



Effect of Two Strains of Probiotics on the Antioxidant Capacity, Oxidative Stress, and Immune Responses of *Salmonella*-Challenged Broilers

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■ Keywords

Immune responses, Probiotics, Oxidative stress, *Salmonella enterica*, Total antioxidant capacity (TAC).

ABSTRACT

The present study aimed at evaluating the effect of two strains of probiotics (*Bacillus subtilis* PB6 and *Bacillus Cereus* var. *toyoi*) on selected indicators of the antioxidant status and immune responses of broilers before and after challenge with *Salmonella enterica* subsp. *Typhimurium* (*S. typhimurium*). Birds were distributed into the following five treatments: negative control (CTL-); negative control+*S. typhimurium* (CTL+); antibiotic+*S. typhimurium*, (AB); Toyocerin®+*S. typhimurium* (TOYO); and CloSTAT™+ *S. typhimurium* (CLOS). Birds in all treatments, except for CTL-, were orally challenged with 3×10^9 CFUS. *S. typhimurium* at 16 days of age. The results showed that total antioxidant capacity (TAC), superoxide dismutase activity (SOD), and hydrogen peroxide (H₂O₂) values were similar among all groups. The antibody titers against *S. typhimurium* and Newcastle disease (ND) were not affected by the treatments ($p > 0.05$). However, *S. typhimurium* and ND titers were influenced by the interaction between week and treatment ($p < 0.01$, $p < 0.05$, respectively) and by week ($p < 0.001$, $p < 0.001$, respectively). In conclusion, *S. typhimurium* challenge had an impact on anti-*Salmonella* and NC titers. Both probiotics did not affect the broilers' antioxidant capacity.

INTRODUCTION

Salmonella enterica serovar *Typhimurium* (*S. typhimurium*) is an enteric bacterium that can colonize chickens; it is a common serovar causing salmonellosis in broiler chickens (Ribeiro *et al.*, 2007).

Probiotics have been reported to prevent the colonization of the gut by pathogenic bacteria like *Clostridium perfringens* and *Salmonella* through the mechanism of competitive exclusion (Teo and Tan, 2007). A *Bacillus subtilis*-based probiotic was capable of producing an antimicrobial factor against many bacteria (Yurong *et al.*, 2005). Other reports showed that a probiotic based on *Bacillus Cereus* var. *toyoi* reduced the prevalence of *Salmonella* in poultry (Vilà *et al.*, 2005).

Sahin *et al.* (2004) reported Japanese quails submitted to stress presented higher oxidative stress and lower antioxidant status than non-stressed ones. Probiotic supplementation was shown to modulate the dynamics of oxidants and antioxidants in the body. For example, probiotics based on *Bifidobacterium* and *Lactobacillus* showed good antioxidant effect by inhibiting lipid peroxidation *in vitro* using intact cells and intracellular cell-free extracts (Lin and Yen, 1999). Similarly, probiotics have been shown to increase antioxidant levels and reduce glutathione concentrations and intestinal oxidative injury (Yadav *et al.*, 2007). Sohail *et al.* (2011) demonstrated that a probiotic reduced the total antioxidant concentration. In another study, rats fed a probiotic showed increased SOD and glutathione peroxidase concentrations (D'Souza *et al.*, 2010).



Probiotics supplementation was reported to enhance disease resistance and improve the immune response of broiler chickens (Huang *et al.*, 2004). A study by Haghighi *et al.* (2006) suggested that probiotics have an immune-boosting role due to enhanced induction of natural antibodies in chickens.

Therefore, the objective of the present study was to evaluate the effect of two strains of probiotics (*Bacillus subtilis* PB6 and *Bacillus Cereus* var. *toyoi*) on selected indicators of antioxidant status and immune responses of broilers before and after challenge with *Salmonella enterica* subsp. *Typhimurium* (*S. typhimurium*).

MATERIALS AND METHODS

Animals and treatments

Two hundred day-old Cobb 500 broilers, vaccinated against Newcastle disease, infectious bronchitis and

Marek's disease, were allotted to 50 experimental cages (four birds per cage) in a controlled-temperature environment, under 24-h lighting, and were offered feed and water *ad libitum*. The experiment was carried out from day one to day 42.

A two-phase feeding program was applied (starter: 0–14 days of age; finisher: 15–42 days of age). Diet formulation is shown in Table 1. Five treatments were tested, each with ten replicates as follows: T1 = control group, non-medicated diet (CTL–); T2 = non-medicated diet and challenged with *S. typhimurium* (CTL+); T3 = antibiotic (0.05 gneoxyval/kg diet) and challenged with *S. typhimurium*, (AB); T4 = probiotic (1 g/kg diet Toyocerin®) and challenged with *S. typhimurium* (TOYO); and T5 = probiotic (1 g/kg diet CloSTAT™) and challenged with *S. typhimurium* (CLOS). Toyocerin® consists of 1×10^9 CFU/g viable spores of a microorganism *Bacillus Cereus* var. *toyoi*

Table 1 – Dietary ingredients and chemical composition of starter and finisher diets

Ingredients	Starter				Treatment ^a			
	CTL+ CTL-	AB	TOYO	CLOS	CTL+ CTL-	AB	TOYO	CLOS
	%							
Corn	63.01	63.01	62.91	62.91	70.09	70.09	69.99	69.99
Soybean meal	31.15	31.15	31.15	31.15	23.08	23.08	23.08	23.08
Palm oil	1.72	1.72	1.72	1.72	2.98	2.98	2.98	2.98
Dicalcium phosphate	1.96	1.96	1.96	1.96	1.87	1.87	1.87	1.87
Limestone	0.73	0.73	0.73	0.73	0.59	0.59	0.59	0.59
Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
VM Mix ¹	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DL-Methionine	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Lysine-HCL	0.18	0.18	0.18	0.18	0.17	0.17	0.17	0.17
Threonine	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.07
Sodium bicarbonate	0.12	0.12	0.12	0.12	0.11	0.11	0.11	0.11
Choline chloride	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.04
Neoxyval	0	0.005	0	0	0	0.005	0.005	0
Toyocerin	0	0	0.1	0	0	0	0.1	0
Clostat	0	0	0	0.1	0	0	0	0.1
Total	100	100	100	100	100	100	100	100
Chemical composition								
ME, kcal/kg	3000				3150			
Crude protein, %	20.5				17.28			
Methionine, %	0.55				0.51			
Lysine, %	1.2				0.98			
Sulfur amino acids, %	0.89				0.8			
Threonine, %	0.85				0.73			
Calcium, %	0.95				0.85			
Phosphorus, %	0.41				0.38			

¹Vitamin-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 B₂, 1600 mg; vitamin B₆, 1000 mg; vitamin B₁₂, 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.

^aT1=control group, non-medicated diet, unchallenged birds, (CTL+); T2=non-medicated diet + bacterial challenge (CTL-); T3=medicated diet with neoxyval (0.05 g/kg diet) + bacterial challenge (AB); T4= toyocerin (1 g/kg diet) + bacterial challenge (TOYO); and T5=CloSTAT (1 g/kg diet) + bacterial challenge (CLOS).



(Rubinum, Spain). CloSTAT™ contains 2×10^7 CFU/g of *Bacillus subtilis* PB6 (CloSTAT, Kemin Industries Inc., Des Moines, IA). At day 16 of age, all birds except for those in the CTL-treatment were orally inoculated with 3×10^9 CFU of *Salmonella* Typhimurium ATCC13311, according to the procedure described by Abudabos *et al.* (2014).

Measurements

Serum total antioxidant capacity (TAC), superoxide dismutase activity (SOD), and hydrogen peroxide (H_2O_2) levels were measured using commercial kits (Cell Biolabs, Inc., San Diego, USA). For this purpose, blood samples were collected from 10 randomly selected birds per treatment via brachial venipuncture and poured into plain tubes on day 42. Samples were centrifuged at 5 °C and 3,000 rpm for 10 min, and then the serum was harvested.

Anti-*S. typhimurium* and Newcastle disease virus (ND) antibodies were titered in the serum from 10 birds per treatment before (at 7 days of age) and after (at 21, 28 and 35 days) *S. typhimurium* challenge. Anti-*S. typhimurium* antibody titers were determined using a commercial ELISA kit (BioChek, London, UK). Microtiter plates pre-coated with inactivated *S. typhimurium* antigen were used. Serum samples were diluted (1:100) in diluent reagent and added to the wells of the microtiter plates to allow the formation of antigen-antibody complexes in the presence of anti-*S. typhimurium* antibodies. Anti-chicken IgG labelled with the enzyme alkaline phosphatase was then added to the wells. The chromogen added was para-nitrophenylphosphate (pNPP), and absorbance was read at 405 nm by using a microtiter plate reader.

A commercial Newcastle disease virus antibody test kit (IDEXX Laboratories, Inc., Westbrook, Maine, USA) was used for the detection of antibodies against ND. Serum samples were diluted (1:500) with diluent reagent and 100 μ L of samples were added to the microtiter wells. Plates were incubated for 30 minutes at 18-26 °C, and then washed with 350 μ L of distilled water, 100 μ L of conjugate were dispensed into each well, and after incubation for 30 minutes at 18-26 °C, 100 μ L of TMB substrate solution were dispensed into each well and 100 μ L of stop solution were dispensed to stop the reaction. Absorbance was read at 650 nm.

On day 42, 10 birds per treatment were slaughtered, and their liver, bursa, spleen, and thymus were collected and weighed. Their weights relative to carcass weight were calculated and expressed as percentages.

Statistical Analysis

Data were analyzed according to a completely randomized block design, with five treatments of 10 replicates each. The general linear model procedure of SAS (version 9.1. SAS Institute, Cary, NC) was used. The effects of week of collection, treatment and week x treatment interaction were tested. Means showing significant differences in the analysis of variance were compared using the PDIFF option. The overall statistical significance level was set at $p < 0.05$. All values were expressed as statistical means \pm standard error of the mean (SEM).

RESULTS AND DISCUSSION

Table 2 shows the serum TAC, SOD and H_2O_2 results of the birds submitted to the experimental treatments. Serum TAC, SOD, and H_2O_2 values were similar ($p > 0.05$) among all groups, suggesting that neither *S. typhimurium* challenge nor probiotics had an impact on total antioxidant capacity or oxidative stress. These results agree with Erdoğan *et al.* (2010), who concluded that the probiotics tested did not promote any significant improvement in the oxidant/antioxidant status in broilers. On the other hand, several other reports provided evidence that probiotics positively modulate the dynamics of oxidants and antioxidants in the body of chickens, Japanese quails, and rats (Lin and Yen, 1999; Sahin *et al.*, 2004; Yadav *et al.*, 2007; Sohail *et al.*, 2011). This difference may be explained by several factors, such as probiotic strain and challenge type. Lin and Yen (1999) hypothesized that beneficial intestinal bacteria produce certain factors that have the ability to chelate free radicals, capturing ROS and inhibiting their cytotoxic activity.

Table 2 – Total antioxidant capacity and oxidative stress (SOD and H_2O_2 levels) in serum of broiler chickens fed the experimental diets.

Treatment	TAC Mm	SOD μ L	H_2O_2 μ M
CTL+	0.54	0.17	78.78
CTL-	0.63	0.16	71.80
AB	0.61	0.29	68.44
TOYO	0.59	0.24	91.98
CLOS	0.57	0.32	75.10
SEM \pm	0.028	0.04	10.39
<i>P value</i>	NS	NS	NS

The mean corresponds to 10 birds per treatment.

Oxidative stress can be defined as a physiological disorder where there is an imbalance between concentrations of reactive oxygen species (ROS), such



as hydrogen peroxide (H₂O₂), and an antioxidant. Sahin *et al.* (2002) reported that low endogenous antioxidant production is the causative factor of oxidative stress in birds. Excessive ROS accumulation leads to cell injury, including damage to the DNA, proteins, and lipid membranes. Generally, ROS are continually produced by aerobic cells and are instantly removed by endogenous antioxidant scavengers. It is established that changes in the activity of several antioxidant enzymes can be used to assess the level of oxidative stress and total antioxidant status in birds. TAC accurately reflects the antioxidative status of the organism. SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide, and molecular oxygen is considered as one of the most potent antioxidant. Hydrogen peroxide (H₂O₂) is one of the most well-documented ROS produced under oxidative stress. It is a toxic product of normal aerobic metabolism and pathogenic ROS production involving oxidase and superoxide dismutase reactions. Under normal physiological conditions, cellular ROS generation is counter balanced by the action cellular antioxidant enzymes and redox molecules.

The results for anti- *S. typhimurium* responses in the different treatments are presented in Table 3. There was a significant treatment*week interaction ($p < 0.01$). The *S. typhimurium* titer continued to increase in birds in treatments CTL+, AB, and TOYO from week one through week five. In the CTL– birds, *S. typhimurium* titer increased from week 1 to week 3, then decreased in week 4 and increased again in week 5 (0.6, 1.17, 1.02 and 2.38, for weeks 1, 3, 4, and 5, respectively). In the birds in the CLOS group, the titer increased until week 4, then decreased in week 5 (1.09, 1.42, 1.62 and 0.92, for weeks 1, 3, 4, and 5, respectively). On the other hand, week showed a significant effect on anti-*S. typhimurium* titer, which increased from week 1 to week 5 (0.73, 1.85, respectively; $p < 0.001$), while treatment had no significant effect on anti-*S. typhimurium* titers ($p > 0.05$). The beneficial effects of probiotics of boosting the immune system of broilers is due to enhanced induction of natural antibodies, as previously reported (Haghighi *et al.*, 2006).

The Newcastle disease (NC) titer results are shown in Table 4. Treatment showed no effect on NC titer; however, there was a significant treatment*week interaction ($p < 0.05$) and a strong effect of week ($p < 0.001$). Birds in all treatments presented lower NC titers after *S. typhimurium* challenge (week 3) as compared to week 1 (before the challenge). However, the response after week 3 varied according to the treatment. For example, the NC titer of birds in groups

Table 3 – Serum anti-*Salmonella* antibody titers according to treatment and week

Treatment		Titer
CTL+		1.29
CTL-		1.33
AB		1.25
TOYO		1.31
CLOS		1.26
SEM		0.11
P value		NS
Week	Challenge	
1	Before	0.73 ^d
3	After	1.14 ^c
4	After	1.45 ^b
5	After	1.85 ^a
SEM		0.09
p value		0.001
Week	Treatment	
1	CTL+	0.6
1	CTL-	0.69
1	AB	0.48
1	TOYO	0.77
1	CLOS	1.17
3	CTL+	1.18
3	CTL-	1.12
3	AB	1.02
3	TOYO	0.98
3	CLOS	1.42
4	CTL+	1.02
4	CTL-	1.65
4	AB	1.39
4	TOYO	1.55
4	CLOS	1.62
5	CTL+	2.38
5	CTL-	1.86
5	AB	2.13
5	TOYO	1.93
5	CLOS	0.93
SEM		0.22
Treatment*week		**

Mean results of 35 birds per treatments, and 50 birds per week.

^{abcd}Means in the same column with different superscripts differ significantly, ** $p < 0.01$, *** $p < 0.001$.

CTL–, CTL+, and TOYO continued to increase after week 3, but not in the birds fed AB and CLOS. Sadeghi *et al.* (2013) found that *S. typhimurium*-infected birds had higher antibody titers in response to NC vaccination when compared to uninfected birds. Holt *et al.* (1999) concluded that broilers exposed to *Salmonella* at a young age may remain infected upon maturity and do not develop significant immunity against *Salmonella*. Seo *et al.* (2000) evaluated the effects of the challenge with *Salmonella* Enteritidis, enrofloxacin and/or normal avian gut flora in layers. They found that antibodies titers (IgG) were similar among treatments at two days



Table 4 – Serum anti-Newcastle disease titers according to treatment and week

Treatment	Titer	
CTL+	2.4	
CTL-	2.28	
AB	2.29	
TOYO	2.27	
CLOS	2.47	
SEM	0.11	
P value	NS	
Week	Challenge	Titer
1	Before	2.67 ^a
3	After	1.75 ^b
4	After	2.44 ^a
5	After	2.42 ^a
SEM		0.1
P value		0.0001
Week	Treatment	Titer
1	CTL+	2.77
1	CTL-	2.6
1	AB	2.78
1	TOYO	2.63
1	CLOS	2.45
3	CTL+	1.94
3	CTL-	1.88
3	AB	2.17
3	TOYO	1.47
3	CLOS	1.18
4	CTL+	2.43
4	CTL-	2.09
4	AB	2.27
4	TOYO	2.47
4	CLOS	2.93
5	CTL+	2.46
5	CTL-	2.55
5	AB	1.83
5	TOYO	2.50
5	CLOS	2.75
SEM		0.22
Treatment*week		*

Mean results of 35 birds per treatments, and 50 birds per week.

^{abcd}Means in the same column with different superscripts differ significantly, **p<0.01, ***p<0.001.

of age and only 8% of samples presented detectable anti-SE antibody titers after four weeks. Ribeiro *et al.* (2007) found that a probiotic had no effect on the production of anti-*Salmonella* Enteritidis antibodies in *Salmonella*-challenged birds.

Also, NC titers were high during week 1 (before the challenge) and then significantly dropped in week 3 after the challenge (2.67 vs. 1.75, respectively) (p<0.05). In weeks 4 and 5, the titers increased to a level similar to those determined in week 1 (p>0.05). Sedeghi *et al.* (2013) concluded that *Salmonella* challenge decreased antibody titers against ND.

Average organ relative weights are documented in Table 5. Treatments did not influence liver, bursa, spleen or thymus relative weights (p>0.05). Peyer's patches, cecal tonsils, and the bursa of Fabricius are sites of lymphoid tissue accumulation in poultry. These organs capture antigens that circulate in the digestive tract and stimulate the production of B and T immune cells. Changes in immune tissue weight affect immune cell phenotypes, immune cell proliferation, and antibody production. Sedeghi *et al.* (2013) reported that *Salmonella* challenge reduced the relative weights of immune organs as well as antibody titers against Newcastle. Ciraci *et al.* (2010) evaluated the effects of *Salmonella* on the global gene expression of chicken immune tissues or cells. Coble *et al.* (2013) evaluated transcriptomic profiles from livers of old 10 days old broilers which were infected with *S. enteritidis* and reported a down regulation of genes that function in metabolic pathways, inflammation and mitochondria apoptosis.

Table 5 – Relative organ weight according to treatment.

Treatment	Liver %	Bursa %	Spleen %	Thymus %
CTL+	2.91	0.22	0.13	0.54
CTL-	2.62	0.19	0.13	0.59
AB	2.91	0.18	0.13	0.54
TOYO	2.71	0.20	0.13	0.68
CLOS	2.87	0.24	0.15	0.60
SEM	± 0.14	0.02	0.01	0.06
p value	NS	NS	NS	NS

CONCLUSIONS

Neither *S. typhimurium* challenge nor probiotics affected broilers' total antioxidant capacity or oxidative stress. Antibody titers against *S. typhimurium* and Newcastle disease (ND) were not affected by treatments. However, both measurements showed a significant week x treatment interaction.

ACKNOWLEDGEMENTS

The authors would like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the Research Group Project No. RGP-VPP-273.

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