Probiotic mixture ameliorates heat stress of laying hens by enhancing intestinal barrier function and improving gut microbiota

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ABSTRACT

Heat stress (HS) is a major stressor for laying hens. It causes enormous financial losses worldwide annually. The purpose of this study was to investigate the effect and mechanism of a probiotic mixture of Bacillus subtilis and Enterococcus faecium on the performance of laying hens under HS. Eight hundred and fifty-six commercial laying hens (Hy-Line Brown, aged 40 weeks) were randomly allocated to three groups, Group C (26°C), Group H (33°C) and Group H + PM (33°C + probiotic mixture), respectively. The trial lasted for 20 days. Compared with Group H, we demonstrated that treatment with the probiotic mixture significantly increased the egg production rate, average daily feed intake (ADFI), and egg weight of laying hens under HS (p < .05). In addition, the eggshell thickness, eggshell strength and albumen height also improved. We further showed that these probiotics improved gut microbiota as well as enhanced intestinal integrity, which may be responsible for inhibiting the invasion of bacteria and improving the performance indices. The study suggests that this probiotic mixture may be an effective strategy for treating laying hens under HS.

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Introduction

Over the past decades, prolonged hot seasons in many geographical regions have been reported due to global warming (Mack et al. 2013). The increasing temperature has inevitably impacted human health and agriculture. The poultry industry, for example, faces a very deleterious effect related to heat stress (HS), which has led to significant losses of production every year (St-Pierre et al. 2003). Impacts of the hot environment on various poultry species have been extensively studied recently, including broilers (Quinteiro-Filho et al. 2012), broiler breeders (Sharifi et al. 2010) and laying hens (Deng et al. 2012).

In addition to their unique metabolic characteristics and high rate of egg production (Lin et al. 2008), laying hens lack sudoriparous glands and inefficient heat dissipation, which make them more susceptible to HS (Wolfenson et al. 2001). Elevated temperatures also cause reduced feed intake, egg production, egg weight, and shell thickness (Rozenboim et al. 2007; Song et al. 2012).

Numerous strategies have been employed to reduce the HS effects on laying hens, including the addition of vitamin C (Torki et al. 2014), γ-aminobutyric acid (Zhang et al. 2012), regulatory peptides (Song et al. 2012), and mannan-oligosaccharides (Bozkurt et al. 2012) to the diet.

Another potential food supplement is probiotic (Deng et al. 2012; Abdelqader et al. 2013), which can provide health benefits to the host when ingested in sufficient amounts (FAO 2001). Recently, the probiotic bacteria Lactobacillus (Sohail et al. 2010) and Bacillus subtilis (Abdelqader et al. 2013) have been applied to birds. The two strains benefit animals by suppressing pathogens directly or indirectly and reduce the occurrence of diseases (Tsaruk’ianova & Smirnova 2005; Bhardwaj et al. 2008). But single species often exposed limited functionality, which led to instability in the aspect of using effect. And a probiotic mixture consists of two or more live strains according to certain proportion made from composite, which possesses the characteristics of full-featured and synergies.
Moreover, aerobic and anaerobic bacteria strains usually cooperate with reasonable. Currently probiotic mixture has been reported to have a better therapeutic effect than a single species (Timmerman et al. 2004; Wang et al. 2014), providing novel insights for relieving HS.

Therefore, in this study we explored the effect and mechanism of probiotic mixture of *Enterococcus faecium* (anaerobe) and *Bacillus subtilis* (aerobe) on HS-induced laying hens by analysing the apparent indicators (production performance and egg quality) and inherent indicators (serum profiles, intestinal barrier and HSP70).

**Materials and methods**

**Birds and housing**

A total of 856 Hy-Line Brown commercial laying hens (aged 40 weeks) were purchased from Zhangdian Chicken Co., Ltd. (Wuhan, China). They were randomly distributed into three groups and housed in cages (2–3 birds/cage). Photoperiodic lighting was set at 16 h (from 5 a.m. to 9 p.m. daily) and 8 h of dark throughout the experiment. All the hens were initially kept under appropriate environmental conditions (28°C; 7 days) and fed a basal diet (Table 1) to adjust there was no significant difference in egg production among the three groups before the formal experiments started. They were then randomly transferred to two different temperature rooms with three replicates each: Group C (N = 50, 16–17 hens per replicate, control) was kept at room temperature (26°C); Group H (N = 405, 135 hens per replicate, high temperature) was kept at 33°C to induce HS; and Group H + P_M (N = 401,133–134 hens per replicate, high temperature + probiotic mixture) was kept at 33°C and treated with the probiotic mixture (5.0 × 10^5 cfu of *E. faecium* and 4.0 × 10^6 cfu of *B. subtilis* per gram of complete feed). The relative humidity was continuously monitored and ranged from 60 to 70% in both conditions. *E. faecium* (China Centre for Type Culture Collection, Wuhan, China, CCTCC NO: M2011031, 5.0 × 10^9 cfu/g) and *B. subtilis* (China Centre for Type Culture Collection, Wuhan, China, CCTCC NO: M2011034, 1.0 × 10^10 cfu/g) were provided by Huada-real Technology Co., Ltd. (Wuhan, China). The compound with a ratio of 1:4 was added to the basal diet. The experiment began when the hens were assigned to the temperature rooms, and it lasted for 20 days. The experimental protocols were performed according to the institutional guidelines of the China Laboratory Committee for Animal Care and Welfare.

**Production parameters**

During the experiment, the number of eggs laid, egg weight, feed intake, and cracked eggs of each group were recorded daily at 4:00 p.m. to calculate the egg production rate, average egg weight, average daily feed intake (ADFI), feed–egg ratio (total feed intake/total egg weight), and broken egg ratio. Furthermore, diarrhoea of the laying hens was recorded based on the state of the faeces: (1) normal: shaped faeces, soft and easy to cut; (2) diarrhoea: faeces falling out of shape, liquid (Zhang et al. 2010). The diarrhoea status of each group was detected and recorded at 10 and 20 days, respectively. The mortality of the hens was recorded as it occurred. The production performance was adjusted with hen death.

Twelve eggs from each group were randomly selected to measure egg quality at 10 and 20 days. Eggshell thickness was measured using an Eggshell Thickness Gauge (ESTG-1) (Orka Technology Co., Ltd., Ramat-Hasharon, Israel) at three different locations on the eggs (top, middle and bottom) as previously described (Song et al. 2012). Eggshell strength and albumen height were measured by employing an Egg Force Reader (EFR-1) and Egg Analyser (Orka), respectively.

**Sample collection**

Nine hens from each group (with three hens each replicate) were randomly selected for fresh blood collection at 10 and 20 days. Approximately 3–4 mL of blood was taken by jugular puncture using a disposable syringe. The blood samples were kept at 25°C for 1 h and then at 4°C for 2 h. Serum was separated by centrifugation at 3000 × g for 15 min at 4°C. The supernatants of the serum were collected and stored at −80°C until further analysis. The hens were slaughtered, and the liver and intestinal tissues were cut.

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**Table 1. Composition and nutrient analysis of experimental basal diets.**

| Ingredient, % of DM | Nutrienta |
|---------------------|-----------|
| Maize               | 62        |
| Soybean meal        | 27        |
| Limestone powder    | 7         |
| Soybean oil         | 0.9       |
| Mineral and premix  | 3         |
| α-methionine        | 0.1       |

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bObtained by calculation.

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[^1]: Premix provided per kilogram of diet: 260–330 KIU of vitamin A, 13–16 KIU of vitamin D_3, 330 IU of vitamin E, 50 IU of vitamin K_3, 55 mg of vitamin B_12, 1.5 mg of vitamin B_6, 5 mg of vitamin B_12, 2.0 mg of vitamin B_6, 1000 mg of vitamin B_12, 900 mg of vitamin B_6, 30 mg of vitamin B_12, 340–580 mg of Cu, 1700–2300 mg of Fe, 2900–4000 mg of Zn, 3000–4500 mg of Mn, 7.0–14.0% NaCl, and 9% H_2O.

[^2]: Obtained by calculation.
rapidly (approximately 0.1 g/hen) and placed into a small RNase-free centrifuge tube perfused with Sample Protector (TaKaRa Biotechnology Co., Ltd., Dalian, China) to analyse the mRNA levels of heat shock protein 70 (HSP70), occludin, zona occudens (ZO)-1, and junction adhesion molecule (JAM)-A. Slices from the centre portion of the ileum and caecum were gathered and fixed in a 4% (vol/vol) paraformaldehyde solution. The ileum and caecum (160 mg) were collected and kept on ice for DNA extraction. In order to reduce the differences from individual animals, equal amounts of samples within each replicate were pooled as previously described (Sun et al. 2013).

**Serum cytokine and endotoxin determination**

Levels of interleukin (IL)-1, IL-10, and endotoxin (Product Numbers: CHE70, CHE79 and CHE71, respectively) were measured using commercial kits (Bogu Technology Co., Ltd., Shanghai, China) following the manufacturer’s instructions. The kits were specific for chicken and no cross reactivity of ELISA test used to quantify the cytokines.

**Intestinal morphology**

The formaldehyde-fixed tissue samples were dehydrated with a series of ethanol solutions and embedded in paraffin. Sections (4 μm) were cut and stained with hematoxylin-eosin (HE) for light microscopy observation using a Nikon DS-Fil microscope (Nikon Co., Ltd., Tokyo, Japan) equipped with a digital camera and imaging software (Nikon DS-Fil-U2). The variables measured were villus height and crypt depth of 10 well-oriented villi per section, and the mean value for each tissue was calculated from 5 sections (Deng et al. 2012).

**Real-time quantitative polymerase chain reaction (PCR) analysis**

Total DNA was extracted from ileal and caecal samples as previously described (Li et al. 2003). The isolated DNA was used as templates for amplification using SYBR Premix Ex Taq™ II (TaKaRa) and a Bio-Rad IQ5 Real-Time Polymerase Chain Reaction (PCR) System software version 1.0CR (Bio-Rad, Hercules, CA). The partial 16S rRNA genes of Lactobacilli and *E. coli* were amplified with the real-time PCR primers described above (Sun et al. 2013) and inserted into the pMD 18-T vector (TaKaRa). The plasmid was extracted using a kit (Omega Biotechnology Co., Ltd., Norcross, GA) following the manufacturer’s instructions, and its concentration and integrity were examined by a NanoDrop spectrophotometer (ND-2000, Thermo Scientific, Wilmington, DE). The copy number per microliter of plasmid was calculated according to the formula of 6.02 $\times 10^{23}$ (copies/mol) × concentration (ng/μL) $\times 10^{-9}$/MW (g/mol) as previously described (Whelan et al. 2003). The standard curve was constructed by matching the plasmids with 10-fold dilutions ranging from $10^9$ to $10^3$ copies, which is formed by inverse correlation between the threshold cycle values and copy numbers per microliter using IQ5 Optical System software version 1.0CR (Bio-Rad, Hercules, CA). Finally, the bacterial populations were determined according to the standard curves.

The mRNA levels of occludin, ZO-1, JAM-A, and HSP70 were measured by reverse-transcription (RT)-PCR. Total RNA was isolated from the frozen tissues using the TRizol Reagent (Life Technologies Co., Ltd., Carlsbad, CA) according to the manufacturer’s protocols. The primers were designed using Primer Express software (Applied Biosystems). The primer sequences are listed in Table 2. The quality and integrity of the total RNA was determined by a NanoDrop spectrophotometer (Thermo Scientific). Reverse transcription was carried out using a Prime Script™ RT reagent Kit (TaKaRa) with random hexamers as recommended by the manufacturer. The expression of the reference gene GAPDH was used as a control and was amplified in parallel with the target genes. The PCR conditions were optimised at 95 °C for 30 s, followed by 40 cycles of 95 °C for 20 s, 60 °C (52 °C for HSP70) for 20 s, and 72 °C for 30 s. All reactions were carried out in triplicate. The partial 16S rRNA genes of Lactobacilli and *E. coli* were amplified with the real-time PCR primers described above (Sun et al. 2013) and inserted into the pMD 18-T vector (TaKaRa). The plasmid was extracted using a kit (Omega Biotechnology Co., Ltd., Norcross, GA) following the manufacturer’s instructions, and its concentration and integrity were examined by a NanoDrop spectrophotometer (ND-2000, Thermo Scientific, Wilmington, DE). The copy number per microliter of plasmid was calculated according to the formula of 6.02 $\times 10^{23}$ (copies/mol) × concentration (ng/μL) $\times 10^{-9}$/MW (g/mol) as previously described (Whelan et al. 2003). The standard curve was constructed by matching the plasmids with 10-fold dilutions ranging from $10^9$ to $10^3$ copies, which is formed by inverse correlation between the threshold cycle values and copy numbers per microliter using IQ5 Optical System software version 1.0CR (Bio-Rad, Hercules, CA). Finally, the bacterial populations were determined according to the standard curves.

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| Gene    | Accession No. | Primer Sequences (5’-3’) | Product (bp) |
|---------|---------------|--------------------------|--------------|
| Occludin| NM_205128.1   | F: ATGGCCTTCCATGCTACATC  | 90           |
| ZO-1    | XM_413773.4   | R: GCTGCACATGGCCAACAAG   | 118          |
| JAM-A   | EF_102433.1   | R: GGGCTCATTGATAATACGCATC| 90           |
| HSP70   | FJ_217667.1   | R: GGCAGGTCAGGTCAACAACA  | 91           |
| GAPDH   | NM_204305.1   | R: GGAAGAGCCCTTCTGGAACTT | 90           |
72 °C for 10 s. The mRNA expression of gene was compared between samples by using the $^{2-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008).

**Statistical analysis**

The results are presented as mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed using SPSS Statistics version 18.0 (SPSS Inc., Chicago, IL). The statistical significance was measured by one-way analysis of variance followed by Duncan’s multiple comparison tests. $p$ values $<.05$ were considered to be statistically significant.

**Results**

**Effect of the probiotic mixture on the performance of HS-induced laying hens**

As shown in Table 3, HS caused a significant decrease in the average egg production rate, ADFI, and average egg weight compared to the room temperature control group (group H vs. group C) ($p < .01$). Addition of the probiotic mixture (group H + PM) significantly recovered the decrease, especially for egg production ($p < .01$). In addition, the feed-egg ratio, broken egg ratio, and mortality in group H were higher than those in group C. Group H + PM had the ability to achieve or exceed the performance levels found in group C. The diarrhoea rate of group H remained approximately 62% over the experimental period. In contrast, the diarrhoea rate of group H + PM dropped from 54% (10 days) to 32% (20 days) (Table 4).

At 10 days, the egg quality of group C and group H + PM was higher compared to group H, but the difference was not statistically significant ($p > .05$). At 20 days, however, most of the indicators were notably improved ($p < .05$), especially between group H and group H + PM.

**Modification of the intestinal microbiota by probiotic mixture administration**

Next, we investigated the effect of the probiotic mixture on the composition of the intestinal microbial

| Table 3. Effects of heat stress and the probiotic mixture (E. faecium and B. subtilis) over the 20-days experimental period on the performance of laying hens. |
| Parameter | Group |
|-----------|-------|
| | C | H | H + PM |
| Egg production rate, % | $85.96 \pm 0.05^A$ | $80.70 \pm 0.03^B$ | $83.92 \pm 0.03^A$ |
| Average daily feed intake, g | $102.00 \pm 1.51^A$ | $88.10 \pm 6.25^{Bb}$ | $93.64 \pm 8.56^{Ba}$ |
| Average egg weight, g | $58.01 \pm 1.31^A$ | $52.18 \pm 2.62^{Bb}$ | $53.90 \pm 1.07^{Ba}$ |
| Feed-egg ratio, % | $2.05 \pm 0.14$ | $2.09 \pm 0.17$ | $2.05 \pm 0.23$ |
| Broken egg ratio, % | $0.30$ | $0.40$ | $0.21$ |
| Mortality, % | $0$ | $2.30$ | $1.00$ |

Results are shown as mean ± SD. $N = 20$ per group. $^a,b$Mean values within a row with different superscript letters are significantly different ($p < .05$), and $^A,B$indicate highly significant ($p < .01$).

| Table 4. Effects of heat stress and the probiotic mixture (E. faecium and B. subtilis) over 10 and 20 days of the experimental period on the diarrhoea rate and egg quality of laying hens. |
| Parameter | Group |
|-----------|-------|
| | C | H | H + PM |
| Diarrhoea rate, % | 46.34 (19/41) | 61.71 (245/397) | 54.06 (213/394) |
| 20 days | 34.37 (11/32) | 63.02 (242/384) | 32.47 (125/385) |
| Eggshell thickness, mm | 0.47 ± 0.03 | 0.45 ± 0.03 | 0.46 ± 0.03 |
| 20 days | 0.48 ± 0.02$^a$ | 0.46 ± 0.02$^b$ | 0.48 ± 0.03$^A$ |
| Eggshell strength, kg/cm² | 7.01 ± 0.97 | 6.16 ± 0.99 | 6.72 ± 1.04 |
| 20 days | 7.32 ± 0.76$^A$ | 6.22 ± 0.57$^{Bb}$ | 7.26 ± 1.39$^{Aa}$ |
| Albumen height, mm | 4.88 ± 1.29 | 4.61 ± 1.50 | 5.33 ± 0.45 |
| 20 days | 5.37 ± 0.78$^{ab}$ | 5.07 ± 0.69$^b$ | 5.84 ± 0.92$^A$ |

Results are shown as mean ± SD. $N = 12$ per group. $^{a,b}$Mean values within a row with different superscript letters are significantly different ($p < .05$), and $^{A,B}$indicate highly significant ($p < .01$).
flora in response to HS. We found that the abundance of caecal *E. coli* in group H + P_M were lower at 20 days (*p* < .05; Figure 1(b)); while the amount of *Lactobacilli* in ileal (10 and 20 days) samples were higher (*p* < .05; Figure 1(c)), compared with group H. A similar but more profound trend was observed in group C (Figure 1).

**Variation of probiotic mixture on serum profiles**

Serum profile analysis showed that IL-1 levels of the C and H + P_M groups were lower (*p* < .05) at 20 days, compared with group H. A similar trend also occurred on the level of endotoxin. The IL-10 levels in group H remained similar, but those for group C and group H + P_M were higher (*p* < .05). These results strongly suggest that the probiotic mixture helps to stimulate the cytokine response and to reduce inflammation in response to HS.

**Protective effect of the probiotic mixture on the HS-induced morphological change of the intestine**

Next, we examined whether HS induced any morphological changes on the intestine. As shown in Figure 2, the intestinal villi of group H were extensively desquamated, mostly located at the tip (Figure 2(a)), compared to the normal structure of the intestinal villi in group C (Figure 2(d)). Some villi in group H appeared with typical fractures and largely exposed the lamina propria (Figure 2(b)). The villus structure of group H + P_M was relatively normal, and no obvious damage was observed (Figure 2(c)). We also found that the villus height in the ileum (10 days and 20 days) and caecum (20 days) of group H was significantly reduced, compared with those of group C and group H + P_M (*p* < .01; Table 5). No pronounced variation of the crypt depth among the three groups was observed (*p* > .05). The villus/crypt of the ileum (10 days and 20 days) and caecum (20 days) in group C and group H + P_M were much higher than that of group H (*p* < .01).

We further examined the intestinal compactness for a possible protective effect of the probiotic mixture to HS. Our results showed that the expression levels of occludin, ZO-1, and JAM-A in the ileum and caecum were notably downregulated in group H compared with group C and group H + P_M (*p* < .05; Figure 3(a–f)), particularly at 20 days (*p* < .01). But the differences of ileal JAM-A (20 days) between group H and group H + P_M were up-regulated expression (*p* < .05; Figure 3(e)), the area still needs further exploration.

**Effect of the probiotic mixture on the mRNA levels of HSP70**

It has been reported that probiotics have the ability to reduce the mRNA level of HSP70 (Gan et al. 2013). Therefore, we examined whether the expression of HSP70 was affected by the probiotic mixture. Our results showed that for group C and group H + P_M, the mRNA levels of HSP70 in the liver were markedly downregulated compared to group H at 10 days, particularly at 20 days (*p* < .01; Figure 4).

![Figure 1](image-url)

**Figure 1.** qRT-PCR analysis of the effects of heat stress and the probiotic mixture (*E. faecium* and *B. subtilis*) over 10 and 20 days of the experimental period on total *E. coli* (a) and *Lactobacilli* (b) in the ileum (left) as well as *E. coli* (c) and *Lactobacilli* (d) in the caecum (right) of laying hens. *N* = 3 per group, a,b: Mean values within the columns with different superscript letters are significantly different (*p* < .05).
Discussion

HS is a hazard that influences the performance of laying hens (Bozkurt et al. 2012), and it has been demonstrated that for every 1°C increase in temperature above 32°C, the feed intake decreases by 4.6% (Song et al. 2012). And more than 32°C will lead to physiological function disorder of laying hens, producing various non-specific responses. Such as lower egg production rate and feed intake, increase diarrhoea rate and mortality, etc. It has been subjected to heat stress, which is consistent with the results.

Figure 2. Protective effect of the probiotic mixture (E. faecium and B. subtilis) on the intestinal morphology of HS-induced laying hens. Image a and b, group H (fed the basal diet only and maintained at 33°C); image c, group H + PM (fed the basal diet supplemented with 5 x 10⁵ cfu/g of E. faecium and 4 x 10⁶ cfu/g of B. subtilis and maintained at 33°C); image d, group C (fed the basal diet and maintained at 26°C). The arrow in image a indicates that the intestinal villi had more desquamation, mostly located at the tip. The arrow in image b indicates that the villi presented typical fractures and largely exposed the lamina propria. The arrow in image d indicates the structure of normal intestinal villi. Original magnification of all images = 20x.

Table 5. Effects of heat stress and the probiotic mixture (E. faecium and B. subtilis) over 10 and 20 days of the experimental period on the intestinal morphometric parameters of laying hens.

| Parameter                  | Group          |
|----------------------------|----------------|
|                            | C             | H             | H + PM         |
| Ileum: Villus height, µm   | 10 days       | 20 days       | 10 days        | 20 days       |
| 504.18 ± 26.30              | 507.92 ± 20.81| 458.73 ± 34.48| 494.51 ± 33.49 |
| 507.92 ± 20.81              | 462.72 ± 42.37| 494.51 ± 33.49| 525.44 ± 26.34 |
| Crypt depth, µm             | 10 days       | 20 days       | 10 days        | 20 days       |
| 172.85 ± 14.03              | 166.39 ± 7.58 | 174.85 ± 15.02| 173.27 ± 22.19 |
| 174.85 ± 15.02              | 172.87 ± 19.85| 170.78 ± 8.66 |                |
| Villus:Crypt                | 10 days       | 20 days       | 10 days        | 20 days       |
| 2.93 ± 0.18                 | 3.06 ± 0.18   | 2.93 ± 0.18   | 3.06 ± 0.18    |
| 2.62 ± 0.07                 | 2.68 ± 0.19   | 2.86 ± 0.09   | 3.08 ± 0.17    |
| Caecum: Villus height, µm   | 10 days       | 20 days       | 10 days        | 20 days       |
| 120.64 ± 15.20              | 135.62 ± 13.94| 118.14 ± 14.34| 126.92 ± 23.31 |
| 118.14 ± 14.34              | 115.34 ± 14.44| 126.92 ± 23.31| 138.82 ± 17.27 |
| Crypt depth, µm             | 10 days       | 20 days       | 10 days        | 20 days       |
| 63.23 ± 8.50                | 64.35 ± 7.40  | 67.12 ± 6.37  | 66.14 ± 12.34  |
| 60.00 ± 5.17                | 69.00 ± 5.17  | 66.73 ± 6.93  |                |
| Villus:Crypt                | 10 days       | 20 days       | 10 days        | 20 days       |
| 1.91 ± 0.13                 | 2.12 ± 0.17   | 1.78 ± 0.09   | 2.08 ± 0.13    |
| 1.87 ± 0.09                 | 2.08 ± 0.13   | 1.78 ± 0.09   |                |

Results are shown as mean ± SD. N = 5 per group.
A,B Mean values within a row with different superscript letters are highly significant (p < .01).
Moreover, it has been reported that the enzymatic activities of the alimentary canal (i.e. amylase, lipase and trypsin) are inversely related to temperature (Zhang et al. 2012) and further affect the feed intake. Furthermore, reduced feed intake limits dietary calcium ingestion and availability (Abdelqader et al. 2013), thus indirectly contributing to a decrease in egg quality. However, adding the probiotic mixture could effectively improve the production performance and egg quality of heat stress-induced laying hens. But how probiotics to alleviate heat stress is still the focus of attention.

In our study, we found that HS leads to dysbiosis of the good gut bacteria (Figure 1), consistent with previous reports (Burkholder et al. 2008), which increases disease susceptibility easily. DuBose et al. (2002) have shown that endotoxin released from disordered commensal flora could invade the blood through the HS-damaged intestinal mucosa, and apply to produce pyrogen cells (mostly mononuclear macrophages) releasing harmful pro-inflammatory factor caused the body response of heat stress (Bouchama & Knochel 2002). And excessive levels may lead to maladjustment for feed intake and energy expenditure, which affect the productivity (Klasing 1988). IL-1, is a pivotal pro-inflammatory factor (Peera & James 2013), stimulating the body of the temperature regulating centre of the hypothalamus, which prompt the body temperature. However, IL-10 is an anti-inflammatory factor, and has a central role in downregulating inflammatory cascades (Steidler et al. 2000). To further explore the effect of cytokines during HS, we investigated the levels of serum IL-1, IL-10, and endotoxin. We found that...
the probiotic mixture has significantly reduced the content of IL-1 and endotoxin, increased the level of IL-10 (Table 6). But whether probiotic form the intestinal barrier to protect laying hens against the dangers of endotoxin and inflammatory factor are also need to be further confirmed.

Several studies have shown that HS damages the integrity and morphology of the intestine (Quinteiro-Filho et al. 2012), resulting in poor nutrient absorption and lower animal performance (Xu et al. 2003). Increased intestinal permeability also induces inflammation and pathogen load (Awad et al. 2009; Chappell et al. 2009). In this study, we showed that the probiotic mixture improved the intestinal villus height and the villus:crypt ratio in HS-induced laying hens (Table 5 and Figure 2). The epithelial cells serve as the first line of defence against bacterial infection (Quinteiro-Filho et al. 2012), toxicity (Deng et al. 2012). Therefore, we further observed that the probiotic mixture ameliorates permeability of intestinal epithelial cell barrier reflected by a strong up-regulation of occludin, ZO-1 and JAM-A for HS-induced laying hens (Figure 3), which could fundamentally prevent the invasion of endotoxin and relieve symptoms of heat stress. These results are consistent with a previous report (Mennigen et al. 2009).

HS results in upregulation of the heat shock protein HSP70 (Franco-Jimenez & Beck 2007; Gan et al. 2013). We found that probiotics significantly downregulated the HSP70 mRNA levels in the liver (Figure 4). This result suggests that probiotics have the ability to ameliorate HS-induced changes in the HSP70 mRNA levels.

**Conclusions**

In conclusion, we demonstrated that the concomitance of probiotics remedied the HS-induced laying hens by two aspects: first, probiotics contributed to the maintenance of gut microbiota; second, probiotics helped to improve the intestinal integrity, which may be responsible for inhibiting the invasion of bacteria and improving the performance indices. This research may provide insights for selecting applicable probiotic species for heat or other stresses.

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**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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**Table 6.** Effects of heat stress and the probiotic mixture (E. faecium and B. subtilis) over 10 and 20 days of the experimental period on the serum IL-1, IL-10, and endotoxin levels of laying hens.

| Parameter          | Group          |
|--------------------|----------------|
| C                  | H              | H + Pm          |
| **IL-1, pg/ml**    |                |                |
| 10 days            | 102.02 ± 5.78⁸ | 151.52 ± 10.42⁸ | 126.96 ± 11.42⁸ |
| 20 days            | 128.00 ± 8.00⁹ | 190.07 ± 12.28⁹ | 136.12 ± 12.41⁹ |
| **IL-10, pg/ml**   |                |                |
| 10 days            | 15.35 ± 4.57⁸  | 4.76 ± 1.65⁸   | 5.39 ± 1.05⁸   |
| 20 days            | 13.38 ± 3.95⁸  | 3.24 ± 1.15⁸   | 7.69 ± 0.98⁸   |
| **Endotoxin, EU/ml** |                |                |
| 10 days            | 0.67 ± 0.10⁸   | 1.94 ± 0.49⁸   | 1.30 ± 0.38⁸   |
| 20 days            | 0.61 ± 0.10⁸   | 1.48 ± 0.24⁸   | 0.80 ± 0.11⁸   |

Results are shown as mean ± SD. N = 3 per group. ⁸Mean values within a row with different superscript letters are significantly different (p < .05), and ⁹indicate highly significant (p < .01).
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