Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Application of Probiotic *(Bacillus subtilis)* to Enhance Immunity, Antioxidation, Digestive Enzymes Activity and Hematological Profile of Shaoxing Duck

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ARTICLE HISTORY

Received: May 24, 2012
Revised: July 24, 2012
Accepted: August 06, 2012

Key words:
Bacillus
Enzymes
Homeostasis
Immunity
Immunoglobulin

ABSTRACT

The study was designed to evaluate the effects of probiotics (*Bacillus subtilis)* to enhance immunity, antioxidation, digestive enzymes activity and hematological profile of Shaoxing duck. A population of 200 laying ducks (160 days old) was divided into two groups each further divided in five replications. The control (G_1) were fed on basal diet and (G_2) with *B. subtilis* 1×10^8 cfu/kg in addition of basal diet for thirty five days. The results showed that, ducks were treated with probiotics (*B. subtilis*), their serum IL-2 increased and IL-10 decreased ($P<0.05$). The concentrations of IgG, IgA and sIgA were observed significantly higher in (G_2) as compared to (G_1). Treatment group (G_2), showed significantly improvement in (SOD), T-AOC and ASAFR activity in serum and liver. However, digestive enzymes amylase and trypsin activity also improved ($P<0.05$) in (G_2). The blood chemistry analysis showed significant decrease in FT3 and no other significant change observed in hematological profile as compared to (G_1). In conclusion, application of *B. subtilis* (1×10^8 cfu/kg) may be beneficial to improve antioxidation response, supportive in innate immunity and digestibility of fowls (Shaoxing duck).

To Cite This Article: Rajput IR, WF Li, YL Li, L Jian and MQ Wang, 2013. Application of probiotic *(Bacillus subtilis)* to enhance immunity, antioxidation, digestive enzymes activity and hematological profile of Shaoxing duck. Pak Vet J, 33(1): 69-72.

INTRODUCTION

Probiotics are live microbial feed/or food supplements or components of bacteria, which have beneficial effects on animal and human health (Rajput and Li, 2012). Probiotics bacteria have been used in poultry as an alternative source of antibiotics against the pathogens, to support microbiota and improve animal growth and productive performance. They act as restrainer of the damage caused by pathogenic micro-organisms, affirmative to balance digestive microflora and increase in antioxidation functioning (Shen *et al*., 2011). The secretions of probiotics improve the continuous interaction of healthy intestinal linings with pathogens and also play a critical role in the development of immunity (Rajput and Li, 2012). Therefore, interest is growing in feed sciences for the use of probiotics due to beneficial effects to improve innate immunity, intestinal structural, support to digestive enzymes and homeostasis (Sanders, 1993). Before study was focused to evaluate the ability of probiotics to observe the beneficial effects to gut microflora (Saulnier, 2007) and the results confirmed that health-promoting bacteria inhabiting the gastrointestinal tract invading pathogens and sustain activities of microbiota (Gong *et al*., 2002). The current literature demonstrated that supplementation of probiotics to poultry diets increased performance of the birds, stabilized the microbial flora and also reduced incidences of disease (Hooge *et al*., 2004). Furthermore, the probiotics application in vivo illustrated that modern approaches could activate innate and adaptive immunity (Li *et al.*, 2011). However, rapid scarce of waterfowls, and limited information about the subject, the present study had been carried out to investigate the application of probiotic (*B. subtilis*) to enhance immunity, antioxidation, digestive enzymes activity and hematological profile of Shaoxing ducks.

MATERIALS AND METHODS

Bacterial Culturing and Experiment Design: The *B. subtilis* B10 used during experiment was isolated and identified by Institute of Feed Science, Zhejiang University. The bacterial strain was cultured in Luria Bertani (LB) broth (Oxoid; England) in aerobic condition at 30°C for 12 to 14 hrs. The latter was centrifuged (6000 × g for 5 minutes to separate the bacterial strain. Moreover, bacteria

were washed twice with Phosphate-Buffered Saline (PBS, pH 7.3), and suspended in skim milk powder to prepare required concentration (1×10^8 cfu/g). The prepared mixture was added in to the feed (Table 1) and maintained (1×10^8 cfu/kg). A total of 200 shaoxing ducks, 160-days-old and at laying, (average weigh 1.72±0.02kg) were randomly divided into two groups, with five replications of each group having 20 ducks per replication. Control group (G_1) was only fed on basal diet (Table 1), while treatment group (G_2) was fed on basal diet with addition of *B. subtilis* (1×10^8 cfu/kg) for 35 days. Ducks were reared in standard farm conditions, spread litter (wheat brown 3-4 inches thick), and free access of feed and water along with facility of playground and fresh water pond.

Table 1: Ingredients and nutrient levels of basal diet

Ingredients	(G/kg)	Nutrients	Content (g/kg)
Corn	465	CP	180
Soybean meal	230	Ca	30.9
Rapeseed Cake	80	P	7.5
Wheat flour	100	Lys	9.5
Mono calcium phosphate	15	Met	3.5
Limestone	67		
Sodium Chloride	3	DE(MJ/kg)	11.4%
Premix compound[1]	10		

Premix compound each kilogram contained: vitamin A, 5,000 IU; cholecalciferol, 1,500 IU; tocopheryl acetate, 11 IU; menadione, 1.1 mg; thiamine HCl, 3.0 mg; riboflavin, 5.0 mg; pyridoxine HCl, 2.2 mg; cyanocobalamin, 0.66 meq; niacin, 44 mg; Ca pantothenate, 12 mg; choline chloride, 220 mg; folic acid, 0.55 mg; D-biotin, 0.11 mg; Mn, 80.0 mg; Zn, 60.0 mg; Fe, 30.0 mg; Cu, 5.0 mg; I, 2.0 mg; and Se, 0.15 mg.

Blood, Digesta and liver sampling: After feeding trial of 35 days, ducks were scarified, as per recommendations of Zhejiang University Animal Centre (ZUAC) and blood samples were obtained from wing vein using 23 gauge needles. Serum was separated and purified using centrifugation (5,500 x g), for 10 minutes. Serum was aspirated by pipette and transferred into 1.5 ml, sterilized eppendorf tubes at -80°C for further analysis. The major ramifications of digestive tract were collected, and the parts were opened longitudinally with micro scalpel and transferred into a separate sterilized tubes containing 10 M PBS (7.4 pH) phosphate buffer saline and applied ultrasonic treatment for 4 mints in order to separate the GUT contents from the GIT tissue which were accomplished by centrifugation (5000xg, 25mints at 4°C). After centrifugation, supernatant was utilized to analyze enzymatic activities. The liver was collected and sample (10 g) was crushed with addition of normal saline (0.75%) with the ratio of 1:4. Furthermore, sample was centrifuged (3000xg 30 mints, at 4°C) and supernatant was collected and freezed for further analysis.

Determination of cytokines by ELISA: The ELISA test of IL-2 IL-6 and IL-10, were performed as per manufacturer's (ELISA Kit; R&D Systems, Inc) instructions. In brief, polyclonal goat anti-human IL-2, IL-6 and IL-10 antibodies were applied as capturing antibodies, biotinylated polyclonal rabbit anti-human IL-2, IL-6 and IL-10 antibodies as detecting antibodies. Streptavidin- RP and TMBS were used as color indicator. Well plates were read at (450 nm) wavelength, right after color reaction was stopped with acid.

Metabolites and immunoglobulin (Ig): The nitric oxide (NO), Free thyroxine (FT4), free triiodothyronine (FT3), corticosterone (CORT), creatine kinase (CK), lactate dehydrogenase (LDH), total protein (TP), albumin (ALB), globulin (GB), IgG, IgM, IgA, secretory IgA (sIgA). Metabolites and immunoglobulins in serum were analyzed using kits by following the instructions provided by Jiancheng Bioengineering Institute (Nanjing, China).

Antioxidtive indicators measurement: The glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), total antioxidation capacity (T-AOC), catalase (CAT), anti-superoxide anion free radical (ASAFR) and malondial-dehyde (MDA), were determined in serum and liver. Briefly, serum was obtained from blood by centrifugation at 5500 x g for 10 mints. The liver supernatant was collected after, (10 grams) of tissue grinding with addition of NaCl (0.75%), using super mixture machine. The centrifugation force 6000 x g for 15 mints was applied and supernatant collected for further analysis. All the indicators were detected by the instruction of manufacturers provided by (Jiancheng Bioengineering Institute Nanjing, China).

Digestive enzymes assay: Amylase, lipase (LPS), trypsin (TPS) and total protein hydrolase (TPH) were analyzed following the instruction using kit, provided by Jiancheng Bioengineering Institute (Nanjing, China). In brief, digesta was transferred into sterilized tubes containing 10 M PBS (7.4 pH) phosphate buffer saline, then ultrasonic treatment was applied for 4 mints to dissociate the tissues. The letter procedure was accomplished by centrifugation (5000 x g for 25 mints). The supernatant was used to determine the enzymatic activates.

Statistical analysis: Data were analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, USA). The intergroup variation was assessed by Paired-samples t test followed by Fischer's least significant difference (LSD) test. Statistical significance of the results was calculated at $P<0.05$.

RESULTS

Serum cytokines and immunoglobulin: The probiotic group showed, ($P<0.05$) lower concentration of anti-inflammatory cytokine IL-10 (Fig.1) compared with control group. While, the secretion of pro-inflammatory cytokine IL-2 increased ($P<0.05$) in treatment group (G_2), whereas no significant difference was observed in production of IL-6 between treatment and control groups (G_1).

The results showed, (Fig 2) that, significant increase observed in IgG and IgA in probiotics addition group (G_2) as compared to control group (G_1). However, there was no significant difference observed in TP, ALB, GB, IgM and sIgA concentration in between treatment (G_2) and control groups (G_1).

Serum and liver antioxidant activities: Our findings manifested (Table. 2), that significant improvement was observed in SOD, T-AOC and ASAFR activities in treatment group (G_2), conversely MDA activity was increased ($P<0.05$), in control group. While, no significant change was observed in GSH-PX and CAT activities in between control (G_1) and treatment groups (G_2).

Antioxidant activity of liver showed that SOD and T-AOC significantly increased in treatment group (G_2) as

compared to control group (G_1) However, non-significant change was observed in the GSH-PX, CAT, ASAFR activities and MDA contents in between control (G_1) and treatment groups (G_2).

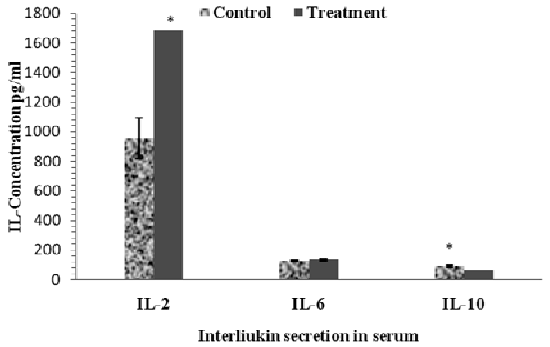

Fig. 1: The figure shows concentration of Interleukin-2, Interliukin-6 and interleukin-10 in each group (mean±SE). Error bars represent standard errors of the means of optical densities.*, statistically significant (P<0.05).

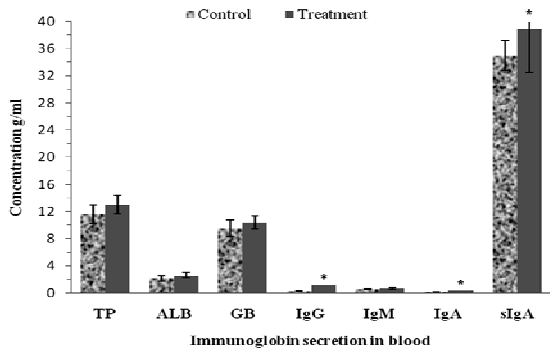

Fig. 2: The figure shows secretion (mean±SE) proteins and immunoglobulins. *,Indicates statistically (P<0.05) difference.

Digestive enzyme activity analysis: Specific enzymes activity (Table 2) among the digestive enzymes of Shaoxing duck, LPS, TPH, and LE activity showed no variation between experimental groups with probiotic supplement (G_2) and control group (G_1) without probiotics addition. Treatment group had high levels of AMS (P<0.05) while, TPS enzyme showed remarkable decrease (P<0.05) in G_2 compared with control group (G_1).

Metabolites of serum: Serum metabolites changes showed (Table 4) that treatment group (G_2) significantly decreased free triiodothyronine FT3 (P<0.05) as compared to (G_1) control group. Whilst, there was no significant difference manifested in nitric oxide (NO), free thyroxine (FT4), corticosterone (CORT), creatine kinase (CK) and lactate dehydrogenase (LDH) concentration between treatment (G_2) and control groups (G_1).

Table 2: Antioxidant levels (mean±SE) in Shaoxing duck serum and liver

Contents	Units	Serum		Liver	
		Control	*B. subtilis*	Control	*B. subtilis*
GSH-PX	U/ mgprot	353.5±61.9	345.1±43.4	281.5±62.7	368.5±75.4
SOD	U/ mgprot	106.7±5	130.4±7.8*	123.7±10.2	141.9±6.4
T-AOC	U/ mgprot	9.7±1.1	19.2±3.1*	328.9±32.8	520.4±70.7*
CAT	U/ mgprot	12.9±3.1	24±2.8	40.5±8.4	62.5±7.4
ASAFR	U/ mgprot	137.5±33.3	250.6±29.2*	443.3±16.6	438.6±18
MDA	nmol/mgprot	1.41±0.19	0.76±0.13*	3.80±0.33	2.53±0.44

*Indicates statistically (P<0.05) difference in a row.

Table 3: Digestive enzymatic activity in Shaoxing duck

Contents	Control	Treatment
Amylase (μ/mg)	7.7±0.37	12.2±1.3*
Lipase (U/g)	296.8±14.7	313.5±21.1
Trypsin (U/mL)	1359.1±182.1	2204.5±153.8*
Total Protein Hydrolase (U/mL)	2.6±0.08	2.8±0.08

Values (Mean+SE) bearing asterisk in a row differ significantly (P<0.05).

Table 4: Metabolites level in shaoxing duck serum

Contents	Control	Treatment
Nitric oxide (μmol/L)	149.4±25.6	155.3±20.7
Free thyroxine (pg/mL)	250.9±15.3	266.01±9.04
FT3 free triiodothyronine (pg/mL)	885.8±24.6	740.1±27.5*
Corticosterone (ng/mL)	536.4±54.1	519.6±58.06
Creatine kinase (U/mL)	0.86±0.2	1.05±0.08
Lactate dehydrogenase (U/L)	2513.9±46.8	2809.3±198.4

Values (Mean+SE) bearing asterisk in a row differ significantly (P<0.05).

DISCUSSION

Although, the functions of probiotics to enhance immunity and their responses against pathogens have been well demonstrated in mammals, but poorly implicit in fowls. However, the recent studies are in progress to establish the information about avian immune response to probiotics. IL-2 is an inflammatory cytokine which plays an important role to endorse cell mediated immunity related to Th_1 cells (Rajput and Li, 2012). IL-6 cytokine has pro-inflammatory activity via the induction of acute phase protein synthesis, and is important in the development of adaptive immune responses (Hirana, 1994). IL-10 inhibits the synthesis of pro-inflammatory cytokines, thus down-regulating inflammatory Th_1 responses (Groux and Powrie, 1999).

Our current findings suggested that probiotics (*Bacillus subtilis*) can support to enhance the production of inflammatory cytokine IL-2 and decrease the amount of IL-10 and no significant change was observed in serum IL-6 level. The supportive findings of (Huang *et al.*, 2012) described that concentrations of pro-inflammatory cytokines increased and response varies depends on probiotic species. While in contrast (Hong *et al*., 2006) found that, *S. enteritidis* as an infection has little effect on IL-2 cytokine production and down-regulates IL-2 mRNA expression. Another study (Weinstein *et al*., 1998) reported that, invasion of *S. typhi* into human or murine epithelial cells resulted in the production of high levels of IL-6. Our finding revealed that, application of *Bacillus subtilis* B10 may have in favor of application to develop immunity, antioxidation response and digestive enzyme improvement.

Probiotics enhance the systemic antibody response to soluble antigens, in serum and participate in the development of immunity (Christensen *et al*., 2002). In one study, ducks supplemented with *B. subtilis* showed enhanced and IgG IgA and sIgA response (Koenen *et al*., 2004). In another study, the administration of probiotics containing *Lactobacillus acidophilus* and *Lactobacillus casei* enhanced the serum IgA and IgG response, while the treatment did not influence other immunoglobulins (Huang *et al*., 2004). Our results are in agreement of previous studies, and found significant increase in IgG and IgA in probiotic addition group as compared to (G_1) control group. The possibility of the effects by probiotics, is stimulation of immune cells subsequently cytokine production (Lammers *et al*., 2003) and in possible cytokines IL-4 and IL-10, have important functions and modulate immune response

(Rakoff *et al*., 2004). Our results revealed application of probiotics stimulate immunity development in waterfowls.

The important components of the antioxidative enzymes including GSH-Px, SOD, T-AOC, CAT and ASAFR, play a vital function for self defense (Miller and Britigan, 1997). Our results indicated that, T-AOC activities significantly increased in (G_2) as compared to (G_1). These findings are in agreement with the previous observations of (Capcarova *et al*., 2010). *Enterococcus faecium* also has oxidation resistance, scavenges hydroxyl radical and increases antioxidant capacity (Wen *et al*., 2011). With regard to antioxidant activity, *L. acidophilus* supernatant showed DPPH radical scavenging activity (Lee *et al*., 2008). Our results demonstrate the potential use of probiotics *B. subtilis* is health-promotion.

The amylase, lipase, trypsin and total protein hydrolase play an important role in the, digestion, and/or fermentation of relative nutrient materials with ultimate improvement in animal performance and health. The results of present study demonstrated that amylase and trypsin activity was significantly higher in G_2 but lipase and TPH was not affected when compared with G_1 after 35 days of feeding. Current results were similar to the finding of Jin *et al*. (2000), who reported that inclusion of a probiotics (a single strain of *L. acidophilus* or a mixture of 12 Lactobacillus strains) resulted in significantly higher enzyme activities in the small intestine of broilers.

Bacteria, particularly members of the genus *Bacillus* secrete a wide range of exoenzymes (Pugsley and Schwartz, 1985). We could not distinguish between activity due to enzymes synthesized by the fowl and due to enzymes synthesized by the probiotics. However, the exogenous enzymes produced by the probiotics represents, only a small contribution to the total enzyme activity of the intestine (Bedford and Schulze, 1998). The application of probiotics might stimulate the production of endogenous enzymes which contribute to develop harmonious environment of intestine for better digestion in fowls.

The brids treated with probiotics in addition to basal diet (G_2) serum chemistry profile showed no significant effects, except the free triiodothyronine (FT3) statistically decreased than control group. There is positive correlation in between adhesion of probiotic bacteria to epithelium and decrease in FT3. Our findings proved probiotic *B. subtilis* is a beneficial bacteria to overall development of immunity of birds, and several studies in agreement that, different probiotics have immune-stimulatory, anti-inflammatory and antiviral effects (Klocking *et al*., 2002; Joone *et al*., 2003; Joone and van Rensburg, 2004).

In conclusion, the current study manifested several prospective effects of probiotic (*B. subtilis*) B10 could induce innate immunity, enhanced antioxidation and improved digestibility via digestive enzymes and beneficial modulation in hematological profile, of waterfowl (Shaoxing) duck.

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