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Bacillus subtilis PB6 Based-probiotic (CloSTAT™) Improves Intestinal Morphological and Microbiological Status of Broiler Chickens under *Clostridium perfringens* Challenge

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Abstract

The aim of the present study was to investigate the influence of a commercial *Bacillus subtilis PB6* based-probiotic, CloSTAT™, as an alternative to in-feed antibiotic, Enramycine, on growth parameters, intestinal morphology and ileal bacterial count of broilers during pre- and post-challenge with *Clostridium Perfringens* (*C. perfringens*) challenge. One hundred, 0-d- old Ross 308 male broilers were allocated in four experimental treatments for 30 days. The experimental treatments received a corn-soybean basal diet and were as follows: T1 = positive control (+CON): unmediated diet, unchallenged birds; T2 = negative control (-CON): unmediated diet, challenged birds; T3 = mediated diet with Enramycin, challenged birds (ENRA); and T4 = mediated diet with probiotic CloSTAT™, challenged birds (CS). Chicks in treatment 2 to 4 were challenged with *C. perfringens* on d 18. Five birds per treatment were sampled at 16 and 30 d of age for morphometric measurements of the small intestine and ileal bacterial count. Overall, feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) were not significantly different among the four treatments ($P>0.05$). On the other hand, CS supplementation caused some changes in the intestinal mucosa morphometrics, birds, which had received CS had longer jejunal and ileal villi as compared to other treatments ($P<0.05$). There was a significant ($P<0.05$) reduction in ileal *C. perfringens* count due to CS supplementation. The results from this study indicated that CS under the condition of this trail had a positive influence on broilers performance.

Keywords: Broiler; CloSTAT; Enramycine; Intestinal mucosa morphometrics; Performance

Introduction

The use of antibiotic in poultry feed at subtherapeutic levels as antimicrobial growth promoters (AGPs) have been beneficial for the improvement of growth performance and reducing the populations of potentially-pathogenic organisms such as *C. perfringens* and thus diseases associated with pathogenic bacteria (Butaye *et al*., 2003; Hume *et al*., 2011; Hafez, 2011). This positive effect for AGPs was explained by several mechanisms such as nutrients may be protected against bacterial destruction, absorption of nutrients may improve because of a thinning of the small intestinal barrier, the antibiotics may decrease the production of toxins by intestinal bacteria, and there may be a reduction in the incidence of subclinical intestinal infections (Hosoi *et al*., 2000; Butaye *et al*., 2003). However, recently there are increasing concerns about the risk of developing cross-resistance and multiple antibiotic resistances in pathogenic bacteria, which could result in proliferation of antibiotics-insensitive bacteria; this could lead to a decrease in the therapeutic effectiveness of antibiotics used by humans (Smith *et al.*, 2003; Castanon, 2007). Current trends in poultry production point to reduce use of AGPs and increase use of non-antibiotic feed additives. Probiotics are among the alternative growth promoters that are already used in practice (Teo and Tan, 2006, 2007; Talpur *et al*., 2012).

According to the manufacturer CS contains a unique strain of *Bacillus subtilis PB6* that inhibits the proliferation of *Clostridium* (CloSTAT, Kemin Industries Inc., Des Moines, IA). Several reports showed that *Bacillus subtilis* are capable of producing an antimicrobial factor against many bacteria, including *C. perfringens*, a causative agent of necrotic enteritis in broilers through the immunestimulating activity of the *Bacillus subtilis* (Muscettola *et al.*, 1992; Seah *et al.*, 2002; Yurong *et al.*, 2005; Teo and Tan, 2006, 2007; Rajput and Li, 2012).

The effect of *Bacillus subtilis* on BWG and FCR is well documented in broilers (Hooge *et al.*, 2004; Gil *et al.*, 2005; Teo and Tan, 2006, 2007; Melegy *et al.*, 2011; Javaid *et al.*, 2012). However, some of these reports tested the probiotic without induced challenge. Therefore, in the

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present study, the objective was to investigate the efficacy of *B. subtilis PB6* based-probiotic on the performance of broilers raised in cages under *C. perfringens* challenge.

Materials and Methods

Animals, Husbandry and Treatments

Ross 308 chicks were obtained from a commercial hatchery (Al-Wadi Poultry Farm Co., Riyadh, Saudi Arabia). Chicks were sexed and grouped by weight to reduce variation in mean body weight. A total of 100, 0-d old male broiler chicks allotted to 20 cages (50 cm length, 60 cm width and 36 cm depth) in a four-deck cage system and received the experimental diets in electrically heated battery brooders with raised wire floors. The chicks had been vaccinated for Marek's disease, Newcastle and Infectious Bronchitis. Birds were maintained at 24 h light schedule.

The *B. subtilis PB6* based-probiotic used in the current study was CS (CloSTAT, Kemin Industries Inc., Des Moines, IA). The product is commercially available and contains 10^9 cfu/t *B. subtilis PB6* according to the manufacturer. Birds received one of the following four treatments: T1 = Control diet (+CON); T2 = T1 + *C. perfringens* challenge (-CON); T3 = T2 + Enramycin 0.1 g/kg of feed (ENRA); T4 = T2 + 0.05% CloSTAT (CS). On d 16, birds which had received T2, T3 and T4 were challenged by *C. perfringens* by using an overdose of 10-fold dose of the anticoccidial vaccine, Paracox-8, followed by 1 ml of a cocktail containing *C. perfringens* inoculations (4×10^8 CFU) on days 18 and 20 according to Gholamiandehkordi *et al*. (2007). Culture of *C. perfringens* was obtained commercially (MicroBiologics, Cloud, MN, U.S.A.) for oral gavages of chicks.

A typical isocaloric and isonitrogenous starter (0-16 d) and finisher (17-30 d) diets based on corn-soybean meal diets were formulated in mashed form which met or exceeded the recommendations in commercial practice in Saudi Arabia (Table 1). Ambient temperature and relative humidity were concurrently and continuously recorded at 3 hours interval using two data loggers (HOBO Pro Series Data Logger, Model H08-032-08, Onset Co., USA) placed inside the chamber. The average temperature and relative humidity for the whole period were 24.95°C ± 0.26 (SD) and 26.63% ± 3.30 (SD), respectively. The study was conducted under a protocol approved by King Saud University and complies with the current laws of Saudi Arabia.

Carcass and Morphometric Measurements

At d 30, five birds per treatment were selected, after euthanasia, feather and skin, heads, necks, and shanks were removed, and the remaining carcasses were dissected to breast and leg quarter and were weighed. The percentage of yield of each part was calculated on the basis of dressed weight. At 16 and 30 d of age, the entire small intestine tract from five birds per treatment was removed aseptically, weighed and the total length was measured then was separated into duodenum, jejunum and ileum and for each part measurements of length and weight were taken. A 2-cm-long sample from each portion of the small intestine was collected for histology measurements. Samples were fixed in phosphate-buffered formalin for at least 48 h, after which they were embedded in paraffin. Sections of 5 mm were cut and stained with haematoxylin and eosin. Measurements of height and width were based on at least 5 well-oriented villi per section per broiler using an IX71 Inverted Olympus Microscope (Eyepiece: WH10X, Objective Lens: 4X) and a PC-based image analysis system (Olympus DP72 Microscope Digital Camera; Olympus NV, Aartselaar, Belgium) with software Analysis (Cellsens Digital Imaging Software for Research Application).

Enumeration and Identification of Bacterial Cells

Ileal digesta contents were aseptically emptied in a new sterile bag and kept in ice until time of analyses. One gram of each sample was diluted 1:9 (wt/vol) in sterile saline. All samples were subjected to 10 sequential dilutions 1:9 (vol/vol), and 0.1 mL of each sample was plated on duplicates by using selective agar media for enumeration of target bacterial groups. *C. perfringens* was counted by using tryptose sulfite-cycloserine (TSC) agar (Oxoid CM587, Basingstoke, Hampshire, England). Colonies on TSC agar that were suspected to be *C. perfringens* were plated by using blood agar (Garridol *et al.*, 2004). Enterobacteriaceae were isolated on MacConkey agar (Oxoiod CM7) after an incubation time of 24 h in an aerobic atmosphere at 37°C (Garridol *et al.*, 2004). Isolates of *Enterobacteriaceae* and *Salmonella* were identified by API 20E. The API 20E strips (bioMerieux Vitek) were inoculated, incubated at 37°C for 18 to 48 h, and interpreted as recommended by the manufacturer. Results were expressed as log10 colony-forming units per ml of ileal digesta ($\log_{10}$ CFU/ g).

Statistical Analysis

Data were analyzed by using the general linear model procedure of SAS (SAS, 2002-2003). A cage constituted the experimental unit. Four treatments were replicated 5 times in a randomized complete block design. Means for measurements showing significant differences in the analysis of variance were tested using the PDIFF option. Means ± SEM are presented in the tables and differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Table 2 shows the effect of treatment on broilers performance. During the starter and the finisher periods, BWG, FI and FCR were not influenced ($P>0.05$) by

treatment. As a result, cumulative FCR was not affected by treatment (P>0.05). However, there was a trend for the cumulative FCR, birds which had received CS had the lowest FCR value, while those which had received –CON had the highest value (P=0.07). The mean percentage of carcass parts in different treatments is documented in Table 3. Treatment had no effect on dressing percentage breast muscle yield, leg quarter yield and abdominal fat (P>0.05). However, relative liver weight was influenced by diet; birds which had received +CON had the heaviest weight as compared to all other treatments (P<0.05). The results of this study are in general agreement with previous report (Kwakernaak *et al*., 2007) who reported that *B. subtilis* spores supplemented to wheat-SBM diet did not improve cumulative BWG and FCR at 36 d. Other reports showed that *Bacillus subtilis PB6* based-probiotic improve performance of broiler chickens through different mechanisms such as stimulating the immune system (Teo and Tan, 2006, 2007).

Our findings correspond to findings by Pedroso *et al.* (2003, 2006), they tested several antibiotics including ENRA on the performance of broilers raised in batteries and on floor and reported that ENRA improved FCR and BWG when chickens were kept on the floor pens but not in batteries. We raised chicks in batteries and we did not see any positive effect for the ENRA.

The morphometric measurements of the intestinal epithelium samples at 16 d of age are given in Table 4. None of the parameters measured at 16 d was influenced by dietary treatment (P>0.05). At 30 d of age (Table 5), higher intestinal weight (g/cm) was obtained from birds which had received CS as compared to those which had received +CON (P<0.05). Longer jejunal villi were obtained from birds which had received CS and –CON as compared to the other two treatments (P<0.01). On the other hand, ileal villi were influenced by treatment; birds which had received CS had longer villus as compared to those received +CON or ENRA (P<0.05) but with no difference from those received the –CON. On the other hand, ileal villus width was not influenced by treatment (P > 0.05). Similar result was reported by Oliveira *et al.* (2008) who found that antibiotic supplementation caused low villi height; they explained that by the suppressing effect of the antibiotic for the beneficial bacteria in the gut, as *Lactobacillus* and *bifidobacteria*. Long villi are usually equated with excellent gut health, high absorptive efficiency and healthier intestinal tract of chickens (Alfaro *et al.* 2007). According to Cera *et al.* (1988), maximum absorption and digestion capacity is provided by a large luminal area with villus height and mature enterocytes and is essential to animal development.

Data related to ileal bacterial count in broilers at 16 and 30 d of age are presented in Table 6. Similar bacterial count of *C. Perfringens* and gram negative *Bacilli* were found in the starter period (before the challenge) (P>0.05). After the challenge, a significant decrease in *C. perfringens* was reported (P<0.05) in birds, which had received CS.

Table 1: Dietary ingredient (%) of broiler chick starter and finisher diets

Ingredients	Starter			Finisher		
	Treatment					
	1 & 2	3[A]	4[A]	1 & 2	3[A]	4[A]
Yellow corn	56.00	55.99	55.95	57.75	57.74	57.70
Soybean meal	36.10	36.10	36.10	34.0	34.0	34.0
Palm oil	3.80	3.80	3.80	4.80	4.80	4.80
DCP	2.30	2.30	2.3	2.0	2.0	2.0
Ground limestone	0.72	0.72	0.72	0.64	0.64	0.64
Choline chloride	0.10	0.10	0.10	0.05	0.05	0.05
DL-methionine	0.23	0.23	0.23	0.16	0.16	0.16
L-lysine	0.15	0.15	0.15	-	-	-
Salt	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin premix[B]	0.25	0.25	0.25	0.25	0.25	0.25
Trace mineral mix[C]	0.05	0.05	0.05	0.05	0.05	0.05
Enramycin	-	0.01	-	0.00	0.01	0.00
CloStat	-	-	0.05	-	-	0.05
Total	*100*	*100*	*100*	*100*	*100*	*100*
Calculated analysis						
ME, kcal/kg	3000	3000	3000	3100	3100	3100
Crude protein, %	22.0	22.0	22.0	21.0	21.0	21.0
Non phytate P, %	0.45	0.45	0.45	0.40	0.40	0.40
Calcium, %	1.0	1.0	1.0	0.9	0.9	0.9
Lysine, %	1.25	1.25	1.25	1.1	1.1	1.1
Methionine, %	0.55	0.55	0.55	0.47	0.47	0.47

[A]Diet 3 had 0.01% Enramycin, diet 4 had 0.05% CloStat on the expense of corn during starter and finisher

[B]Vitamin mix is supplied in the following per kg of diet: Retinyl acetate, 3.41 mg; cholecalciferol, 0.07 mg; DL-α-tocopheryl acetate, 27.5 mg; menadione sodium bisulphate, 6 mg; riboflavin, 7.7 mg; niacin, 44 mg; pantothenic acid, 11 mg; cyanocobalamin, 0.02 mg; choline 496 mg; folic acid, 1.32 mg; pyridoxine HCl, 4.82 mg; thiamine mononitrate, 2.16 mg; D-biotin, 0.11 mg

[C]Mineral-mix is supplied in the following per kg of diet: manganese, 67 mg; zinc, 54 mg; copper, 2 mg; iodine, 0.5 mg; iron, 75 mg; and selenium, 0.2 mg

Table 2: Body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) of broiler chickens given experimental diets at different ages

Characteristics	Treatment				SEM	p
	T1	T2	T3	T4		
Performance at 16 d						
BWG (g)	479.2	481.2	515.2	494.0	±14.3	NS
FI (g)	638.0	645.6	652.6	620.8	±22.8	NS
FCR(g: g)	1.333	1.342	1.267	1.257	±0.03	NS
Performance at 30 d						
BWG (g)	886.3	832.1	869.5	846.9	±26.8	NS
Feed (g)	1489.6	1468.9	1432.5	1377.5	±36.1	NS
FCR (g: g)	1.718	1.770	1.656	1.630	±0.05	NS
Cumulative						
BWG (g)	1345.3	1313.3	1384.6	1340.9	±35.5	NS
Feed (g)	2127.4	2114.5	2084.8	19998.3	±46.1	NS
FCR(g: g)	1.583	1.611	1.511	1.492	±0.03	0.07
Livability (%)	100.0	92.0	92.0	96.0	±6.0	NS

A 3.4 $\log_{10}$ reduction in the cell count of *C. perfringens* was found in CS as compared to the +CON. Challenged birds without any antibiotic or probiotic produced the higher counts all over the other groups. Our results clearly show that CS supplementation controlled the proliferation of *C. perfringens* in the broiler intestine. Several other studies used the same probiotic supplement as in this experiment

Table 3: Effect of different treatments on parts yield as percentages of broiler dressed weight at d 30

Yield parameters	Treatment				SEM	p*
	T1	T2	T3	T4		
Dressed yield (%)	60.9	60.7	62.1	61.7	±1.52	NS
Breast (%)[1]	35.6	35.2	34.2	33.6	±0.71	NS
Leg quarter (%)[1]	40.4	40.3	40.2	40.1	±0.64	NS
Abdominal fat (%)	1.05	0.80	1.26	1.78	±0.26	NS
Liver (g/100 g)	0.31[a]	0.24[c]	0.28[ab]	0.26[bc]	±0.01	*

[1]Breast and leg quarter were expressed as percentage of the carcass weight
[abc]means in the row with different superscripts differ significantly (* $p < 0.05$)

Table 4: Intestinal morphology and histology of broilers at d 16

Intestine characteristics	Treatment				SEM	p*
	T1	T2	T3	T4		
Intestine length (cm)	129.7	132.0	127.0	130.3	±7.20	NS
Intestine weight (g/ cm)	0.28	0.21	0.27	0.24	±0.02	NS
IRW[A] (g/100g BW)	7.4	7.6	7.2	8.0	±0.74	NS
Ileal *Villus height*[B] *(µm)*	4509	3765	3804	4053	±234.9	NS
Ileal *Villus width*[B] *(µm)*	693	766	653	640	±60.5	NS

[A]IRW: intestine relative weight
[B]Measurements of height and width were based on at least 5 well-oriented villi per ileum per broiler for a total of 5 birds per treatment

Table 5: Intestinal morphology and histology of broilers at 30 d of age

Intestine characteristics	Treatment				SEM	p*
	T1	T2	T3	T4		
Intestine length (cm)	152.0	178.0	176.2	174.3	±7.09	NS
Duodenum length (%)	15.7	16.6	14.8	15.3	±0.59	NS
Jejunum length (%)	52.1	41.8	43.2	41.1	±4.12	NS
Ileum length (%)	32.2	41.7	42.1	43.6	±4.18	NS
Intestine weight (g/ cm)	0.36[b]	0.44[ab]	0.41[ab]	0.51[a]	±0.03	*
IRW[A] (g/100g BW)	4.7	5.7	5.5	6.4	±0.35	NS
Villus height[B] *(µm)*						
Duodenum	8964	8488	8480	8924	±161	NS
Jejunum	7208[b]	8449[a]	7434[b]	8903[a]	±202	**
Ileum	5869[b]	6643[ab]	4692[b]	8004[a]	±616	*
Villus width[B] *(µm)*						
Duodenum	1245	1298	1635	1317	±102	NS
Jejunum	1109	938	1088	1004	±54	NS
Ileum	843	882	851	1088	±140	NS

[A]IRW: intestine relative weight
[B]Measurements of height and width were based on at least 5 well-oriented villi per section per broiler for a total of 5 birds per treatment
[abc]means in the row with different superscripts differ significantly (* $p < 0.05$, ** $p < 0.01$)

Table 6: Ileal bacterial count in broilers at 16 and 30 d of age

Bacterial species	Treatment				SEM	p*
	T1	T2	T3	T4		
Starter	Mean (log_{10} CFU/ g)					
C. Perfringens	4.2	3.9	4.3	4.4	±0.16	NS
Gram negative Bacilli	3.8	4.3	4.3	4.0	±0.22	NS
Finisher						
C. Perfringens	4.7[a]	6.0[a]	4.8[a]	1.3[b]	±0.72	*
Gram negative Bacilli	4.8	4.7	5.0	4.8	±0.15	NS

Measurements of were based on 5 broilers per treatment
[abc]means in the row with different superscripts differ significantly (*$p<0.05$)

and came to same findings (Jack *et al.*, 1995; Teo and Tan, 2006, 2007). The antimicrobial factor produced by *Bacillus subtilis PB6* is typical of gram positive bacteriocins in being broadly active against various strains of *Clostridium* species.

To check the anticlostridial effects of CS, a necrotic enteritis (NE) challenge was induced artificially by using *C. perfringens* to mimic a challenge that a bird could face in rearing facilities. According to Porter (1998) *C. perfringens* among the most gut-specific pathogens which is assumed to be the main health problem associated with removing the antibiotics from feed. *C. perfringens* infection of broilers may cause impairment of production performance (Lovland and Kaldhusdal, 2001), which was explained by the high level of bile salt hydrolase activity in the *C. perfringens* which causes growth depression (Feighner *et al*., 1987). Also, *C. perfringens* can cause a subclinical disease associated with NE which is characterized by damage to the intestinal mucosa that decreases digestion, absorption and reduces weight gains (Kaldhusdal *et al.*, 2001). Wilson *et al.* (2005) suggested that the growth suppressing effect of intestinal bacteria was due to the production of toxic metabolites that irritate the gut mucosa, thereby inhibiting nutrient absorption. On the other hand, our results regarding the effect of ENRA on bacterial counts relate to those of Pedroso *et al.* (2006) who reported that the number of bacterial genotypes found in the intestinal tract of chickens was not reduced by ENRA supplementation as compared to non-medicated diet in broilers.

None of the challenged birds produced overt clinical signs of NE and there were no mortalities associated with oral exposure to high doses of *C. perfringens*. This finding may be due to the absence of stress related to other diseases, diets or environment, all these factors with the presence of *C. perfringens* could cause an outbreak of NE (Ficken and Wages, 1997). However, many of the challenged birds showed distinctly pronounced pathological changes in the intestinal tissue. The gross examination of the responses in birds challenged orally with *C. perfringens* showed sub-clinical inflammatory responses throughout various sections of gizzard, duodenum, jejunum, ileum and ceca associated with intestinal lesions and hemorrhages.

Conclusion

In summary, CS reduced significantly the bacterial count of C. perfringens and improved the morphological status of the small intestine, as a result the cumulative FCR improved numerically in the group which had received the CS.

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