Effects of dietary probiotic (*Bacillus subtilis*) supplementation on productive performance, immune response and egg quality characteristics in laying hens under high ambient temperature

Moataz Fathi, Ibrahim Al-Homidan, Abdelaziz Al-Dokhail, Tarek Ebeid, Osama Abou-Emera, and Ahmed Alsagan

Department of Animal Production and Breeding, College of Agriculture and Veterinary Medicine, Qassim University, Al-Qassim, Saudi Arabia; Department of Poultry Production, Faculty of Agriculture, Ain Shams University, Cairo, Egypt; Department of Poultry Production, Faculty of Agriculture, Kafrelsheikh University, Kafr El-Sheikh, Egypt; King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia

ABSTRACT

A two level factorial experiment (3 × 3) was designed to evaluate the effect of dietary inclusion level of probiotic and breed on productive performance, immune status and egg quality characteristics in laying hens reared under high ambient temperature. A total of 216, 32-week-old laying hens representing three different breeds (White Leghorn, Saudi black and Saudi brown) were randomly assigned to three dietary treatments (0, 200 and 400 g/t feed) in a 90-d experiment. The current results indicated that the inclusion of probiotics in layer diets did not appear to cause any adverse effects on egg production traits compared with non-treated hens. Dietary supplementation with probiotics at 400 ppm level significantly (*p* < .05) increased shell thickness compared to non-treated laying hens. Moreover, an improvement in eggshell quality and breaking strength in hens fed a diet containing probiotics was observed under high ambient temperature. Also, plasma cholesterol and triglyceride were reduced in laying hens fed a diet supplemented with probiotics. Furthermore, probiotic inclusion significantly increased IgM immunoglobulin concentration in laying hens fed a diet containing either 200 or 400 ppm compared with untreated hens. Concerning breed effect, Saudi black laying hens recorded significantly (*p* < .02) higher egg mass compared with the other breeds. Also, both Saudi chicken breeds exhibited significantly higher cell-mediated response and IgM concentration compared with Leghorn hens.

ARTICLE HISTORY

Received 12 October 2017
Revised 2 January 2018
Accepted 4 January 2018

KEYWORDS

Probiotic; egg quality; laying hens; immunity; Saudi chickens

Introduction

Probiotics as feed additives are increasingly used in animal and poultry feeding in commercial manner. Improved growth performance, reduced mortality and enhanced immunocompetence broiler chickens are well defined (Attia et al. 2011). The effectiveness of probiotic administration in laying hens may depend on several factors, including microbial species composition, supplemental dose, method and frequency of administration, diet composition, bird’s age, genotype (breed) and environmental stress factors (Mikulski et al. 2012). It is well known that heat stress in laying hens has a severe negative impact on eggshell quality. Many investigators have reported that dietary probiotic supplementation could reduce heat stress in birds (Männer and Wang 1991; Zulkifli et al. 2000). It has been shown that probiotic supplementation is more beneficial during stressful conditions (Jin et al. 1997).

The inclusion of probiotic in the diet has been found to improve egg production and food conversion ratio in several studies (Panda et al. 2008; Youssef et al. 2013; Chung et al. 2015). Furthermore, giving probiotics to laying hens has been found to improve eggshell quality and reduced the number of damaged eggs (Mikulski et al. 2012; Zhang et al. 2012). Likewise, Zhang et al. (2013) reported that the dietary supplementation of 0.01% probiotic improved egg production and egg quality. Conversely, some contradictorily results have been reported on the effects of probiotic supplementation on egg yield and feed efficiency

CONTACT Prof. Moataz Fathi mmfathi@fulbrightmail.org Department of Animal Production and Breeding, College of Agriculture and Veterinary Medicine, Qassim University, Al-Qassim 51452, Saudi Arabia

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The supplementation of probiotics to diet of laying hens may play an important role in altering the lipid metabolism of chickens. Many investigators have pointed out that probiotics could reduce the cholesterol content of egg yolk (Li et al. 2006; Mikulski et al. 2012) and serum (Kalavathy et al. 2003).

Probiotics have been used to stimulate the immune system in poultry. Numerous studies have concluded that using probiotics as feed additives exerts a beneficial influence on immune response and health status in both layer- and meat-type strain of chickens (Willis and Reid 2008; Vila et al. 2009; Shivaramaiah et al. 2011). Fong et al. (2015) observed an improved immune response in the cells of healthy subjects treated with Lactobacillus rhamnosus in vitro. An increase in serum bactericidal activity was observed in laying hens given a diet supplemented with probiotics (Forte et al. 2016). Therefore, the present study aimed at evaluating productive performance, immune status and egg quality of laying hens from different chicken breeds fed a diet supplemented with varied concentrations of probiotic under high ambient temperature.

Materials and methods

Experimental design, birds and dietary treatments

A total of 216, 36-week-old laying hens were allocated in a two-level factorial experimental design (3 × 3), consisting of three concentrations of dietary probiotic (0, 200 and 400 g/t feed) containing 4 × 10^9 cfu/g of Bacillus subtilis and three different breeds (White Leghorn, Saudi black and Saudi brown). Each subgroup had eight replicates (experimental cages) of three hens each. The birds were placed in wire cages (60 cm × 45 cm × 43 cm, L × W × H) under lighting schedule of 17 h/d light cycle. The average of high ambient temperature and relative humidity during the schedule of 17 h/d light cycle. The average of high ambient temperature and relative humidity during the whole experimental period was 34 ± 1.5 °C and 55%, respectively. Feed and water were provided ad libitum throughout the 90-d experimental period. The basal diet was formulated to contain approximately 16.6% crude protein and 2875 ME kcal/kg in a typical layer diet (Table 1). Three probiotic levels (0, 200 and 400 ppm) were mixed with diet every week. Conventional breeding and management procedures were applied throughout the experimental period. All birds were provided with similar environmental and hygienic conditions. The care and handling of the laying hens were in accordance with regulations of animal care committee of Qassim University.

Table 1. Ingredients and composition of the basal diet.

| Ingredient | (%) |
|------------|-----|
| Yellow corn | 60.2 |
| Soybean meal (44% CP) | 26.0 |
| Limestone | 8.7 |
| Di-calcium phosphate | 1.7 |
| Vitamin–mineral Premix | 0.3 |
| Sodium bicarbonate | 0.23 |
| Salt (NaCl) | 0.24 |
| DL-Methionine | 0.13 |
| Vegetable oil | 2.5 |
| **Calculated analysis (% unless otherwise noted)** | |
| Metabolisable energy (kcal/kg diet) | 2875 |
| Crude protein | 16.6 |
| Ca | 3.77 |
| Available phosphorous | 0.45 |

*Dicalcium phosphate contained: 16% phosphorous and 23% calcium. *Premix provided per kilogram of diet: vitamin A (transretinyl acetate), 10,000 IU; vitamin D₃ (cholecalciferol), 3000 IU; vitamin E (all-rac-α-tocopherol), 30 IU; menadione, 1.3 mg; thiamine, 2.2 mg; riboflavin, 8 mg; nicotinamide, 40 mg; choline chloride, 400 mg; calcium pantothenate, 10 mg; pyridoxine, 4 mg; biotin, 0.04 mg; folic acid, 1 mg; vitamin B12 (cobalamin), 0.013 mg; Fe (from ferrous sulphate), 80 mg; Cu (from copper sulphate), 8.0 mg; Mn (from manganese sulphate), 110 mg; Zn (from zinc oxide), 60 mg; I (from calcium iodate), 1.1 mg; Se (from sodium selenite), 0.3 mg.

Laying performance

Feed intake was recorded on a replicate basis for the whole experimental period. Feed conversion ratio (FCR) was calculated at the end of the experiment on the basis of the amount of feed consumed in gram divided by egg mass in gram. Damaged eggs, including broken, cracked and shell-less eggs, were recorded as they occurred. All eggs were collected and recorded on a daily basis. Eggs were individually weighed and egg mass was calculated for the whole experimental period.

Egg quality

At 48 weeks of age, 48 eggs from each dietary treatment per breed were collected to assess internal and external egg quality characteristics. Egg width and egg length were measured in mm using electronic digital Vernier calliper (±0.01 mm). Egg shape was calculated according to the following formula:

\[
\text{Egg shape} = \frac{\text{egg width}}{\text{egg length}} \times 100
\]

Following collection, the breaking strength for intact eggs was determined in kg/cm² using Egg Force Reader™, Orka Food Technology Ltd, St. Paul, MN. Also, egg weight, Haugh unit and yolk colour were measured automatically using Egg Analyzer™ manufactured by Orka Food Technology Limited, St. Paul, MN. The liquid contents were put aside and the shell plus membranes were washed under running water to remove adhering albumen. The wet eggshell was left for 24 h at room temperature for drying and then weighed to the nearest 0.01 g. The relative weight of...
dry eggshell was calculated on the basis of egg weight. To measure shell thickness, pieces from three different regions (two poles and equator) of each eggshell with intact membranes were measured with a dial gauge micrometer to the nearest 0.01 mm.

Concentrations of plasma immunoglobulins

The effects of probiotic administration and breed type on the humoral status of the bird by quantifying IgA, IgM, and IgY levels in blood plasma were measured. At the end of the experiment, 10 hens per sub-group were randomly chosen and blood samples were collected from the wing vein in heparinised tubes. Blood samples were subsequently stored in ice, centrifuged at 2500×g for 10 min at 4°C, and the harvested plasma was stored at −20°C until antibody analyses. Plasma IgA, IgM and IgY concentrations were determined in appropriately diluted samples by a sandwich ELISA using microtitre plates and chicken-specific IgA, IgM, and IgY ELISA quantitation kits (GenWay Biotech Inc., San Diego, CA).

Cell-mediated immunity

Cell-mediated immunity in vivo was evaluated by injection of the mitogen phytohemagglutinin-P (PHA-P) into the wattle. At 48 weeks of age, 15 laying hens from each treatment per breed were randomly assigned. Each hen was intradermally injected in the right wattle with 100 µg phytohemagglutinin-P (Sigma Chemical Co., St. Louis, MO) in 0.1 mL of sterile saline. The initial wattle thickness was measured with a constant tension calliper before injection and at 24, 48 and 72h after PHA-P injection. The resultant swelling response in the wattle was calculated as the difference between its thickness before and after injection.

Blood biochemical analysis

At the end of the experimental period, 10 blood samples from each treatment group per breed were withdrawn for blood biochemical analysis. Plasma was harvested as mentioned above. Plasma total protein, albumen, total cholesterol and triglyceride were spectrophotometrically determined using commercial kits (Stanbio Laboratory, Boerne, TX). The globulin was calculated as the difference between the total protein and albumen. Serum triiodothyronine (T₃) and thyroxine (T₄) concentrations were measured using commercial ELISA kits (BioCheck®, Foster City, CA).

Statistical analysis

Data were subjected to a two-way ANOVA with probiotic level and breed as fixed effects using JMP Ver. 11 (SAS Institute 2013). The model applied is as follows:

\[ Y_{ijk} = \mu + P_i + B_j + (PB)_{ij} + e_{ijk} \]

where \( Y_{ijk} \) is the observation taken on the kth individual, \( \mu \) is the overall mean, \( P_i \) is a fixed effect of the ith probiotic level, \( B_j \) is a fixed effect of the jth breed, \( (PB)_{ij} \) is the interaction between probiotic level and breed, \( e_{ijk} \) is the random error assumed to be independent normally distributed with mean = 0 and variance = \( \sigma^2 \).

Data given in percentage were subjected to arcsine transformation before statistical analysis, however, the actual data are listed. All results are presented as mean and the pooled SEM. The significance of difference among the groups was assessed using Tukey’s test. Significance was set as \( p < .05 \).

Results

Egg production traits and damaged egg ratio as affected by dietary probiotic level and breed are shown in Table 2. At the beginning of the experiment, there were no statistically differences in the average body weight-laying hens between dietary treatments within each breed (data not shown). During the experimental period, no hen died in each treatment within each breed. As shown in Table 2, there was no statistically significant difference in egg production performance among dietary treatments. Insignificant decreased feed intake (\( p < .08 \)) was observed in both groups fed a diet supplemented with probiotics compared to group receiving the control diet. The lowest feed intake (9789 g) was found in group that consumed diet containing 200 ppm probiotics, while the highest (10,406 g) was recorded in the control group. There was no significant difference in FCR between hens fed probiotic-supplemented diets and hens fed the control diet. Probiotic supplementation did not affect damaged egg ratio. However, non-significant reduction in damaged eggs was detected (22% and 39% in groups supplemented with 200 and 400 ppm probiotics, respectively). With respect to breed effect, the Saudi black laying hens recorded significantly (\( p < .02 \)) higher egg mass compared with the other breeds. The worst performance was found in Saudi brown breed.

The effects of dietary probiotic supplementation and breed type on internal and external egg quality
measurements of laying hens are presented in Table 3. No significant differences were found between the dietary groups with respect to yolk%, yolk colour and albumen quality expressed as Haugh unit score. Both probiotic levels significantly (p < .01) increased relative weight of eggshell compared with the control group. Likewise, shell thickness significantly (p < .05) improved in laying hens fed a diet containing 400 ppm probiotics when compared with those fed a basal diet. While the shell thickness of hens fed on 200 ppm probiotic was intermediate. Therefore, as a consequence, the inclusion of probiotic in both levels significantly (p < .05) increased eggshell strength compared with the control group (4.27, 4.17 and 3.82 kg/cm², respectively).

Regarding breed effect, it could be noticed that the native breeds (black and brown) laid eggs with significantly (p < .01) darker yolk colour than those of Leghorn hens. Also, they produced eggs with rounded shape compared with those of Leghorn. The brown breed recorded lower relative yolk and higher HU compared to the other two breeds. In terms of eggshell quality characteristics, the results revealed that there was no significant difference due to breed effect. As shown in Figure 1, a significant interaction between probiotic level and breed was observed for shell weight, shell thickness and egg-shape index. The highest shell weight and shell thickness were detected in Leghorn given 200 ppm probiotics and in black given 400 ppm probiotics.

Cellular and humoural immune responses of the laying hens as affected by probiotic administration level and breed are shown in Table 4. The laying hens fed a diet with the level of 400 ppm inclusion resulted in a numerical improvement in cellular immune response (PHA-P) at all tested times compared with the other groups. Moreover, probiotic inclusion significantly (p < .01) increased IgM immunoglobulin concentration at both probiotic levels (200 and 400 ppm). On the contrary, this trend did not exist in both IgA and IgY immunoglobulins. In terms of breeds, the results obviously revealed that the Saudi native breeds (black and brown) had a significant increase in swelling response, particularly at earlier stage of PHA-P test. Concerning the interaction between breed and

### Table 3. Effect of probiotic level and breed on internal and external egg quality characteristics of laying hens.

| Trait                  | Factor | Yolk % | Yolk colour | Haugh unit | Egg shape | Shell weight, g | Eggshell % | Shell thickness (mm x 10²) | Shell strength (kg/cm²) |
|------------------------|--------|--------|-------------|------------|-----------|-----------------|------------|-----------------------------|------------------------|
| Probiotic level (P)    |        |        |             |            |           |                 |            |                             |                        |
| 0 ppm                  |        | 30.9   | 9.1         | 58.5       | 76.5      | 5.0             | 9.5b       | 39.7b                       | 3.8b                   |
| 200 ppm                |        | 30.7   | 9.3         | 59.0       | 76.0      | 5.2             | 10.2a      | 40.1ab                      | 4.3a                   |
| 400 ppm                |        | 30.9   | 9.1         | 56.1       | 75.3      | 5.2             | 10.2a      | 40.8a                       | 4.2*                   |
| Breed (B)              |        |        |             |            |           |                 |            |                             |                        |
| Leghorn                |        | 30.9ab | 8.7b        | 53.8b      | 74.8b     | 5.5a            | 10.2       | 40.3                        | 4.0                    |
| Black                  |        | 31.9a  | 9.4a        | 57.9ab     | 76.4a     | 5.1ab           | 9.8        | 39.8                        | 4.1                    |
| Brown                  |        | 29.7b  | 9.4a        | 61.9a      | 76.6a     | 4.8b            | 9.9        | 40.5                        | 4.2                    |
| SEM                    |        | 0.27   | 0.14        | 0.84       | 0.250     | 0.05            | 0.09       | 0.200                       | 0.05                   |
| p Value                |        | .9     | .7          | .3         | .1        | .1              | <.01       | .05                         | <.01                   |
| P × B                  |        | .01    | <.01        | <.01       | <.01      | .3              | .03        | A                           | .9                     |

**a,b**Means in the same column with no common letters differ significantly (p < .05).

### Table 2. Effect of probiotic dietary inclusion and breed on egg production traits.

| Trait                  | Factor | Egg No | Egg weight, g | Egg mass, g | Feed intake, g | FCR | Damaged egg ratio |
|------------------------|--------|--------|---------------|-------------|----------------|-----|------------------|
| Probiotic level (P)    |        |        |               |             |                |     |                  |
| 0 ppm                  |        | 62.8   | 51.9          | 3252.8      | 10405.9        | 3.2 | 2.71             |
| 200 ppm                |        | 58.2   | 51.5          | 2966.6      | 9789.4         | 3.3 | 2.11             |
| 400 ppm                |        | 65.4   | 51.8          | 3380.7      | 10283.0        | 3.0 | 1.66             |
| Breed (B)              |        |        |               |             |                |     |                  |
| Leghorn                |        | 56.9b  | 55.6a         | 3173.0b     | 9605.9b        | 3.0b| 1.21b            |
| Black                  |        | 69.1a  | 50.7a         | 3499.6a     | 10637.0b       | 3.0b| 1.94b            |
| Brown                  |        | 60.4b  | 48.9b         | 2927.5b     | 10235.3a       | 3.5a| 3.34a            |
| SEM                    |        | 1.38   | 0.53          | 67.7        | 158.6          | 0.05| 0.34             |
| p Value                |        | .46    | .96           | .28         | <.01           | .59 | .42              |
| B × P                  |        | .04    | .01           | .02         | <.01           | .04 | .03              |

**a,b**Means in the same column with no common letters differ significantly (p < .05).

FCR: feed conversion ratio (g feed: g egg mass).
probiotic level, a significant effect on cell-mediated immunity was found. The best swelling responses at 24, 48 and 72 h were detected in black × 400 and brown × 0 (Figure 2). The highest IgY concentration was detected in Leghorn × 200, black × 0 and black × 400 (Figure 3).

Biochemical blood plasma parameters as affected by probiotic concentration and type of breed in laying hens are presented in Table 5. Plasma total protein, albumen and globulin concentrations were not significantly affected due to dietary probiotic supplementation. On the basis of breed factor, there were significant differences among breeds for albumen and globulin, while no significant difference was observed in total protein. After 90-d of probiotic supplementation, a significant difference was found in blood cholesterol. As shown in Table 5, cholesterol level was significantly (p < .05) reduced by probiotic inclusion. This decrease was associated with increased dietary probiotic concentration. On the one hand, blood cholesterol level was significantly affected by the interaction between probiotic level and breed (Figure 4). The lowest cholesterol level was found in brown breed fed a diet supplemented with 400 ppm probiotics. On the other hand, probiotic supplementation had no effect on serum triglyceride level. There were no

![Figure 1. Interaction between probiotic level and breed of eggshell traits.](image)

![Table 4. Effect of probiotic level and breed on cell mediated response and plasma immunoglobulins concentration of laying hens.](table)

| Factor | Swelling response | Immunoglobulin |
|--------|-------------------|----------------|
|        | Difference 24 h   | Difference 48 h | Difference 72 h | IgA | IgM | IgY |
| Probiotic level (P) | | | | | | |
| 0 ppm  | 0.88             | 0.55            | 0.45            | 0.43 | 0.87 c | 4.61 |
| 200 ppm| 0.74             | 0.52            | 0.42            | 0.43 | 0.96 p | 4.65 |
| 400 ppm| 0.96             | 0.65            | 0.50            | 0.45 | 1.06 a | 4.69 |
| Breed (B) | | | | | | |
| Leghorn| 0.57 b           | 0.44 b          | 0.37            | 0.44 | 0.68 c | 4.62 |
| Black  | 1.00 a           | 0.61 a          | 0.47            | 0.41 | 1.29 a | 4.70 |
| Brown  | 1.01 a           | 0.67 a          | 0.53            | 0.46 | 0.92 a | 4.63 |
| SEM    | 0.05             | 0.04            | 0.03            | 0.0098 | 0.0301 | 0.0266 |
| p Value|                  |                 |                |  .62 | <.01 | .41 |
| P      | .14              | .26             | .53             | .19 | <.01 | .40 |
| B      | <.01             | .02             | .07             | .90 | .74 | <.01 |
| P × B  | .04              | .04             | .03             | | | |

a,b,c Means in the same column with no common letters differ significantly (p < .05).
significant differences in cholesterol and triglyceride due to breed effect. The statistical analysis revealed that there was no interaction between dietary treatment and breed for all blood parameters except for cholesterol level (Table 5). In terms of thyroid hormones, no significant differences in blood plasma T₃ and T₄ concentrations were observed among treatments or breeds.

**Discussion**

**Laying performance**

In congruent with our findings, it was speculated that FCRs were not affected by probiotics in some reports (Nahashon et al. 1994; Mohan et al. 1995; Tortuero and Fernández 1995). Similarly, numerous studies have concluded that probiotics used as feed additives did
not affect egg production traits (Arpášová et al. 2012; Afsari et al. 2014; Sobczak and Kozłowski 2015). Beneficial effects on the damaged egg ratio resulting from probiotic supplementation are in agreement with the report of Balevi et al. (2001), who indicated a similar probiotic supplementation. On the one hand, we suggest that the increase in shell thickness percentage and in turn, the reduction in number of damaged eggs in hens receiving probiotics may have been caused by increase calcium retention. This hypothesis was suggested by Balevi et al. (2001). On the other hand, several studies confirmed that the inclusion of probiotic to the diet has been found to improve egg production and feed conversion ratio (Panda et al. 2008; Youssef et al. 2013; Chung et al. 2015). However, the difference between the present study and the previous reports may be related to differences in probiotic level, breed type and the hen’s age. Significant interaction between probiotic level and breed type in egg mass confirms that performance of laying hens receiving probiotics may differ according to genotype or breed used in the current experiment.

**Egg quality assessment**

Our findings have been confirmed by many reports (Mikulski et al. 2012; Zhang et al. 2012; Sobczak and Kozłowski 2015). Conversely, Mahdavi et al. (2005) and Mohebbifar et al. (2013) did not find significant effects for dietary probiotic inclusion on egg quality. Probiotic supplementation seems to have a positive effect on the eggshell percentage. A positive effect of probiotic

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**Table 5.** Effect of probiotic level and breed on biochemical blood plasma parameters of laying hens.

| Parameter | T. Protein, g/dL | Albumen, g/dL | Globulin, g/dL | Cholesterol, mg/dL | Triglyceride, mg/dL | T3 | T4 |
|-----------|------------------|---------------|---------------|-------------------|-------------------|----|----|
| Probiotic level (P) |       |               |               |                   |                   |    |    |
| 0 ppm     | 6.7              | 4.7           | 2.0           | 166.2             | 1660.8            | 1.9| 5.5|
| 200 ppm   | 6.5              | 4.4           | 2.4           | 152.8             | 1643.5            | 1.9| 5.6|
| 400 ppm   | 7.0              | 4.6           | 2.0           | 145.5             | 1626.8            | 1.9| 5.6|
| Breed (B) |       |               |               |                   |                   |    |    |
| Leghorn   | 6.9              | 4.1           | 2.8           | 154.8             | 1541.3            | 1.9| 5.7|
| Black     | 6.7              | 4.7           | 2.0           | 157.9             | 1722.9            | 1.9| 5.5|
| Brown     | 6.5              | 4.9           | 1.7           | 151.6             | 1658.8            | 1.9| 5.4|
| SEM       | 0.11             | 0.10          | 0.12          | 4.31              | 47.8              | 0.01|0.10|

**p Value**

| P         | .1 | .5 | .2 | .05 | .30 | .72 | .95 |
| B         | .4 | <.01 | <.01 | .57 | .96 | .71 | .43 |
| P × B     | .2 | .07 | .8 | .02 | .26 | .21 | .25 |

*a,b,c* Means in the same column with no common letters differ significantly (p < .05).
supplementation on eggshell quality characteristics has been reported (Li et al. 2006; Abdelqader et al. 2013). Świątkiewicz et al. (2010) attributed the positive effects of probiotics on eggshell quality parameters to the increased intestinal availability of Ca. The beneficial effects on eggshell thickness and strength observed in the current study were directly associated with the reduction in the number of damaged eggs. Under heat stress, it is well known that the eggshell strength is impaired and in turn, the incidence of cracked eggs exceeded. Therefore, many researchers have focussed their investigations toward improving eggshell quality using genetic, nutritional and biological approaches. Probiotic supplementation is the effective applied procedure in birds suffering from several stressors. Insignificant results of eggshell quality due to breed effect are in consistence with the results of Najib and Al-Yousef (2014) and Al-Homod (2016).

**Immune response**

Inflammatory response to PHA-P injected in wattle, as an indication for cell-mediated immunity, did not differ significantly as affected by probiotic supplementation level. Immunoglobulin (chicken IgY, IgA and IgM) levels in serum are indicative of the humoural immune status of chickens (Mountzouris et al. 2010). As well established, IgM is a potent complement activating antibody and since complement is needed for the generation of a normal antibody response (Parmentier et al. 2004). Generally, enhancing immunity resulting from probiotic administration has been reported by many investigators (Fong et al. 2015; Attia et al. 2017). Laying hens received a diet containing multi strains of probiotics exhibited a higher antibody production against SRBC compared with non-treated hens during high environmental temperature (Asli et al. 2007). Higher antibody production against Newcastle disease virus (NDV) was observed in the group fed a diet supplemented with *Lactobacillus acidophilus* compared to the control group (Forte et al. 2016). Under heat stress, dietary supplementation of probiotics improved humoural immunity against Newcastle disease virus and infectious bursal disease virus (Sohail et al. 2010). Contrarily, probiotic supplementation did not affect specific antibody synthesis to ND vaccine antigen administered via drinking water (Balevi et al. 2001). This non-stimulation of humoural immunity by probiotic may be attributable to the non-host specific strains, species or even genera of the microorganisms. Perdigon et al. (1990) showed that treating lagers with *Lactobacillus* supplementation increased cellularity of Peyer’s patches and this indicated a stimulation of the mucosal immune system, which responds to antigenic stimuli by secreting immunoglobulin (IgA). Diet supplemented with 0.1% *Lactobacillus acidophilus* induced the best immune response compared with 0.05% *Bacillus subtilis* in laying hens (Forte et al. 2016). The improvement in immune response found in Saudi chicken breeds compared with Leghorn chickens is in agreement with the results of Fathi et al. (2017). Likewise, Saudi laying hens significantly \((p<.01)\) recorded higher IgM concentration compared to Leghorn counterparts. In consistent with our results, Osei-Amponsah et al. (2013) confirmed that the local Ghanaian ecotypes of chickens were superior to exotic breeds in terms of their ability to respond to SRBC antigens. In a comparison of humoural immunity due to breed, Fathi et al. (2017) reported that there was no significant difference among breeds (Saudi, Leghorn and Lohmann) for antibody levels against Newcastle disease virus vaccine. Regarding the other types of immunoglobulins (IgA and IgY), the current results did not show significant differences among breeds. Contrary to our results, Mountzouris et al. (2010) reported that the use of probiotics may modulate the systemic immune system by increasing the total levels of serum IgG in broilers and be indicative of the overall humoural immune status of the bird. Koenen et al. (2002) also explored the effects of probiotics in the systemic humoural immune response, and found that different *Lactobacillus* spp. increase the levels of IgG in laying hens.

**Blood plasma biochemical concentrations**

The present findings are in agreement with those of Dimcho et al. (2005), who found that probiotic inclusion did not affect the serum total protein concentration of chickens. Also, Alkhalf et al. (2010) postulated that the serum concentrations of total protein and albumin were not significantly affected by dietary probiotic supplementation. The levels of total protein, albumin and globulin obtained in the present study are within the normal physiological values reported by Meluzzi et al. (1992) and Attia et al. (2011, 2016). It is generally known that blood plasma total protein plays key roles in the maintenance of colloid osmotic pressure, as a rapid substitute for indispensable amino acids, assuring glucose through gluconeogenesis, in transport of minerals and hormones, in forming enzymes and the immune system in the organism. Therefore, blood plasma proteins have an exceptional significance in homeostasis maintenance. Moreover, albumen serves as the most favourable source of amino acids for synthesis of tissue proteins in the
period of quick somatic growth of birds, especially under feed restricted conditions (Yaman et al. 2000; Filipoviae et al. 2007).

Reducing blood cholesterol level due to probiotic inclusion has also been reported in broilers fed a diet supplemented with prebiotic and probiotic (Sohail et al. 2010) and layers supplemented with probiotic (Kurtoglu et al. 2004; Sobczak and Kozlowski 2015). On the one hand, manipulation of enteric microflora with probiotics may also play an important role in altering the lipid metabolism of chickens as various studies have shown that probiotics could reduce cholesterol content in egg yolk and serum (Jin et al. 1998; Kalavathy et al. 2003; Li et al. 2006). On the other hand, Kurtoglu et al. (2004), Zarei et al. (2011) and Mohebbifar et al. (2013) reported that the dietary probiotic supplementation did not affect serum cholesterol or triglyceride. Insignificantly decreased serum triglyceride level is in consistency with Zhang et al. (2012) who reported that using Bacillus subtilis at level of 400 g/t of diet significantly (p < .05) reduced serum triglyceride level (1760.6 mg·dL⁻¹) compared with untreated group (2158.8 mg·dL⁻¹). Our results concerning blood plasma T₃ and T₄ concentrations in laying hens fed a diet supplemented with probiotics are in agreement with Chotinsky and Mihaylov (2013), who noted that the quantity of T₄ did not change significantly, while blood serum T₃ concentration increased with the supplementation of Lactobacillus acidophilus in the diets of broiler chickens. Plasma thyrotrophin levels were not significantly affected due to supplementation of Lactobacillus acidophilus in the diets of broiler chickens (Chotinsky and Mihaylov 2013). On the contrary, Sohail et al. (2010) revealed that dietary probiotic supplementation significantly increased (p < .05) serum T₄ concentration without affecting T₃ concentration. Also, Aluwong et al. (2013) reported that there was highly significant (p < .05) difference in T₄ level for probiotic treated group when compared with the control one. According to breed effect, it could be noticed that there were no significant differences in plasma T₃ and T₄ concentrations among breeds. The thyroid gland is an endocrine organ found in all vertebrates. The thyroid hormones are primarily involved in energy production by increasing the metabolic rate. It is well known that thyroid activity is important in controlling metabolic rate (Reyns et al. 2002). Bobek et al. (1976) showed that T₃ is the main thyroid hormone regulating oxygen consumption (bio-oxidation processes in cells), particularly in young chickens. Klandorf et al. (1981) confirmed that T₃ is, in chickens, a metabolically more active substance than T₄.

Conclusions

In conclusion, diet supplementation with probiotics did not improve laying performance. A beneficial effect resulting from probiotic supplementation on eggshell quality was observed without affecting internal egg quality traits under high ambient temperature. Also, plasma cholesterol and triglyceride reduced in laying hens fed a diet supplemented with probiotics. Furthermore, probiotic inclusion significantly increased IgM immunoglobulin concentration in laying hens fed a diet containing either 200 or 400 ppm compared with unsupplemented hens. Concerning breed effect, Saudi black laying hens recorded significantly higher egg mass compared with the other breeds. Also, both Saudi chicken breeds exhibited higher cell-mediated response and IgM concentration compared with Leghorn hens.

Disclosure statement

The authors alone are responsible for the content and writing of this article.

ORCID

Moataz Fathi http://orcid.org/0000-0001-9207-3861

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