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Supplementary Effects of *Saccharomyces boulardii* and *Bacillus subtilis* B10 on Digestive Enzyme Activities, Antioxidation Capacity and Blood Homeostasis in Broiler

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Abstract

The research was conducted to evaluate the feed supplementary effect of *Saccharomyces boulardii* and *Bacillus subtilis* on digestive enzyme activities, antioxidation capicity and blood homeostasis in broiler. A total of 300 day-old Sanhuang broilers (Chinese cross breed) were randomly divided into three groups with five replications in each group (n =20). The control group was fed basal diet contained antibiotic and experimental groups were supplemented with *S. boulardii* and *B. subtilis* in addition (1×10^8 cfu/kg) to basal diet for 72 days, respectively. The results revealed that activities of jejunal Na^+-K^+ ATPase (ATP), lipase (LP) and Gamma glutamyl transpeptidase (γGT) concentration increased ($P<0.05$) in probiotic supplementary groups. Moreover, results from ileum showed high levels of γGT in *B. subtilis* supplemented group only. The serum glutathione peroxidase (GSH-PX), peroxidase (POD), glutathione (GSH), glutathione reductase (GR) and catalase (CAT) concentration were significantly higher, while there was a significant decrease ($P <0.05$) in malondialdehyde (MDA) content in supplementary groups. The blood biochemical analysis showed significant decrease in uric acid and triglycerides of *S. boulardii* and *B. subtilis* groups. Conversely, albumin and low-density lipoprotein concentration increased in *S. boulardii* and *B. subtilis* groups as compare to control. The present research revealed that, supplementation of *S. boulardii* and *B. subtilis* B10 could be applied to enhance digestive enzyme activities, antioxidation and blood profile of broilers. In addition it might be considered as a natural antioxidant feed additive for broiler. © 2013 Friends Science Publishers

Keywords: Probiotics; Digestive enzyme; Blood biochemistry; Antioxidation

Introduction

The scope of the probiotics is developing rapidly as evidenced by expansion in research, and increasing demand in humans and livestock since last two decades (Reid *et al*., 2003; Huang *et al.*, 2012). Probiotics exert beneficial effects on the host by activation of local mucosal protective mechanisms, anti-oxidation, and immune response modulation at all the mucosal sites (Takahashi *et al*., 2004). Sanders (1993) reported that supplementation of probiotics could improve digestive enzymes activity in intestine, enhance antioxidation and maintain homeostasis, and secretions of probiotics play critical roles to improve the intestinal linings, and would continuously interact with microorganisms (Rajput and Li, 2012). In addition, probiotics could inhibit excess of oxidative free radicals that can cause cell damage and finally effects on performance (Li *et al*., 2012). In order to protect against oxidative stress, living organism use their non-enzymatic antioxidants that are present in the cytosolic and membrane compartments of the cell (Vossen *et al*., 2011). And antioxidant enzymes, such as glutathione peroxidase (GSH-Px), superoxide anion (O_2^-), total anti-oxidation capacity (T-AOC), malondialdehyde (MDA), peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione (GSH) have capacity to break down the free-radical effects by using a chain reaction mechanism. Different synthetic antioxidants have been used to evaluate beneficial effects but their toxicological safety has always been questioned. Thus it has become desirable to replace these conventional antioxidants with some natural antioxidative substances (Formanek *et al*., 2001). In this regard, natural antioxidant source *Saccharomyces Boulardii* is considered as a useful probiotics, and its oral administration could improve enzymatic activity and anti-oxidation functions and protect intestinal mucosa during transitional time in the intestine (Kotowska *et al.*, 2005). Similarly in recent study, *Bacillus subtilis* was used as a probiotics and it has been illustrated through modern approaches *in vivo* studies that, probiotics support in anti-oxidation and enhance enzymatic activity during nutrients transportation in the intestine (Li *et al*., 2011a). Meanwhile, another report also demonstrated that, probiotics stimulate biotherapeutic protection mechanism through heat shock

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protein Hsp27 via oligopeptide transporter OCTN2 that protects the cells from oxidant mediated damage. These evidences inspired us to focus our research on the role of probiotics on the digestive enzyme activities and antioxidative capacity. Several studies has been conducted on antioxidative effects of probiotics in various species but effect of *S. boulardii* and *B. subtilis* B10 on broiler chickens is not reported. Therefore, the present study was designed to evaluate the feed supplementation effects of *S. boulardii* and *B. subtilis* on the enzyme activities, antioxidative capacity and blood chemistry of the broiler chickens.

Materials and Methods

Culturing of Probiotics

S. boulardii and *B. subtilis* B10 used during experiment were isolated and identified by Institute of Feed Science, Zhejiang University. The yeast and bacterial strains were cultured in Yeast Peptone Dextrose (YPD) and Luria Bertani (LB) broth (Oxoid; England) in aerobic condition at 30°C for 24 and 12 h respectively. Centrifugation at (6000 × g) for 5 min, used to separate the yeast and bacterial strains. Moreover, yeast/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), respectively. The prepared mixture was added in to basal diet (Table 1), and maintained (1×10^8 cfu/kg).

Experiment Design and Feeding Method

A total of 300 day old Sanghuang broilers (Chinese cross breed) were randomly divided into three groups, each group with five replications (n =20). Control group fed basal diet (Table 1) containing antibiotic, meanwhile broiler in experimental groups were fed basal diet in addition to *S. boulardii* and *B. subtilis* (1×10^8 cfu/kg), devoid antibiotics for 72 days.

Blood Collection

After feeding trial of 72 days, broilers 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 × g), for 10 min. Serum was aspirated by pipette and transferred into 1.5 mL, Eppendrof tubes sterilized at -80°C for further analysis.

Sampling of Digesta and Liver

The major parts of broiler digestive tract, jejunum and ileum were collected and opened longitudinally with micro scalpel, then transferred into a separate sterilized tubes containing 10 M Phosphate buffer saline (7.4 pH) and applied ultrasonic treatment for 4 min in order to separate the GUT contents from the GIT tissue, which were accomplished by centrifugation (5000 rpm, 25 min at 4°C). After centrifugation, supernatant was utilized to analyze enzymatic activates. The liver was collected and sample (10 g) was homogenized in normal saline (0.75%) by vortex mixture. Then, sample was centrifuged (3000 rpm 30 min, at 4°C) and supernatant was collected and freezed at -80°C for analyses.

Enzymatic Activities Analysis

The homogenates with PBS (7.4 pH), were collected and centrifuged at (5000, 25 min at 4°C min at 4°C) and the supernatant was stored at -80°C for enzyme assays. In brief, After thawing the homogenates, adjusting to room temperature, the activities of α-amylase (AMS), Na^+ K^+ ATPase (ATP), gamma glutamyl transpeptidase (γGT), lipase (LPS), trypsin (TPS), were analyzed by a microplate reader (Spectra Max M5, Molecular Devices, USA) using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufacturer.

Antioxidation Capacity Assays

For antioxidation assays, liver tissues (10 g) were homogenized in ice-cold normal saline (NaCl) to form homogenates were centrifuged and serum was separated and purified using centrifugation (5,500 x g), for 10 min. The supernatants and serum were prepared, subjected to the analyses of glutahinone peroxidase (GSH-Px), superanion oxide (O^{2-}), total antioxidation capacity (T-AOC), malondialdehyde (MDA), peroxidase (POD) superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione (GSH) levels by absorbance methods using a spectrophotometer (UV-2000, Unico Instruments Co. Ltd., Shanghai, P.R. China). All of the assays followed the instructions of the kits (Jiancheng Bioengineering Institute Nanjing, China).

Statistical Analysis

Data was analyzed using SPSS 16.0 for Windows (SPSS Inc., Chicago, USA). Paired-samples t test was chosen as compare mean to analyze index differences between groups. The probability ($P<0.05$) was considered as statements of statistical significance.

RESULTS

Digestive Enzymes Activity Assay

Digestive enzymes play an important role to digest the feed components that is directly proportionate with growth and performance. In the present study specific enzymes activities were determined (Fig. 1) among the digestive enzymes of broiler. The activity of ATPase (ATP) and lipase (LP) in jejunum was found significantly higher in *S. boulardii* group. However, Gamma glutamyl transpeptidase (γGT), concentration improved ($P<0.05$) in

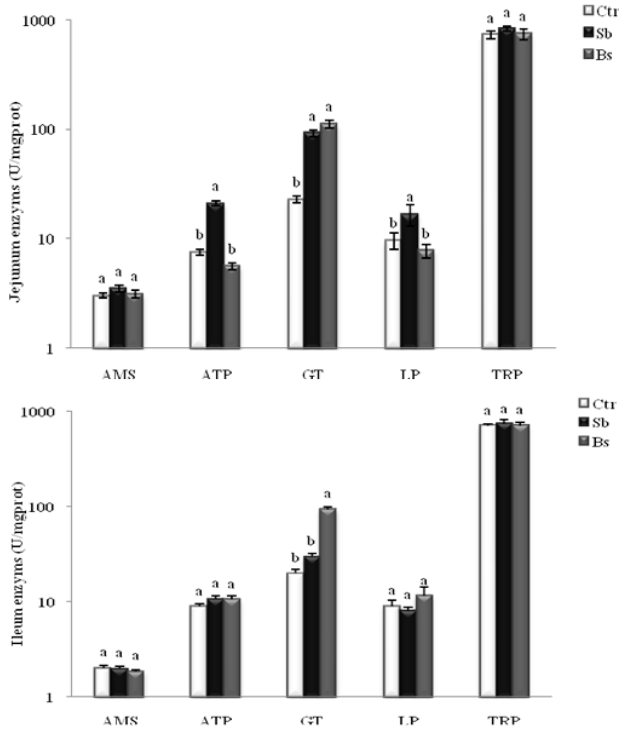

Fig. 1: The figure shows concentration of AMS, ATP, GT, LP and TRP in each group (mean±SE). Error bars represent standard errors of the means of optical densities **[a,b,c]**, statistically significant (P<0.05)

both *S. boulardii* and *B. subtilis* groups. However, no significant improvement was observed in amylase (AMS), and trypsin (TPS) activities in jejunum. Moreover, γGT significantly increased in Ileum of *B. subtilis* supplemented group. However, AMS, ATP, LP and TRP remained unaltered in ileum of *S. boulardii* and *B. subtilis* groups in comparison to control group.

Serum Antioxidation Analysis

The endogenous antioxidant defense mechanism of the animals depends upon the external sources and this can be easily supported by the probiotics. The present results revealed (Fig. 2), significantly higher serum levels of GSH-PX, POD, GSH, GR and CAT and conversely prominent (P <0.05) decrease was noted in MDA contents in *S. boulardii* group. Meanwhile, *B. subtilis* group improved the CAT and GSH-PX activity, conversely decreased MDA contents significantly. Besides, no significant change was appeared statistically in T-AOC, O_2^- and SOD activities amongst groups.

Liver Antioxidation Functioning

Liver is a vital body organ and oxidation in it might be prevented by the supplementation of probiotics as a feed additive. Our findings showed (Fig. 3), significantly higher response of T-AOC, POD, CAT and GSH activities in *S.*

Table 1: Composition and nutrition of the basal experimental diet (%)

Contents	1-36d	37-72d
Corn	55.90	61.60
Soybean meal	31.00	27.00
Wheat shorts	3.00	4.00
Imported fish meal	5.00	2.00
Rapeseed oil	1.50	2.00
Salt	0.30	0.30
Dicalcium phosphate	1.20	1.00
Limestone	1.00	1.00
DL-Met	0.10	
Lysine		0.10
Premix	1.00	1.00
Total	100.00	100.00
Nutrient		
ME（MJ/kg）	12.78	13.05
Crude protein	22.86	19.14
Lys	1.07	0.98
Met+Cys	0.86	0.72
Ash	7.38	6.41
Ca	0.93	0.91
Total phosphorus	0.64	0.56

Premix compound: Each kilogram contained: VA 7 000 IU; VD3, 2 500 IU; VE, 30 mg; of VK3 1 mg; VB1 1.5 mg; VB2, 4 mg; VB6, 2 mg; VB12, 0.02 mg; niacin, 30 mg; folic acid, 0.55 mg; pantothenic acid,10 mg; biotin, 0.16 mg; choline chloride, 400 mg; Cu 20, mg; Fe 70, mg; Mn, 100 mg; Zn, 70 mg; I, 0.4 mg and Se,0.5 mg

Table 2: Blood biochemical analysiss of broilers

Contents	Ctr	*Sb*	*Bs*
Calcium	2.64±0.040	2.738±0.054	2.696±0.031
Phosphorus	2.024±0.146	2.124±0.131	2.126±0.079
Uric acid	516.78±31.13^{a}	465.42±6.96^{ab}	43 4.74±3.52^{b}
Globin	15.94±0.693	18.04±0.793	16.58±1.137
Albumin	22.44±0.604^{ab}	23.62±0.681^{a}	21.9±0.306^{b}
Albumin/globulin ratio	1.414±0.035	1.314±0.036	1.342±0.086
Total protein	38.38±1.267	41.66±1.413	38.48±1.306
Alanine aminotransferase	3.4±0.244	4.2±1.019	3.2±0.583
High-density lipoprotein	1.94±0.091	1.936±0.131	1.936±0.079
Low-density lipoprotein	0.66±0.115^{b}	0.714±0.036^{ab}	0.928±0.079^{a}
Triglycerides	0.968±0.148^{a}	0.734±0.109^{ab}	0.572±0.089^{b}
Total cholesterol	3.462±0.193	3.258±0.793	3.43±1.137

Values (Mean+SE) bearing alphabets **[a, b, c]** in a row differ significantly (P<0.05)

boulardii group. Moreover, *B. subtilis* group improved GSH-PX, O_2^-, T-AOC, SOD and CAT activities and significantly drop in MDA contents of *S. boulardii* and *B. subtilis* groups (P <0.05) as compare with control group.

Blood Biochemical Examination

Blood homeostasis is the initial sign to know physiological and biochemical status and to evaluate supplementation effects on the health of subject. The current findings showed (Table 2) decreased level of uric acid in *B. subtilis* supplemented group followed by *S. boulardii* group (P <0.05). However, biochemical component albumin concentration was found significantly higher in *B. subtilis* group, conversely no significant change observed in *S. boulardii* group. Meanwhile, low-density lipoprotein concentration level increased (P <0.05) in *B. subtilis* group

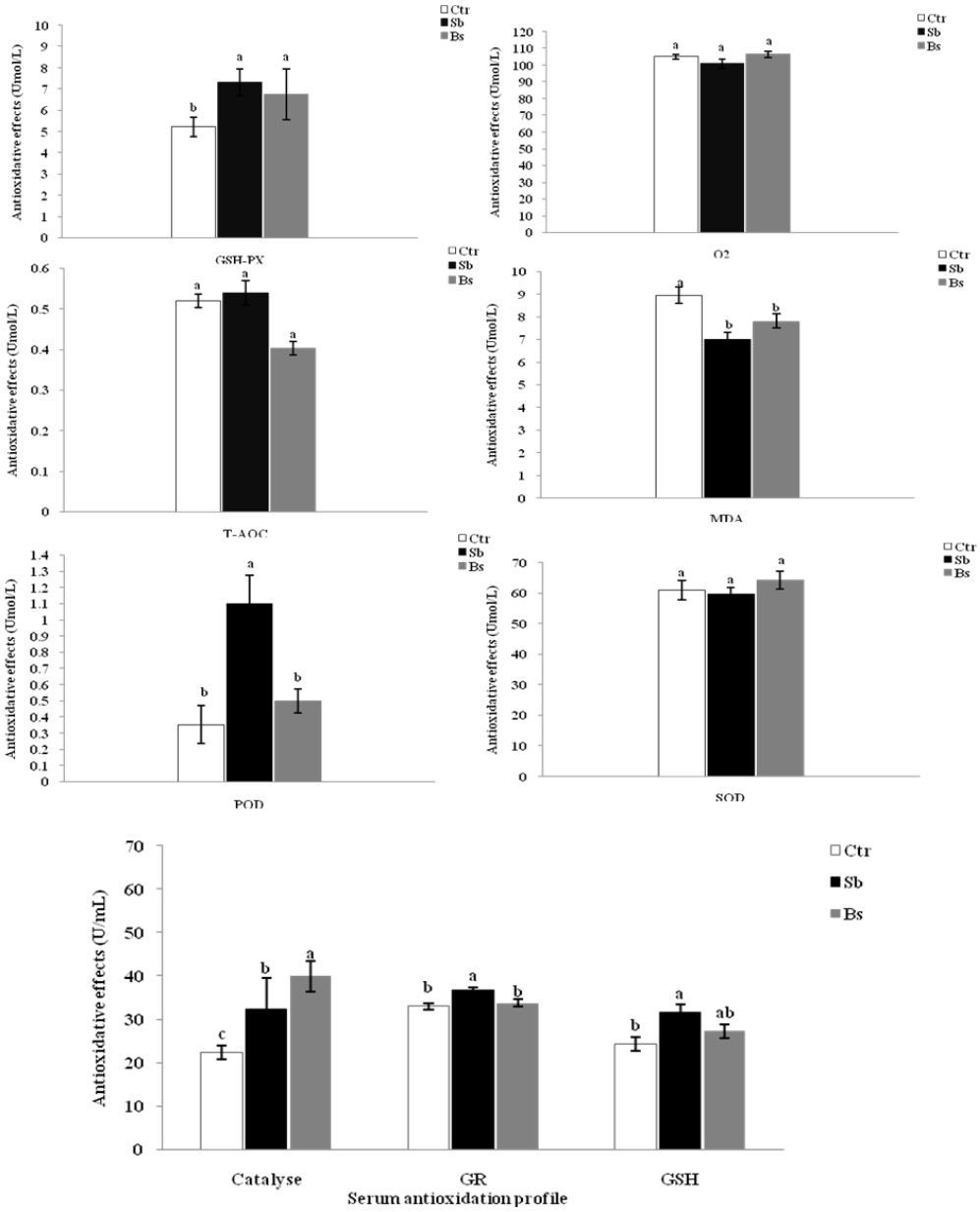

Fig. 2: The figure shows levels of antioxidants in serum GSH-Px, O^{2-}, T-AOC, MDA, POD SOD, CAT, GR, and GSH (mean±SE). **[a,b,c]**, Indicates statistically ($P<0.05$) difference amongst group

and *S. boulardii* group showed numerically improvement. However, triglycerides level dropped in *S. boulardii* and *B. subtilis* both groups as compare to control. Conversely, calcium, phosphorus, total protein, globulin, albumin and globulin ratio, alanine aminotransferase, high-density lipoprotein and total cholesterol levels were found unaltered among the groups.

Discussion

Probiotics exerts beneficial effects to enhance the digestive enzymes activities, improve anti-oxidation and homeostasis (Sanders, 1993). They inhibit the excess of oxidative free radicals that may cause cell damage and finally affect on performance (Li *et al.*, 2012). Moreover, secretions of probiotics play a critical role to recover the intestinal linings that are in continuous interaction with the microorganisms (Rajput and Li, 2012). Previous studies have evaluated the effects of various probiotics but information about *S. boulardii* and *B. subtilis* is still lacking and/or incomplete. In the present study for the first time, we evaluated the feed supplementary effect of *S. boulardii* and *B. subtilis* B10 on the digestive enzyme, anti-oxidation and blood homeostasis in broiler chicken.

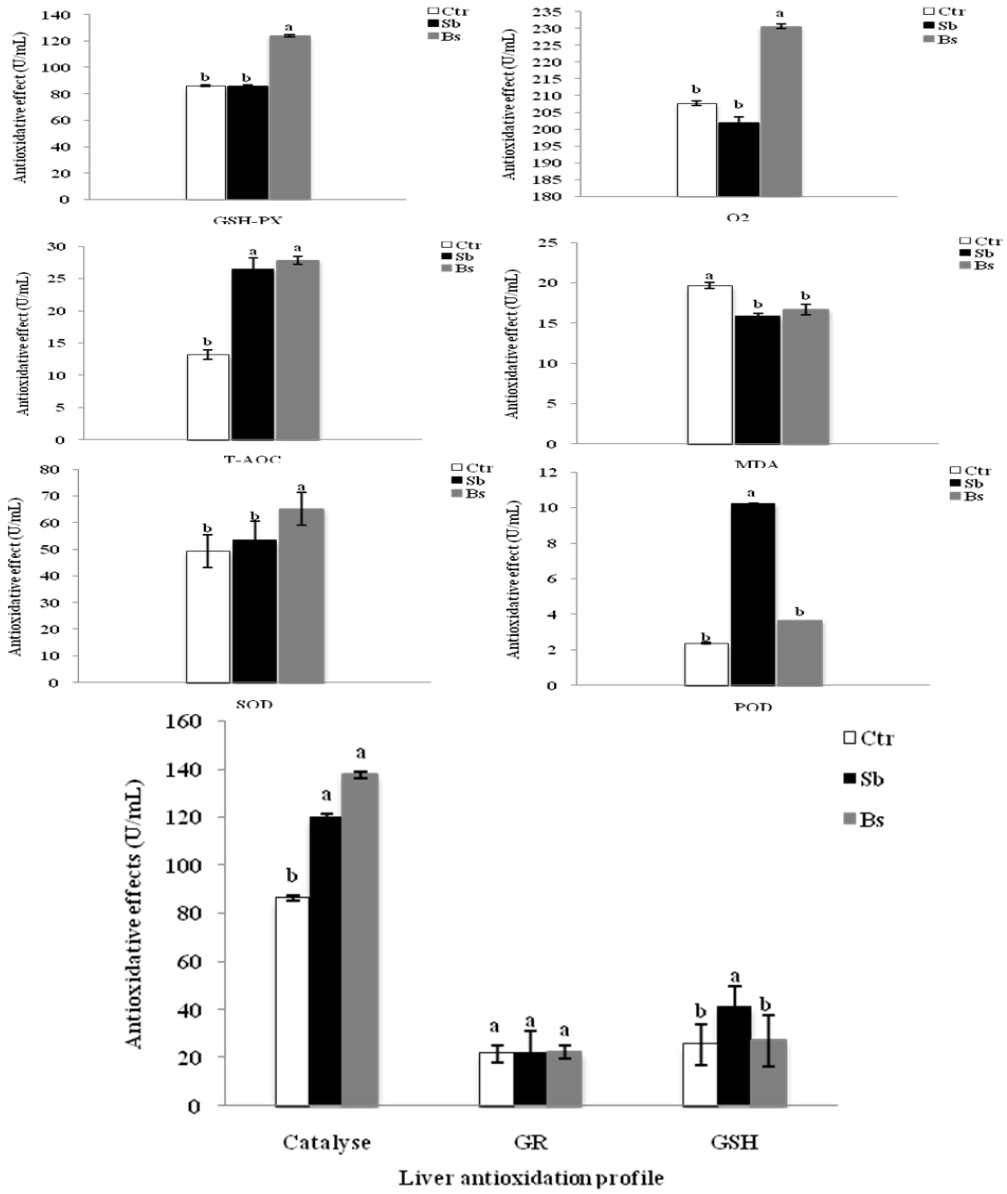

Fig. 3: The figure shows levels of antioxidants in liver GSH-Px, O^{2-}, T-AOC, MDA, POD SOD, CAT, GR, and GSH (mean±SE). **[a,b,c]**, Indicates statistically ($P<0.05$) difference amongst group

Presently, we found that ATP and γGT activity significantly increased in the jejunum followed by the LP activity in comparison to control group, while *B. subtilis* B10 group showed significant effect merely in the γGT activity in ileum as compared to the other groups. Thus our present findings are in agreement with the results of Chevalier *et al*. (1999), who illustrated that prokaryotic (yeast) could enhance the activity of γGT and ATP in the intestine. In another study on rat has also reported that, lipase activity significantly increased in treatment group supplemented with *S. Boulardii* (Chu *et al*., 2002). Moreover, a similar study showed improvements in the digestive enzyme activity in case of *B. subtilis* B10 supplementation in ducks (Rajput *et al*., 2012). Digestive enzymes are very important to metabolize the feed or food containing polypeptide chains in the intestine into simple amino acids and the transport of the amino acids in the intestine is mainly achieved by a brush border enzyme γGT (Smith *et al*., 1991) that supports the catalysis of the decomposition of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and a free phosphate ion that releases energy, which is used by the enzymes to drive other chemical reactions and to help in the transportation of amino acids (Cotgreave and Schuppe-Koistinen, 1994). Thus, our study on the enzymes activity modulation in the intestine suggested that *S. boulardii* and *B. subtilis* B10 might be supporting in the action of fluid or

protein metabolism in broiler intestine.

The important components of the antioxidative enzymes are CAT, GSH, GSH-Px, GR, T-AOC, SOD, POD and MDA contents, which play key roles to perform fundamental functions of self defense mechanism (Rajput *et al*., 2012) however, probiotics were described a natural source to enhance anti-oxidation functioning activity of animals (Li *et al*., 2012). Our findings manifested significant increase in POD, GSH, GR and prominent decrease in MDA contents of serum in *S. boulardii* group ($P < 0.05$). Conversely, *B. subtilis* group enhanced the CAT activity and decreased MDA contents in serum significantly. Meanwhile, activities of liver antioxidant enzymes T-AOC, POD, GSH and GR increased in *S. boulardii* group. Moreover, GSH-PX, O_2^-, T-AOC SOD and CAT activities were higher in *B. subtilis* group, however significantly decrease in MDA contents was found in both *S. boulardii* and *B. subtilis* groups. Our findings were in agreement with the previous observations of (Capcarova *et al*., 2010; Wen *et al*., 2011a) who reported that some microorganisms could help in the oxidation resistance, scavenge hydroxyl radical and increase antioxidant capacity. In another study (Lee *et al*., 2008) with regard to antioxidant response *L. acidophilus* supernatant showed DPPH radical scavenging activity. Normally cells generate small amounts of free radicals or reactive oxygen species (ROS), while performing their normal metabolic functions. Although low levels of ROS are essential in many biochemical processes, accumulation of ROS may damage biological macromolecules (Santos *et al*., 1999). In the situations, where the level of free radicals exceeds the antioxidant capacity of the cell, then oxidative stress might happen. Oxidative stress would essentially give rise to toxic accumulation of high levels of free radicals whereby the free radicals may damage the cells by oxidizing fatty acids of the cell membrane or interacting with DNA or protein (Russel *et al*., 2000). Consequently, oxidative stress may compromise the health status and impair the production performance of broilers (Douglas *et al*., 2011). The endogenous antioxidant defense mechanism of the animals also depends upon the other external sources and this can easily be supported by the probiotics, which are the natural source to prevent from the effects produced by the oxidative stress. Here, our findings demonstrated the benefits of *S. Boulardii* and *B. subtilis* B10 in ROS removing and health-promotion in broiler.

Blood homeostasis is the key to know physiological and biochemical states of the subject and to evaluate the effects of supplementation. In the present study, significant decrease in the uric acid and triglycerides were observed, conversely significant improvement ($P < 0.05$) in albumin concentration. Moreover, low-density lipoprotein concentration level also increased ($P < 0.05$) in *S. boulardii* and *B. subtilis* groups. The findings of Victor *et al*. (1993) illustrated that homeostasis susceptibility of the broilers increased after supplementation of *S. ceravaesea* and significantly decrease was observed in uric acid. Similar effect was found by Rajput *et al*. (2012) in ducks fed with *B. subtilis* B10. The findings of Li *et al*. (2011b) also revealed that uric acid level decreased and albumin concentration increased significantly in blood. Moreover, reduction of blood uric acid and improvement in low density lipo-protein concentrations due to supplemental dietary probiotics had been reported in poultry (Balog *et al*., 1994). Previously Becker (1993) reported uric acid as marker of oxidative stress and its level in plasma had also been associated as an antioxidant.

In conclusion, *S. boulardii* and *B. subtilis* B10 could be applied as probiotics to boost sustaining homeostasis and blood profile of broilers. In addition findings revealed these probiotics enhance digestive enzyme activities and are alternative sources of synthetic antioxidants and potentially useful to apply as natural antioxidants.

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