Effect of a synbiotic supplement as an antibiotic alternative on broiler skeletal, physiological, and oxidative parameters under heat stress

J. Y. Hu,* A. A. Mohammed,*† G. R. Murugesan,†,§ and H. W. Cheng ⊗‡,†

*Department of Animal Sciences, Purdue University, West Lafayette IN 47907, USA; †Department of Animal and Poultry Behavior and Management, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt; ‡BIOMIN America Inc., Overland Park, KS 66210, USA; §BIOMIN Holding GmbH, Getzersdorf, Austria; and †USDA-Agricultural Research Service, Livestock Behavior Research Unit, West Lafayette, IN 47907, USA

ABSTRACT The aim of this study was to examine if synbiotics can function as alternatives to antibiotics in broiler production under heat stress (HS). Day-old broiler chicks (528 birds) were randomly placed in floor pens within 2 identical temperature-controlled rooms (11 birds/pen and 24 pens/room). The pens of each room were evenly divided among 3 treatments (n = 8): basal diet (CON), the basal diet mixed with 50 ppm of bacitracin methylene disalicylate (BMD) or a synbiotic (50 ppm of PoultryStar meUS, SYN). From d 15, room 2 was under thermoneutral (TN) conditions (TN-CON, TN-BMD, and TN-SYN), while HS was applied to room 1 at 32°C for 9 hrs/d (0800 to 1700) (HS-CON, HS-BMD, and HS-SYN). Treatment effects on footpad dermatitis and gait score were measured on 5 birds/pen, and latency to lie (LTL) test was measured on 2 birds/pen at d 27 and d 41; and 1 broiler/pen was sampled on d 28 and d 42, respectively. Body, liver, and spleen weight were determined. Plasma levels of interleukins (IL), heat shock protein 70, immunoglobulin (Ig)Y, liver superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzyme activities were examined. Heat stress suppressed BW and IgY concentrations on both d 28 and d 42, while suppressed plasma IL-6 concentrations, SOD activities, and LTL duration on d 28 only (P < 0.05). Among all treatments, SYN birds had the best foot and skeletal health scores on both d 27 and d 41 (P < 0.05). On d 42, SYN increased BW, and TN-SYN birds had higher relative spleen weight than both TN-BMD and TN-CON birds (P < 0.05). Antibiotic BMD increased BW (P < 0.05) but decreased SOD activities (P < 0.05) on d 42. These results indicate that the SYN supplementation decreases HS negative effect on broilers by improving BW, foot, and skeletal health, while BMD improves BW but also increases oxidative stress in broilers. The data suggest that synbiotic supplement may function as an alternative to antibiotics in broiler production during summer seasons, especially in the tropical and subtropical regions.

Key words: broiler, heat stress, synbiotic, antibiotic, welfare

INTRODUCTION

Heat stress (HS) is one of the top welfare issues affecting global poultry production. Chickens’ thermoneutral zone is relatively narrow (16–25°C) and is vulnerable to HS due to the lack of sweat glands and feather coverage (Liu et al., 2020). Ambient temperature elevated above the upper limit of thermoneutral zone negatively affects production performance and skeletal health, compromises gut integrity, and related immune function, in severe cases, leading to morbidity and mortality (Lara and Rostagno, 2013). Excessive heat reduces calcium (Ca) absorption and decreases the metabolic conversion of vitamin D3 (cholecalciferol) to its biologically active form, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) (Petruk and Korver, 2004), by which it reduces bone density (lowing Ca storage in bone), leading to poor skeletal health (Christakos et al., 2010). Femoral lesions have been found to be more severe in broilers under HS, which accelerates the development of lameness (Wideman and Pevzner, 2012). In addition, HS damages the integrity of intestinal tight junctions, increasing the permeability of pathogenic bacteria and related toxins from the gut to the systemic circulation (leaky gut), consequently elevating the incidence of bone diseases such as lameness, resulting in poor walking ability and footpad dermatitis (FPD) (Wideman, 2016).
Heat exposure suppresses both cell mediated and humoral immune responses, leading to alterations of cytokines and immunoglobulin syntheses. Under HS, there is an increase in the production of reactive oxygen species (ROS), causing oxidative stress and related inflammatory reactions in injured tissues (Montilla et al., 2014; Slimen et al., 2014). Excessive amounts of ROS enhance metabolic disorders of lipids, proteins, and nucleic acids, resulting in heat-induced oxidative damage by suppressing antioxidative enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPx), ultimately causing cell death (Slimen et al., 2014; Akbarian et al., 2016; Ghosh et al., 2018). The synthesis of heat shock proteins (HSPs) is associated with cytoprotective effects against HS, which prevents protein improper folding or damage and inhibits apoptosis of cells (Richter et al., 2010).

Subtherapeutic antibiotics, such as bacitracin methylene disalicylate (BMD), have been used as feed additives to prevent inflammation and infectious diseases and to promote growth performance in animal production since 1920s (Engberg et al., 2000; Castanon, 2007). One of the active models of antibiotics is to directly inhibit certain intestinal pathogens for reducing inflammation (Shin et al., 2020; Strzepa et al., 2017). However, use of antibiotics in animal husbandry has caused public concerns about drug residues in meat products and the development of antibiotic-resistant bacteria (Kirchhelle et al., 2020; Nadeem et al., 2020). Many countries have already taken actions to control antibiotic usage, and the use of antibiotics for growth promotion (AGPs) has been banned in European Union since 2006 (Millet and Maertens, 2011). In 2012, the United States Food and Drug Administration (FDA) issued a proposal that requires veterinarians’ oversight for using antibiotics in livestock to limit preventive use of antibiotics (Kuehn, 2014). Eliminating antibiotics in poultry production with no reliable alternatives has raised considerable consequences, such as comprised production performance and increased incidence of infectious diseases and associated mortality (Cervantes, 2015). Restoration of disrupted gut microbiota and impacted intestinal homeostasis without medical intervention has become a critical issue in the poultry industry. Several alternatives of non-therapeutic antibiotics have been tested, where prebiotics and probiotics have been considered for replacing antibiotics as growth promoters and prevention of diseases in poultry productions (Redweik et al., 2020). A synbiotic is defined as “a mixture of probiotics and prebiotics that aids the host by improving the survival and activity of beneficial microorganisms in the gut” (Gyawali et al., 2019). The beneficial effects of supplementing synbiotics include improvement of growth performance, gut integrity, and immune function as well as inhibition of colonization of pathogens in broilers with and without stress challenges (Awad et al., 2009; Hassanpour et al., 2013; Min et al., 2016; Chen et al., 2018; Yan et al., 2019). Currently, there are limited studies comparing the effects of synbiotics and antibiotics on growth and skeletal health of broilers under hot ambient conditions. Particularly, climate change in the recent decades has resulted in more hot days with more frequent and unexpected heat waves. Therefore, the objective of this study was to determine if effects of synbiotics on production, skeletal health, immune response, and oxidative status are comparable with antibiotics in broilers subjected to HS. We hypothesize that synbiotics would perform at the levels similar to antibiotics in HS broilers due to their multiple beneficial effects on gut health and immune function.

**MATERIALS AND METHODS**

**Birds and Treatment**

Day-old Ross 708 male broiler chicks (528 birds) were obtained from a commercial hatchery (Townline Poultry Farm, Zeeland, MI). The chicks were weighed in a group of 11 birds each, then randomly assigned to floor pens (0.17 m²/bird) with similar average BW within 2 identical temperature- and lighting-controlled rooms (24 pens/room). Birds were reared for 42 d. The pens of each room were divided among 3 dietary treatments (n = 8): basal diets (CON), the basal diets mixed with antibiotic (BMD, 50 ppm, bacitracin methylene disalicylate, BMD50, Zoetis, Kalamazoo, MI) or a synbiotic (SYN, 50 ppm, PoultryStar meUS, BIOMIN America Inc., Overland Park, KS; Table 1). The SYN contains a prebiotic, fructo-oligosaccharides, and a probiotic mixture of 4 microbial strains, *Bifidobacterium animalis*, *Pediooccus acidilactici*, *Enterococcus faecium*, and *Lactobacillus reuteri*. The level of the SYN was recommended by the company (BIOMIN America Inc., San Antonio, TX) and tested in our previous studies (Yan et al., 2019). The antibiotic dose was adapted from the previous studies conducted in broilers (Koltes et al., 2017; de Oliveira et al., 2019; Zoetis, 2020). One room was maintained under thermoneutral condition (TN, 24 ± 2°C); while HS was applied to another room (32°C for 9 h, 0800–1700) from d 15 to d 42. There were 6 treatments defined as: TN-CON, TN-BMD, TN-SYN, HS-CON, HS-BMD, and HS-SYN, respectively. The bird management was performed according to the guidelines of Ross broilers (Aviagen, 2018). Feed and water were provided with free access. The diets were mixed at the Purdue University feed mill (West Lafayette, IN) using a step-increase procedure (Mohammed et al., 2018). Room temperature and humidity were monitored by data loggers (HOBO, Onset Computer Corporation, Bourne, MA) fixed 30 cm above the litter surface. All procedures of animal care used in this experiment were approved by the Purdue Animal Use and Care Committee (PACUC Number: 1712001657).

**Physiological Sampling**

On d 28 and 42, 1 bird per pen was used for sample collection, respectively (8 birds/treatment/time point). To balance sampling effects on the measured parameters, the sampled birds of each treatment were taken by
repeating the cycle of the TN-CON, TN-BMD, TN-SYN, HS-CON, HS-BMD, and HS-SYN until the end. Each sampled bird was weighed and sedated with sodium pentobarbital (30 mg of pentobarbital/kg of BW) injected into the brachial vein; then 10 mL blood was collected into an EDTA (Ethylenediaminetetraacetic acid) coated tube through cardiac puncture. The birds were euthanized via cervical dislocation immediately after blood collection. Whole liver and spleen were weighed and reported as both absolute and relative organ weight (absolute organ weight/body weight). Approximately 1 cm² sample of each liver were collected from the same location for oxidative status analysis. Blood samples were centrifuged at $700 \times g$ for 20 min at $4^\circ C$ for plasma collection. Plasma and liver tissue samples were stored at $-80^\circ C$ until further analysis.  

**Plasma Cytokines and Immunoglobulin Y**

Plasma levels of interleukin (IL)-2, IL-6, IL-10, HSP 70 (Catalog #: MBS005165, MBS037319, MBS007312, MBS017726, MyBioSource, San Diego, CA), and immunoglobulin (Ig) Y (Catalog #: E33-104, Bethyl, Montgomery, TX) were measured using the commercially available ELISA kits following the individual manufacturer’s protocols. The optical density of each sample was read at 440 nm and 340 nm, respectively, using a microplate spectrophotometer (BioTek). All samples were measured in duplicates with CV ≤15%.

**Liver Superoxide Dismutase Enzyme and Glutathione Peroxidase Enzyme Activities**

The liver SOD (ESOD-100, BioAssay Systems, Hayward, CA) and GPx (EGPX-100, BioAssay Systems) enzyme activities were measured using commercially available kits following the corresponding manufacturer’s protocols. The optical density of SOD and GPx was read at 440 nm and 340 nm, respectively, using a microplate spectrophotometer (BioTek). All samples were measured in duplicates with CV ≤15%.

**Gait Score, Footpad Dermatitis, and Latency to Lie**

Gait Score (GS), FPD, and LTL were tested on d 27 and 41, respectively. Gait score was determined from the marked 5 birds (labeled with leg bands) per pen (40 birds/treatment: 5 birds/pen £ 8 pens/treatment) using a 3-point gait scoring system adopted from Webster et al. (2008): score 0, birds with no impairment of walking ability; score 1, birds with obvious impairment but still ambulatory; and score 2, birds with severe impairment and not able to walk. The numbers collected from the 5 birds were averaged for statistical analysis.

Footpad dermatitis was scored from the left foot of the same 5 birds per pen using a 0 to 2 scale, 0 indicates no blemish or discoloration of foot pad, 2 indicates severe lesion with black coloration, and 1 intermediate. The numbers collected from the 5 birds were averaged for statistical analysis (Kjaer et al., 2006).

The LTL test was performed by modified the protocol published previously (Webster et al., 2008). Briefly, birds were made to stand in a container (size 45 $\times$ 40 $\times$ 30 cm) with shallow warm water inside the testing room. The time it took for the bird to first attempt to sit down was recorded. The test was terminated after 600 s if the bird was still standing. Two birds per pen were randomly selected for the testing, and the
results were averaged for statistical analysis (16 birds/treatment). To avoid reuse of the birds in the following test and sample collection, the tested bird was marked for identification.

**Statistical Analysis**

A 3 × 2 factorial design was used in the study, and the main factors were diet (CON, BMD, and SYN) and room temperature (TN and HS). Each pen was considered as an experiment unit. Physiological data were subjected to two-way ANOVA using the MIXED method of SAS 9.4 software (SAS Institute Inc., Cary, NC). A one-way ANOVA was used to partition treatment difference under each environmental condition, Tukey-Kramer test was used to partition the difference among the treatments if there was significance. A two-way non-parametric analysis Aligned Ranks (ART) was used for LTL, FPD, and GS results. Transformation of data (Box-Cox or log transformation) was performed if the data were not normally distributed or not homoscedastic. Statistical trend was similar for both transformed and untransformed data, thus untransformed data were reported. The data were presented as least square means (LSMeans) ± standard error of the mean (SEM). Statistical difference was reported when \( P \leq 0.05 \), and a trend was reported when 0.05 < \( P \leq 0.10 \).

**RESULTS**

### Production Performance and Physical Parameters

On d 28, HS suppressed BW (\( P_{\text{temperature}} = 0.01 \)) and absolute liver weight (\( P_{\text{temperature}} = 0.05 \)) without dietary and temperature interaction effect (Table 2). No overall dietary influence was found in liver weight, but under TN condition, CON birds tended to have lower relative liver weights than SYN (\( P = 0.08 \)) and BMD (\( P = 0.08 \)) supplemented birds. Among HS treatments, HS-SYN birds had greater relative spleen weight than HS-CON (\( P = 0.01 \)) but not HS-BMD birds. Absolute

### Table 2. Effect of the dietary synbiotic inclusion on body and organ weights of 28-day-old and 42-day-old broilers under thermoneutral and heat stress conditions.

|                | Absolute liver weight (g) | Relative liver* | Absolute spleen weight (g) | Relative spleen* | BW (kg) |
|----------------|---------------------------|-----------------|-----------------------------|-----------------|--------|
| **28-d**       |                           |                 |                             |                 |        |
| TN-CON         | 28.83                     | 1.90X           | 1.19                        | 0.83            | 1.44   |
| TN-BMD         | 32.20                     | 2.30B           | 1.44                        | 1.04            | 1.39   |
| TN-SYN         | 29.76                     | 2.08X           | 1.26                        | 0.89            | 1.42   |
| HS-CON         | 28.91                     | 2.12            | 1.14                        | 0.84X           | 1.36   |
| HS-BMD         | 27.58                     | 2.11            | 1.29                        | 0.99XY          | 1.32   |
| HS-SYN         | 25.46                     | 2.08            | 1.34                        | 1.09X           | 1.22   |
| **Feed additives** |                        |                 |                             |                 |        |
| CON            | 28.87                     | 2.06B           | 1.16                        | 0.84X           | 1.40   |
| BMD            | 29.89                     | 2.21A           | 1.37                        | 1.02A           | 1.35   |
| SYN            | 27.61                     | 2.08B           | 1.30                        | 0.99B           | 1.32   |
| **Temperature** |                          |                 |                             |                 |        |
| TN             | 30.26D                    | 2.13            | 1.30                        | 0.92            | 1.42   |
| HS             | 27.32B                    | 2.10            | 1.26                        | 0.98            | 1.30B  |
| SEM            | 0.99                      | 0.05            | 0.06                        | 0.06            | 0.03   |
| **P-value**    |                           |                 |                             |                 |        |
| Diet           | 0.41                      | 0.08            | 0.18                        | 0.10            | 0.50   |
| Temperature    | 0.05                      | 0.74            | 0.58                        | 0.33            | 0.01   |
| Diet × temperature | 0.34                    | 0.17            | 0.47                        | 0.14            | 0.36   |
| **42-d**       |                           |                 |                             |                 |        |
| TN-CON         | 41.58X                    | 1.54            | 2.24XY                      | 0.84V           | 2.70V  |
| TN-BMD         | 54.26X                    | 1.90            | 2.11Y                      | 0.74V           | 2.86V  |
| TN-SYN         | 53.74X                    | 1.78            | 2.81A                      | 0.93V           | 3.00V  |
| HS-CON         | 43.87                     | 1.69            | 1.98                        | 0.76            | 2.59V  |
| HS-BMD         | 43.73                     | 2.23            | 0.96                        | 2.49V           |        |
| HS-SYN         | 46.85                     | 1.70            | 2.19                        | 0.79            | 2.75V  |
| **Feed additives** |                        |                 |                             |                 |        |
| CON            | 42.72B                    | 1.61            | 2.11                        | 0.80            | 2.65B  |
| BMD            | 49.00A                    | 1.83            | 2.17                        | 0.82            | 2.67B  |
| SYN            | 50.30A                    | 1.74            | 2.50                        | 0.86            | 2.67B  |
| **Temperature** |                          |                 |                             |                 |        |
| TN             | 49.86D                    | 1.74            | 2.39A                      | 0.84            | 2.85A  |
| HS             | 44.82B                    | 1.72            | 2.13B                      | 0.82            | 2.61B  |
| SEM            | 2.56                      | 0.11            | 0.19                        | 0.06            | 0.08   |
| **P-value**    |                           |                 |                             |                 |        |
| Diet           | 0.10                      | 0.17            | 0.17                        | 0.70            | 0.04   |
| Temperature    | 0.08                      | 0.83            | 0.08                        | 0.68            | 0.0003 |
| Diet × temperature | 0.17                    | 0.37            | 0.10                        | 0.02            | 0.20   |

*Least squares means within a column for the 3 dietary treatments under 2 temperature conditions lacking a common superscript differ (\( P \leq 0.05 \), \( n = 8 \) per treatment) and **a,b** least square means represents a trend difference (0.05 < \( P \leq 0.1 \)).

**Statistical trend was similar for both transformed and untransformed data, thus untransformed data were reported. The data were presented as least square means (LSMeans) ± standard error of the mean (SEM). Statistical difference was reported when \( P \leq 0.05 \), and a trend was reported when 0.05 < \( P \leq 0.1 \).**
liver and spleen weight were not different among the 6 groups (Table 2).

On d 42, SYN and BMD supplemented birds had increased BW ($P_{diet} = 0.04$) with a trend to have greater absolute liver weights ($P_{diet} = 0.10$) compared with CON birds, mostly due to the changes under TN condition. Heat stress suppressed BW ($P_{temperature} = 0.0003$) and had a trend to decrease absolute liver ($P_{temperature} = 0.08$) and spleen weights ($P_{temperature} = 0.08$). The beneficial effect of dietary supplements on reducing HS induced negative effects on production performance was only found in HS-SYN birds compared to HS-CON ($P = 0.04$). In addition, a diet by temperature interaction was determined in relative spleen weight ($P_{diet*temperature} = 0.02$) due to the effects of SYN under TN condition, in which TN-SYN birds had a higher relative spleen weight than both TN-BMD and TN-CON birds ($P < 0.05$).

**Physiological and Oxidation Parameters**

On d 28, birds exposed to HS had increased plasma IL-6 ($P_{temperature} = 0.05$) with decreased IgY ($P_{temperature} = 0.04$) concentrations than birds raised under TN condition (Table 3). There was a trend overall dietary effect on plasma IgY concentration ($P_{temperature} = 0.10$) that was driven primarily from birds raised under TN condition, where IgY concentrations were decreased in TN-CON birds compared to both TN-BMD ($P = 0.03$) and TN-SYN birds ($P = 0.03$), while no difference was found among diet treatments under HS. There was no diet ($P_{diet} > 0.05$) or temperature ($P_{temperature} > 0.05$) effect on IL-2 and IL-10 concentrations. Liver SOD enzyme activity tended to decrease with heat exposure ($P_{temperature} = 0.06$, Table 4) due to the effects mainly found in CON birds. The SOD enzyme activity in HS-CON birds was significantly reduced compared to TN-CON birds (1.02 vs. 0.65, $P = 0.01$); while there were no dietary supplement effects under both TN and HS ambient conditions ($P_{diet} > 0.05$). No dietary ($P_{diet} > 0.05$) or temperature ($P_{temperature} > 0.05$) effect was found on GPx and HSP70 concentrations.

On d 42, HS decreased the concentrations of plasma IgY ($P_{temperature} = 0.01$, Table 3), while no difference was found in measured cytokines ($P_{temperature} > 0.05$). In addition, there were no dietary effects on the measured

### Table 3. Effect of the dietary synbiotic inclusion on plasma cytokines and immunoglobulin Y of 28-day-old and 42-day-old broilers under thermoneutral and heat stress conditions.

|        | IL-2 (pg/mL) | IL-6 (pg/mL) | IL-10 (pg/mL) | IgY (mg/dL) |
|--------|--------------|--------------|---------------|-------------|
|        | 28-d         | 42-d         | 28-d          | 42-d        |
| TN-CON | 145.18       | 153.49       | 130.02        | 132.31      |
| TN-BMD | 162.37       | 150.86       | 117.38        | 126.29      |
| TN-SYN | 142.40       | 153.49       | 131.46        | 131.46      |
| HS-CON | 153.49       | 160.86       | 160.86        | 160.86      |
| HS-BMD | 160.86       | 153.49       | 160.86        | 160.86      |
| HS-SYN | 183.21       | 160.86       | 160.86        | 160.86      |
| Feed additives | | | | |
| CON    | 149.34       | 153.49       | 132.31        | 132.31      |
| BMD    | 161.61       | 160.86       | 150.86        | 150.86      |
| SYN    | 162.80       | 153.49       | 131.46        | 131.46      |
| Temperature | | | | |
| HS     | 132.31       | 132.31       | 132.31        | 132.31      |
| TN     | 126.29       | 126.29       | 126.29        | 126.29      |
| SEM    | 18.5         | 18.5         | 18.5          | 18.5        |

**P-value**

- Diet: 0.74
- Temperature: 0.29
- Diet x temperature: 0.48

**Table 3.** Effect of the dietary synbiotic inclusion on plasma cytokines and immunoglobulin Y of 28-day-old and 42-day-old broilers under thermoneutral and heat stress conditions.

**Abbreviations:** IL-2, Interleukin 2; IL-6, interleukin 6; IL-10, interleukin 10; IgY, immunoglobulin Y.

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**ANCOVA Results**

- Least squares means within a column for the 2 temperature conditions lacking a common superscript differ ($P \leq 0.05$, n = 8 per treatment), and
- Least square means represents a trend among treatments ($0.05 < P \leq 0.1$).
- Least square means represents a trend among treatments within 1 environmental condition ($0.05 < P \leq 0.1$).
- Abbreviations: IL-2, Interleukin 2; IL-6, interleukin 6; IL-10, interleukin 10; IgY, immunoglobulin Y.
cytokines and IgY levels in broilers ($P_{\text{diet}} > 0.05$). BMD additive decreased SOD activities in broilers compared to CON and SYN broilers under both TN and HS conditions ($P_{\text{diet}} = 0.04$, Table 4). SYN birds tended to have lower HSP70 than CON birds but not BMD birds ($P_{\text{diet}} = 0.08$), while the difference was mainly contributed from HS groups as HS-SYN birds tended to have lower HSP70 concentrations than HS-CON birds ($P = 0.06$, Table 4). GPx enzyme activity was not different among the 6 groups ($P > 0.05$, Table 4).

Latency to Lie, Footpad Dermatitis, and Gait Score

On d 27, main effects of both diet and temperature were significant for LTL test ($P_{\text{diet}} < 0.05$, $P_{\text{temperature}} = 0.05$) and effect of diet was significant for FPD and GS ($P_{\text{diet}} < 0.05$, Table 5).

Under TN condition, TN-SYN birds had lower levels of FPD and GS compared with TN-CON birds ($P < 0.05$). TN-SYN birds also had longer durations during LTL tests than TN-BMD birds ($P < 0.05$), while TN-BMD birds had similar levels of LTL duration, FPD, and GS as those of TN-CON birds ($P > 0.05$). Under HS condition, HS-SYN birds had longer LTL durations and lower GS than both HS-BMD and HS-CON birds ($P < 0.05$). HS-SYN birds as well as HS-BMD birds had fewer incidences of FPD than HS-CON birds ($P < 0.05$).

On d 41, there was no temperature effect on the measured parameters ($P_{\text{temperature}} > 0.05$, Table 5), while dietary effects on leg health were persisted. There was an overall diet effect ($P_{\text{diet}} = 0.02$) on LTL duration between SYN and BMD treatments but not CON treatment due to TN-SYN birds had a greater LTL duration compared with TN-BMD birds ($P < 0.05$). GPx enzyme activity was not different among the 6 groups ($P > 0.05$, Table 4).

### Table 4. Effect of the dietary synbiotic inclusion on heat shock protein 70 (HSP70) and oxidative status indicators of 28-day-old and 42-day-old broilers under thermoneutral and heat stress conditions.

|          | 28-d |          |          |
|----------|------|----------|----------|
|          | HSP70 (pg/mL) | GPX activity (U/L) | SOD activity (U/mL) |
|          |          |          |          |
| 28-d     |          |          |          |
| TN-CON   | 748.75  | 613.55   | 1.02     |
| TN-BMD   | 931.62  | 575.10   | 0.83     |
| TN-SYN   | 723.21  | 509.25   | 0.82     |
| HS-CON   | 743.54  | 620.09   | 0.65     |
| HS-BMD   | 740.74  | 502.09   | 0.78     |
| HS-SYN   | 777.18  | 591.87   | 0.73     |
| Feed additives | CON | 746.14  | 616.82   | 0.83     |
|          | BMD   | 836.18   | 538.59   | 0.80     |
|          | SYN   | 750.19   | 550.56   | 0.78     |
| Temperature | HS | 753.82  | 571.35   | 0.72$^{A}$ |
|          | TN   | 801.19   | 565.97   | 0.89$^{A}$ |
|          | SEM  | 109.65   | 50.53    | 0.09     |
| $P$-value | Diet | 0.70     | 0.27     | 0.70     |
|          | Temperature | 0.58   | 0.91     | 0.06     |
|          | Diet $\times$ temperature | 0.49  | 0.43     | 0.30     |
| 42-d     |          |          |          |
| TN-CON   | 936.61  | 642.64   | 0.86$^{a}$ |
| TN-BMD   | 766.10  | 575.49   | 0.58$^{a}$ |
| TN-SYN   | 853.28  | 587.64   | 0.72$^{a}$ |
| HS-CON   | 848.54$^{X}$ | 602.32   | 0.75$^{X}$ |
| HS-BMD   | 853.68$^{X}$ | 573.11   | 0.54$^{X}$ |
| HS-SYN   | 708.35$^{X}$ | 587.35   | 0.94$^{X}$ |
| Feed additives | CON | 892.57$^{A}$ | 622.48  | 0.81$^{a,b}$ |
|          | BMD   | 809.89$^{AB}$ | 574.30  | 0.56$^{b}$ |
|          | SYN   | 780.94$^{A}$ | 587.49  | 0.83$^{a}$ |
| Temperature | HS | 803.60  | 587.39   | 0.77     |
|          | TN   | 852.00   | 601.92   | 0.75     |
|          | SEM  | 114.76   | 34.29    | 0.12     |
| $P$-value | Diet | 0.08     | 0.49     | 0.04     |
|          | Temperature | 0.64  | 0.50     | 0.85     |
|          | Diet $\times$ temperature | 0.10  | 0.69     | 0.63     |

$^{a,b}$Least squares means within a column for the 3 dietary treatments lacking a common superscript differ ($P \leq 0.05$. n = 8 per treatment), and $^{A,B}$least square means represents a trend among treatments ($0.05 < P \leq 0.1$).

$^{a,b}$Least squares means within a column for the 1 temperature condition (HS or TN) and 3 dietary treatments ($P \leq 0.05$) and $^{X,Y}$least square means represents a trend among treatments within 1 environmental condition ($0.05 < P \leq 0.1$).

1 Abbreviations: GPX, glutathione peroxidase; HSP70, heat shock protein 70; SOD, superoxide dismutase.

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improved in TN-SYN birds ($P < 0.05$) but reduced in TN-BMD birds ($P < 0.05$). Under HS conditions, the GS level of HS-SYN birds was similar to that of HS-CON birds but was lower compared with HS-BMD birds ($P < 0.01$).

**DISCUSSION**

Heat stress is one of the detrimental factors that lead to impaired health and welfare in poultry species (Lara and Rostagno, 2013). In the current study, broilers exposed to HS had impaired growth performance compared to broilers raised under TN ambient conditions regardless of dietary treatments. Similar negative effects have been reported in multiple studies conducted in broilers under different HS severities (degree of temperature and its duration; Liu et al., 2020; Ruff et al., 2021). The outcomes from the current study indicate that the inclusions of SYN and BMD were able to improve broiler growth performance under the TN rearing conditions; while under the HS environments, the beneficial effects were only seen in SYN fed birds during the 42-d trial due to its functions on the antioxidative system and regulating HSP70 synthesis and immunity.

One of the strategies for using BMD in broiler production is to preserve gut integrity and homeostasis by targeting primarily gram-positive bacteria through inhibiting synthesis of bacterial proteins in the intestinal tract (Singh et al., 2008). However, long-term use of antibiotics could lead to disrupted beneficial bacteria causing undesired shift in the gut commensal microbiome composition, which adversely affects host health (Konstantinidis et al., 2020). Synbiotic supplements can improve intestinal integrity by providing beneficial bacteria, which enables the gastrointestinal tract to absorb nutrients more efficiently and releases various biochemical factors with antimicrobial, antioxidant, and immunomodulatory activities to prevent translocation of pathogens, and ultimately to improve animal growth performance (Huang et al., 2019; Shini et al., 2020). In the current study, however, BMD was only able to improve BW of broilers reared under TN conditions,
without heat exposure. Similar effect of dietary BMD was shown by Song et al. (2011), who reported that BMD does not counteract the negative effects of HS on growth performance in finishing pigs. The BMD effects could be related to increased villus height and crypt depth in the duodenum, resulting in a great surface for nutrient digestion and resorption, and through reducing harmful gut bacteria and related subclinical disorders. However, few published studies have documented the response of farm animals fed BMD in a HS environment. In addition, long-term use of BMD could cause imbalance of gut microbiota, clear out normal gut microflora, and promoting growth of resistant strains. Antimicrobial resistance has become a global threat to the one health approach (Aslam et al., 2018).

Broilers’ foot health was improved with the SYN inclusion in the current study, which could be related to SYN improving litter quality. Previous studies have shown that the SYN prevents growth of Campylocacter jejuni, a common pathogen leading to bacterial foodborne infection in broilers (Ghareeb et al., 2012). Campylobacter jejuni caused diarrhea (enteritis) in broilers, which not only affects growth performance but also leads to damage of the feet and legs due to standing and lying on wet litter (Humphrey et al., 2014). Wet litter is a major factor causing FPD, a thickness and discoloration with or without lesions of the footpad (Shepherd and Fairchild, 2010). The severity of FPD is positively correlated with Campylobacter infection in broilers (Colles et al., 2008). In the present study, broilers supplemented with the SYN had the best foot condition on both d 27 and d 41, which could directly result from the dryer litter with provision of beneficial bacteria against pathogens. Although the quality of litter was not examined in this study, previous studies have shown that the intensity of FPD is correlated with Campylobacter infection in broilers (Toppel et al., 2019) which is affected by diet nutrition with various supplements (Swiatkiewicz et al., 2017).

Broilers fed with the SYN were able to stand longer during LTL test (a common indicator of leg strength), indicating that SYN fed birds had a stronger leg musculoskeletal system. It has been reported previously that broilers fed with the SYN product (Wideman et al., 2012) have a lower incidence of bacterial chondronecrosis with osteomyelitis lameness as well as lower incidence of femoral head transitional degeneration and tibial head necrosis due to prevention of gut bacterial translocation and hematogenous distribution. The direct link between prebiotic and probiotic supplements and bone health has been demonstrated in a variety of animal models (zebrafish, rodents, and chicken) and humans (Scholz-Ahrens et al., 2007; Tu et al., 2021). In this aspect, it could be argued that broilers fed with the SYN have improved skeletal health under both TN and HS conditions. The similar results have been reported in one of our previous studies, the SYN improves broiler bone mineral density, bone mineral content, GS, and LTL (Yan et al., 2019). Several other beneficial bacteria strains, such as Bacillus subtilis, Saccharomyces cerevisiae, and Lactobacillus acidophilus, with the similar effects on skeletal health including the improvement of bone mineral density and breaking force have been reported previously (Saleh et al., 2012; Abdelqader et al., 2020; Li et al., 2020; Khan et al., 2019).

Heat stress activates the hypothalamus-pituitary-adrenal (HPA) axis, stimulating release of glucocorticoids which play an important role in regulation of the immune system (Bae et al., 2019; Bagath et al., 2019). Glucocorticoids regulate the synthesis and release of both proinflammatory and anti-inflammatory cytokines to modulate the healing process of stress-induced inflammatory damage (Siddiqui et al., 2020). Generally, T lymphocyte subtype Th1 is responsible for releasing IL-2, IL-8, IFN-α, and TNF-α, while Th2 subtype functions in synthesizing and releasing IL-4, IL-5, IL-10, and IL-13, playing important roles in the development of humoral immune response (Romagnani, 1997). Among the immune regulators, IL-6, as a proinflammatory cytokine, is produced mainly by macrophages in response to various stimulations (Tanaka et al., 2014). High IL-6 levels have been found in association with many diseases and related tissue damage (Tanaka et al., 2014). In the present study, plasma proinflammatory cytokine IL-6 was elevated in broilers exposed to HS on d 28, which may indicate HS-induced inflammation in broilers. In agreement with the current results, increased plasma concentrations and upregulated mRNA expressions of several proinflammatory cytokines including IL-6 as well as TNF-α, IL-2, IL-1β, and IL-8 have been reported previously in HS broilers (Alhenaky et al., 2017; Baxter et al., 2020; Humam et al., 2021).

Heat stress suppressed the concentrations of total plasma IgY, the major immunoglobulin subclass in broiler chickens, on both d 28 and d 42. This finding agrees with the data reported by Honda et al. (2015), who observed decreased plasma IgY after 19 d of heat exposure. On d 28, both SYN and BMD supplemented birds tended to have higher IgY than CON birds, suggesting an increased immune response in these birds. The reasons for this change could be due to the protective effect of BMD and SYN in against intestinal inflammation. Heat stress disturbs commensal bacteria and increases intestinal permeability allowing for translocation of pathogens, endotoxin, and biochemical factors, leading to systematic inflammation (De Boever et al., 2008; Alhenaky et al., 2017). Antibiotics target certain pathogens to reduce infection, however, chronic antibiotic administration can lead to commensal microbe depletion and microbiota composition imbalance, ultimately affecting host health (Ubeda and Pamer, 2012). This hypothesis has been evidenced by the reconstruction of disrupted intestinal flora after administration of commensal gut microbiota in germ free mice (Mazmanian et al., 2005). In addition, immunological effects of probiotics have been discovered in different hosts, including broilers (Azad et al., 2018; Tuo et al., 2018; Wang et al., 2018). Results of higher antibody titers against new castle disease (An et al., 2008;
A. Shahir et al., 2014) and infectious bursal disease (Rehman et al., 2020) have been determined in broilers fed probiotic and/or probiotic supplements. In the current study, SYN supplemented birds had a higher relative spleen weight compared with both BMD and CON birds on d 42 under TN condition. The spleen is a major immune organ in birds, and weight changes have been used as an indicator of immunity in response to stress (Scanes, 2020). Greater relative spleen weight may indicate an enhanced immune response and lower stress reaction in broilers fed with the SYN supplements. These results may indicate that provision of beneficial bacteria via dietary supplementation of probiotics or synbiotics could be a harmless strategy for restoring commensal bacterial balance, protecting against pathogens, and stimulating the immune responses in broilers as well as other animals.

Heat stress causes a surge of ROS, which contributes to oxidative stress (Slimen et al., 2014). Oxidative stress occurs when overproduced ROS overcomes the capacity of antioxidant enzymes such as SOD activity (Slimen et al., 2014). The drop of SOD activity between TN-CON and HS-CON birds on d 28 evidenced that the HS induced oxidation, while SYN supplements were able to attenuate the negative effect. In addition, HS-BMD birds had reduced SOD enzyme activity compared with both HS-CON and HS-SYN birds. The decreased SOD enzyme activities in the BMD supplemented group indicated a higher oxidative status in the broilers. Antioxidative administration has been shown to increase intracellular oxidation by potentially increase ROS production due to gut bacteria death (Liu et al., 2016; Sannassimuthu et al., 2020). Oxidative stress negatively affects broiler performance as well as meat quality, causing profound economic loss (Altan et al., 2003). Moreover, oxidative stress is associated with expression of HSP70 (Grunwald et al., 2014). Our data showed plasma levels of HSP 70 tended to be lower in HS-SYN but not HS-BMD broilers compared to it of HS-CON birds on d 42. Heat shock proteins have cytoprotective activities, which aid protein assembling and folding as well as degradation of improperly folded proteins to prevent cell apoptosis (Jee, 2016; Ikwegbue et al., 2018). As a chaperone protein, HSP70 is synthesized rapidly after the animals being exposed to high environmental temperature (Feder and Hofmann, 1999). Increased circulating HSP70 and upregulated gene expression have been reported in HS broilers (Tang et al., 2018; Siddiqui et al., 2020). The lower HSP70 found in the SYN supplemented birds could be an indicator for attenuated HS response and oxidative stress by increasing the birds’ adaptation to HS via the gut-immune and gut-brain axes.

In conclusion, our results showed that antibiotics BMD can promote broiler growth performance but may lose efficacy under HS and potentially lead to oxidative stress. The SYN supplements attenuate negative effect of HS by improving BW, foot health, leg strength, immunity, and antioxidant capability. The dietary supplementation of SYN may be a viable BMD alternative for broilers production, especially in the tropical and subtropical regions.

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DISCLOSURES

J. Y. Hu, A. A. Mohammed, G. R. Murugesan, and H. W. Cheng declare that they have no conflict of interest.

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