Effect of Dietary Supplementation of *Bacillus subtilis* PB6 (CLOSTAT™) on Performance, Immunity, Gut Health and Carcass Traits in Broilers

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Abstract: A feeding trial was conducted to study the effect of *Bacillus subtilis PB6* (CLOSTAT™) on performance, intestinal bacterial count, immunity and carcass traits in broilers. A total of 900 day-old broiler chicks (Cobb 500) were randomly assigned into two experimental groups. Each group was subdivided into six replicates with 75 birds each. Birds of group I served as a control and were fed on basal diet. Birds of group II were fed the basal diet plus 500 g CLOSTAT™ (2×10^7 CFU/g) / ton of feed. Results indicated that the CLOSTAT-supplemented group showed a significantly better ($P < 0.05$) final body weight (BW), body weight gain (BWG) and feed conversion ratio (FCR) compared to the birds in the control group. The CLOSTAT-supplemented group showed significantly higher ($P < 0.05$) dressing percent. However, there was a significant increase ($P < 0.05$) in lymphocyte count as well as Newcastle disease (ND) antibody titer in the CLOSTAT -supplemented birds. Bacteriological evaluation of the fecal samples revealed a significant reduction ($P< 0.05$) in total aerobic bacteria and *Clostridium perfringens* count in CLOSTAT- supplemented group. In conclusion, dietary supplementation with *PB6* could improve the performance, improve dressing percent, improve immune response, and have an antimicrobial effect against *C. perfringens in broiler*s.

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Key words: broiler, *Bacillus subtilis*, immunity, carcass

1. Introduction

Subtherapeutic use of antibiotics in poultry feeds has become undesirable because of the residues in meat products and development of antibiotic-resistant bacterial populations in humans (Barbosa and Levy 2000, Levy, 2002). This has prompted the European Union to phase out the use of these compounds as animal feed additives by the first of January 2006 (Perreten, 2003). As a consequence of the ban, feed additive-producing companies, have been looking for alternative solutions such as, acidifiers, prebiotics, probiotics and phytogenic extracts. Therefore, probiotic use has gained widespread interest. And of using living bacteria to replace antibiotics in poultry (Tortuero, 1973). Although there have been some conflicting studies, probiotics have been shown to improve weight gain and feed conversion ratios (FCR) and to reduce mortality (Jin et al., 1997a).

The use of competitive exclusion (CE) agents and probiotic feed additives in the livestock industry is therefore attracting increased attention as a cost-effective alternative to controlling animal disease and improving birds performance (Reuter, 2001). Probiotics are selected preparations of beneficial microbials, mainly *Lactobacilli*, *Streptococci*, and *Bacillus* species. Although modes of action are not entirely clear, probiotics are thought to influence the intestinal flora by CE and antagonistic activity to pathogenic bacteria for the host (Jin et al., 1997b), but to use correctly these "additives", we need to know better the environment of the gut and more precisely, the gut flora. Improving the knowledge about the gut flora is really important; because it appears that a slight modification of the balance between the different bacteria and other microorganisms of the gut can be the source of gut health issues causing economical losses. Therefore, in order to maintain a proper and healthy gut flora leading to better broiler productivity, our objectives were to study effect of CLOSTAT™ as a probiotic on broiler performance, intestinal bacterial count, immunity and carcass traits.

2. Materials and Methods

In this feeding trial, the CLOSTAT™ *(Bacillus subtilis* PB6), Kemin Europe, NV. Herentals, Belgium was added to one day old broiler chick (Cobb500) diets, (starter and grower- finisher) at a rate of 500 g / ton of feed for 6 weeks of experimental period, in order to investigate the effects of dietary supplementation of this product on performance, immunity (cellular and humoral), fecal microbiology and carcass traits.

This study was carried out at the Animal and Poultry Research Center, Faculty of Veterinary Medicine, Cairo University, Egypt. Care of the birds was in compliance with applicable guidelines from

Cairo University Policy on Animal Care and Use.

Birds and Diets

A total of 900 day-old broiler chicks (Cobb 500) were weighed individually. Chicks were randomly assigned into two experimental groups. Each group was subdivided into six replicates with 75 birds each. Birds of group I served as control and were fed on the basal diet (Table 1), while the birds of group II were fed the basal diet fortified by 500 gram CLOSTAT^{TM} / ton of feed. Chicks were floor reared in an electrically heated experimental room bedded by a layer of wood shavings, with a constant lighting program employed during the whole experimental period.The birds were provided with clean water and fed ad-libitum on the starter diet for the first three weeks and on grower-finisher diet up to 40 days**.** The Birds in the two experimental groups were fed ad-libitum on 2 stages basal diets formulated to meet all the nutrient requirements of the Cobb 500 broilers according to the recommendations established by the breed producers. Composition, calculated and chemical analysis of different diets were performed according to AOAC (1990) are illustrated in (Table 1). Diets were formulated by using UNE Form software linear programming, (1999). Diets were calculated based on the nutrient composition for the feeds published by the Central Lab for Food and Feed (CLFF), Ministry of Agriculture, Agricultural Research Center, Giza, Egypt (Technical Bulletin Nr.1, 2001)**.** All birds were kept under standard hygienic conditions.

Measurements

Performance Data

The weekly body weight and feed intake were recorded, to calculate, body weight gain (BWG) and feed conversion ratios (FCR) along the whole experimental period for each replicate. Mortality and morbidity were observed and recorded daily. FCR was adjusted accordingly.

Whole Blood Parameters

Whole blood samples (with soluble EDTA) were collected from five birds from each replicate via wing vein on the last day of the experiment and used for determinations of Total Leukocytic Count (TLC) and Differential Leukocytic Count (DLC): Lymphocytes %, Neutrophiles %, Eosinophiles %, Basophiles % and Monocytes % according to the method described by Feldman et al., (2000).

Immune Parameters

Haemagglutination (HA) and Haemagglutination Inhibition Test (HI)

The test was performed according to description of the Council of European Communities (1992). The test was carried out in a U-shapped bottom microtiter plates to determine the haemagglutination activity of Newcastle Disease Vaccine (NDV) (La Sota) in order to determine the number of haemagglutinating units of the virus to be used in the haemagglutination inhibition test. The geometric mean titer of haemaagglutination inhibition (HI) against Newcastle Disease Virus (NDV) at 21, 35 and 40 d of age was recorded.

Fecal Microbiology

Ten fecal samples were collected under aseptic conditions from each replicate by pressing the outer wall of cloaca to push its content into sterile tubes in both control and experimental groups and used for cultivation and counting.

Total Aerobic Bacteria

Cultivation and counting of total aerobic bacteria were done according to the method described by El-Afifi and Moustafa (2008).

Anaerobic Bacteria (Clostridia perfringens)

Clostridium perfringens count was done according to method described by Health Protection Agency (2004).

Carcass Traits

At the end of the experimental period, five birds from each replicate in both control and experimental groups were randomly chosen, left overnight in the waiting yard where only water was allowed. Each bird was weighed then hanged, slaughtered, scalded at 55-65°C, defeathered, eviscerated and washed with tap water. The carcass was then placed on a processing table where the breast meat (deboned breast meat yield without skin) was cut from the remaining upper back and rib cage of the carcass, washed, cooled in ice water tank for two hours, dried for ten minutes and the dressing yield (DY) and breast muscle yield (BMY) were recorded. The weight of liver, spleen, bursa and heart for each bird was recorded, and then the indices were calculated. The dissection of carcass was performed according to the procedures described by Jensen (1984).

Statistical Analysis

All data were processed by the t-test that evaluates the significance of differences among the treatment according to Cockran and Cox (1957) by using M stat-C software (1989) and the level of significance was set at a minimum at ($P< 0.05$).

3. Results and Discussion

Performance Parameters

Data concerning the performance represented by body weight (BW), body weight gain (BWG), feed consumption (FC) and feed conversion ratio (FCR) of birds in the control and CLOSTAT^{TM} - supplemented groups are illustrated in Table (2). Results of performance revealed that dietary supplementation of CLOSTAT^{TM} resulted in a significantly ($P<0.05$) improved body weight gain, feed efficiency and Low mortality. However, the feed consumption in both groups was not affected. The positive impact of dietary supplementation with CLOSTAT^{TM} on broiler performance could be associated with improved the total gut health, and are thought to be caused better absorption of different nutrients by the birds. These findings have been reported by other authors (Jiraphocakul et al., 1990; Maruta et al., 1996; Pelicano et al., 2005; Teo and Tan, 2007; El-Afifi and Moustafa, 2008 and Chen et al., 2009) who found that broilers treated with *B. subtilis* PB6 could improve the FCR up to 42 d of age, particularly in those that were challenged with *E. coli* (1.81; $P<0.05$). Furthermore, the previous researchers reported that there was a significant increase in the body weight of infected 42-d old broilers treated with *B. subtilis* PB6 when compared with the birds from the negative control group ($P<0.05$) and also found that these beneficial effects of CLOSTAT^{TM} include CE of pathogenic strains of *E. coli, Campylobacter jejuni* and *Salmonella enterica* serovar Enteritidis); are thought to be caused enhancing the growth and viability of beneficial gut microflora and could be associated with improved digestion and absorption of nutrients and viability in chickens.

Blood Profile

Results of blood profile concerning total (TLC) and differential leukocytic count (DLC) are shown in Table (3). CLOSTAT^{TM} supplementation resulted in leukocytosis manifested by a significant ($P<0.05$) increase of TLC and highly significant ($P<0.05$) increase in total lymphocytes percentage, however monocytes, eosinophils and basophils were not affected by dietary treatment. Our findings could be attributed to the immunostimulatory effects of CLOSTAT^{TM} on different subsets of immune cells to produce cytokines, which may in turn play a role in the induction and regulation of the immune response, (Pollman et al., 1980; Fais et al., 1987; Kirj avainen et al.1999; Christensen *et al.,* 2000; Lammers et al., 2003 and Rahimi et al., 2003).

Humoral Immune Parameters

Data concerning the geometric mean titer of haemaagglutination inhibition (HI) against Newcastle Disease Virus (NDV) at 21, 35 and 40 d of age are illustrated in Table (4). Results showed a significant increase ($P<0.05$) in NDV titer in birds fed the CLOSTAT^{TM} – supplemented diets at the age of 40 days. The results are thought to be associated with the humoral immune-stimulatory effect of CLOSTAT^{TM}. These findings have been reported by other authors (Fiorini et al. 1985; Muscettola et al. 1992; Havenaar and Spanhaak 1994 and Rowghani et al. 2007). While, Talebi et.al., (2008) found that the administration of the PrimaLac probiotic improved the antibody responses to NDV and Infectious Bursal Disease vaccination, but the antibody titers of the probiotic-treated group were not significantly different from control one.

Fecal Microbiology

Total Aerobic Bacteria

Microbiological examinations of fecal samples of each replicate in both control and experimental groups at different intervals throughout the experimental period are shown in Table (5). A highly significant ($P<0.01$) reduction in total aerobic bacterial count was noticed in the fecal samples collected from birds in the CLOSTAT^{TM} supplemented group. This could be associated with the inhibitory effect of *Bacillus subtilis* PB6 against aerobic harmful microflora. The inhibition mechanism of CLOSTAT^{TM} against such bacteria may include competition with pathogens for nutrients and binding sites of intestinal epithelium that are needed for growth and proliferation (CE) and prevent adhesion of pathogenic microorganisms to the intestinal wall. Adhesion is one of the most important virulence factors. These findings are in agreement with Maruta et al., 1996; Patterson and Burkholder, 2003; Mountzouris et al., 2007; Teo and Tan, 2007 and El-Afifi and Moustafa, 2008 who reported that broilers provided feed supplemented with *B. subtilis* PB6 experienced no reduction in the counts of beneficial intestinal bacteria, *Lactobacillus* species and *Bifidobacterium* species, examined. On the contrary, a 1- to 2-log^{10} reduction in the cell counts of *Clostridium* species and *E. coli* was observed in broilers provided feed supplemented with *B. subtilis* PB6.

Clostridium Perfringens Count

Effect of dietary supplementation of CLOSTAT^{TM} on *Clostridium perfringens count* (cfu/g) in fecal samples collected from each replicate in both control and experimental groups at different intervals throughout the experimental period are shown in Table (6). There was a highly significant ($P<0.01$) reduction in *Clostridium perfringens* count due to CLOSTAT ^{TM} supplementation. The antimicrobial factor produced by *Bacillus subtilis*

PB6 is typical of gram positive bacteriocins in being broadly active against various strains of *Clostridium* species. These findings are in agreement with Schuller et al., 1989; Jack et al., 1995; Teo and Tan, 2005 and Teo and Tan, 2007.

Carcass Traits

Data concerning the carcass traits at the end of experiment in the control and CLOSTAT^{TM} supplemented groups are presented in Table (7). Results revealed that there was a significant ($P<0.05$) increase in dressing yield of birds receiving CLOSTAT^{TM} - supplemented diets in comparison to those of the control group. However, breast muscle yield (BMY) and internal organ indices were not affected by *Bacillus subtilis* supplementation. The improvement observed in some of the carcass traits of birds receiving CLOSTAT^{TM} could be attributed to the overall improvement in growth, carcass yield and the yield of edible parts of broiler chickens as reported by Pelicanto et al., (2003), Haj Ayed et al., (2004) and Racevičiūtė-Stupelienė1 et al., (2007). In contrast, other studies have reported that probiotic supplementation had no effect on the carcass yield,. (Moreira et al., 2001; Karaoglu and Durdag, 2005 and Anjum et al., 2005).

Table 1. Composition and chemical analysis of basal control diets

Ingredient	Starter	Grower-Finisher
	%	%
Yellow corn	55.78	60.40
Soybean meal (44%CP)	29.19	23.83
Corn gluten meal (60% CP)	7.50	7.50
Soy oil	3.04	4.10
Common Salt (NaCl)	0.32	0.35
Sodium Bicarbonate	0.04	0.06
L-Lysine	0.20	0.18
DL-Methionine	0.06	0.07
Monocalcium phosphate	1.76	1.55
Limestone, ground	1.81	1.66
Broiler premix[1]	0.30	0.30
Total	100	100
Calculated analysis		
ME Kcal/kg	3050.00	3175.00
CP %	22.00	20.00
EE %	6.33	7.35
CF %	3.45	3.15
Lysine %	1.28	1.10
Methionine %	0.50	0.48
Met+Cys %	0.89	0.84
Ca %	1.00	0.90
P (total) %	0.81	0.74
P (available) %	0.50	0.45
Ca/P ratio	2.00	2.00
Chemical analysis		
CP %	22.20	20.030
EE %	6.46	7.5
CF %	3.51	3.3
Ca %	1.1	1.00
P (total) %	0.71	0.70

[1]Vitamin and mineral mixture contained: 13000000 IU vitamin A; 5000000 IU vitamin D_3; 80000 mg vitamin E; 4000 mg vitamin K; 5000 mg vitamin B_1; 9000 mg vitamin B_2; 4000 mg vitamin B_6; 20 mg vitamin B_{12}; 15000 mg pantothenic acid; 60000 mg Nicotinic acid; 2000 mg Folic acid; 150 mg Biotin; 400000 mg choline chloride; 20000 mg Copper sulphate; 1000 mg calcium Iodide; 50000 mg ferrous sulphate; 100000 mg Manganese oxide; 100000 mg Zinc oxide and 300 mg sodium selenite.

Table 2. Effect of dietary supplementation of CLOSTAT™ on broiler performance

Parameter	Control group[1]	CLOSTAT™ [2] -supplemented group	P value [4]
Initial body weight (g)	48.64 ± 2.1	48.56 ±1.9	0.18
Final body weight (g)[3]	1810.93 ± 47.8	1902.23 ± 20.2	0.002
Body weight gain (g)	1762.29 ± 44.4	1853.67 ± 17.2	0.001
Total feed consump./chick (g)	3421.43 ± 96.8	3432.85 ± 59.6	0.731
FCR (Feed:gain)	1.94± 0.025	1.85± 0.024	0.0004
Mortality %	3.56 ± 0.31	2.67 ±0.22	0.022

[1]Control group was fed the basal diet.
[2]CLOSTAT-supplemented group was fed the basal diet fortified by 500 gm CLOSTAT™ (2×10^7CFU/g)/Ton of feed.
[3]Data are Means ± SD for 6 replicates of 75 chicks per pen in each group.
[4]The level of significance was set at (P< 0.05).

Table 3. Effect of dietary supplementation of CLOSTAT™ on total and differential leukocytic count of broilers

Parameter	Control group[1]	CLOSTAT™ -supplemented group[2]	P value[5]
TLC[3] ($\times10^3$ / µl)	16.070 ± 0.87	17.757± 0.79	0.0055
DLC[4] Lymphocytes %	63.23 ± 1.5	70.96± 1.6	0.0001
Neutrophiles %	28.3 ± 1.5	20.83± 1.2	0.0001
Monocytes %	3.2 ± 0.3	3.13± 0.2	0.7009
Eosinophiles %	3.97 ± 0.3	3.83± 0.5	0.6084
Basophiles %	1.3± 0.2	1.23± 0.3	0.64

[1]Control group was fed the basal diet.
[2]CLOSTAT-supplemented group was fed the basal diet fortified by 500 gm CLOSTAT™ (2×10^7CFU/g)/Ton of feed.
[3]Whole blood samples were collected from 5 birds from each replicate.
[4]Data are Means ± SD for 6 replicates of 75 chicks per pen in each group.
[5]The level of significance was set at (P< 0.05).

Table 4. Effect of dietary supplementation of CLOSTAT™ on titer of haemagglutination inhibition (HI) against Newcastle Disease Virus (NDV) at different experimental periods

Age[3]	Control group[1]	CLOSTAT™ -supplemented group[2]	P value[5]
21 d[4]	3.27± 0.6	3.39± 0.5	0.20
35 d	6.26± 0.13	6.61± 0.2	0.022
40 d	6.22 ± 0.49	7.36 ± 0.48	0.038

[1]Control group was fed the basal diet.
[2]CLOSTAT-supplemented group was fed the basal diet fortified by 500 gm CLOSTAT™ (2×10^7CFU/g)/Ton of feed.
[3]Heparinized blood samples were collected from 5 birds from each replicate. LaSota (Intervet International B.V. Company, Boxmeer, Holland) was used as standard antigen to perform HI test.
[4]Data are Means ± SD for 6 replicates of 75 chicks per pen in each group.
[5]The level of significance was set at (P< 0.05).

Table 5. Effect of dietary supplementation of CLOSTAT™ on total aerobic bacterial count (CFU/g) in fecal samples collected from broilers at different times

Age[3]	Control group[1]	CLOSTAT™ -supplemented group[2]	P value[5]
21 d[4]	$1.20\times10^9\pm8.3$	$4.32\times10^8\pm3.2$	0.041
28 d	$1.68\times10^9\pm8.7$	$4.44\times10^8\pm2.7$	0.006
35 d	$1.86\times10^9\pm6.1$	$4.39\times10^8\pm2.9$	0.0004
40 d	$1.60\times10^9\pm4.8$	$4.28\times10^8\pm2.9$	0.0005

[1]Control group was fed the basal diet.
[2]CLOSTAT-supplemented group was fed the basal diet fortified by 500 gm CLOSTAT™ (2×10^7CFU/g)/Ton of feed.
[3]Ten fecal samples were collected under aseptic conditions from each replicate in both groups used for cultivation and counting of total aerobic bacteria.
[4]Data are Means ± SD for 6 replicates of 75 chicks per pen in each group.
[5]The level of significance was set at (P< 0.05).

Table 6. Effect of dietary supplementation of CLOSTAT™ on *Clostridium perfringens count* (cfu/g) in fecal samples collected from broilers at different times

Age[3]	Control group[1]	CLOSTAT™ -supplemented group[2]	P value[5]
21 d[4]	$1.9 \times 10^4 \pm 0.19$	$8.8 \times 10^3 \pm 0.17$	0.001
28 d	$4.5 \times 10^4 \pm 0.15$	$1.4 \times 10^4 \pm 0.2$	0.0001
35 d	$5.4 \times 10^4 \pm 0.36$	$1.0 \times 10^4 \pm 0.28$	0.0001
40 d	$5.4 \times 10^4 \pm 0.38$	$9.4 \times 10^3 \pm 0.24$	0.0001

[1]Control group was fed the basal diet.
[2]CLOSTAT-supplemented group was fed the basal diet fortified by 500 gm CLOSTAT™ (2×10^7CFU/g)/Ton of feed.
[3]Ten fecal samples were collected under aseptic conditions from each replicate in both groups used for cultivation and counting of *Clostridium perfringens*.
[4]Data are Means ± SD for 6 replicates of 75 chicks per pen in each group.
[5]The level of significance was set at ($P < 0.05$).

Table 7. Effect of dietary supplementation of CLOSTAT™ on carcass traits of broilers

Parameter	Control group[1]	CLOSTAT™ -supplemented group[2]	P value[5]
Dressing Yield [3]	75.58 ± 0.05	76.25 ± 0.06	0.048
Breast Muscle Yield (BMY) [4]	26.70± 1.2	27.20 ± 0.7	0.385
Liver Index	2.39 ± 0.1	2.23± 0.1	0.026
Spleen Index	0.10 ± 0.01	0.10 ± 0.01	0.562
Heart Index	0.57 ± 0.03	0.58 ± 0.03	0.834
Bursa Index	0.12 ± 0.02	0.13 ± 0.01	0.228

[1]Control group was fed the basal diet.
[2]CLOSTAT-supplemented group was fed the basal diet fortified by 500 gm CLOSTAT™ (2×10^7CFU/g)/Ton of feed.
[3]Five birds from each replicate in both groups were randomly chosen for carcass traits
[4]Data are Means ± SD for 6 replicates of 75 chicks per pen in each group.
[5]The level of significance was set at ($P < 0.05$).

Conclusion

Dietary supplementation of *B.subtilis PB*6 could be used to improve the performance, dressing yield and immune response as well as have an antimicrobial effect against *C. perfringens* in broiler chicks, helping to maintain an overall healthy gut microflora.

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