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Effect of *Bacillus subtilis* Pb6 on Growth and Gut Microflora in *Clostridium perfringens* Challenged Broilers

A. Khalique, T. Naseem, N. Haque, Z. Rasool

***Abstract*—**The objective of current study was to investigate the effect of *Bacillus subtilis* PB6 (CloSTAT) as a probiotic in broilers. The corn-soybean based diet was divided into four treatment groups; T1 (basal diet with no probiotic and no *Clostridium perfringens*); T2 (basal diet challenged with *C. perfringens* without probiotic); T3 (basal diet challenged with *C. perfringens* having 0.05% probiotic); T4 (basal diet challenged with *C. perfringens* having 0.1% probiotic). Every treatment group had four replicates with 24 birds each. Body weight and feed intake were measured on weekly basis, while ileal bacterial count was recorded on day-28 following *Clostridium perfringens* challenge. The 0.1% probiotic treatment showed 7.2% increase in average feed intake (P=0.05) and 8% increase in body weight compared to T2. In 0.1% treatment body weight was 5% higher than T3 (P=0.02). It was also observed that 0.1% treatment had improved feed conversion ratio (1.77) on 6th week. No effect of treatment was observed on mortality and ileal bacterial count. The current study indicated that 0.1% use of probiotic had positive response in *C. perfringens* challenged broilers.

Keywords*—Bacillus subtilis* PB6, antibiotic growth promoters, *Clostridium perfringens*, CloSTAT, broilers.

I. Introduction

PROBIOTICS are viable bacterial cell preparations which manipulate gut micro flora in a way that their beneficial activities are stimulated and harmful activities are suppressed [1]. The emergence of cross resistance in pathogenic bacterial strains has led to the banning of antibiotic growth promoters (AGPs) all across the world. Incidence of necrotic enteritis has increased in areas that have stopped using AGPs [2]. Therefore, the need for alternative approaches is increasing and 'probiotics' has been proved to be one of the best alternatives. Many microbes have been used commercially, like *Lactobacillus* species, *Bacillus* species, *Enterococcus* species and *Saccharomyces* species [3]. Various species of *Bacillus* genus have been found effective for application in food and agricultural industries [4]. Due to spore forming, there is a huge advantage in the strain survival during pellet formation [5]. Spores of various bacillus species including, *Bacillus subtilis, Bacillus cereus, Bacillus clausii* are being used as probiotics [6]. *Bacillus subtilis* PB6 has been shown to produce a bacteriocin which is effective against both gram-positive and gram-negative bacteria that are potentially pathogenic for both humans and animals [7]. Many studies have evaluated the effect of this particular strain on intestinal health improvement in broilers against *Clostridium perfringens* induced necrotic enteritis [8]-[10]. To the best of our knowledge, no data is available locally in Pakistan to assess the efficacy of *Bacillus subtilis* PB6, so the objective of present study was to evaluate the effect of *Bacillus subtilis* PB6 on performance and intestinal health of Hubbard classic broiler birds with induced necrotic enteritis.

Anjum Khalique is with the Department of Animal Nutrition, University of Veterinary and Animal Sciences Lahore, Pakistan (e-mail: akhalique@uvas.edu.pk).

II. Materials and Methods

Three hundred and eighty-four Hubbard classic one-day-old broilers of average weight (37g) were procured from a local hatchery. Chicks were sexed on arrival day by vent sexing method and were randomly allotted to four treatment groups with four replicates with 24 birds in each replicate (384 birds). Each treatment group had equal number of males and females.

A. Dietary Treatments

A standard iso-nitrogenous and iso-caloric corn soya based diet for broilers was formulated without adding any growth promoter. The treatments in the study was divided into four groups; T1 (basal diet with no probiotic and no *Clostridium perfringens*, uninfected control); T2 (basal diet challenged with *C. perfringens* without probiotic, infected control); T3 (basal diet challenged with *C. perfringens* having 0.05% probiotic); T4 (basal diet challenged with *C. perfringens* having 0.1% probiotic). A commercial product with trade name CloSTAT containing *Bacillus subtilis* PB6® was used as probiotic with inclusion rate 500gm/ton and 1000 gm/ton for 0.05 % and 0.1 %, that gave 5×10^8, and 1×10^9cfu of beneficial bacteria per ton feed, respectively. Experimental diets were fed in two phases, starter crumbles (days: 1-21) and finisher pellets (days: 21-42). The diets were free of AGP and coccidiostat. The composition of basal diet has been presented in Table I.

B. Experimental Induction of Necrotic Enteritis

Necrotic enteritis (NE) was induced according to the disease model of [11]. On day 16, the birds of Treatment 2, 3 and 4 were given 10-fold dose of anticoccidial vaccine, Immucox, followed by inoculation of *Clostridium perfringens* culture contains 7×10^8 cfu/ml on days 18.

C. Performance Parameters

The parameters recorded were body weight gain, feed intake, mortality, feed conversion ratio, and feed efficiency.

Body weight and feed consumption were measured on weekly basis while the mortality was recorded on daily basis. Postmortem examination was carried out to investigate the cause of death.

TABLE I
COMPOSITION OF THE BASAL DIET (%)

Ingredients and Composition	Starter	Grower
Maize	44.38	46.1
Rice tips	20.7	20.8
Canola meal	11.16	11.2
Soybean meal	15	12
Corn Gluten 60%	2	2
Poultry by product meal	3.2	3.2
Chips	0.8	0.8
Salt	0.32	0.32
Di-calcium phosphate	0.8	0.8
Vitamin mineral Premix	0.1	0.1
Lysine sulphate	0.62	0.6
DL-Methionine	0.22	0.18
L-Threonine	0.12	0.1
Vegetable oil	0.5	0.18
Calculated Composition		
Crude Protein, (%)	22	20
ME (kcal/kg)	3000	3100

Vitamin mineral premix provided following per kg of diet

D. Enumeration of C. perfringens and lactobacilli

For enumeration of *C. perfringens* and lactobacilli, ileal contents (digesta content from the Meckel's diverticulum to ileo-caecal-colon junction) were taken into sterile bag and transferred immediately to university diagnostic laboratory. One gram of each sample was diluted ten- folds with sterile saline solution and subjected to 10 sequential dilutions. 0.1 ml of each sample was plated on duplicates by using selective media for enumeration of target bacteria. For *C. perfringens* enumeration, dilutions were plated on Reinforced Clostridial agar and perfringens agar base (PAB) and incubated at 37°C anaerobically overnight. Presumptive lactobacilli were enumerated on deMan Rogosa Sharpe agar. Birds were evaluated for Clostridium perferingens and lactobacillus content on day 28.

E. Statistical Analysis

The data collected for quantitative parameters was analyzed using analysis of variance technique (ANOVA) under Completely Randomized Design. Significant difference among the treatments was measured by using Duncan Multiple Range test.

III. RESULTS AND DISCUSSION

A. Feed Intake, Body Weight, FCR and Feed Efficiency

The present findings showed that T4 (0.1% probiotic) had 7.2% higher feed intake as compared to T2 (Table II). Higher level of probiotic containing sufficient number of *Bacillus subtilis* produced a positive effect on gut health by increasing lactobacillus count which aided better digestion and metabolism hence increased the feed intake. Also, T_4 and T_1 had comparative feed intake which showed that this level of probiotic had positive effect on feed intake. It was reported linear improvement in feed intake with increasing level of *Bacillus subtilis* based probiotic diets [12]. The results indicated that T_4 (0.1% probiotic), T_3 (0.05% probiotic) and T_1 (un-infected birds) had higher body weights in comparison with T_2 (negative control) as shown in Table II (P= 0.02). Both probiotic levels had comparable feed conversion ratios but T_4 showed better feed conversion ratio in later stages of development (Table III, P= 0.09). Highest feed efficiency was observed in T_4 in 6th week (Table IV, P= 0.1). However, no effect of treatment was observed on cumulative feed conversion ratio, feed efficiency and mortality but T_4 showed remarkable improvement in feed conversion ratio as compared to other treatments in 6th week. In agreement with the previous studies significant improvement in feed conversion ratio has been observed with the supplementation of probiotics at the end of rearing period. There was significant improvement in body gain of broilers after 4 weeks of feeding probioic [13] whereas, [14] reported improvement in feed conversion ratio during 21 to 42 days period. In contrast [9] did not find any effect on feed intake, body weight gain and feed conversion ratio using *Bacillus subtilis*.

B. Microbial Count and Antibody Titer

Antibody titer both in T3 and T4 was high on day 28 as compared to day 14 (Table V, P <0.05). However, the birds have not achieved the protective geometric mean titer of haem-agglutination inhibition against Newcastle Disease Virus (NDV) but in comparison the birds supplemented with probiotic showed better results. These results showing the immune-stimulatory effect of probiotic are in agreement with [15], [16].

In our study, T_4 and T_3 had numerically less number of Clostridium though statistically non-significant (Table V). These results are in line with [17] that *Bacillus subtilis* interferes with the colonization and persistence of bacterial pathogens in young chicken. A numerical increase in lactobacilli counts have been observed in *Bacillus subtilis* PB6 supplemented birds (Table VI). Beneficial effects of *Bacillus subtilis* PB6 on lactobacilli are well documented [7], [9], [10], [15], [18].

The overall performance of 0.05% probiotic treatment (T_3) was not different than the negative control (T_2) which showed that this level of probiotic was not sufficient enough to prevent the performance depression associated with sub clinical necrotic enteritis. However, T_4 (0.1 % probiotic) showed even better performance than T_1 (non-infected healthy birds).

In conclusion, *Bacillus subtilis* PB6 (CloSTAT) at 0.1 % level was effective to improve the performance and intestinal microbial balance in the gut.

TABLE II
EFFECT OF PROBIOTIC SUPPLEMENTATION ON PERFORMANCE OF BROILER FROM DAY 1-42

Parameters	T1 (Positive Control)	T2 (Negative Control)	T3 (0.05% probiotic)	T4 (0.1% probiotic)	Probability
Body Weight (g)	2209.75[a]	2053.71[b]	2112.83[ab]	2216.10[a]	0.02
Weight Gain (g)	2168.31[a]	2014.13[b]	2071.39[ab]	2176.04[a]	0.02
Feed Intake (g)	3686.69[a]	3464.22[b]	3529.29[ab]	3712.86[a]	0.05
FCR (g)	1.7011	1.722	1.704	1.706	0.96
Feed Efficiency	0.588	0.582	0.587	0.586	0.98
Mortality %	5.898	1.136	5.898	2.380	0.29

TABLE III
EFFECT OF PROBIOTIC SUPPLEMENTATION ON WEEKLY FEED CONVERSION RATIO

Weeks	T1 (Positive Control)	T2 (Negative Control)	T3 (0.05% probiotic)	T4 (0.1% probiotic)	Probability
First week	1.160	1.163	1.203	1.220	0.24
Second week	1.389	1.413	1.442	1.442	0.13
Third week	1.485	1.507	1.458	1.538	0.19
Fourth week	1.988	1.746	1.800	1.810	0.43
Fifth week	1.721	1.881	1.801	1.844	0.19
Sixth week	2.421 [a]	2.461 [a]	2.154 [ab]	1.835 [b]	0.09
Cumulative	1.701	1.721	1.703	1.706	0.96

TABLE IV
EFFECT OF PROBIOTIC SUPPLEMENTATION ON FEED EFFICIENCY

Weeks	T1 (Positive Control)	T2 (Negative Control)	T3 (0.05% probiotic)	T4 (0.1% probiotic)	Probability
First week	0.844	0.859	0.830	0.819	0.3
Second week	0.711	0.708	0.685	0.687	0.09
Third week	0.673	0.663	0.670	0.650	0.71
Fourth week	0.516	0.573	0.556	0.553	0.43
Fifth week	0.581	0.514	0.541	0.542	0.26
Sixth week	0.425 [ab]	0.409 [b]	0.475 [ab]	0.574 [a]	0.12
Cumulative	0.588	0.582	0.587	0.586	0.98

TABLE V
EFFECT OF PROBIOTIC SUPPLEMENTATION ON ANTIBODY HI TITER

Treatments	Day 14	Day 28
T1 (Positive Control)	4.25±0.25	4.25[b]±0.25
T2 (Negative Control)	3.5±0.5	4.5[b]±0.29
T3 (0.05%)	3.75±0.25	5[ab]±0.41
T4 (0.1%)	4.00±0.41	5.75[a]±0.25
Probability	0.53	0.02

TABLE VI
EFFECT OF PROBIOTIC SUPPLEMENTATION ON MICROBIAL COUNT ON DAY 28 POST HATCH (CFU/G)

Parameters	T1 (Positive Control)	T2 (Negative Control)	T3 (0.05% probiotic)	T4 (0.1% probiotic)	Probability
Clostridium	7.008×10^{6}	1.014×10^{7}	5.9123×10^{6}	6.855×10^{6}	0.93
Lactobacillus	9.325×10^{6}	8.828×10^{6}	1.315×10^{7}	1.376×10^{7}	0.64

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