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Effects of Probiotic (*Bacillus subtilis*) on Laying Performance, Blood Biochemical Properties and Intestinal Microflora of Shaoxing Duck

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Abstract: The study was conducted to evaluate the effects of probiotic supplementation (*Bacillus subtilis*) in diet on performance, blood properties and microflora of Shaoxing duck. A population of 200 laying ducks (160 days old) was divided into two groups each having five replications. The control (T_0) were fed on basal diet while T_1 with *Bacillus subtilis* 1×10^9 CFU/kg in addition to basal diet for thirty five days. The results showed that for T_1 group, the egg laying rate increased (3.79%) significantly, while a decrease of about 12.60%, 23.52% and 40.14% was observed in egg triglyceride, total cholesterol and malondialdehyde, respectively. However, the blood glucose content and alkaline phosphatase activity increased by 15.13% and 53.41%, significantly. Moreover, there was a decrease of blood cholesterol, uric acid and ammonia in T_1 by 28.86%, 22.39% and 62.5%, respectively. As compared to T_0 , T_1 showed a significant increased in the amount of caecal microflora, i.e. general aerobic bacteria (18.68%) and *Lactobacillus* (9.92%). In conclusion, supplementation of *Bacillus subtilis* in diets had significant effects on some aspects of egg composition, biochemical properties of blood and few microflora of Shaoxing ducks.

Key words: Probiotics, *Bacillus subtilis*, total cholesterol, shaoxing duck

INTRODUCTION

Probiotics are living microbes given orally to proliferate in the Gastrointestinal Tract (GI) of the host and create beneficial conditions for nutrients' utilization (Nahashon *et al.*, 2005; Jin, *et al.*, 1996; Jin *et al.*, 2000). Probiotic produce a positive balance of digestive microflora and limiting the damage caused by pathogenic bacteria, improves epithelial cell integrity and increased immune response (Vanbella *et al.*, 1990; Jin *et al.*, 2000; Wenk, 2000; Panda *et al.*, 2001; Linge, 2005). However, if microorganisms and/or substances, which contribute to the proper microbial balance, were added to the diet, the animal would continually receive a "boost" to establish the proper microbial population. Many investigators obtained data in the literature relating to the use of bacterial cultures to control and/or promote the proper environmental conditions for the establishment of an ideal microbial population in an animal's digestive tract through application of cultured bacteria. Species that have traditionally been regarded safe probiotics are *Enterococcus*, *Bifidobacterium* and *Bacillus* (Ishibashi and Yamazaki, 2001). Preventive application of probiotics achieved better utilization of nutrients and they have a positive effect on environment of gastrointestinal tract (Capcarova *et al.*, 2009). These act as growth promoters, feed savers, nutritional bioregulators and help in improving the performance and health (Vanbella *et al.*, 1990; Jin *et al.*, 1998), by releasing of proteases,

beta-mannanase and many other enzymes, these germinating and outgrowing spores assist in the feed digestion process (Hooge, 2003). Several studies demonstrated that the supplementation of probiotics to poultry diets increased performance of the birds, stabilized the intestinal microbial flora and also reduced incidence of disease (Samanya and Yamauchi, 2002; Hooge *et al.*, 2004). Therefore keeping in view the importance of the subject and rapid scarce of waterfowls the present study had been designed to evaluate the effects of probiotic (*Bacillus subtilis*) on performance, blood properties and caecum microbial population of the shaoxing ducks.

MATERIALS AND METHODS

The study was conducted to investigate the effects of supplementation (*Bacillus subtilis*) on the performance, biochemical changes in blood and caecum microflora of Shaoxing Ducks. A total of 200 shaoxing ducks (160-day-old at laying, (average weigh 1.72 ± 0.02 kg) were randomly divided into two groups, with five replications each group and 20 ducks per replication. Ducks were rare; in standard farm with spreaded litter (wheat brown 2-3 inches thick) ducks had free access of feed and water with facility of playground and fresh water pond. Control group (T_0) was only fed on basic diet (Table 1) for 35 days while treated group (T_1) was fed on basic diet with *Bacillus subtilis* (1×10^9 cfu/kg). The nutrients in the

Table 1: Ingredients and nutrient levels of basal diet

Ingredients	Percentage	Nutrients	Content (%)
Corn	46.5	DE (MJ/kg)	11.40
Soybean meal	23.0	CP	18.00
Rapeseed cake	8.0	Ca	3.09
Wheat flour	10.0	P	0.75
Mono calcium phosphate	1.5	Lys	0.95
Limestone	6.7	Met	0.35
Sodium chloride	0.3		
Premix compound ¹	1.0		

¹Premix compound each kilogram contained: vitamin A, 5,000 IU; cholecalciferol, 1,500 IU; tocopheryl acetate, 11 IU; menadione, 1.1 mg; thiamine-HCl, 3.0 mg; riboflavin, 5.0 mg; pyridoxine-HCl, 2.2 mg; cyanocobalamin, 0.66 meq; niacin, 44 mg; Ca pantothenate, 12 mg; choline chloride, 220 mg; folic acid, 0.55 mg; D-biotin, 0.11 mg; Mn, 80.0 mg; Zn, 60.0 mg; Fe, 30.0 mg; Cu, 5.0 mg; I, 2.0 mg; and Se, 0.15 mg. Premixes were formulated to meet recommended levels for minerals and vitamins (NRC, 1994). Composition was analyzed by AOAC (1990)

basal feed were analyzed by the method of Naumann and Bassler (1993) (Table 1). Nutrient levels of the basal diet were analyzed for CP, Lys, Met, Ca and P by the methods of AOAC (1990).

Sampling, testing and observations: Feed consumption, Egg number and weight were recorded daily during the whole test period. Egg laying rate, mean egg weight, daily egg mass, feed-egg ratio were determined using formula, 1, 2, 3 and 4 respectively.

$$\text{Egg laying rate} = \frac{\text{Total egg number}}{\text{Total duck number} \times \text{days}} \times 100\%$$

$$\text{Mean egg weight} = \frac{\text{Total egg mass}}{\text{Total egg number}}$$

$$\text{Daily egg mass} = \text{Egg laying rate} \times \text{Mean egg weight}$$

$$\text{Feed - egg ratio} = \frac{\text{Total feed consumption}}{\text{Total egg weight}}$$

Collection of eggs was performed weekly and three eggs of each replicate were randomly selected to analyze egg quality for egg weight, shell thickness, horizontal-vertical ratio, yolk colour and haugh unit. Concentrations of triglyceride, cholesterol and malondialdehyde in egg yolk were measured using commercial kits (Roche Diagnostics GmbH, Mannheim, Germany) on a biochemistry autoanalyzer (RX Daytona, Shining Sun Technology, Beijing, China).

At the end of experiment, three ducks with similar body weight of each replicate were selected, then weighed and slaughtered after fasting for 12 h. The heart, liver, spleen, pancreas of each duck were collected, weighed and kept at -80°C. Relative organ weight was determined according to the following formula: relative organ weight = organ weight/live body weight. Moreover,

After merciful killing, weight of internal organs, such as liver, spleen, pancreas and heart was recorded. Ten eggs from each replicate were randomly selected to collect the yolk. The egg yolk cholesterol was extracted by the method of Folch *et al.* (1956) as modified by (Washburn and Nix, 1974) and their chemical composition was determined according to the methods of Association of Official Analytical Chemists (AOAC, 1990). Caecal contents were also collected for microbial analysis.

Blood samples were collected from carotid artery blood-letting using 23 gauge needle. Serum was separated and purified by 10 minutes centrifugation (5,500 x g), then aspirated by pipette and kept in 1.5 ml, ependrof tubes at -80°C for further analysis. Biochemical blood indexes i.e. triglyceride, total cholesterol, glucose, uric acid, serum ammonia, alkaline phosphatase, glutamic oxaloacetic transaminase, Glutamic pyruvic transaminase were also analyzed by RX Daytona auto analyzer using (Roche Diagnostics commercial) kits.

Microbial analysis

Preparation of test sample: Ceacum content (1 g) was diluted in sterile diluents peptone water solution (9 ml) to make primary dilution (10^{-1}). Then a series up to 10^{-5} dilution was prepared by transferring primary dilution (1 ml) into test tube containing sterile diluents (9 ml) to obtain 10^{-2} dilution and repeating the operations with sterile diluents (9 ml) using the 10^{-2} and further dilutions to obtain 10^{-3} , 10^{-4} and/or 10^{-5} . The caecal content samples from each group were used to enumerate the count of total aerobic bacteria, total anaerobic bacteria, *Bifidobacterium*, *E. coli*, *Lactobacillus* and *Bacillus* by selective culture medium after collection of caecal contents.

Pre prepared test sample (1 ml) of 10^{-3} , 10^{-4} and/or 10^{-5} dilutions was transferred into petri dishes containing selective media in duplicate through dispensing pipette (100 µl) with sterile plastic tips and warm sterile agar medium (15 ml) was mixed with inoculums. The mixture was allowed to solidify and incubated. Parallel to that, control plates were also prepared using similar medium (15 ml) to check the sterility.

Counting of cecal microflora: The dishes containing more than 30 and/or fewer than 300 colonies were selected and counted using colony counter. The result was calculated using following formula:

$$N = \frac{\sum c}{(n_1 + 0.1 \times n_2) \times d}$$

$\sum c$: Sum of colonies counted on all the dishes retained.

n_1 : Number of dishes retained in the first dilution.

n_2 : Number of dishes retained in the second dilution.

d : Dilution factor corresponding to the first dilution.

Table 2: Effect of supplemental *Bacillus subtilis* on laying performance of Shaoxing ducks

Group	Live body weight (kg)	Egg laying rate (%)	Mean egg weight (g)	Daily egg mass (g)	Feed-egg ratio
T ₀	1.710±0.037 ^a	84.767±0.6 ^a	63.466±1.371 ^a	53.798±1.162 ^a	3.135±0.039 ^a
T ₁	1.699±0.054 ^a	88.100±0.9 ^b	64.182±1.157 ^a	56.544±1.019 ^a	3.020±0.093 ^a

Means in columns with different letters are significantly different from each other (p<0.05)

Statistical analysis: Data was processed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Paired-samples t test was chosen as compare mean to analyze index differences between group T₀ and T₁. The probability of (p<0.05) was considered as statements of statistical significance.

RESULTS AND DISCUSSION

The effect of supplemental probiotic *Bacillus subtilis* on the laying performance of Shaoxing ducks is presented in Table 2. Live body weight of birds was not significantly improved comparatively to control group; results showed that the supplementation of 1×10^8 *Bacillus subtilis*/kg diet brought about a significant (p<0.05) improvements in intensity of egg laying in supplemented group as compared to control groups. However, slight numerical improvements were observed in means of daily egg collection, feed conversion ratio and average egg weight, when birds were fed with 1×10^8 *Bacillus subtilis*/kg diet. It is of interested to note that egg weight did not decline by the increase of intensity of egg laying in *Bacillus subtilis* fed ducks. These results are in conformity with the findings of Zeweil and Ismail (1998) and Abdel-Azeem *et al.* (2005), who also indicated that egg production, egg weight and egg mass were improved in laying hens fed probiotic-supplemented diets. The positive effects of *Bacillus subtilis* supplementation to Shaoxing ducks could be due to decrease in the multiplication of harmful bacteria resulting from improvement in gut environment and enhanced nutrient utilization (Miles, 1993). *Bacillus subtilis* may, also, enhance enzymatic activity in the digestive tract resulting in improving nutrient utilization. The positive effects of probiotic supplementation, observed in the present study, are in agreement, with by El-Sheikh (2006).

The effects of the dietary supplementation with probiotic on relative weight of some internal organs of Shaoxing ducks are summarized in Table 3. These results suggested that the effect of probiotic supplementation as a feed additive has not significantly (p>0.05), affective on liver, spleen, heart and pancreas weights

Table 3: Effect of supplemental *Bacillus subtilis* on weights of selected organs

Organs(g)	T ₀	T ₁
Liver	47.03±2.39 ^a	48.81±2.64 ^a
Spleen	0.53±0.06 ^a	0.73±0.11 ^a
Heart	10.36±0.60 ^a	10.67±0.53 ^a
Pancreas	5.24±0.27 ^a	5.19±0.15 ^a

Means in rows with same letters are non-significant (p>0.05). T₀ = Control, T₁ = *B. subtilis* treated

Table 4: Effect of supplemental *Bacillus subtilis* on egg quality of Shaoxing ducks

Contents	T ₀	T ₁
Egg weight (g)	63.26±2.38 ^a	62.63±1.57 ^a
Shell thickness (mm)	0.39±0.01 ^a	0.40±0.00 ^a
Horizontal-vertical	0.77±0.01 ^a	0.76±0.01 ^a
Egg yolk color	11.20±0.37 ^a	11.00±0.00 ^a
Haugh unit	82.16±3.93 ^a	83.37±2.12 ^a
Triglyceride (mmol/L)	712.45±22.12 ^a	622.66±28.95 ^b
Total cholesterol (mmol/L)	126.96±2.79 ^a	97.09±2.29 ^b
Malondialdehyde	943.92±38.68 ^a	564.99±39.99 ^b

Means in rows with different letters are significantly different from each other (p<0.05). T₀ = Control, T₁ = *B. subtilis* treated

respectively. Jin *et al.* (1998) and Bozkurt *et al.* (2005) also found that dietary prebiotic and probiotic supplementation did not stimulate the internal organ weight of broilers. The present results are in disagreement with the findings of Hill *et al.* (1957), Visek (1978), Henry *et al.* (1986) and Engberg *et al.* (2000) indicated that dietary inclusion of feed grade antibiotics, given as growth promoters, reduced liver, spleen and intestine weight by thinning the intestinal wall evoked particularly by antimicrobial activity in gut lumen. However, a series of reports suggested similar antimicrobial mode of action for prebiotics (Iji *et al.*, 2001; Shane, 2001; Ferket, 2004), organic acids (Broek, 2000; Dibner, 2004) and probiotics (Vanbella *et al.*, 1990; Yeo and Kim, 1997; Wenk, 2000). Moreover, definitive data are lacking with respect to effects of dietary probiotics on the internal organs of poultry.

Analysis of the egg quality traits of shaoxing ducks after supplementation of *Bacillus subtilis*, data showed in Table 4. Probiotic (*Bacillus subtilis*) inclusion did not influence significantly on the egg weight, shell thickness, horizontal-vertical egg yolk color and haugh unit (p>0.05), are in agreement with the findings of Cerniglia *et al.* (1983), Mohan *et al.* (1995), Haddadin *et al.* (1996) and Chen and Chen (2003). Complementary reports by the Nahashon *et al.* (1996) and Haddadin *et al.* (1996) suggested that additional concentration of biological additives did not influence the egg quality significantly (p>0.05). But, present results are entirely different as reported by Nahashon *et al.*, 1992; Tortuero and Fernandez, 1995, showed that using vital biomass of probiotic supplements affects the egg quality significantly (p<0.05) that might be related to the concentration, strain of bacteria, induction root and the form of application (viability, dry or Liquid and their products). Damron *et al.* (1976) and Jensen *et al.* (1978) also found significant (p<0.05) improvements in interior

Table 5: Effect of supplemental *Bacillus subtilis* on biochemical parameters of blood in Shaoxing ducks

Contents	T ₀	T ₁
Glucose (GLU, mmol/L)	25.841±2.679 ^a	30.455±1.159 ^b
Triglyceride (TG, mmol/L)	4.886±0.200 ^a	3.958±0.345 ^a
Total cholesterol (T-CHL, mmol/L)	3.435±0.121 ^a	2.446±0.299 ^b
Uric acid (UA, mg/L)	90.270±4.044 ^a	70.054±1.698 ^b
Serum ammonia (SA, umol/L)	168.000±30.108 ^a	63.000±13.096 ^b
Glutaminoxaloacitate Transaminase (GOT)	31.767±2.203 ^a	33.587±1.548 ^a
Glutamic Pyruvic Transaminase (GPT)	43.324±1.752 ^a	43.050±0.892 ^a
Alkaline Phosphatase (ALP/100 mL)	11.547±0.895 ^a	24.774±4.587 ^b

Means in rows with different letters are significantly different from each other (p<0.05). T₀ = Control, T₁ = *B. subtilis* treated

egg quality as measured by hough units in hens fed distillers feeds and corn fermentation solubles. In some reports, presented by Nahashon *et al.* (1992) and Tortuero and Fernandez (1995) the dosages of probiotic and concentration of bacteria used in the diet produces significant effects on egg quality. In diets there are more bacteria per gram of feed comparing with Nahashon *et al.* (1996) and Haddadin *et al.* (1996), 2200 mg/kg vs 1100 mg/kg; 10⁹ cfu/gr feed vs 10⁶ - 10⁷ cfu/g feed respectively. Thus, increase of quality might be related to the vital form with higher doses up to 10⁹ cfu/g feed of probiotic.

In addition, probiotics had significant (p<0.05) effect on egg yolk cholesterol, shown in Table 4. Haddadin *et al.* (1996); Sutton *et al.* (1984) and Marks and Washburn (1991) also reported that inclusion of *Lactobacillus acidophilus* in three ages (40, 44 and 48 week) affects egg cholesterol in 40 week of production not 44 and 48. The results of present study revealed that the dietary probiotic *Bacillus subtilis* suppressed the bacterial growth that produce urease which correlate to blood uric acid and ammonia showed (Table 5), significantly decreased in treatment group (p<0.05) as compare to control. A similar effect was found when broiler chicks were fed a diet supplemented with *Lactobacillus acidophilus* (Tortuero, 1973; Francis *et al.*, 1978). In the present study, depressed net ammonia production in blood contents by dietary supplementation of probiotics (Table 5) might be due to absorption of ammonia by *Bacillus subtilis* (Headon and Dawson, 1990) and suppressing urease activity can be beneficial for improving health of waterfowls. Reduction of blood urea and ammonia concentrations due to supplemental dietary probiotics has been reported in rats (Duffy and Brooks, 1998), poultry (Balog *et al.*, 1994), rabbits (Hussain *et al.*, 1996; Al-Bar and Al-Aghbari, 1996) and steers (Hussain and Cheeke, 1995). However, Lowe and Kershaw (1997) observed that blood urea significantly increased in the cats. The Table 5 showed significant (p<0.05) increase in glucose and decrease in total cholesterol of blood. The present results and the findings of Yoon *et al.* (2004); Al-Kassie and Abd-Aljaleel (2007) are similar. However, Kim *et al.* (2003) reported that broiler fed on a diet contained *A. oryzae* caused a reduction in cholesterol level of blood serum of broiler. Same results were obtained by Al-Sodini (2005) by using probiotic *Aspergillus niger* as feed supplement.

Table 6: Effect of supplemental *Bacillus subtilis* on cecal microbe's concentration of Shaoxing ducks

Microbes (cfu/g)	T ₀	T ₁
Aerobic bacteria	8.317±0.161 ^a	10.226±0.121 ^b
Anaerobic bacteria	10.546±0.452 ^a	10.426±0.276 ^a
<i>Bifidobacterium</i>	10.341±0.123 ^a	10.772±0.565 ^a
<i>E. coli</i>	8.271±0.105 ^a	7.613±0.294 ^a
<i>Lactobacillus</i>	7.977±0.120 ^a	8.856±0.244 ^a
<i>Bacillus</i>	9.624±0.079 ^a	9.696±0.248 ^a

Means in rows with different letters are significantly different from each other (p<0.05). T₀ = Control, T₁ = *B. subtilis* treated

Addition of probiotics in feed improves the overall body biochemical factors, but stress of additional dietary supplement may be cause of increase on glucose in blood. Triglyceride in blood did not show significant difference in control and treatment group. However, alkaline phosphatase was significantly higher in treatment group. No significant difference was observed in glutaminoxaloacitate transaminase and glutamic pyruvic transaminase between control and treatment groups. The present results are in agreement with Abdel-Fattah *et al.* (2008), who reported non significant (p>0.05) difference in the SGPT and SGOT levels. Application of *Bacillus subtilis* as a feed supplement affects the microbes of caecum significantly (p<0.05) increased the population of aerobic microbes and *lactobacillus* (Table 6). The presented results are in agreement with the findings of Dimcho *et al.* (2005). In addition, no numerical improvement and degradation was observed in caecum general anaerobic bacteria, *Bifidobacterium*, *E. coli* and *Bacillus*. These results resemble with Hristev *et al.* (2004) who found that, probiotics did not influence on concentration of *Eenterococci* growth in Muskovy ducks. The results of current study gives line to observe further study about the probiotics on quality improvements. Moreover, Pandey *et al.* (2010) concluded that dietary supplementation of probiotics in the diet of poultry could be given without producing any adverse effect.

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