height : crypt depth ratio ($P = 0.088$) in 1.0× group but not in 0.5× group compared to controls. In the ileum, the villus height ($P = 0.005$) was higher in both synbiotic treated groups compared to controls, while villus height:crypts depth ratio ($P = 0.013$) was higher in 1.0× group only. In the jejunum, there was no difference between the experimental groups for the villus height, crypts depth, and the ratio of villus height and crypts depth ($P > 0.05$).

Levels of HSP70 and HSP70 mRNA in the Liver and Hypothalamus

The synbiotic effects on the HSP70 concentrations and HSP70 mRNA expressions in the liver and hypothalamus are presented in Table 3. Regardless of dose, liver HSP70 levels were significantly lower for the synbiotic birds (0.5×: 0.33 ± 0.06; 1.0×: 0.30 ± 0.06) compared to controls (0.95 ± 0.06; $P < 0.001$). Following a similar trend, the hypothalamus HSP70 levels were also lower in synbiotic birds (0.5×: 0.72 ± 0.12; 1.0×: 0.35 ± 0.12) compared to controls (1.55 ± 0.12; $P < 0.001$). However, the levels of HSP70 mRNA expression in both organs were not affected by the synbiotic supplementation regardless of its dose ($P = 0.756$ and 0.994, respectively).

DISCUSSION

Heat stress has profound negative effects on health and welfare of broiler chickens. The results of this study showed that the dietary synbiotic supplement improved HSP70 expression and some aspects of intestinal morphology in broiler chickens under HS conditions. Therefore, supplementation of broiler chickens with the synbiotic has shown promise in mitigating the negative effects of HS.

In this study, heat stimulation at 32°C for 9 h daily (from days 15 to 42) was used as an environmental stressor. A similar ambient condition has been used previously to activate stress reactions in broiler chickens (Mahmoud et al., 2015; Mohammed et al., 2018) and laying hens (Mack et al., 2013). In a concurrent study,

Table 2. Effect of dietary supplementation of different levels of the synbiotic on the intestinal morphometry of heat stressed broiler chickens at 42 D of age.

Treatment[1]	Control	0.5×	1.0×	SEM	*P*-value
Duodenum					
Villus height (μm)	1,995.48[a]	1,982.54[a]	2,134.89[b]	25.50	0.009
Crypt depth (μm)	199.37	180.81	158.01	9.57	0.195
Villus height: crypt depth ratio	10.33	11.51	13.56	0.64	0.088
Jejunum					
Villus height (μm)	1,325.09	1,430.13	1,369.65	31.46	0.681
Crypt depth (μm)	146.98	168.19	162.09	6.3	0.352
Villus height: crypt depth ratio	9.3	8.5	8.5	0.3	0.429
Ileum					
Villus height (μm)	819.39[a]	934.01[b]	1016.09[b]	27.65	0.005
Crypt depth (μm)	155.28	154.78	150.40	4.3	0.893
Villus height: crypt depth ratio	5.32[a]	6.08[ab]	6.9[b]	0.22	0.013

Least square means with different superscripts in the same row differ significantly ($P \leq 0.05$).
[1]Basal dietary supplemented with 0 (Control), 0.5 (0.5×), and 1 (1.0×) g kg-1synbiotic (PoultryStar me[US]). n = 5 (1 broiler chicken was randomly selected from 5 pens per treatment).

broiler chickens experiencing the HS condition exhibited heat stress-associated behaviors, such as panting and wing spreading (Mohammed et al., 2018). The collective results of these studies indicate that poultry are sufficiently heat stressed under this described conditions.

Thyroid hormones (T_3 and T_4) play a vital role in controlling thermoregulation and nutrient metabolism, which is influenced by internal and external factors, including exposed to various stressors (Mancini et al., 2016; Garasto et al., 2017). Regulation of thyroid functions, such as T3 and T4 concentration, is essential for maintenance of body temperature via energy metabolism in homoeothermic animals including chickens. Significantly reduced blood T3 concentrations have been reported in broilers exposed to thermal stress at various conditions, such as 36°C for 4 to 6 h daily from days 22 to 42 (Zaglool et al., 2019), 38°C for 3 h daily from days 35 to 40 (Tollba and Hassan, 2003); and 38°C for 1 h at day 40 (Iqbal et al., 1990). Reduced T3 state may prevent undesirable catabolic effects in stressed broilers. On the other hand, Bowen and Washburn (1985) reported that both serum T3 and T4 concentrations were significantly increased in broilers exposed to acute heat stress at 50°C for 1 h at 5 D of age. While May et al. (1986) found that plasma T3 and T4 levels were not affected in broilers exposed to heat at 41°C for 4 to 6 h on the fourth day. These results indicate that HS effect on broiler thyroid hormones is affected by multiple factors including stress level and duration and bird age. Similarly, nutrient levels and contents affect the structure and function of the nervous system (Bourre, 2006), including the thyroid hormonal synthesis, release, and metabolism (Ventura et al., 2017), via the gut-brain axis (Grant et al., 2018). In the current study, under the current experimental condition, there was no treatment effect on thyroid hormones in heat-stressed broilers; blood T3 and T4 concentrations and T3/T4 ratio were not significant difference in synbiotic fed broilers compared to controls. Similarly, Spaggiari et al. (2017) reported that probiotic (containing *Lactobacilli* and *Bifid bacteria*) did not alter thyroid hormonal parameters (thyroid-stimulating hormone, free T3 and free T4 levels) in humans. In addition, Kolodziejski et al. (2018) found that 2 synbiotics (*Lactobacillus salivarius* plus *galactooligosaccharide* and *Lactobacillus plantarum* plus *raffinose family oligosaccharides*) improved digestive proteolytic and lipolytic ability without effect on thyroid hormones in broilers. Furthermore, Fathi et al. (2018) reported that *Bacillus subtilis* did not significantly change the concentrations of plasma T3 and T4 in the laying hens under high ambient temperature. In contrast, Sohail et al. (2010) found that dietary probiotic supplementations (*Lactobacillus*) significantly increased serum T4 but not T3 concentrations in broilers exposed to heat stress at 35°C for 8 h daily from days 22 to 42. Hosseini et al. (2013) also reported that both prebiotics (BIO-MOS of *Saccharomyces cerevisiae* yeast) and probiotic (BIO-SAF) as well as their combination normalized HS reduced thyroid hormones (T3 and T4) in broilers (exposed to heat at 34.5°C for 4 h daily during the first week, then reduced and maintained constantly at 26.5°C until 42 D of age). Anwar et al. (2018) also reported that synbiotics and probiotics increased T3 concentrations but decreased T4 concentration in molted hens. Taken together, the current and previous results may suggest that the regulation of thyroid hormones may not be the major function of the synbiotic in heat-stressed broilers, and the effect of synbiotics on thyroid hormones may be affected by the components of synbiotics, treatment duration, bird species and age, and stress condition.

The small intestine is composed of 3 parts: the duodenum, jejunum, and ileum; each component contributes to various aspects of nutrient absorption and digestion. The duodenum is the principal place of food breakdown (Klasing, 1999), the jejunum primarily absorbs and assimilates nutrients (Damron, 2005), and the ileum plays an essential role in fermentation (Ewing and Cole,

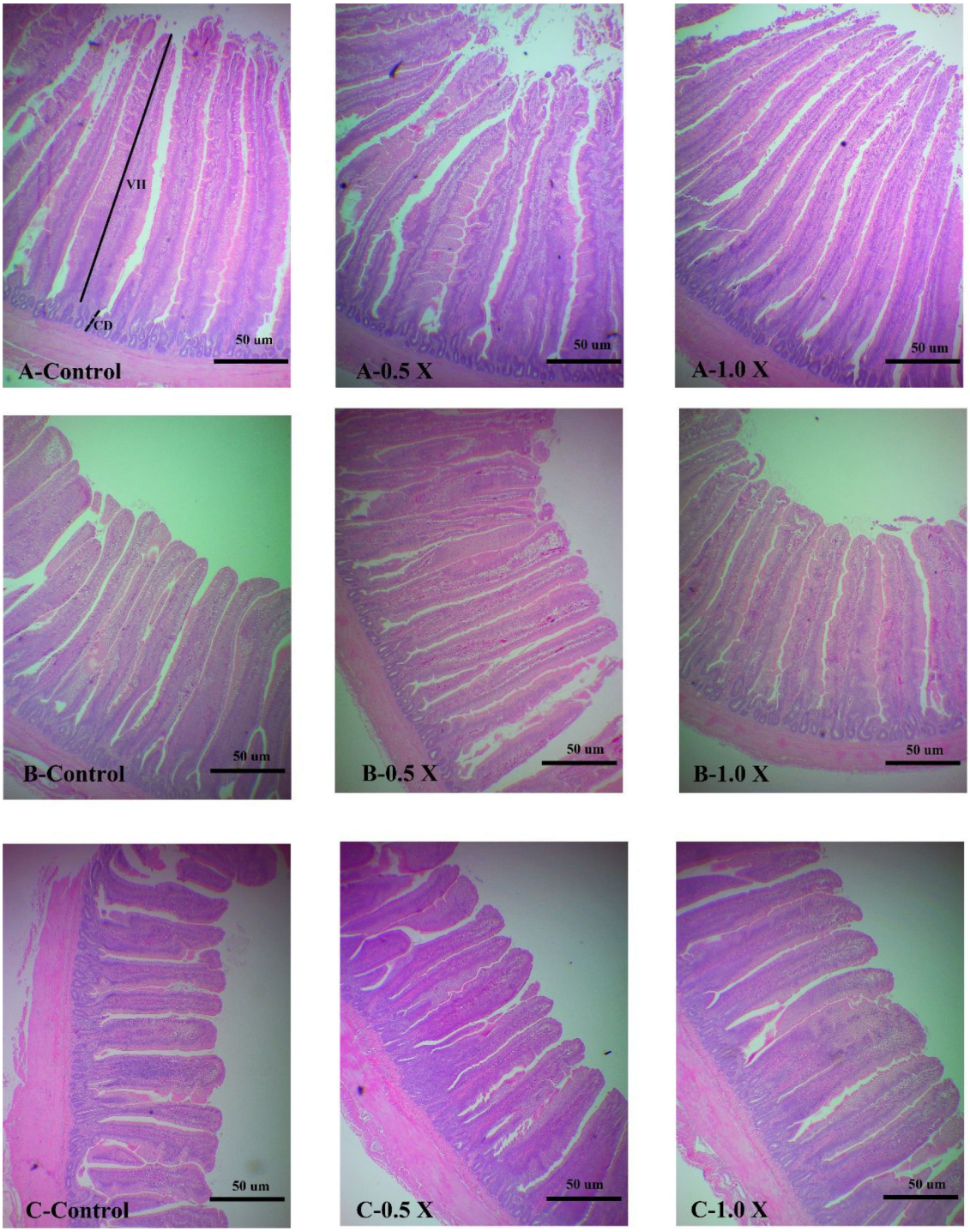

Figure 1. The examples of the morphological changes of the villus height and crypts depth in the duodenum (A), jejunum (B), and ileum (C) of heat-stressed broiler chickens fed different levels of the synbiotic (PoultryStar me^{US}). Basal dietary supplemented with 0 (Control), 0.5 (0.5×) and 1 (1.0×) g kg^{-1}synbiotic. –VH: Villus height; —CD: Crypts depth.

1994). The morphology of these components is one of the main indicators of the gut health overall (Awad et al., 2009; Ducatelle et al., 2018), and HS has severe consequences to the structure of the small intestine, including villi fracture, shortening of the villus height, deeper crypts, and mucosal epithelial cell exfoliation (Yu et al., 2010). Our findings suggest that the synbiotic can protect the structure of the duodenum and ileum during stressful conditions. In our study, synbiotic fed birds had longer villi in the duodenum with tendency higher villus height: crypt depth rations and longer villi in the ileum with higher villus height: crypt depth ratios compared to birds fed control diet. The similar results have been reported previously (Quinteiro-Filho et al., 2010; Sohail et al., 2012; Al-Fataftah and Abdelqader, 2014; Song et al., 2014, Wu et al., 2018). The improvement in the intestinal morphometry may be due to the colonization

Table 3. Effect of dietary supplementation of the synbiotic in 2 concentrations on HSP70 and HSP70 mRNA expression in liver and hypothalamus of the heat stressed broiler chickens from 15 to 42 D of age.

Treatment[1]	Control	0.5×	1.0×	SEM	*P*-value
Liver					
HSP70	0.95[a]	0.33[b]	0.30[b]	0.06	0.001
HSP70 mRNA	1.18	1.16	1.15	0.02	0.756
Hypothalamus					
HSP70	1.55[a]	0.72[b]	0.35[b]	0.12	0.001
HSP70 mRNA	1.02	1.02	1.01	0.06	0.994

Least square means with different superscripts in the same row differ significantly ($P \leq 0.05$).

[1]Basal dietary supplemented with 0 (Control), 0.5 (0.5×), and 1 (1.0×) g kg^{-1} synbiotic (PoultryStar me^{US}). n = 8 (as each pen was considered as an experimental unit, each pen included 15 birds, each treatment included 8 pens).

of administrated probiotic bacteria in the small intestine, effectively protecting the villi from toxins and pathogens (Song et al., 2014). In addition, the probiotic bacteria may allow for better nutrient absorption, expression of intestinal protective factors (Lutgendorff et al., 2009), competitive exclusion of harmful bacteria (Vicente et al., 2008), and support of epithelial cell cytoskeleton and tight junctions, by which further contributes to the villus protection (Song et al., 2014). In the current study, the synbiotic had no obvious effects on the microstructure of the jejunum, which is similar to the previous findings that yeast nucleotides improved the morphological changes in the ileum but not in the jejunum of chickens vaccinated intranasally with inactivated infectious bronchitis virus (Wu et al., 2018). A potential cause could be related to that the functions and microbiota compositions are different between the jejunum and ileum (Ewing and Cole, 1994; Damron, 2005; Stanley et al., 2012).

HSP70 prevents cells from death mediated by reactive oxygen species (**ROS)** and free radicals (Jacquier-Sarlin et al., 1994), thus the levels of HSP70 are typically elevated when birds are exposed to HS conditions (Felver-Gant et al., 2012). In our study, HSP70 levels in both the liver and hypothalamus were significantly lower in synbiotic groups compared to control group, which agrees with the previous reports on the effect of prebiotics and probiotics on HSP70 levels during HS (Varasteh et al., 2015; Khan et al., 2016). These findings may reflect the ability of synbiotic-induced microbial changes to protect against HS-induced protein damage via the microbiota-gut-brain axis (Varasteh et al., 2015). However, surprisingly, HSP mRNA expression was not different between treatments; this may be attributed to the major changes are at the translation rather than transcription level (Lund et al., 2003). Previous research has reported similar results between the levels of HSP70 and HSP mRNA expression in heat-stressed chickens followed probiotic supplementation (Varasteh et al., 2015). The current and previous results indicate that one of the pathways of probiotics and synbiotics improving the hosts' health is through regulating HSP70 expression.

CONCLUSION

In the current study, both doses of the synbiotic supplement significantly inhibited expression of HSP70 of broiler chickens reared in heat stress environment. The effect of dosage also resulted in several notable differences between the measurements, namely, the 1.0× concentration resulted in a significantly greater villus height and villus height:crypts depth ratio in the duodenum and ileum. These results indicate the synbiotic at 1.0× level may be more effective. Overall, our findings suggest that supplementing the broiler chickens with the synbiotic (PoultryStar me^{US}) may be useful in ameliorating the negative effects of HS, particularly when exposed to hot climates.

ACKNOWLEDGMENTS

We would like to express our gratitude to the scientists and staff of the USDA-ARS unit, farm staff, and the graduate students at Purdue University who contributed to the development and sample collection of this study. Research reported in this publication was supported by the National Natural Science Foundation of China (No. 31702307), Fundamental Research Funds for the Central Universities (XDJK2019B013), the USDA-NIFA- AFRI (Award No. 2017-67015-26567), and the Egyptian Cultural and Educational Bureau (ECEB) in Washington DC., Ministry of Higher Education and Scientific Research, Egypt. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement of the USDA. The USDA is an equal opportunity provider and employer.

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