Supplementation of *Bacillus subtilis*-based probiotic reduces heat stress-related behaviors and inflammatory response in broiler chickens[1]

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ABSTRACT: Probiotics reduce stress-related inflammation and abnormal behaviors in humans and rodents via regulation of the microbiota-gut-brain axis. The objective of this study was to determine if probiotic, *Bacillus subtilis*, has similar functions in broiler chickens under heat stress (**HS**). Two hundred forty 1-d-old broiler chicks were assigned to 48 pens with 4 treatments: Thermoneutral (**TN**)-RD (regular diet), TN-PD (the regular diet mixed with 1×10^6 CFU/g feed probiotic), HS-RD and HS-PD. Probiotic (Sporulin) was fed from day 1; and HS at 32°C for 10 h daily was initiated at day 15. The data showed that final BW, average daily gain , and feed conversion efficiency were improved in PD groups as compared to RD groups regardless of the ambient temperature ($P < 0.01$). Heterophil to lymphocyte ratio was affected by treatment and its value was in the order of HS-RD > HS-PD > TN-RD > TN-PD birds ($P < 0.01$). Compared to TN birds, HS birds spent more time in wing spreading, panting, squatting close to the ground, drinking, sleeping, dozing, and sitting but spent less time in eating, standing, and walking ($P < 0.05$ or 0.01). In addition, HS birds had greater levels of hepatic IL-6, IL-10, heat shock protein (HSP)70, and HSP70 mRNA expression ($P < 0.01$) and greater levels of cecal IgA and IgY ($P < 0.01$) compared to TN birds. Within TN groups, TN-PD birds had greater concentrations of hepatic IL-10 ($P < 0.05$) and cecal IgA ($P < 0.01$) than TN-RD birds. Within HS groups, HS-PD birds spent less time in wing spreading, panting, squatting close to the ground, drinking, sleeping, dozing, and sitting but spent more time in eating, foraging, standing, and walking than HS-RD birds ($P < 0.05$ or 0.01). The HS-PD birds also had lower concentrations of hepatic IL-6 and HSP70 ($P < 0.01$), whereas greater levels of IL-10 ($P < 0.05$) and lower concentrations of cecal IgA and IgY ($P < 0.01$). These results indicate that broilers fed the probiotic, *B. subtilis*, are able to cope with HS more effectively by ameliorating heat-induced behavioral and inflammatory reactions through regulation of microbiota-modulated immunity.

Key words: *Bacillus subtilis*, behavior, broiler chicken, heat stress, inflammation, probiotic

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INTRODUCTION

Heat stress (**HS**) has been recognized as a critical environmental stressor reducing performance, health, and well-being of farm animals including poultry (Lara and Rostagno, 2013). In broiler chickens, body temperature above the thermoneutral zone (TNZ) disturbs physiological homeostasis (Pawar et at., 2016) and suppresses the function of both the immune (Sugiharto et al., 2016) and digestive system (Quinteiro-Filho et al., 2012), which leads to gut inflammation and dysfunction, reducing health status and increasing

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mortality (Strong et al., 2015). As the ambient temperature increases beyond the TNZ, chickens alter their behaviors to prevent body core temperature changes; so, the temperature modulation is shifted from sensible heat loss (such as radiation via the wattle, comb, feet, and wing spreading) to evaporative heat loss (such as panting) (Lustick, 1983). However, excessive panting decreases the partial pressure of carbon dioxide and calcium availability, while increases pH value in blood, leading to the risk of respiratory alkalosis (Hamano, 2012) and lameness (Hothersall et al., 2014). Recent studies have shown that gut microbiota are able to alleviate inflammatory response and reduce stress-induced behaviors in humans and rodents via regulation of both the microbiota-gut-brain axis (Yano et al., 2015) and the microbiota-gut-immunity axis (Brandsma et al., 2015).

Probiotics, as beneficial direct-fed bacteria (FDA 2003), regulate a host's behavior and health via immunomodulation (Goto et al., 2013), metabolic homeostasis (Thaiss et al., 2014), and neuroendocrine loops (O'Mahony et al., 2015). *Bacillus subtilis,* as a probiotic, has been used in poultry, which inhibits pathogenic proliferation and maintains gut integrity, resulting in the improvement of performance in broilers exposed to *Eimeria* spp. and *Clostridium perfringens* (Lee et al., 2010, 2015). Therefore, we examined whether dietary *B. subtilis* supplementation ameliorates broiler performance, behaviors, and immunity under HS conditions.

MATERIALS AND METHODS

The project was approved by the Animal Care and Use Committee of Purdue University (PACUC #: 1111000262) and animals were housed in accordance with the guidelines of the Federation of Animal Science Societies (2010) at the Animal Research and Education Center.

Birds and Management

Two hundred and forty 1-d-old broiler chicks (Ross 708 strain) were obtained from a commercial hatchery (Pine Manor/Miller Poultry, Goshen, IN). Based on their BW, the chicks were evenly assigned to 48 floor pens (152 cm × 81 cm) within 2 identical, temperature controlled rooms at the Poultry Research Farm of Purdue University. The pens were evenly assigned into 4 dietary treatments: 1) TN (thermoneutral condition)-RD (regular diet); 2) TN-PD (the regular diet mixed with 250 ppm Sproulin); 3) HS-RD; and 4) HS-PD (n = 12 per treatment). The concentration of *B. subtilis* (1×10^6 CFU/g feed) was recommended by the company, and the feeding was started from day 1. The birds were fed the starter diet from day 1 to 14, the grower diet from day 15 to 28, and the finisher diet from day 29 to 43 (Table 1). Feed and water were free-access.

The chicks were maintained at 34°C on day 1, decreased 0.5°C per day until 21°C in one room and maintained at the temperature until the end of this study. In another room, heat stimulation was started at day 15, at 32°C for 10 h (0700–1700) daily up to day 43. The lighting program was as follows: 30 lux for 24 L: 0 D at day 1; 23 L: 1 D at day 2 and 3; 22 L: 2 D at day 4 and 5; and 21 L: 3 D at day 6; then 10 lux for 20 L: 4D until day 43.

Behavioral Observations

Video cameras were set up inside the rooms and bird behaviors were observed according to the ethogram developed previously (Blokhuis, 1984; Santos et al., 2015) (Table 2). The observations of panting, wing spreading, and squatting close to the ground were conducted daily at 1700 h (immediately after HS and before the room temperature was cool down, it took approximately 2 h), and sitting, standing, walking, sleeping, and dozing behaviors observations were conducted daily at 0100 h (immediately before the room light off) during the finisher phase (from day 29 to 42) using the instantaneous scanning sampling method (Altmann, 1974). Eating, drinking, and foraging observations were conducted daily at 1000 h during the heat stimulation (heater was turned on at 0700 daily) using the 5 min scan sampling method (Mack et al., 2013). Scan sampling of posture (sitting, standing, walking, sleeping, or dozing) and heat related behaviors (panting, wing spreading, or squatting) were calculated with the following formula: the number of birds spent in each behavior/the total birds number during the observation time × 100%. All occurrence sampling of feeding behaviors (eating, drinking, and foraging) were calculated with the following formula: the time spent in one behavior/the total time spent in all behaviors during the observation time. For each behavior, the data collected daily were averaged for the statistical analysis.

Sample Collection

The sampled birds (1 bird per pen, n = 12 per group) at day 43 during the HS period was sedated by injection of sodium phenobarbital

Table 1. Nutrient specification of the diets[1]

Item	Starter (day 1–14)	Grower (day 15–28)	Finisher (day 29–43)
Ingredient (% of diet)			
Corn	52.00	52.30	62.80
Soybean meal (48% CP)	40.00	39.10	29.70
Soy oil	3.59	4.97	4.11
Sodium chloride	0.51	0.46	0.43
DL-Methionine	0.30	0.24	0.23
L-Lysine HCL	0.13	–	0.07
Threonine	0.06	–	–
Limestone	1.29	1.15	1.12
Monocalcium phos	1.75	1.48	1.17
Vitamin/mineral premixa[1]	0.35	0.35	0.35
Calculated nutrient composition			
Crude protein (%)	23.40	22.80	19.20
Poultry ME (kcal kg^{-1})	3,050	3,151	3,200
Calcium (%)	0.95	0.85	0.75
Available phosphorus (%)	0.50	0.44	0.36
Methionine (%)	0.66	0.59	0.53
Methionine + Cysteine (%)	1.04	0.97	0.86
Lysine (%)	1.42	1.29	1.09
Threonine (%)	0.97	0.89	0.74
Na (%)	0.22	0.20	0.19

[1]Provided per kilogram of diet: vitamin A, 13,233 IU; vitamin D3, 6636 IU; vitamin E, 44.1 IU; vitamin K, 4.5 mg; thiamine, 2.21 mg; riboflavin, 6.6 mg; pantothenic acid, 24.3 mg; niacin, 88.2 mg; pyridoxine, 3.31 mg; folic acid, 1.10 mg; biotin, 0.33 mg; vitamin B12, 24.8 µg; choline, 669.8 mg; iron from ferrous sulfate, 50.1 mg; copper from copper sulfate, 7.7 mg; manganese from manganese oxide, 125.1 mg; zinc from zinc oxide, 125.1 mg; iodine from ethylene diamine dihydroidide, 2.10 mg; selenium from sodium selenite, 0.30.

(30 mg/kg BW, iv; Sigma-Aldrich, MO) within 2 min of removal from its pen. After sedation, BW was weighted, and blood sample (10 mL) was collected by cardiac puncture. The blood samples were centrifuged at 700× *g* for 15 min at 4°C. Plasma samples were kept at −80 °C until further analyses.

The sampled birds were immediately euthanized after blood collection via cervical dislocation, and liver samples (approximately 1 cm^3) were collected from the same location among the sampled birds and the cecal tonsils of the right cecum were dissected. Cecal content of the left cecum were also collected at both day 14 (before HS) and 43 (the end

Table 2. Behavioral ethogram

Behavior	Definition
Panting	Bird is breathing hard and quickly, constantly shallow respiration with beak open.
Wing spreading	A space can be seen between a bird's wings (both wings) and body.
Squat close to the ground	Lie, at least, the ventral region of a bird's body in contact with ground.
Drinking	Bird's beak is in contact with drinker.
Eating	Bird's head is located inside feeder.
Foraging	Bird is searching food from outside feeder.
Standing	Both feet but without other body part are in contact with the floor.
Sitting	Most of the ventral region of the bird's body in contact with the floor. No space is visible between the bird and floor.
Walking	Bird is in the process of taking at least 2 steps including scratching the litter.
Sleeping[1]	Bird has eyes closed and the head is tucked into the feathers above the wing base or even behind the wing.
Dozing[2]	Bird has eyes closed and appears to be awake with the neck is more or less withdrawn

Adapted from Blokhuis, 1984; De Queiroz et al., 2014; Mahmoud et al., 2015; McDougald & McQuistion, 1980; Santos et al., 2015.

[1]Sleeping: The head is tucked into the feathers above the wing base or even behind the wing. Feathers are slightly fluffed and sometimes the wings are drooping. It can be performed in a sitting as well as a standing position. While standing, a slight crouching posture is shown. The tail is down.

[2]Dozing consisting of 2 stages: 1) The neck is more or less withdrawn. The head is moving regularly and the eyes are open. The tail is slightly down. 2) The neck is withdrawn. The head is motionless and sometimes drooped, the eyes are closed or are slowly opened and closed. Feathers are slightly fluffed and sometimes the wings are drooping and the tail is down. Dozing can be performed in a sitting as well as a standing position (Blokhuis, 1984; De Queiroz et al., 2014).

of this study). All the samples were snap frozen on dry ice and then stored at −80°C until analysis.

Sample Analysis

Body weight of birds was measured at day 14 and 43. Average daily gain (ADG) was: (BW at day 43 – BW at day 14)/29 d. Average daily feed intake (ADI) was recorded weekly, started from day 15 (the beginning of week 3): (Daily total grams of feed per pen – daily grams of feed wasted and leftover per pen)/number of birds per pen. Feed conversion ratio (FCR) was: ADI/ADG.

Blood smear (2 smear slides per bird) was stained with Wright's stain (Walberg, 2001). Heterophils and lymphocytes were differentiated and quantified (100 white cells per slide, total 200 cells per bird) through a light microscopy at 2,000× (Walberg, 2001), and heterophil to lymphocyte (H/L) ratios were calculated (Cheng et al., 2001).

Total RNA of each liver sample was prepared by using RNeasy Mini Kits (Qiagen Inc., Valencia, CA) and cDNA was synthesized by using the Maxima First Strand cDNA Synthesis Kits (Applied Biosystems, Foster City, CA). Chicken *Hsp70* primers were designed and synthesized by the company and RT-PCR was performed by using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the reference gene. Standards and samples were measured in duplicates with a standard deviation of less than 2.0. Data of the HSP70 gene were expressed as relative quantification (RQ), compared the Ct value of targeted gene to reference control gene using the formula: $2^{\Delta\Delta CT}$ and a coefficient of variation less than 2.0%.

Interleukin (IL)-6, IL-10, and HSP70 in the livers as well as immunoglobulins (Ig)A and IgY in the cecal tonsils were measured by using the commercially available ELISA kits (Neo Biolab, Cambridge, MA; Catalog No. CKI0013, CKI0030, CKH0029, CKI0028. Abcam, Cambridge, UK; Catalog No. ab157693) following each company's protocol. Protein samples were analyzed in duplicate with optical density (OD) at 450 nm. Standard curve was constructed by using the Gen5 (BioTek Inc., Winooski, VT). The protein concentrations were obtained by tracking the corresponding OD values with the standard curve. Targeted proteins were reported as mg/mL or ng/mg.

Each cecal content was diluted at 1:10 with PBS and heated on water-bath at 80°C for 10 min. The cecal solution was then serially diluted in PBS, and 10 µL of each serial dilution was spotted on to tryptic soya agar (BD Difco, Franklin Lakes, NJ) in triplicate wells (Multiple Well Plate size 12 wells, Corning CellBIND), then incubated at 37°C for 12 h as previously described (Barbosa et al., 2005). After incubation, bacteria were enumerated from each countable dilution and averaged; the results were reported as Log_{10} CFU.

Statistical Analyses

The experimental design was an incomplete block design with temperature and diet as fixed effects and pens arrayed within temperature by diet as the random effect. Data were analyzed with a mixed model using the MIXED procedure of SAS 9.2 (SAS Institute Inc., Cary, NC). The pen was the experimental unit. The data were normally distributed and reported as least square means ± SEM. The SLICE option was used to examine the effect of one independent variable within a level of the second independent variable. The Benjamini-Hochberg method was used to control the false discovery rate (FDR) due to multiple comparisons. The FDR was set at 0.05, and all *P*-values shown have been adjusted for multiple comparisons accordingly (Benjamini and Hochberg, 1995). Statistical significance was set at adjusted (adj) $P \leq 0.05$ and with a trend at $0.05 < P_{adj} \leq 0.10$.

RESULTS

Behavioral Changes

There was no temperature × diet interaction on heat-induced behaviors. However, HS birds spent more time in panting, wing spreading, and squatting close to the ground than TN birds ($P < 0.01$) regardless of diet treatment (Table 3). Between HS birds, the HS-RD birds spent more time in panting, wing spreading, and squatting close to the ground (Table 3) than the HS-PD birds ($P = 0.01$). However, there were no diet treatment effects between the TN-RD and TN-PD birds ($P > 0.05$). There was also no temperature × diet interaction on posture behaviors. Under the high temperature, standing was increased ($P < 0.05$), whereas both sitting ($P = 0.03$) and sleeping ($P = 0.04$) were decreased in the HS-PD birds compared with the HS-RD birds (Table 4). Between RD groups, the HS-RD birds spent less time in standing and walking ($P = 0.04$, respectively) but more time in sleeping ($P < 0.01$), sitting ($P < 0.01$), and dozing ($P < 0.05$) than TN-RD birds (Table 4). There were

Table 3. The effect of heat stress and probiotic on broilers' cooling behaviors[1]

Treatment[2]	Panting, %	Wing spreading, %	Squatting close to the ground, %
TN-RD	11.76 ± 2.21^{B}	6.87 ± 2.59^{C}	3.89 ± 1.55^{C}
TN-PD	10.99 ± 2.19^{B}	6.76 ± 2.60^{C}	3.64 ± 1.44^{C}
HS-RD	97.04 ± 3.16^{Aa}	82.58 ± 3.74^{A}	60.44 ± 6.18^{A}
HS-PD	82.16 ± 2.80^{Ab}	58.96 ± 2.20^{B}	35.45 ± 5.24^{B}
Adjusted *P*-values			
Diet	0.007	0.006	0.009
Temperature	<0.0001	<0.0001	<0.0001
Diet × Temperature	0.121	0.093	0.354
TN-RD vs. TN-PD	0.896	0.951	0.984
HS-RD vs. HS-PD	0.011	0.008	0.012
TN-RD vs. HS-RD	<0.0001	<0.0001	<0.0001
TN-PD vs. HS-PD	<0.0001	<0.0001	<0.0001

$^{a–b}$Means within a column with different superscripts are different at $P < 0.05$.

$^{A–C}$Means within a column with different superscripts are different at $P < 0.01$.

[1]All means reported as means ± SEM created by mixed model analysis, $n = 12$/treatment.

[2]HS = heat stress condition; PD = a regular diet mixed with probiotic; RD = a regular diet; TN = thermoneutral condition.

no differences in overall posture behaviors between the TN-PD and HS-PD birds ($P > 0.05$).

Performance and Behavioral Measures Associated With Feeding

Overall, final BW, ADG, AFI, and FCR were improved in PD groups as compared to RD groups regardless of ambient temperature ($P < 0.01$, Table 5) but there were no temperature × diet interactions. Between RD groups, the HS-RD birds spent less time in eating and foraging ($P < 0.05$, respectively, Table 5), resulting in tendency of reduced AFI compared with the TN-RD birds, while there were no temperature effects on ADG and feeding-associated behaviors between the HS-PD and TN-PD chickens ($P > 0.05$). There was also no temperature × diet interaction on drinking. However, between RD groups, drinking was greater in the HS-RD birds than the TN-RD birds (Table 5, $P = 0.05$). There were no differences in the drinking behavior between TN-PD and HS-PD ($P > 0.05$). However, under high temperature, the HS-RD birds spent more time drinking than the HS-PD birds ($P < 0.05$).

Changes of Cytokines and HSP70 in the Liver

In the liver, there was no temperature × diet interaction on the concentrations of IL-6 and IL-10, and HSP70 concentration and mRNA expression. However, compared to its corresponding partner of TN groups, the HS birds had greater concentrations of IL-6, IL-10, concomitant with greater concentrations and mRNA expression of HSP70 regardless of diet treatment (HS-RD vs. TN-RD and HS-PD

Table 4. The effect of heat stress and probiotic on broilers' posture and locomotion[1]

Treatment[2]	Sitting, %	Standing, %	Walking, %	Sleeping, %	Dozing, %
TN-RD	14.87 ± 1.45^{B}	19.78 ± 1.72^{a}	15.67 ± 1.40^{a}	21.79 ± 1.73^{B}	3.85 ± 0.78^{b}
TN-PD	14.05 ± 1.45^{B}	21.74 ± 1.74^{a}	16.72 ± 1.40^{a}	20.09 ± 1.73^{B}	3.03 ± 0.78^{b}
HS-RD	21.83 ± 1.65^{Aa}	13.81 ± 1.97^{b}	8.76 ± 1.33^{b}	30.78 ± 1.86^{Aa}	7.20 ± 1.00^{a}
HS-PD	15.82 ± 1.5^{ABb}	18.89 ± 1.70^{a}	12.59 ± 1.37^{ab}	24.68 ± 1.80^{ABb}	5.54 ± 0.79^{ab}
Diet	0.018	0.024	0.057	0.025	0.632
Temperature	0.001	0.012	0.011	0.004	0.042
Diet × Temperature	0.975	0.946	0.993	0.723	0.889
TN-RD vs. TN-PD	0.773	0.956	0.996	0.879	0.923
HS-RD vs. HS-PD	0.027	0.048	0.124	0.036	0.656
TN-RD vs. HS-RD	0.002	0.037	0.037	0.008	0.047
TN-PD vs. HS-PD	0.506	0.885	0.107	0.566	0.753

$^{a–b}$Means within a column with different superscripts are different at $P < 0.05$.

$^{A–B}$Means within a column with different superscripts are different at $P < 0.01$.

[1]All means reported as means ± SEM created by mixed model analysis, $n = 12$/treatment.

[2]HS = heat stress condition; PD = a regular diet mixed with probiotic; RD = a regular diet; TN = thermoneutral condition.

Table 5. Effect of probiotic on broilers' BW, ADG, AFI, FCR, and eating, drinking, foraging behaviors under different temperatures[1]

Treatment[2]	BW, g		ADG, g/day	AFI, g/day	FCR	Eating, %	Drinking, %	Foraging, %
	Day 14	Day 43						
TN-RD	238.10 ± 10.56	2,295.98 ± 31.87^{B}	71.17 ± 0.96^{B}	133.92 ± 2.81	1.88 ± 0.04^{C}	36.89 ± 1.92^{a}	7.62 ± 0.76^{b}	3.86 ± 0.31^{a}
TN-PD	247.99 ± 10.55	2,493.39 ± 33.80^{A}	77.43 ± 1.05^{A}	139.01 ± 2.86	1.80 ± 0.04^{D}	39.56 ± 1.89^{a}	6.72 ± 0.84^{b}	3.90 ± 0.32^{a}
HS-RD	236.44 ± 9.74	1,921.09 ± 26.18^{D}	58.09 ± 0.85^{D}	124.90 ± 2.33	2.15 ± 0.05^{A}	28.32 ± 2.41^{b}	15.02 ± 1.89^{a}	1.77 ± 0.32^{b}
HS-PD	247.35 ± 10.21	2,176.78 ± 28.28^{C}	66.53 ± 0.92^{C}	132.87 ± 2.30	2.00 ± 0.04^{B}	33.16 ± 2.05^{ab}	9.98 ± 1.88^{b}	3.30 ± 0.31^{a}
Adjusted *P*-values								
Diet	0.3458	<0.0001	<0.0001	0.0526	<0.0001	0.086	0.033	0.040
Temperature	0.9734	<0.0001	<0.0001	0.0378	<0.0001	0.046	0.036	0.026
Diet × Temperature	0.6735	0.3716	0.3645	0.7217	0.1121	0.873	0.893	0.984
TN-RD vs. TN-PD	0.3864	0.0003	<0.0001	0.2148	0.0077	0.297	0.781	0.996
HS-RD vs. HS-PD	0.3691	0.0001	<0.0001	0.0813	0.0035	0.097	0.049	0.046
TN-RD vs. HS-RD	0.9867	<0.0001	<0.0001	0.0727	0.0012	0.048	0.041	0.042
TN-PD vs. HS-PD	0.9984	<0.0001	<0.0001	0.1175	0.0383	0.672	0.359	0.792

[a–b]Means within a column with different superscripts are different at $P < 0.05$;

[A–D]Means within a column with different superscripts are different at $P < 0.01$.

[1]All means reported as means ± SEM created by mixed model analysis, n = 12/treatment.

[2]ADG = average daily body weight gain; AFI = average daily feed intake; FCR = feed conversion ratio; HS = heat stress condition; PD = a regular diet mixed with probiotic; RD = a regular diet; TN = thermoneutral condition.

vs. TN-PD; $P < 0.01$, respectively, Fig. 1a and b, Fig. 2a and b). Within HS groups, the HS-PD birds had lower concentration of IL-6, and lower concentration and mRNA expression of HSP70 ($P < 0.01$, respectively; Fig. 1a, Fig. 2a and b), whereas greater concentrations of IL-10 ($P < 0.01$; Fig. 1b) compared to the HS-RD birds. Within TN groups, IL-10 concentration was increased in the TN-PD birds compared to the TN-RD birds ($P < 0.05$; Fig. 1b).

Heterophil to Lymphocyte (H|L) Ratio and Changes of Antibodies in the Cecal Tonsils

There was no temperature × diet interaction on cecal IgA and IgY concentrations. However, concentrations of IgA and IgY were increased in the HS exposed birds regardless of diet treatment ($P < 0.01$, respectively; Fig. 3a and b). Within HS groups, the concentrations of IgA ($P < 0.01$; Fig. 3a) and IgY ($P < 0.01$; Fig. 3b) were lower in the HS-PD birds compared to the HS-RD birds. Between TN groups, IgA ($P < 0.01$) but not IgY ($P > 0.05$) concentrations were greater in the TN-PD birds.

There was no temperature × diet interaction on H/L ratio. However, greater H/L ratios were observed in the HS exposed birds regardless of diet treatment ($P < 0.01$; Fig. 4a). In addition, probiotic attenuated H/L ratios under both HS and TN conditions ($P < 0.01$). The levels of H/L ratios were in the following order: HS-RD (0.56) > HS-PD (0.43) > TN-RD (0.36) > TN-PD (0.27) ($P < 0.01$).

Spore Numbers in the Cecal Contents

The concentration of *B. subtilis* was analyzed in the feed at day 15 and 29 immediately before feeding, and 43 immediately end of the study, respectively. The results indicated that the targeted dose

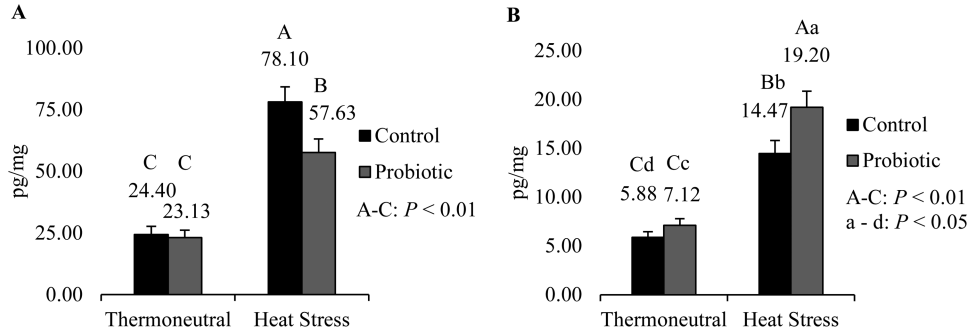

Figure 1. Hepatic IL-6 and IL-10 protein concentrations. Temperature and diet effects on hepatic IL-6 (A) and IL-10 (B) concentrations in broilers at 43 d of age. All means reported as means ± SEM (n = 12/treatment).

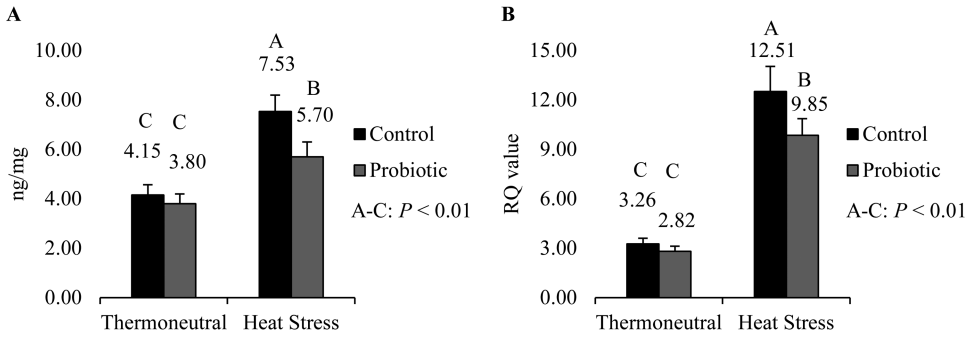

Figure 2. Hepatic HSP70 protein concentrations and mRNA expression. Temperature and diet effects on hepatic HSP70 protein concentrations (A) and mRNA levels (B) in broilers at 43 d of age. All means reported as means ± SEM (n = 12/treatment).

(1×10^6 CFU/g feed) was reached: Its concentrations were: regular diet (RD) vs. probiotic diet (PD) at 0.027 vs. 1.10×10^6 CFU/g feed (day 15); 0.0013 vs. 1.30×10^6 CFU/g feed (day 29), and 0.006 vs. 0.84×10^6 CFU/g (day 43).

Probiotic-fed birds, compared to RD-fed birds, had greater numbers of spore forming *Bacillus* in the cecal content at both day 14 and 43 ($P < 0.01$; Fig. 4b). Among PD birds, spore number increased from day 14 to 43 regardless of temperature condition ($P < 0.05$). There were no effects of temperature and temperature × diet interaction on spore numbers in the PD birds (i.e., TN-PD vs. HS-PD birds, $P > 0.05$). There were also no temperature and time differences in spore numbers in the RD broilers ($P > 0.05$).

DISCUSSION

The data from the current study revealed that the dietary *B. subtilis* supplementation improved broiler performance and feed conversion efficiency regardless of the ambient temperature. *B. subtilis* supplementation also alleviated inflammatory response and ameliorated behavioral health in broilers under HS.

In the current study, *B. subtilis* improved the final BW, ADG, and FCR of birds under both thermoneutral and heat stimulated conditions, which is consistent with the findings from a previous study that *B. subtilis* improves growth in broilers under HS (Roy et al., 2015). The current and previous findings could be related to *B. subtilis* inhibits bacterial pathogenic reproduction and promotes feed utilization (van Immerseel et al., 2006) via increased gut flora diversity with high incidence of beneficial lactic acid bacteria (Knarreborg et al., 2008). In addition, chronic HS reduces digestive enzyme activities in chickens (Lin et al., 2006), whereas *B. subtilis* accelerates the feed metabolization by improving the production of digestive enzymes in the small intestine (Chen et al., 2009). Probiotics in broilers lessen HS-caused intestinal damages with greater villus height and surface areas compared with the broilers exposed to HS only (Quinteiro-Filho et al., 2010).

When subjected to high ambient temperatures, both broilers and laying hens spend less time eating to diminish metabolic heat production (Syafwan et al., 2012), thereby, coping with HS or reducing HS caused damage (Mack et al., 2013). The current results indicated that feeding of probiotic, *B. subtilis*, increased the time of birds spent on foraging and tended to increase eating under HS. These changes could be attributed to improved body heat metabolism and improved appetite (Mack et al., 2013; Mahmoud et al., 2015). Probiotic-fed birds tended to have greater feed intake ($P = 0.08$) with

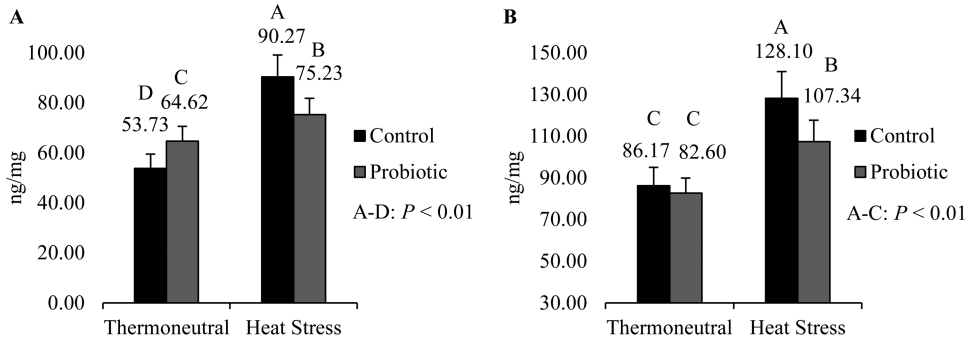

Figure 3. Cecum IgA and IgY concentrations. Temperature and diet effects on cecum IgA (A) and IgY (B) concentrations in broilers at day 43 of age. All means reported as means ± SEM (n = 12/treatment).

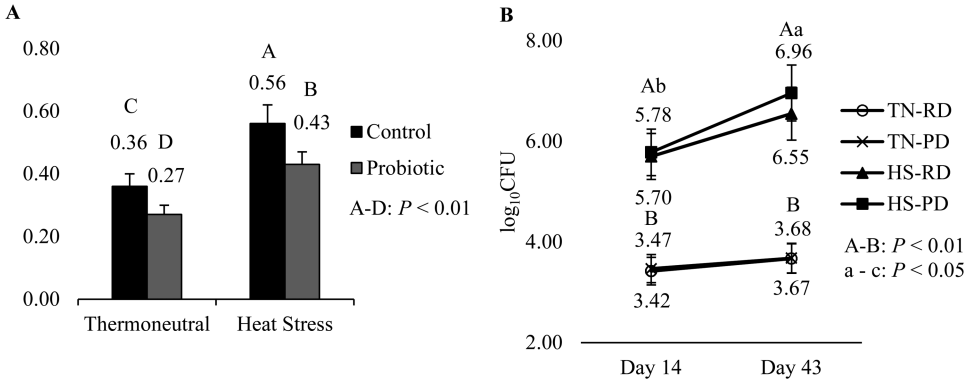

Figure 4. Heterophil to lymphocyte (H/L) ratio and cecal spore forming Bacillus number[1]. Temperature and diet effects on heterophil to lymphocyte (H/L) ratio (A) and cecal spore forming *Bacillus* number (B) in broilers at day 43 of age. All means reported as means ± SEM (n = 12/treatment). [1]HS = heat stress condition; PD = a regular diet mixed with probiotic; RD = a regular diet; TN = thermoneutral condition.

an improved FCR compared to the controls under HS. The mechanisms of probiotic improved foraging behavior were not examined in this study, but it could be similar to the ones reported previously (Neves et al., 2014). In general, chickens under chronic HS enhance the deposition of abdominal fat pad (Lu et al., 2007) due to altered lipid metabolism (He et al., 2015), but probiotic can reduce the fat accumulation to improve body cooling capability (Kalavathy et al., 2003). In addition, short chain fatty acids (SCFAs), one kind of fermentation products of gut probiotics, function increasing energy metabolism and preventing insulin resistance (Shen et al., 2013) to improve tolerance during HS (Rhoads et al., 2013).

High ambient temperature in birds causes heat-associated behaviors including panting, wing spreading, and squatting close to the ground, by which birds attempt to reduce or adapt heat stimulation. These behavioral changes usually happened before body core temperature increase and physiological changes. Panting, a method used for cooling in animals including poultry, is associated with water loss, which may lead to dehydration in chickens (Vanderhasselt et al., 2014), and dehydration may further result in decrease of enzyme activities (Tan et al., 2010). Another adverse effect of panting is decrease of partial pressure of carbon dioxide (Toyomizu et al., 2005). The imbalance of blood chemicals may lead to alkalosis (Etches et al., 2008) and reduce ionic calcium (Han et al., 2010). Consequently, chickens have to drink more water under HS for the body water replenishment (Gowe and Fairfull, 2008). Wing spreading increases radiation heat loss, however, too frequent spreading, as one of the reasons, may cause pale, dry, and exudative meat in broilers (Sandercock et al., 2001; Spurio et al., 2016). Squatting closely to ground increases heat conduction (Li et al., 2017), but also increases the frequency of foot-pad dermatitis (Part et al., 2016). In the current study, the behavioral reactions of HS birds were far greater than TN birds, but the HS-PD birds exhibited significantly less panting than the HS-RD birds, which is consistent with the less time spent drinking water in the PD birds compared to RD birds under high temperature. These results indicate that dietary *B. subtilis* supplement alleviates HS-induced panting and related dehydration in birds. Previous finding that probiotics reduce activation of the hypothalamic-pituitary-adrenal (HPA) axis may explain to the decrease of HS-induced panting in HS-PD birds (Mohammed et al., 2018). Cortisol, for example, inhibits cholinergic vasodilation (Mangos et al., 2000). As one of the mechanisms, the probiotic supplement may attenuate the HPA response, leading to vasodilatation, which concomitants with the redistribution of blood flow throughout the body (Fedde, 1998). Therefore, nonevaporative cooling efficiency in chickens via body surface is improved, and the needs of evaporative heat loss via panting are reduced (Strong et al., 2015). The hypothesis that probiotic ameliorate heat-related behavior via blunting HPA response will be examined in the future studies.

Additionally, the current results indicated that standing and walking were increased, whereas sitting, dozing, and sleeping were decreased in the probiotic-fed birds reared under HS. These behavioral alterations could attribute to the function of the probiotic in improving skeletal health and decreased stress via the microbiota-gut-brain and brain-bone axes (Santisteban et al., 2016). A previous study showed that probiotics enhance bone health of chickens under HS, as indicated by greater bone mineral contents in both the tibia and femur (Yan, 2016). In addition, the behavioral changes may relate to the probiotic preventing high blood

pH that is caused by HS-induced excessive panting (Etches et al., 2008); therefore, more blood calcium is available for skeletal bone ossification (Han et al., 2010). Similarly, Wideman (2016) reported that probiotics reduce pain, mobility, and lameness in overweight broilers.

In the liver, the lower IL-6 but greater IL-10 concentrations were found in the HS-PD birds compared to the HS-RD birds, which may reveal that HS-induced systemic inflammation was repressed in birds fed the probiotic. IL-6 is a proinflammatory cytokine that involves in the cellular immunity to mediate infection (Rajput and Li, 2012), and IL-10 is an anti-inflammatory cytokine that protects cells from oxidative stress (He et al., 2012). It has reported that *B. subtilis* B10 dietary supplementation increases IL-6 concentrations in the jejunum and ileum but has little effect on IL-10 release in *Sanhuang* broilers, a Chinese cross breed (Rajput et al., 2013). Fujiwara et al. (2009) also reported that *Tsukuba jidori* meat chickens fed *B. subtilis*-fermented soybean for either 53 d or 80 d had little effect on cytokine expression in the spleens. These results indicate that the effects of probiotics on a host's health may be affected by multiple factors, such as animal species, age and experience; types of stressor and its duration; and environmental condition.

Compared to the HS-RD group, the probiotic, *B. subtilis*, decreased HSP70 concentration and mRNA expression in the livers of the HS-PD birds. Under high temperature, when a chicken attempts to maintain its thermal homeostasis, it overproduces reactive oxygen species (ROS) in multiple organs, including the liver, brain, and heart (Zhen et al., 2006; Yu et al., 2008). Heat shock proteins including HSP70 are chaperone proteins which can be induced by ROS to protect tissue cells from the oxidative injuries (Lin et al., 2006). Numerous studies have shown that HSP70 is inducible in broilers and laying hens when exposed to HS, and the HSP70 expression is positively correlated with the degree of heat stimulation (Felver-Gant et al., 2012). The mechanism resulting in low levels of both liver HSP70 protein and mRNA expression in probiotic-fed birds is not clear but could be similar to previously reported that probiotics alleviate stress reactions in birds by blunting the activity of the HPA axis (Sohail et al., 2010, 2012). In addition, the probiotic may reduce heat stimulation by decreasing the trigger point for HSP70 synthesis and release in the HS-PD birds.

However, at present, cellular mechanisms that hereditarily regulate productivity between the selected lines are unclear. Although identification of mechanisms that underlie the inhibitory effects of dopamine (DA) on productivity in present lines is unclear, previous experimental findings suggest that the regulation might be related to genetic selection induced changes in physiological functions of the neuroendocrine system, including the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-gonadal (HPG) axes. For instance, endogenous DA secreted in the hypothalamus exhibited catecholamine's tonic inhibition of luteinizing hormone-releasing hormone (LHRH) release (Contijoch et al., 1992) and suppression LH secretion (Martin et al., 1981).

In the current study, broilers exposed to high temperatures had high levels of natural antibodies, antibodies without any previous antigen exposure, in the cecal tonsils as a response to heat-stimulated intestinal inflammation. Similar to these findings, previous studies have shown that HS induce local inflammation, resulting in intestinal microstructural injury and pathogenic infection (Quinteiro-Filho et al., 2010, 2012), leading to high natural antibodies (Regnier et al., 1980; Strong et al., 2015). These HS effects were reduced in the HS-PD birds which had lower levels of both IgA and IgY compared to HS-RD. These results indicate that the probiotic, *B. subtilis*, may reduce HS caused gut inflammation and related damage of intestinal integrity and function. IgA plays an important defensive function in preventing pathogens attaching to the mucosal cells and inhibiting bacterial colonization and viral infection (Renegar et al., 2004; Pabst et al., 2016). In the current study, compared to TN-RD birds, TN-PD birds had greater levels of IgA in the cecal tonsils. Similar to the present findings, Rajput et al. (2013) reported that a greater number of IgA-positive cells were observed in the jejunum of chickens fed *B. subtilis* under TN conditions. In the current study, IgA levels were further increased in both HS-RD and HS-PD birds, which could be caused by HS-induced local (the gut) and systemic inflammation, resulting in overexpression of cytokines, such as IL-6 and IL-10. These changes further promote the differentiation of B cells to produce IgA (Suzuki et al., 2010). However, HS-induced the change of IgA level was reduced in HS-PD birds compared to HS-RD birds (116% vs. 168% increase from each relative control).

Heterophil/lymphocyte ratio is another immune and stress indicator of chickens (Lentfer et al., 2015). In this study, lower H/L ratios were found in probiotic-fed birds under both NT (TN-PD > TN-RD) and HS (HS-PD > HS-RD) condition.

The probiotic-reduced H/L ratios could be caused by reduced heterophil cell number. Heterophil cells in birds, similar to neutrophils in mammals, as phagocytes, actively participate in inflammatory damage (Maxwell and Robertson, 1998). The current results may suggest that probiotic-fed birds have a greater adaptive capability by reducing HS-induced inflammation than the control birds. Probiotic supplementation decreased H/L ratio has been found in social stressed broilers (Cengiz et al., 2015) and feed withdrawal molted laying hens (Dastar et al., 2016)

Over the course of the experiment, the numbers of cecal spore forming bacteria were increased in the PD groups regardless of the ambient temperature, which was linearly related to the duration of treatment. The results indicate *B. subtilis* is able to survive and copes with the hosts' intestinal microenvironment.

CONCLUSIONS

Dietary *B. subtilis* supplementation improves broiler production performance, behaviors, and immunity under both thermoneutral and heat conditions. The data suggest that the dietary probiotic-fed birds are able to cope with HS more effectively via microbiota-modulated immunity.

Conflict of interest statement. None declared.

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