The effect of *Bacillus amyloliquefaciens* on productive performance of laying hens

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**ABSTRACT**

This 6-weeks study was conducted to investigate the efficiency of *Bacillus amyloliquefaciens* as probiotic in laying hens. A total of 180 ISA brown laying hens (28-week-old) were randomly allocated into 90 cages (two hens per cage), and then assigned to three dietary treatments with 10 replicates per treatment (each replicate with three adjacent cages): (i) CON, basal diet (antibiotic-free); (ii) P1, CON +1.0 × 10^7 cfu/kg probiotic; (iii) P2, CON +2.0 × 10^7 cfu/kg probiotic. The probiotic was a commercial product. The Hen Day production was daily recorded. The egg quality parameters were measured by using egg multi-tester. The caecal contents and fresh excreta were collected for measuring the caecal microflora and excreta gas emission, respectively. All data were analysed using the GLM procedure of SAS. Differences among treatment means were determined using the Tukey’s range test. During 4–6 weeks, hens fed P1 and P2 diets had a greater \( (p < .05) \) egg production than those fed CON diets. Additionally, birds in P2 treatments had higher \( (p < .05) \) eggshell strength and eggshell thickness than those in CON treatment at 3 and 6 weeks. The caecal *Lactobacillus* numbers were increased \( (p < .05) \) while the caecal *Escherichia coli* and *Salmonella* concentrations and the excreta NH\(_3\) emission were decreased \( (p < .05) \) in the P1 and P2 groups. In conclusion, dietary *B. amyloliquefaciens* supplementation had positive effects on productive performance in laying hens.

**Introduction**

During last decades, the poultry industry has become one of the most dynamic and ever expanding sectors in the world. The intensive systems of rearing led poultry to stressful conditions, which resulted in decreased immunity and productivity (Foley et al. 2008). To prevent the infectious diseases and improve the performance, antibiotics have been widely added to poultry diets for long periods of time. However, the extensive use of antibiotics contributes to the increase in drug-resistant pathogens in animals that can potentially be transmitted to humans and negatively impact human health (Cully 2014). As a result, the increasing concerns about antibiotic resistance led to bans on antibiotics growth promoters (AGP) in the European Union in 2006. Thus, alternatives to AGP are desperately needed.

Probiotics, have been proved to be the most preferred and effective alternative to AGP and pathogens inhibitor in poultry industry (Patterson and Burkholder 2003). Presently, *Lactobacillus*, the genera *Bacillus*, *Bifidobacterium* and yeast are mainly used as probiotic preparations in broilers and laying hens (Khan 2013). Among these direct-fed microbials, the members of *Bacillus* have been considered to be the most promising because of their survival through the digestive process, germination within the digestive tract, and excretion through faecal matter (Cartman et al. 2008; Shivaramaiah et al. 2011). *Bacillus amyloliquefaciens* is a potent *Bacillus* strain that have capacity to produce extracellular enzymes including cellulase, \( \alpha \)-amylases, proteases and metalloproteases, which help to increase the efficiency of digestion and absorption of nutrients (Lee et al. 2008). Moreover, the bacteriocins such as subtilin and barnase produced by *B. amyloliquefaciens* possess antibacterial effects against pathogenic microorganisms (Lisboa et al. 2010). Most recently, several studies have demonstrated that dietary supplementation of *B. amyloliquefaciens* exert beneficial effect on nutrient digestibility, gut morphology and intestinal microflora.
thus improving the growth rate and feed conversion ratio in broilers (An et al. 2008; Ahmed et al. 2014; Latorre et al. 2015; Li et al. 2015a, b). These positive effects indicated that the _B. amyloliquefaciens_ could also reduce the noxious gas emission in chickens. However, as per our knowledge, reports about the role of _B. amyloliquefaciens_ in improving the productive performance and reducing the excreta noxious gas emission of laying hens are scarce. Therefore, the objective of the present study was to evaluate the effect of dietary _B. amyloliquefaciens_ supplementation on productive performance, egg quality, blood cell counts, caecal microbial shedding and excreta odour contents in laying hens.

**Material and methods**

**Birds, diets, and experimental design**

A total of 180 ISA brown laying hens (28-weeks-old) were used in this 6-weeks feeding trial. Hens were randomly allocated into 90 cages (two hens per cage, 38.1-cm width × 50-cm length × 40-cm height), and then assigned to three dietary treatments with 10 replicates per treatment (each replicate with three adjacent cages). The three dietary treatments as follows: (i) CON, basal diet (antibiotic-free); (ii) P1, basal diets +1.0 × 10^7 cfu/kg probiotic; (iii) P2, basal diets +2.0 × 10^7 cfu/kg probiotic. The probiotics used in this study are commercial products, and this probiotics preparation is manufactured by a commercial company (AOFENGGSHENGWU™, Aofeng Biological Technology Co., Ltd., Bingzhou, China). This product is brown powder and composed of complex strain of spray-dried spor forming _B. amyloliquefaciens_. The hens were housed in a windowless and environmentally controlled room that was maintained at 26°C with a daily lighting schedule of 16L:8D. There was one empty cage between every three cages. Hens were provided with free access to water and feed through the nipple of an automatic drinker and a common trough feeder, respectively. The experimental diets were formulated in accordance to recommendations of the breeder’s manual for ISA brown and to meet National Research Council (NRC) (1994) and provided in mash form, and the composition of the experimental diet is shown in Table 1.

**Sampling and measurements**

Daily records of egg production were maintained. Egg production was expressed as average Hen Day production, which was calculated from the total number of eggs divided by the number of days, and summarised on an average basis. A total of 30 saleable eggs (no shell defects, cracks, or double yolks) were randomly collected at 17:00 h from each treatment (three eggs per replicate) on a weekly basis. The egg quality of the collected eggs was then determined at 20:00 h on the day of collection. Egg weight, egg yolk height, egg yolk colour, and Haugh units were measured by using an egg multi-tester (Touhoku Rhythm Co., Ltd., Tokyo, Japan). Eggshell breaking strength was evaluated using a model II egg shell force gauge (Robotmation Co., Ltd., Tokyo, Japan). A dial pipe gauge (Ozaki MFG. Co., Ltd., Tokyo, Japan) was employed for measurements of the egg shell thickness, which was determined on the basis of the average thickness of the rounded end, pointed end, and the middle of the egg, excluding the inner membrane.

Ten birds were selected at random from each treatment (one hen per replication) on the final day of the
experiment. Whole-blood samples were collected from the wing vein of the laying hens using a K$_2$EDTA vacuum tubes. Red blood cells (RBC), white blood cells (WBC) and lymphocyte counts of the whole blood samples were determined using an automatic blood analyser (ADVIA 120, Bayer, Tarrytown, NY).

At the end of experiment, 15 birds per treatment (three birds per replicate, from the first five replicates) were killed by cervical dislocation. Ceca were ligated and caecal contents were immediately obtained and then placed on ice for transportation to the laboratory. The viable counts of microflora were analysed by the method of Wang and Kim (2011). In brief, bacteria were plated on MacConkey agar plates (SS agar; Difco Laboratories, Detroit, MI) and Lactobacillus medium III agar plates (Medium 638, DSMZ, Braunschweig, Germany) to isolate Escherichia coli, Salmonella and Lactobacillus, respectively. The Lactobacillus medium III agar plates were then incubated for 48 h at 39 °C under anaerobic conditions. The MacConkey agar plates were incubated for 24 h at 37 °C. The E. coli, Salmonella and Lactobacillus colony counts were enumerated immediately after removal from the incubator, and the results were presented as log10-transformed data.

For analysis of excreta odour contents, fresh excreta samples from each pen were collected at the end of the experiment. All fresh excreta samples was transferred to a sealed box and incubated for 30 h in an incubator (35 °C). After the fermentation period, the Gastec (model GV-100) gas sampling pump was used for gas detection (Gastec Corp., Kanagawa, Japan) for ammonia (NH$_3$), hydrogen sulphide (H$_2$S) and used detecting tube for total mercaptan (R.SH) (No. 3L, No. 4LT and No. 70L; Gastec, Kanagawa, Japan).

### Statistical analyses

Data were statistically analysed via ANOVA using the GLM procedure of SAS (SAS Institute Inc., Cary, NC). Differences among treatment means were determined using the Tukey’s range test. The mean values and standard error of means (SEM) were reported. Probability values of <0.05 were considered significant.

### Results

#### Productive performance

The effects of _B. amyloliquefaciens_ supplementation on egg production and egg weight in laying hens are shown in Table 2. Hens fed P1 and P2 diets had a higher (p < 0.05) egg production compared with those fed CON diets during 4–6 weeks. There was no difference in egg production between P1 and P2 groups. In addition, there was no difference in egg weight among treatment groups throughout the whole experimental period.

#### Egg quality parameters

Regarding the egg quality, at 3 weeks, birds in P1 and P2 treatments had higher (p < 0.05) eggshell strength and eggshell thickness compared with those in CON treatment (Table 3). At 6 weeks, eggshell strength in P2 groups was greater (p < .05) compared to the CON group, and birds fed P1 and P2 diets had higher (p < .05) eggshell thickness than those fed CON diets. There was no difference in eggshell strength and thickness between P1 and P2 groups. Moreover, yolk height, yolk colour, and Haugh units were unaffected by dietary treatments through the experiment.

#### Blood cell counts, caecal microflora and excreta odour contents

As shown in Table 4, none of blood characteristics (WBC, RBC and lymphocyte) changed in response to the addition of probiotic to the diets. The caecal _Lactobacillus_ spp. numbers were increased (p < .05) in the P1 and P2 groups compared with those of the CON group (Table 5). Escherichia coli and Salmonella spp. concentrations in the caecum were decreased (p < .05) when laying hens fed P1 and P2 diets. Additionally, the excreta NH$_3$ concentration in the P1 and P2 treatments was decreased (p < .05) compared with those in the CON treatment (Table 6). However, there was no difference in caecal microflora and excreta NH$_3$ concentration between P1 and P2 groups. Furthermore, no difference between groups was observed with respect to the H$_2$S and RSH emission of excreta.

### Discussion

Probiotics, including _Lactobacillus_, _Bacillus_, _Bifidobacterium_ and _Enterococcus_, are becoming
Table 3. Effects of dietary B. amyloliquefaciens supplementation on egg quality in laying hens.

| Items                        | CON  | P1     | P2     | SEM  | p value |
|------------------------------|------|--------|--------|------|---------|
| Yolk height, mm              | 8.10 | 8.20   | 8.12   | 0.15 | .91     |
| Yolk colour                  | 5.95 | 6.00   | 5.80   | 0.23 | .78     |
| Haugh unit                   | 87.05| 87.14  | 87.10  | 0.84 | .62     |
| Eggshell strength, kg/cm²    | 3.38 | 3.32   | 3.33   | 0.07 | .75     |
| Eggshell thickness, mm       | 0.378| 0.383  | 0.390  | 0.01 | .64     |
| Eggshell thickness, mm       | 0.381| 0.413  | 0.426  | 0.009| <.05    |
| Haugh unit                   | 87.05| 87.14  | 87.10  | 0.84 | .62     |
| Yolk height, mm              | 8.16 | 8.13   | 8.35   | 0.13 | .49     |
| Yolk colour                  | 5.75 | 6.10   | 6.03   | 0.28 | .88     |
| Eggshell strength, kg/cm²    | 3.21 | 3.55   | 3.61   | 0.08 | <.05    |
| Eggshell thickness, mm       | 0.38 | 0.413  | 0.426  | 0.009| <.05    |

Table 4. Effects of dietary B. amyloliquefaciens supplementation on blood cell counts in laying hens.

| Items                        | CON  | P1     | P2     | SEM  | p value |
|------------------------------|------|--------|--------|------|---------|
| WBC, × 10³/mm³               | 4.83 | 4.66   | 4.50   | 0.42 | .41     |
| RBC, × 10⁶/mm³               | 2.67 | 2.15   | 2.50   | 0.40 | .72     |
| Lymphocyte, %                | 69.57| 71.61  | 70.53  | 2.56 | .75     |

Table 5. Effects of dietary B. amyloliquefaciens supplementation on caecal microflora in laying hens.

| Items                        | CON  | P1     | P2     | SEM  | p value |
|------------------------------|------|--------|--------|------|---------|
| Lactobacillus, spp.          | 7.46 | 7.79   | 7.80   | 0.11 | <.05    |
| Escherichia coli             | 6.68 | 6.35   | 6.29   | 0.10 | <.05    |
| Salmonella, spp.             | 2.06 | 1.62   | 1.64   | 0.08 | <.01    |

Table 6. Effects of dietary B. amyloliquefaciens supplementation on excreta odour contents in laying hens.

| Items                        | CON  | P1     | P2     | SEM  | p value |
|------------------------------|------|--------|--------|------|---------|
| NH₃                          | 49.2 | 35.3   | 36.5   | 2.9  | <.05    |
| H₂S                         | 6.6  | 6.7    | 6.4    | 0.4  | .20     |
| RSH                         | 5.8  | 5.5    | 4.8    | 0.5  | .47     |

CON = basal diet (antibiotic free); P1 = CON + 1 × 10⁷ cfu of B. amyloliquefaciens/kg of diet; P2 = CON + 2 × 10⁷ cfu of B. amyloliquefaciens/kg of diet; SEM = standard error of the means (10 replicates per treatment, and three cages per replicate with two hens per cage).

As we known, eggshell quality was directly associated with egg breakage, and shell deformation decreased as shell weight and shell thickness increased (Chowdhury and Smith 2001). Previously, probiotics were reported to improve eggshell quality in laying hens (Panda et al. 2008). This study confirmed the beneficial effect of probiotics (B. amyloliquefaciens) on eggshell quality (eggshell strength and eggshell thickness). Presumably, the improvements in eggshell parameters were linked to the promoting effect of probiotics on metabolic processes and utilisation of calcium (Abdelqader et al. 2013). Although the mineral digestibility was not measured in this study, it is likely that the B. amyloliquefaciens could improve intestinal calcium digestion and absorption efficiency. Based on the enhancement of eggshell strength and eggshell thickness, which was observed in the current study, it is likely that dietary inclusion of B. amyloliquefaciens was able to reduce the number of unmarketable eggs.

The gut microflora plays a major protective function to keep the integrity of intestinal mucosa. Impairing this integrity leads to a progressive increase of mucosal permeability, which facilitates pathogens infection (Lambert 2009). It is well-accepted that probiotics play an important role in stabilising the gut ecosystem of animals by competing with pathogenic bacteria and improving the growth of beneficial bacteria in the intestine (Higgins et al. 2008). Likewise, the present study demonstrated that dietary supplementation with B. amyloliquefaciens probiotic increased the caecal

2007), especially after the European Union ban on AGP in 2006. Although many studies of the genera Bacillus in poultry have been published (Zhang et al. 2012; Zhang et al. 2013; Ahmed et al. 2014; Park and Kim 2014; Latorre et al. 2015), reports about B. amyloliquefaciens in laying hens are still limited. In this study, there was an improvement in egg production when B. amyloliquefaciens was supplemented to the diets of laying hens. Similar to our study, the beneficial effects of B. amyloliquefaciens probiotics on broiler performance (An et al. 2008; Ahmed et al. 2014; Li et al. 2015a,b) were observed. However, in another experiment on broilers conducted by Jerzsele et al. (2012), inclusion of B. amyloliquefaciens to diets did not even work. These inconsistency results indicated that the effect of probiotics is uncertain depending on various factors, such as the bacterial strains, dose and growth period of experimental animals. As for the mode of action, it is generally agreed that B. amyloliquefaciens have capacity to modify gut microbial composition, improve intestinal health and nutrient digestibility, consequently, the productive performance was enhanced (Ahmed et al. 2014).

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Lactobacillus spp. counts, whereas decreased E. coli and Salmonella spp. numbers. Similar results in broilers were obtained by Mallo et al. (2010), who reported that dietary B. amyloliquefaciens supplementation could increase Lactobacillus spp. population, and reduce E. coli population in the caecal digesta. Ahmed et al. (2014) also found that broilers fed B. amyloliquefaciens diet had lower caecal E. coli numbers. The positive effects of B. amyloliquefaciens on intestinal microbiota balance may be due to the competition for adherence sites and nutrients, and production of antimicrobial peptides. According to Ahmed et al. (2014), the lactic acid produced by B. amyloliquefaciens could cause a severe drop in intestinal pH, under the modulated intestinal environment, Lactobacillus spp. will be easy to colonise and recolonise the gut, while pathogenic bacteria will be inhibited. Furthermore, the B. amyloliquefaciens have capacity to produce bacteriocins, such as lantibiotic subtilin and mersacidin, which belong to peptide antibiotics group and could suppress the growth of potentially pathogenic bacteria (Lisboa et al. 2010; Ulyanova et al. 2011). Therefore, it can be concluded that dietary supplementation of B. amyloliquefaciens effectively modulated the intestinal microbiota and created a stable gut ecosystem, which could account for the significant improvement in egg production in laying hens.

Additionally, it is noteworthy that B. amyloliquefaciens supplementation obviously reduced the excreta NH3 concentrations in laying hens. In agreement with our results, Ahmed et al. (2014) reported that dietary administration of B. amyloliquefaciens linearly decreased the NH3 emission from broilers excreta. Similarly, Zhang et al. (2013) also found that application of Bacillus-based probiotics resulted in 26.5 and 37.9% decrease in excreta NH3 and H2S contents, respectively. It has been reported that probiotics could reduce the levels of pollutants arising from animal manure by improving nutrient utilisation, altering the intestinal microbiota ecosystem, and reducing the pH of manure (Ferket et al. 2002). Therefore, the reduction of excreta NH3 concentrations could be attributed to an improvement in nutrient digestibility, and a healthy modulation of gut microbiota composition, thereby allowing less substrate for fermentation in the large intestine. For poultry industry, this is of great interest because the B. amyloliquefaciens not only could provide positive effects on production, but it also could reduce the environmental pollution.

Finally, it should be noted that the present study failed to reveal any difference in analysed parameters between P1 and P2 groups (1 × 107 cfu/kg versus 2 × 107 cfu/kg B. amyloliquefaciens), this suggested that increased the concentration to 2 × 107 cfu/kg didn’t significantly improve the effect of B. amyloliquefaciens. However, due to there were only two different levels of probiotics in our study, further investigations are still need to evaluate the effects of graded levels of B. amyloliquefaciens in laying hens.

Conclusions

The present study demonstrated that the probiotic (B. amyloliquefaciens) showed positive effects on egg production, eggshell quality, intestinal microbial balance and excreta NH3 contents in ISA brown laying hens. However, this study involved a relatively small number of samples and a short time, so the beneficial effects of B. amyloliquefaciens are still needed to be further investigated in a larger population and over the long term.

Disclosure statement

No potential conflict of interest was reported by the authors.

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