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EFFECT OF ORGANIC ACID BLEND AND *BACILLUS SUBTILIS* ON GROWTH, BLOOD METABOLITES AND ANTIOXIDANT STATUS IN FINISHING BROILERS CHALLENGED WITH *CLOSTRIDIUM PERFRINGENS*

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ABSTRACT

There is a growing concern over the use of antimicrobial drugs in poultry feed due to the risk of potential resistance of pathogens and the accumulation of antibiotic residues in meat. The present study was designed to compare the effect of an antibiotic, an organic acid and a probiotic on performance traits, blood biochemical parameters, ileal histology and antioxidant status in broilers during finishing phase exposed to *Clostridium perfringens* challenge. A total of 480 day-old broiler chicks (Ross 308) was randomly allocated to six treatments with eight replicates as follows: control (basal diet); T1: infected with *Clostridium perfringens;* T2: infected + Avilamycin at the rate of 0.2 g/kg; T3: infected + organic acid blend; T4: infected + consisting of a probiotic, *Bacillus subtilis*; and T5: infected + organic acid + probiotic. Results showed that body weight and feed conversion ratio (FCR) increased significantly ($P<0.05$) in T4 and T1 during fourth and third week, respectively. Further, results revealed that blood globulin and total protein decreased significantly ($P<0.05$) in birds in T1, while albumin increased significantly ($P<0.05$) in T1 and T2 during the third week. During the fourth week albumin, protein and glucose concentration increased significantly ($P<0.05$) in T5. Villus height increased significantly in T4 with no significant change ($P>0.05$) in plasma thiobarbituric acid reactive substances (TBAR) and total antioxidant capacity (TAC). From the results, we concluded that probiotic and organic acid could be successfully used as antibiotic to sustain growth and biochemical profile in broilers challenged with *C. perfringens*.

Key words: Antibiotic, broiler, *Clostridium perfringens*, organic acid, probiotic.

INTRODUCTION

Necrotic enteritis (NE) is one of the most expensive diseases in poultry caused by *Clostridium perfringens* invading the intestinal tract (Ao *et al*., 2012). Dietary and management practices such as stocking density, contaminated feed and other factors causing damage of the intestinal mucosa are linked with the pathogenesis of the disease (Ao *et al*., 2012; Jayaraman *et al*., 2013). For the past few decades, the antibiotics have been successfully used to improve the poultry production; however, the risk of cross resistance is a major concern in animal production, and consequently their use in feed has been either banned or restricted (Pereira *et al*., 2015; Abudabos *et al*., 2016). Ultimately, this scenario has led to the spread of high prevalence of economically important disease such as NE. Therefore, there is an increasing demand to produce more and more chickens under antibiotic free environment. Such demand can be managed by nutriceutical alternatives such as organic acid, phytogenetics, prebiotics and probiotics (Ao *et al*., 2012; Alzawqari *et al*., 2016; Raza *et al*., 2016; Chand *et al*., 2016).

Positive effects like increased growth and maintenance of healthy intestine using the beneficial bacteria such as *Lactobacillus spp., Bifidobacterium spp.,* and *Bacillus subtilis* have been previously reported (Khan and Naz, 2013; Jahromi *et al*., 2016; Alhidary *et al*., 2016). One of the most important bacteria, *B. subtilis* is used extensively in the feed and poultry industry to stimulate the positive changes in the intestine, recovery from diarrhea, enhanced feed efficiency and weight gain (Teo and Tan, 2005). The antimicrobial effect of *B. subtilis* against *C. perfringens* has been previously reported (Teo and Tan, 2006, 2007; Rajput and Li, 2012; Abudabos *et al*., 2013). In addition, many researchers have documented that the use of *Bacillus subtilis* as a probiotic plays an essential role in animal performance and health by adjusting the intestinal ecological imbalance (Abudabos *et al*., 2013; Khan and Naz, 2013; Khan *et al*., 2016). Bacillus species have been considered safe in animal production and can be easily administered to the animal as oral dose for weight gain and feed efficiency (Wu *et al*., 2011; Nguyen *et al*., 2015). The organic acids are considered alternative to antibiotics and improve the performance of birds (Hayat *et al*., 2014; Pereira *et al*., 2015). The exact action mechanism of these organic acids is not fully understood; however, they have been reported for their strong bacteriostatic and bactericidal activities (Pereira *et al*., 2015).

Therefore, the objective of this study aimed to evaluate the effect of an organic acid blend and a probiotic in comparison with an antibiotic on the performance, blood biochemical profile, ileal histology and antioxidant status in broilers during the finishing stage exposed to *Clostridium perfringens*.

MATERIALS AND METHODS

This experiment was approved by the Departmental board of Studies on Ethics, Methodology and Welfare, King Saud University, Kingdom of Saudi Arabia.

Experimental design and bird husbandry: A total 480 day-old broiler chicks (Ross 308) was randomly allocated to six treatments. Each treatment was further divided into eight replicates having twelve birds per replicate. On arrival, all chicks were confirmed for the absence of *C. perfringens*. The experiment was carried out in an environmentally controlled poultry farm at temperature 25±0.1 °C. A standard starter (0-14) and finisher (15-28 days) diets with isocaloric and isonitrogenous contents were offered as mash form as recommended by National Research Council (NRC, 1994). The composition of the basal diet is shown in Table 1. On day 15, the chicks were randomly distributed to one of the six treatments as follows: Control (basal diet), T1: infected with *C. perfringens*, T2: infected + Avilamycin at the rate of 0.2 g/kg (Maxus, Viena, Austria), T3: infected + organic acid blend containing phenolic compounds stimulating release of butyrate, medium chain fatty acids and organic acids at the rate of 1.0 g/kg (Presan, Trouw Nutrition, Ireland), T4: infected + a probiotic having viable spores (2×10^7 CFU/g) of *Bacillus subtilis (ATCC PTA-6737)* (Clostat, Kemin Industries Inc., Des Moines, IA, USA), T5: infected + Presan + clostat.

Challenge inoculum: On day 15, except for control group, birds were challenged by *C. perfringens* (MicroBiologics, Cloud, MN, U.S.A.) at the rate of 4×10^8 CFU/g as oral gavages (Ao *et al*., 2012). Necropsies of all the infected birds from the day of inoculation onwards were carried out to determine the cause of mortality. Typical signs of confluent necrosis and sloughing epithelium of the intestines were considered as the caused by NE. *C. perfringens* was isolated from the birds died as a result of NE.

Performance measurements: Feed intake on a daily basis was calculated by subtracting the amount of feed rejected from the feed offered. Total feed intake was computed for each group at the end of the third and fourth week. Body weight was measured on a weekly basis and feed conversion ratio (FCR) was computed for each group. Production efficiency factor (PEF) as suggested by Griffin (1979) was determined as follows:

PEF= (livability × live weight (kg) / (age in days ×FCR) × 100

Carcass measurements: At day 28, eight birds per treatment were randomly selected. After slaughtering, feather, heads and shanks were removed and the remaining carcass was dissected to separate breast and thigh. Similarly, fat, liver, intestines, heart, spleen and drumstick were also separated and weighed. The percentage of yield of each part was calculated on the basis of dressing weight of each bird.

Biochemical measurements of blood: At the end of the third and fourth week, two blood samples (3 ml) were obtained from the wing vein of the bird per replicate and centrifuged at 3000 rpm ×10 min. Serum was harvested, and then transferred into clean plastic tubes and stored at -20ºC until analysis. Serum total antioxidant capacity (TAC) and Thiobarbituric acid reactive substances (TBARS) were measured by using ELISA kits (Cayman Chemical Company, MI, USA). Total protein, albumin, glucose, alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured by enzymatic calorimeteric kits (M di Europa GmbH Wittekamp 30. D-30163 Hannover, Germany). All the analyses were carried out in duplicate.

Ileal histomorphology: On day 28, a 1 cm section of the lower ileum from two birds per replicate was cut, washed in physiological saline solution, and fixed in 10% formalin (buffered) for 3 days. The fixed intestinal segments were mounted onto slides after cutting up to 5 µm in thickness. The slides were stained using H & E stain. Measurements of height and width of the villi were based on at least 10 well-oriented villi per section using a microscope (IX71 Inverted Olympus) and PC-based image analysis system (Olympus DP72 Microscope Digital Camera; Olympus NV, Aartselaar, Belgium) with software Analysis (Cellsens Digital Imaging Software for Research Application).

Statistical analysis: All statistical analyses were performed using the Statistical Analysis System (SAS, 2003). Means of measurements showing significant differences in the analysis of variance were tested using the PDIFF option. The overall level of statistical significance was set at P<0.05. All values were expressed as statistical means ± standard error of the mean (SEM).

RESULTS

The findings of feed intake (FI), body weight (BW), FCR and PEF during the third week are given in Table 2. Results showed that the performance traits except FCR did not change significantly (P>0.05) during the experiment. In infected birds (T1), the FI increased and the corresponding weight gain decreased numerically. It is further important to note that FI, BW and PEF were not significantly different in antibiotic treated (T2) and other antimicrobial growth promoters suggesting the alternative role of organic acid and probiotics. In contrast, significantly (P<0.05) improved FCR was recorded in T2 compared to the other groups during the third week.

During the fourth week, weight gain was significantly (P<0.05) low in T1 and T5 compared to the other groups. No significant difference was found in FI, FCR and PEF in other groups between the control and the experimental groups as shown in Table 4.

The carcass characteristics of the control and the experimental groups are given in Table 4. Results revealed that dressing percentage decreased significantly (P<0.05) in T1 compared to the other treatments. Similarly, intestine weight also increased significantly (P<0.05) in T1.

Table 1. Dietary composition at finisher phase

Ingredients	Finisher
Yellow corn	59.56
Soybean meal	32.60
Corn oil	3.80
Di-calcium phosphate	1.80
Ground limestone	0.74
Choline chloride	0.05
DL-methionine	0.15
L-lysine	0.16
Salt	0.46
Threonine	0.07
Vitamins and minerals premix[1]	0.50
Chemical analysis	
ME (kcal/kg)	3100
Crude protein (%)	21.0
Non phytate P (%)	0.40
Calcium (%)	0.9
Lysine (%)	1.2
Methionine (%)	0.45
Sulfur amino acids (%)	0.80
Threonine (%)	0.85

[1]Vitamin-mineral premix contains in the following per kg: vitamin A, 2400000 IU; vitamin D, 1000000 IU; vitamin E, 16000 IU; vitamin K, 800 mg; vitamin B1, 600 mg; vitamin B_2, 1600 mg; vitamin B_6, 1000 mg; vitamin B_{12}, 6 mg; niacin, 8000 mg; folic acid, 400 mg; pantothenic acid, 3000 mg; biotin 40 mg; antioxidant, 3000 mg; cobalt, 80 mg; copper, 2000 mg; iodine, 400; iron, 1200 mg; manganese, 18000 mg; selenium, 60 mg, and zinc, 14000 mg.

The effect of treatments on the blood biochemical parameters in broiler during the third week are given in Table 5. The result revealed that blood albumin and total protein decreased significantly (P<0.05) in birds in T1, while albumin increased significantly (P<0.05) in the same group. Blood glucose, triglyceride, AST and ALT concentration did not differ significantly (P>0.05) between the control and treated birds. Similarly, the results of blood biochemical parameters in Table 6 during the fourth week showed that albumin, protein and glucose concentration increased significantly (P<0.05) in T5. However, no significant changes (P>0.05) were observed in globulin, triglyceride, AST and ALT concentration between the control and treated groups, respectively.

The antioxidant status in the form of the TAC and TBAR at the end of the fourth week is shown in Table 7. No significant change (P>0.05) was observed in TBAR and TAC at the end of the experiment.

Results of villus height and width of the control and experimental groups are given in Table 8. Findings showed that villus height decreased significantly (P<0.05) in the T1 and villus width did not differ significantly (P>0.05) in the experimental groups as well as in the control.

Table 2. The effect of treatments on feed intake (FI), body weight (BW), feed conversion ratio (FCR) and performance efficiency factor (PEF) of broiler chickens at the end of third week.

Treatments	FI (g)	BW (g)	FCR	PEF
Control	680.8	382.9	1.780^{ab}	211.1
T1	663.7	348.8	1.903^{a}	198.7
T2	627.9	380.3	1.652^{b}	217.2
T3	652.4	356.4	1.830^{a}	194.7
T4	677.6	376.4	1.803^{a}	205.2
T5	640.1	353.7	1.815^{a}	185.2
SEM	17.1	9.6	0.046	11.9

Mean values bearing different superscripts in a column differ significantly (P<0.05)
T1: Infected; T2: Infected + Maxus; T3: Infected + Presan; T4: Infected + Clostat; T5: Infected + Presan + Clostat

Table 3. The effect of treatments on feed intake (FI), body weight (BW), feed conversion ratio (FCR) and performance efficiency factor (PEF) of broiler chickens at the end of fourth week.

Treatments	FI (g)	BW (g)	FCR	PEF
Control	891.8	491.3^{a}	1.864	255.4
T1	919.7	400.4^{b}	2.384	188.2
T2	856.7	447.1^{ab}	1.920	234.2
T3	946.4	452.7^{ab}	2.110	210.8
T4	945.6	488.0^{a}	1.938	236.3
T5	851.9	406.6^{b}	2.139	194.3
SEM	26.9	22.6	0.154	17.7

Mean values bearing different superscripts in a column differ significantly (P<0.05)
T1: Infected; T2: Infected + Maxus; T3: Infected + Presan; T4: Infected + Clostat; T5: Infected + Presan + Clostat

Table 4. Effect of different treatments on parts yield as percentages of broiler dressed weight at 28 days of age.

Treatments	Dressing (%)	Leg (%)	Breast (%)	Fat (%)	Liver (%)	Heart (%)	Spleen (%)	Intestines (%)
Control	68.9[a]	28.4	37.1	1.2	4.4	1.0	0.2	6.2[d]
T1	63.7[b]	30.5	35.8	0.8	4.3	1.2	0.1	11.0[a]
T2	67.5[a]	28.4	36.1	1.5	4.8	1.0	0.2	8.9[bc]
T3	67.4[a]	28.3	36.1	1.2	4.1	1.0	0.1	9.1[ab]
T4	67.9[a]	28.3	37.9	0.9	4.5	1.0	0.2	8.7[bc]
T5	67.9[a]	28.8	38.3	1.2	4.9	0.9	0.1	6.9[cd]
SEM	0.76	1.40	1.66	0.25	0.27	0.08	0.02	0.87

Mean values bearing different superscripts in a column differ significantly (P<0.05)
T1: Infected; T2: Infected + Maxus; T3: Infected + Presan; T4: Infected + Clostat; T5: Infected + Presan + Clostat;

Table 5. Effect of treatments on blood biochemical parameters and liver enzymes of broiler chickens at the end of the third week.

Treatments	AST (U/L)	ALT (U/L)	TG (mg/dl)	Globulin (g/dl)	Albumin (g/dl)	Protein (g/dl)	Glucose (mg/dl)
Control	196.9	7.70	120.6	1.60[a]	1.78[ab]	3.37[a]	260.2
T1	204.2	6.18	130.2	0.75[b]	1.98[a]	2.73[b]	262.8
T2	209.5	5.28	89.1	1.08[ab]	1.96[a]	3.05[ab]	246.8
T3	216.5	5.62	128.5	1.51[a]	1.50[b]	3.02[ab]	263.2
T4	179.6	6.46	119.0	1.11[ab]	1.42[b]	2.53[b]	241.2
T5	231.0	7.56	84.8	1.21[ab]	1.60[ab]	2.81[b]	231.6
SEM	21.50	0.916	14.97	0.192	0.139	0.177	10.47

Mean values bearing different superscripts in a column differ significantly (P<0.05)
T1: Infected; T2: Infected + Maxus; T3: Infected + Presan; T4: Infected + Clostat; T5: Infected + Presan + Clostat

Table 6. Effect of treatments on blood biochemical parameters and liver enzymes of broiler chickens at the end of the fourth week.

Treatments	AST (U/l)	ALT (U/l)	TG (mg/dl)	Globulin (g/dl)	Albumin (g/dl)	Protein (g/dl)	Glucose (mg/dl)
Control	181.1	5.58	86.2	1.44	1.80[b]	3.24[a]	200.0[b]
T1	181.2	7.54	106.8	1.00	1.70[bc]	2.70[b]	189.8[b]
T2	221.9	7.92	113.5	1.30	1.29[e]	2.59[b]	198.2[b]
T3	195.3	8.08	97.8	1.22	1.45[ed]	2.67[b]	183.8[b]
T4	252.1	5.08	120.5	1.12	1.54[cd]	2.66[b]	195.4[b]
T5	195.8	4.68	99.3	1.03	2.25[a]	3.27[a]	269.4[a]
SEM	25.74	1.13	15.69	0.164	0.079	0.16	16.61

Mean values bearing different superscripts in a column differ significantly (P<0.05)
T1: Infected; T2: Infected + Maxus; T3: Infected + Presan; T4: Infected + Clostat; T5: Infected + Presan + Clostat

Table 7. Effect of treatments on plasma TBAR and TAC of broiler chickens at the end of the experiment.

Treatments	TBARS (mM)	TAC (mM)
Control	4.02	4.98
T1	3.57	4.78
T2	3.85	5.81
T3	4.16	4.98
T4	3.85	3.66
T5	2.66	3.66
SEM	0.46	1.00

T1: Infected; T2: Infected + Maxus; T3: Infected + Presan; T4: Infected + Clostat; T5: Infected + Presan + Clostat

Table 8. Effect of treatments on histological dynamics of broiler chickens given experimental diets at the end of the study.

Treatments	Villus height (µm)	Villus width (µm)
Control	799.6[abc]	63.9[a]
T1	756.5[c]	77.2[a]
T2	861.8[ab]	81.7[a]
T3	887.8[a]	86.9[a]
T4	892.7[a]	84.2[a]
T5	774.6[bc]	86.1[a]
SEM	34.51	4.72

Mean values bearing different superscripts in a column differ significantly (P<0.05)
T1: Infected; T2: Infected + Maxus; T3: Infected + Presan; T4: Infected + Clostat; T5: Infected + Presan + Clostat

DISCUSSION

The poultry researchers are focusing on the increased application of non-antibiotic feed agents to fill the gap of the banned antibiotics for improvement in the growth indices and feed utilization in broiler (Abduabos *et al*., 2013, 2015). The use of antibiotics at sub-therapeutic level has been produced useful outcome in growth of poultry, however, due to the possible antibiotic resistance and their residues in the animal products, a ban has recently been imposed. Several feed additives such as phytogenes, antioxidants, acidifiers, probiotics and prebiotics are being under scrutiny in poultry production to fill the gap left by the feed added antibiotics (Dhama *et al*., 2015).

In the present study, we found that the effect of an organic acid blend and a probiotic was similar to an antibiotic in term of growth traits and carcass characteristics, suggesting their potential to act as alternatives. Improved body weight and feed intake in broilers treated with probiotics in comparison with antibiotics in experimentally infected with different pathogenic bacteria such as *Salmonella*, *Clostridium perfringens* and *Escherichia coli* have been recently reported (Abudabos *et al*., 2013 & 2015; Al-Owaimer *et al*., 2014; Murshed and Abudabos, 2015). However, few information on the comparison of organic acid blend with antibiotic alone or in combination with a probiotic have probably been reported for the first time in our study. The higher performance traits in treated birds with a probiotic have been associated with the mechanism of action through which the useful bacteria exert their action through variety of mechanism such as reduction in the gut pH by the production of organic acids, competitive exclusion, production of antibacterial mucin and antibacterial enzymes (β-glucosidase and β-glucuronidase), potentiating the immune system, competition for nutrients in the gut and others (Khan and Naz, 2013). An improved performance in broilers in response to organic acid has been previously reported (Sultan *et al*., 2015), however, birds were not challenged with any bacterial inoclum. In other studies, it has been documented that the addition of organic acid significantly reduced Salmonella probably by inducing low pH in the gut thwarting the growth and proliferation of harmful bacteria but favors the growth of beneficial bacteria (Samanata *et al*., 2010; Sultan *et al*., 2015). In addition, acidic environment of the gut stimulates the secretion of pepsin, gastrin and cholecystokinin, playing an essential role in the feed utilization, and subsequently improving growth performance (Hayat *et al*., 2014).

In the present study, blood total protein decreased in the treated groups while globulin decreased significantly in T2 during the third week. In addition, albumin increased significantly in the T1 and T2 compared to the other groups. In the fourth week, the combination of organic acid and probiotic (T5) significantly increased the protein profile. No significant effect on blood metabolites particularly glucose in birds fed with organic acid has been previously reported (Mahdavi and Torki, 2009; Adil *et al*., 2010). In the previous studies, an increase in serum protein and albumin have been reported in probiotic treated birds (Arslan and Saatci, 2004; Yesilbag and Copan, 2006; Li *et al*., 2013; Rajput *et al*., 2013), however, in these studies, the birds were free from any bacterial infection. The discrepancy may be due to the genetic, dose and duration of the agents and other experimental conditions. From the improved result of glucose and protein profile during the fourth week, we suggest that these may be due to the fact that an acidic environment in the gut stimulates peptide-2 (Tappenden and McBurney, 1998) which may result in better absorption of glucose and protein in the gut.

In the present study, villus height decreased significantly in the infected group (T1) while villus width did not differ significantly between the control and the treatment groups. The pathogenic microorganisms are associated with the changes in the intestinal wall and absorptive capacity of the intestines. Such modification in the intestines is more pronounced in the upper part of the intestines than the lower part (Ao *et al*., 2012). No significant changes in villus height, width and area were documented in broiler simultaneously treated with *B.*

subtilis and *C. perfringens.* However, improved morphological dimensions were reported in broilers treated with an acidifier without any bacterial challenge (Ao *et al*., 2012). Jayaraman *et al*. (2013) reported improved villus height and width in response to supplementation of *B. subtilus* PB6 in *C. perfringens* challenged birds. The improved intestinal morphology in the treatment groups may be due to the organic acid and probiotic supplementation.

From our findings, we can concluded that probiotic and organic acid could be successfully used as substitutes to antibiotic in order to sustain the growth performance and biochemical profile in broilers challenged with *C. perfringens*.

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