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Research article

Effects of probiotics (*bacillus subtilis* and *bacillus licheniformis*) on the performance and antimicrobial activity of broiler chicken

Md. Imran Hossain[1*], Md. Mufazzal Hossain[1] and Md. Mahfuj Ullah Patoary[1]

[1]Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh
[1*]Master of Science Student, Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka-1207, Bangladesh

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[*]*Corresponding author:*

Cell: +8801521213516
E-mail: imranhossain.an20@gmail.com

A B S T R A C T

This study was conducted to investigate the efficacy of probiotics on the performance and antimicrobial activity of broiler. A total of 300 one-day old of Cobb-500 broiler chicks were divided into five experimental groups (T_1-T_5) in completely randomized design with six replicates as ten chicks in each replication. One of the five experimental groups fed diet without probiotic was as control (T_1) and the remaining four groups fed diet with 4 levels of commercial probiotics were 20g BS/metric ton feed (T_2), 50g BS/metric ton feed (T_3), 20g BL/metric ton feed (T_4) and 50g BL/metric ton feed (T_5). Among the groups, T_5 showed significantly ($P<0.05$) higher body weight and lower feed intake than the others. Feed conversion ratio (FCR) was better in all probiotic treated groups compared to the control and the best FCR was found in T_5 group. The weight of breast, wing and giblet was significantly ($P<0.05$) higher in T_4 group and had no significant difference ($P>0.05$) in thigh and drumstick as compared to others treatment groups and control (T_1). Probiotics had significant difference ($P<0.05$) on percentage of moisture and dry matter but no significance difference ($P>0.05$) on water holding capacity (WHC) and ash percentage. Treatment groups found lower numbers of *Escherichia coli* and *Salmonella* but higher number of *Lactobacilli* rather than control group ($P<0.05$). Based on these results, it is concluded that dietary supplementation of probiotics improves the growth performance and intestinal microbial ecosystem of broiler chicken.

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1. INTRODUCTION

The rapid growth of poultry industry is greatly associated to the demands of the consumers for a healthier diet and meat as its essential component. Various feed additives like enzymes, emulsifiers, antioxidants, antibiotics and synthetic hormone have been randomly used as growth promoters for raising poultry production. Moreover, the concerns about antimicrobial resistant in bacteria from poultry production is increasing due to the existence of antimicrobial residues in meat (Reigh et al., 2008) and eggs (Goetting et al., 2011). Restrictions or total bans on the use of growth promoting antibiotics in poultry feed are currently in place, to limit and prevent harmful effects related with over usage, such as the onset of microbial antibiotic resistance (Hooge et al., 2004). Antimicrobial resistance is increasing and for this reason alternatives are currently being suggested and pursued, specifically targeting probiotics for use in the poultry industry (Zhang et al., 2012).

Probiotics can boost the growth rates of broiler chicken (Afsharmanesh and Sadaghi, 2014; Lei et al., 2015), It also helps to sustainability and enhancement of intestinal micro biota that can increase beneficial colonization against pathogens in the gastro intestinal tract. Probiotics are active in many ways against enter pathogens, including increased immunity-based elimination, fighting for mucosal attachment and vital nutrients, and developing antimicrobial complexes (Patel et al., 2015). In broiler nutrition, probiotic species belonging to *Bacillus*, *Bifidobacterium*, *Aspergillus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Candida* and *Saccharomyces* are commonly used for the prevention of poultry pathogens and diseases. *Bacillus* species are superior probiotic feed additives among the probiotics for poultry and pigs because of their large genomes with relevant characteristics; they are spore producers that keep the product viable for a long time and improve the intestinal integrity and growth efficiency of the bird (Vazquez, 2016). Important progress has been made in recent clinical assessments and trials of the probiotic *Bacillus subtilis*, revealing potential mechanisms of action, such as the antimicrobial effect of synthesis antimicrobial substance, the immune stimulatory effect, the antidiarrheal effect, the competitive elimination of pathogens, the avoidance of inflammation of the intestines and the normalization of intestinal microflora (Suva et al., 2016). Thus, this study was carried out to determine the effect of two probiotics *Bacillus subtilis* and *Bacillus licheniformis* on the performance of broiler chickens observing with caecal microbial population.

2. MATERIALS AND METHODS

Experimental design and birds

This experimental research was performed at the Sher-e-Bangla Agricultural University, Dhaka, on an open-sided poultry farm, with 300 one-day old Cobb-500 broiler chicks for a period of 28 days to assess the probability of using probiotic *(Bacillus subtilis* and *Bacillus licheniformis)* in commercial broiler diet on performance, carcass traits and antimicrobial activity of broiler chickens. The chicks were collected from Kazi farms limited hatchery, Dhaka, Bangladesh and the initial average weight of the chick was 43.4g. Chicks were randomly assigned to five experimental treatments with six replicates of ten chicks in each replication. One of the five experimental group fed diet without probiotic was as control (T_1) and the remaining four groups fed diet with four levels of commercial probiotics were T_2 (20g BS/metric ton feed), T_3 (50g BS/metric ton feed), T_4 (20g BL/metric ton feed) and T_5 (50g BL/metric ton feed).

Management

Prior to beginning of this study the experimental shed, feeder, drinker and other facilities were washed and disinfected. The chicks after arrival at poultry farm were weighed and allocated to different treatments on the basis of physical appearance and unique body weight. The birds were housed in 3ft × 2ft floor pens using fresh rice husk litter of 3 cm with a 24 hours lighting plan. Optimum temperature and humidity was maintained throughout the experiment according to the age of bird. The basal diet fed to the chicks in all groups was the same and were formulated to meet the NRC (1994) recommendations for broiler chickens (Table 1). The commercial probiotic was used in feed according to the manufacturer instructions. *Ad libitum* feeding was allowed for rapid growth of broiler chicks up to the end of the four weeks with fresh clean drinking water. According to the Cobb-500 manual, feeds were supplied four times daily and water two times daily. To measure actual feed consumption, left over feeds were registered. Digital electronic equilibrium was used to record the feed. Each experimental pen contained one feeder and drinker that was made of plastic. The scale of the feeder and drinker was adjusted according to the age of the birds. The feeders and drinkers were washed once regularly at the end of the week. According to the standard procedure of rearing and treatment of farm animals this experiment was carried out that was approved by the ethical committee of Sher-e-Bangla Agricultural University.

Table 1. The ingredients and chemical composition of the basal diet

Item	**Starter phase**	**Grower phase**
	1 to 14 (day)	**15 to 28 (day)**
Maize, 7.4% CP	53.80	56.76
Soybean meal, 44.5 % CP	38.79	35.12
Soybean oil (%)	2.30	3.28
Oyster shell (%)	1.58	1.5
Sodium bicarbonate (%)	0.19	0.17
Dicalcium phosphate (%)	2.02	1.9
Salt (NaCl) (%)	0.20	0.23
Vitamin premix*	0.25	0.25
Mineral premix**	0.25	0.25
DL- Methionine (%)	0.34	0.32
L- Lysine HCL (%)	0.20	0.16
L-Threonine (%)	0.08	0.06
Chemical composition		
ME (kcal/kg)	2900	3000
CP (%)	22.10	20.69
Methionine (%)	0.65	0.9
Lysine (%)	1.26	1.23
Methionine + Cysteine (%)	0.90	0.82
Calcium (%)	0.92	0.84
Available phosphorus (%)	0.41	0.38

***Supplied per kilogram of diet:** Vitamin A 10,000 IU; vitamin D_3 2,000 IU; vitamin E 10 mg; vitamin K 20 mg; vitamin B_1 2 mg; vitamin B_2 10 mg; vitamin B_3 15 mg; vitamin B_6 300 mg; vitamin B_5 10 mg; vitamin B_8 5mg; vitamin B_9 250 mg. ** **Supplied per kilogram of diet:** Manganese 500 mg; iron 250 mg, iodine 10 mg, zinc 600 mg; copper 100 mg, selenium 1 mg and cobalt 1 mg.

Vaccination program

Vaccination was applied to the experimental birds according to the vaccination schedule. One ampoule vaccine was mixed with purified water in compliance with the manufacturer's instructions. The cool vaccine chain was exclusively maintained until vaccination. The birds were vaccinated on proper schedule against New Castle Disease, Infectious Bronchitis and Infectious Bursal Disease.

Measurement of moisture, dry matter and ash percentage of broiler meat

Broiler chickens were slaughtered, bled, scalded, defeathered and carcasses were eviscerated by hand. The chilled carcass was then wiped dry with a cloth towel and divided into different parts such as breast, thigh, wing, drumstick, giblet (liver, heart, neck, gizzard) etc. After weighing, the carcass parts were frozen (-20℃) and stored prior to grinding in preparation for proximate analysis. The meat samples of slaughtered birds were dried in an oven at 70˚C for 24 hours and the meat moisture was determined as follows-

$$\text{Meat moisture (\%)} = \frac{\text{Initial weight-final weight}}{\text{Initial weight}} \times 100$$

The ash composition was measured by charring and then ashing the samples to a white ash at 720°C (Bouton et al., 1971).

Water holding capacity (WHC)

Water-holding capacity (WHC) was assessed by the method described by Jang et al. (2008). Breast meat of the slaughtered birds was ground in a food processor. Around two grams of minced meat was packed into a small polyethylene bag with small holes. The polyethylene bag with meat sample it was then put into a 5-mL glasses tube. The tube was centrifuged at 1500 × g for 5 minutes. The excess water was assessed by drying the samples overnight at 70°C after centrifugation (Jang et al., 2008).

Growth performance and characteristics of carcass

Body weight gains (BWG) of broiler chickens were measured weekly using digital electronic weighing scale in each replication. Feed intake (FI) and feed conversion ratio (FCR) were calculated and analyzed over the entire duration of each week. Breast, thigh, wing, drumstick, giblet and relative weight of each organ were calculated as follows-

$$\text{Relative weight} = \frac{\text{Organ weight}}{\text{Live body weight}} \times 100$$

Collection of sample and microbiological analysis

In each replication, three birds were randomly selected and slaughtered at the end of experiment. Both caecal pouches were collected from each bird and immediately deposited in a portable freezer at -20^{o}C, transported to the laboratory and deposited at -80^{o}C for microbial enumeration. One milliliter of the homogenized suspension was then transferred into 9 mL of anaerobic broth and serially diluted from 10^{-1} to 10^{-6}, in phosphate buffer solution out of which 100 μL were plated on agar plates. In order to isolate *Lactobacillus*, *Salmonella* and *Escherichia coli* bacteria the diluted samples were seeded on De Man, Rogosa and Sharpe (MRS) agar (anaerobic conditions at 39°C for 48 h), *Salmonella Shigella* (SS) agar and Eosin Methylene Blue (EMB) agar, respectively and incubated for 48 hours at 37°C. *Escherichia coli*, *Lactobacillus* and *Salmonella* colonies were counted immediately upon removal from the incubator. Results were expressed as log10 colony forming units per gram of cecum digesta (log10 CFU/g) (Hashemi et al., 2012).

Statistical analysis

All data were analyzed using SPSS 16.0 (SPSS Inc., USA). Significant differences among treatments were tested using one-way analysis of variance (ANOVA) followed by Duncan multiple comparison test. The level of statistical significance was preset at P<0.05 with standard error of the means.

3. RESULTS

Growth performance

The results were determined based on the performance (body weight, body weight gain, feed intake, feed conversion ratio) and carcass performance in broiler are summarized in Tables 2 and 4, respectively. Birds that received probiotics (*Bacillus licheniformis*) significantly consumed less feed and gained maximum body weight. Therefore, the feed conversion ratio was significantly better (P<0.05) where the experimental birds treated with probiotic (*Bacillus licheniformis*) were compared to control since the 4th week until the end of the experiment. There were significant (P<0.05) differences in breast, wing and giblet among the various treatment groups throughout the experimental period.

Table 2. Effects of probiotics on live weight (LW), body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) in broiler chicken

Treatment	LW (g/bird)	BWG (g/bird)	FI (g/bird)	FCR
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
T_1	1472.14^b±38.51	1430.13^b±38.54	2173.93^a±1.44	1.52^a±0.04
T_2	1592.07^a±35.81	1550.07^a±35.81	2150.97^b±1.86	1.39^b±0.03
T_3	1560.00^{ab}±26.70	1518.00^{ab}±26.70	2141.70^{bc}±1.21	1.41^b±0.03
T_4	1601.57^a±29.18	1559.57^a±29.18	2137.77^{bc}±1.57	1.37^b±0.02
T_5	1607.50^a±30.98	1602.17^a±25.39	2128.50^c±9.04	1.33^b±0.02
Level of significance	*	*	*	*

Here, T_1 = (Control), T_2 = (20g *Bacillus subtilis*/metric ton feed), T_3 = (50g *Bacillus subtilis*/metric ton feed), T_4 = (20g *Bacillus licheniformis*/metric ton feed), T_5 = (50g *Bacillus licheniformis*/metric ton feed), n=30, one-way ANOVA (SPSS, Duncan method). * Means within the same column with no common superscripts differ (P<0.05).

Characteristics of carcass

Value of the moisture content of meat, dry matter, ash and water holding capacity (WHC) of experimented broilers are given in Table 3. We observed that there was substantial difference (P<0.05) in moisture and dry matter but WHC and ash percentage didn't reveal any significance difference (P>0.05). In this analysis, the water holding capacity of meat was estimated to be between the range of 46.33-48.33%.

Microbiological assessment

The changes in *Salmonella, Lactobacillus* and *E. coli* bacteria count for the treated and untreated birds are illustrated in Table 4. Probiotics (*Bacillus subtilis* and *Bacillus licheniformis*) was fed in this study influenced (P<0.05) *Salmonella, Lactobacillus* and *E. coli* population in the caecal sample. As presented in Table 5 the control group (T_1) had the highest *Salmonella* and *E. coli* population but lowest number of *Lactobacillus* bacteria. Interestingly, the lowest *Salmonella* and *E. coli* population was observed in T_5 (50g BL/metric ton feed) group but found highest number of *Lactobacillus* population in T_5 group. In this analysis, it was found that the broiler treated with probiotic supplements had lower bacterial counts in caeca (P<0.05). In this experiment it was revealed that probiotic (*Bacillus subtilis* and *Bacillus licheniformis*) increases the beneficial bacteria in caeca but decreases the pathogenic bacteria.

Table 3. Effects of probiotics on water holding capacity (WHC), moisture, dry matter and ash percentage in broiler meat

Treatment	**WHC %**	**Moisture %**	**Dry matter %**	**Ash %**
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
T_1	46.67±1.20	76.33^{a}±0.33	23.67^{b}±0.33	0.90±0.06
T_2	46.33±0.33	75.33^{ab}±0.33	24.67^{ab}±0.33	0.90±0.06
T_3	48.33±0.88	74.67^{b}±0.33	25.33^{a}±0.33	0.87±0.03
T_4	46.67±0.88	75.00^{ab}±0.58	25.00^{ab}±0.58	0.90±0.06
T_5	48.00±0.58	75.00^{ab}±0.58	25.00^{ab}±0.58	0.87±0.03
Level of significance	NS	*	*	NS

Here, T_1 = (Control), T_2 = (20g *Bacillus subtilis*/metric ton feed), T_3 = (50g *Bacillus subtilis*/metric ton feed), T_4 = (20g *Bacillus licheniformis*/metric ton feed), T_5 = (50g *Bacillus licheniformis*/metric ton feed), n=30, one-way ANOVA (SPSS, Duncan method). * Means within the same column with no common superscripts differ (P<0.05). NS = non-significant.

Table 4. Effects of probiotics on organ relative weight of broiler chicken in 28 day

Treatment	**Organ relative weight (% of live body weight)**				
	Breast	**Thigh**	**Wing**	**Drumstick**	**Giblet**
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
T_1	21.7^{b}±1.38	11.17±0.39	7.19^{a}±0.14	11.07±0.24	7.76^{b}±0.15
T_2	23.36^{ab}±0.28	11.02±0.12	6.68^{b}±0.13	10.33±0.52	7.18^{b}±0.19
T_3	22.52^{ab}±0.61	10.91±0.14	6.90^{ab}±0.18	10.71±0.64	7.69^{b}±0.18
T_4	22.79^{ab}±0.37	10.62±0.13	7.14^{ab}±0.11	10.18±0.36	7.70^{b}±0.19
T_5	24.44^{a}±0.13	11.08±0.26	7.18^{a}±0.13	10.97±0.83	8.34^{a}±0.20
Level of significance	*	NS	*	NS	*

Here, T_1 = (Control), T_2 = (20g *Bacillus subtilis*/metric ton feed), T_3 = (50g *Bacillus subtilis*/metric ton feed), T_4 = (20g *Bacillus licheniformis*/metric ton feed), T_5 = (50g *Bacillus licheniformis*/metric ton feed), n=30, one-way ANOVA (SPSS, Duncan method). * Means within the same column with no common superscripts differ (P<0.05). NS = non-significant

Table 5. Effects of the probiotics supplementation on caecal bacterial population (log10 CFU/g) in broiler chickens at 28 days of age

Treatment	***Escherichia coli***	***Salmonella***	***Lactobacillus***
	Mean ± SE	Mean ± SE	Mean ± SE
T_1	$5.96^a \pm 0.01$	$5.92^a \pm 0.01$	$6.53^c \pm 0.01$
T_2	$5.83^b \pm 0.02$	$5.85^b \pm 0.00$	$6.57^c \pm 0.01$
T_3	$5.83^b \pm 0.02$	$5.84^b \pm 0.01$	$6.65^b \pm 0.02$
T_4	$5.81^b \pm 0.01$	$5.84^b \pm 0.01$	$6.68^b \pm 0.01$
T_5	$5.66^c \pm 0.03$	$5.77^c \pm 0.01$	$6.77^a \pm 0.01$
Level of significance	*	*	*

Here, T_1 = (Control), T_2 = (20g *Bacillus subtilis*/metric ton feed), T_3 = (50g *Bacillus subtilis*/metric ton feed), T_4 = (20g *Bacillus licheniformis*/metric ton feed), T_5 = (50g *Bacillus licheniformis*/metric ton feed), n=30, one-way ANOVA (SPSS, Duncan method). * Means within the same column with no common superscripts differ (P<0.05).

4. DISCUSSION

Growth performance

In this study, the present results indicated that inclusion of probiotic supplementation have positive effects on broiler growth performance. These results are consistent with the findings of Ghasemi et al. (2014), who found that probiotic feed supplementation promotes growth, feed efficiency and intestinal health. However, the findings of research on the effect of probiotic supplementation on the growth performance of broiler chickens are contradictory with the results of Edens (2003). At the same time, Ahmad and Taghi (2006) also showed an improvement in body weight gain when broiler diet was accompanied with probiotics (*Bacillus subtilis and Bacillus licheniformis*) during 21-42 days' period. Pelicano et al. (2003) suggest that the use of probiotics in broiler diet increased total body weight gain and improved FCR. Thus, meat quality was better when probiotics were fed in the water and diet instead of only in the diet. In the current research, birds fed higher concentrations of probiotics (*B. subtilis* and *B. licheniformis*) during starter, grower and finisher phases gain higher body weight, and the results are consistent with Tournut (1998) who reported that the effectiveness of probiotics depends on the quantitative and qualitative properties of the microorganisms used in the manufacturing of probiotic growth promoters.

Water holding capacity (WHC)

In this study, we found that supplementation of probiotics (*B. subtilis* and *B. licheniformis*) had no difference in percentage of water holding capacity and ash. These results are consistent with the findings of Pelicano et al. (2003), who showed that there were no disparities in water holding capacity, cooking loss and shearing force among different probiotics or between the treatments and the control group.

Feed consumption

In present experiment lower feed consumption was observed in probiotic (*Bacillus licheniformis*) supplemented group which is concurrence with the outcomes stated by before researcher (Shim et al., 2012) that supplementation of probiotic reduced the feed intake in comparison with the control group (P<0.05). Bansal et al. (2011) showed that addition of probiotics showed increased feed efficiency which is inconsistent with the finding of Al-Khalifa et al. (2019) who reported that dietary supplementation of probiotics did not exhibit any significant difference in feed intake between control and probiotic supplemented groups.

Feed conversion ratio (FCR)

In this analysis, the dietary feed conversion ratio (FCR) was slightly lower in chicks that had received probiotics (*Bacillus subtilis* and *Bacillus licheniformis*) from the first weeks of age to the end of the trial. These results are consistent with the findings of Manoj et al. (2018) who reported that using of probiotics in broiler diet dramatically increased body weight gain and feed efficiency. The better FCR in probiotic supplemented group might be due to the rapid development of beneficial bacteria in the digestive tract of host. According to Bedford (2000), in the intestine a more diverse micro flora population is expected to contribute to greater productivity in the digestibility and consumption of food, resulting in increased growth and better FCR.

Carcass characteristics

The result of the study demonstrated that the use of probiotic (*Bacillus licheniformis*) @ 50g BL/metric ton feed increased the percentage of breast, wing and giblet weight when compared to other group. Molnar et al. (2011) reported that *Bacillus* species supplemented group had significantly (P<0.05) higher breast yield than control group. Supplementation of dietary probiotics improved carcass yield and lowering the abdominal fat significantly without altering other performance parameters in broilers (Yalcin et al., 2016). On the other hand, Mahmoud et al. (2017) reported that birds of probiotic supplemented group and control did not demonstrate any statistically significant difference in carcass yield. Likewise, the current study didn't show statistically any significance difference (P>0.05) in thigh and drumstick weight of birds. However, Mohammad et al. (2016) reported that probiotic didn't show any effects on carcass traits, but the relative weights of drumsticks and wings showed increasing and decreasing linear responses, respectively, to probiotic supplementation level.

Microbiological assessment

In this study it was observed that dietary treatment of probiotic decreases the number of *Escherichia coli* and *Salmonella* population in the caeca but increases the *lactobacillus* population. The results are in line with the finding of Mohammad et al. (2016) who stated that probiotics lowered caecal *Escherichia coli* counts, but had no effects on immunity related organs or immune response. Lin et al. (2006) and Mountzouris et al. (2007) reported that probiotics in the diet of broilers caused higher concentrations of *Lactobacilli* and gram-positive cocci (e.g., *Enterococci*, *Pediococci*) in the caecal microflora compared with the controls. The current findings are in consistent with those of earlier researcher Song et al. (2014) reported that probiotic mixture contained *Bacillus licheniformis*, *Bacillus subtilis* decreased (P<0.05) viable counts of *E. coli* bacteria. It is known that in broiler nutrition, *Bacillus* such as other probiotic species have a beneficial effect on maintaining normal intestinal microflora by competitive exclusion and antagonism (Kabir, 2009). The addition of *B. subtilis* to broiler diets has several pathways in which it may improve production parameters. *B. subtilis* consume oxygen in the gut tract and additionally it produces certain enzymes, knowing subtilisin and catalase, which results in a positive environment for beneficial bacteria such as lactobacilli (Edens, 2003). The various suppositions identified with the effects of probiotics on broiler intestinal microflora in various studies might be attributed to the strain and the form of bacteria used and their concentration in dietary supplements.

5. CONCLUSION

The results of the present study demonstrated that supplementation of broiler diets with *B. subtilis* and *B. licheniformis* (1×10^{11} CFU/g of feed) for 28 days significantly decreased the caecal populations of *Escherichia coli* and *Salmonella* but slightly increased counts of lactobacilli. Body weight gain, feed conversion ratio, feed intake, hot carcass weight, moisture and dry matter percentage were significantly improved. In this study, the caecal microbial population revealed that there was a favorable environment in intestine that increased the nutrient digestion and absorption processes, which may illustrate the positive effects on the broiler growth performance. It can be concluded that dietary supplementation of probiotic 50g BL/Metric ton feed may have a benefit to promote growth performance of broilers.

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