Antioxidant status, serum biochemical profile and Duodenal morphometry of broilers supplemented with Spirulina (Arthrospira) and exogenous protease enzyme

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Abstract

The present study was carried out to assess the effect of dietary supplementation of different levels of spirulina (*Arthrospira*), a blue-green microalgae with or without exogenous protease enzyme on antioxidant status, serum parameters and duodenal morphometry of broiler chicken. Two hundred and forty commercial broiler chicks were randomly distributed into five dietary treatments with four replicates containing 12 birds each. The dietary treatments were: basal diet prepared as per ICAR (2013) specifications (control: T_1), basal diet containing 1% spirulina (T_2), basal diet containing 1% spirulina + 0.05% exogenous protease (T_3), basal diet containing 2% spirulina (T_4) and basal diet containing 2% spirulina+0.05% exogenous protease (T_5). Birds were reared under deep litter system for 35 days and fed with pre-starter, starter and finisher diets. Results revealed that the antioxidant activity of broilers was found to be significantly ($p<0.05$) improved by supplementation of spirulina with or without exogenous protease enzyme. The serum biochemical profile of broilers revealed that total protein, albumin and A/G ratio did not vary significantly whereas significant difference ($p<0.05$) was reported in serum globulin, glucose, urea and AST levels. With regard to serum lipid profile, supplementation of spirulina had no significant effect ($p>0.05$) on serum triglycerides, HDL cholesterol and VLDL cholesterol. While, total cholesterol (mg/dl) and LDL cholesterol (mg/dl) levels were significantly ($p<0.05$) lower in spirulina supplemented groups. Duodenal morphometry was improved ($p<0.05$) as the level of spirulina increased from 0 to 1% and there after it was decreased. These results concluded that supplementation of spirulina up to 1% level can be beneficial in the broiler diets and an additional benefit was observed with exogenous protease supplementation at 0.05% level.

Keywords: Spirulina, Exogenous protease, Antioxidant, Serum profile, Morphometry

Introduction

Broiler production is facing severe constraint due to increasing prices of traditional cereal based feed and its competition with human consumption. Feeding broilers diets containing unconventional feed ingredients would be an attractive and alternative strategy for broiler production that avoids competition between human food and broiler feed (Tsugkieva *et al*., 2021; Gobezie, 2022). Use of Spirulina, a biomass of cyanobacterium, containing high protein, vitamins, minerals, essential amino and fatty acids, phytopigments, reported to have growth promoting, immuno- modulating and anti-inflammatory effects (Farag *et al.* 2016., Mariey *et al*. 2012). In addition to its nutritional value, spirulina contains a wide range of bioactive compounds such as phenolics, flavonoids, saponins, triterpenoids and steroids, which contribute significantly to its strong antioxidant activity. These properties making spirulina a promising natural feed additive in poultry production. Recent interest has focused on spirulina as a potential alternative to antibiotic growth promoters in broiler diets, particularly for improving growth performance, enhancing antioxidant status and supporting gut health. Dietary inclusion of spirulina in broiler nutrition has favourable effects on health and performance (Kharde *et al*. 2012., Shanmugapriya and Saravana babu. 2014). Spirulina has also been associated with increased growth rate (Evans *et al*. 2015., Park *et al*. 2018) and antioxidant enzyme activity (Mirzaie *et al*, 2018., Park *et al*, 2018., El-Bahr *et al*, 2020). However, the structural complexity of spirulina's cell wall limits the release and digestibility of its intracellular nutrients (Shirazi *et al*. 2017). To overcome this limitation, the use of exogenous enzymes, particularly protease, has emerged as a promising strategy. Protease enzymes enhance protein digestion by breaking down complex protein molecules, thereby reducing undigested proteins in the gut, increasing amino acid availability, and potentially lowering the dietary protein requirement (Buttin *et al*. 2016). Enzymatic degradation may also facilitate the breakdown of spirulina's cell wall, improving nutrient accessibility and utilization.

Despite these promising prospects, limited research has explored the combined effects of spirulina and exogenous protease enzyme supplementation in broiler diets. Therefore, the present study was undertaken to evaluate the impact of dietary inclusion of different levels of spirulina, with or without exogenous protease, on antioxidant status, serum profile and duodenal morphometry in broiler chickens.

Materials and Methods

The experiment was carried out at Department of Animal Nutrition, College of Veterinary Science, Tirupati, SVVU after taking approval by the Institutional Animal Ethics Committee vide reference number 281/go/ReBi/S/2000/CPCSEA/CVSc/TPTY/028/Animal Nutrition/2021 dated 24-08-2021.

Two hundred and forty commercial broiler chicks (Cobb - 400 strain) were randomly distributed into five dietary treatments with four replicates containing 12 birds each. The dietary treatments were: basal diet prepared as per ICAR (2013) specification (control: T_1), basal diet containing 1% spirulina (T_2), basal diet containing 1% spirulina+0.05% exogenous protease (T_3), basal diet containing 2% spirulina (T_4), basal diet containing 2% spirulina+0.05% exogenous protease (T_5). Birds were reared under deep litter system for 35 days and fed with pre-starter, starter and finisher diets. Clean and fresh drinking water was made available at all the times. All the birds were vaccinated as per standard vaccination schedule.

Oxidative stress and anti-oxidant status

Eight birds from each group were sacrificed on 35d of experiment and blood samples were collected. Hemolysate was prepared and used for analysis. The concentration of malondialdehyde (MDA) was measured by the method of Niehaus and Samuelsson (1968), using thiobarbituric acid reactive substances by TCA-TBA method. The total antioxidant capacity (TAC) was measured according to the method of Ciuti and Liguri (2017). The Superoxide dismutase (SOD) activity was assessed by the procedure given by Misra and Fridovich (1972) and glutathione peroxidase (GPx) activity according to Rotruck *et al*. (1973).

Serum biochemical profile

The blood sample for biochemical analysis was collected in tubes without anticoagulant. The blood samples were centrifuged at 3000 rpm for 20 minutes to obtain serum, and stored at –20°C until the time of chemical determinations.

The serum biochemical constituents like Glucose, Total protein, Albumin, Urea, Triglycerides, Total cholesterol, High-density lipoprotein cholesterol and the activity of enzymes (Aspartate aminotransferase and Alanine transferase) were estimated in A15 automated biochemical analyser by using Biosystems kits.

Duodenal morphometry

The duodenal segment of small intestine was identified and separated by dissection. Each sample was externally and internally washed with 0.9% NaCl to remove the intestinal contents and individually transferred to jars containing 10% buffered formalin for fixation. After 12-24 h fixation period, samples were embedded in paraffin, sectioned to a 2-5 µm thickness, mounted on glass slides and stained with haematoxylin - eosin (Prophet *et al.,* 1992).

Villi height and crypt depth were then measured. Villus height was defined as the length between the villus basal lamina (which coincides with the upper crypt end) and the villus apex. Crypt depth was measured between the base and the crypt. Measurements were carried out using a trinocular stereoscopic microscope (Quimis^{TM}) under 10x magnifications. Between five and 10 villi and crypts were scored for each bird, and means calculated there from were used in the statistical analysis.

Statistical analysis

Data were analysed by the method of Snedecor and Cochran (1994) and using SPSS version 22.0. Statistical comparisons of the results were made using one way analysis of variance (ANOVA) and significant differences ($p<0.05$) between different experimental groups were analysed by Duncan's test.

Results

Oxidative stress and anti-oxidant status

The concentration of MDA was significantly lower ($p<0.05$) and the Superoxide dismutase activity, Glutathione peroxidase activity and Total Antioxidant Capacity was significantly higher ($p<0.05$) in spirulina supplemented groups compared to control and are presented in Table 1.

Serum biochemical profile

The effect of dietary treatments with Spirulina and exogenous protease on serological parameters are presented in Table 1. The serum total protein content and globulin content was increased ($p>0.05$) as the level of spirulina increased in the rations. The serum albumin content was decreased ($p>0.05$) as the level of spirulina increased in the rations. The serum glucose content, urea content, AST activity was decreased linearly as level of spirulina increased from 1 to 2 % when compared to control. The serum ALT activity was lower ($p>0.05$) in spirulina supplemented groups compared to control. The serum triglyceride content and total cholesterol content was decreased significantly as the level of spirulina increased with and without exogenous protease enzyme increased from 0 to 2 per cent. The serum HDL cholesterol level was increased whereas LDL cholesterol and VLDL cholesterol content was decreased with spirulina treatment groups compared to control.

Table 1. Effect of supplementation of Spirulina and exogenous Protease enzyme on Oxidative Stress and Antioxidant Status, Serum Biochemical Profile and Serum Lipid Profile

Parameter	Treatments				
	T_1	**T_2**	**T_3**	**T_4**	**T_5**
Oxidative Stress and Antioxidant Status					
MDA (µM/ml)*	$2.79^{a} \pm 0.07$	$2.38^{b} \pm 0.05$	$1.90^{d} \pm 0.05$	$2.25^{bc} \pm 0.14$	$2.01^{cd} \pm 0.08$
SOD (U/mg protein)*	$0.44^{b} \pm 0.03$	$0.55^{a} \pm 0.02$	$0.54^{a} \pm 0.02$	$0.52^{ab} \pm 0.03$	$0.53^{ab} \pm 0.03$
GPx (U/mg protein)*	$713.19^{d} \pm 11.70$	$774.96^{bc} \pm 13.73$	$799.39^{ab} \pm 8.36$	$763.61^{c} \pm 10.33$	$817.39^{a} \pm 7.55$
TAC (mM/mg Protein)*	$27.99^{d} \pm 0.57$	$52.88^{b} \pm 1.18$	$56.98^{a} \pm 0.81$	$49.62^{c} \pm 1.15$	$52.66^{b} \pm 2.11$
Serum biochemical profile					
Total protein (g/dl)	2.81 ± 0.06	2.91 ± 0.06	2.91 ± 0.06	2.87 ± 0.04	2.96 ± 0.11
Albumin (g/dl)	1.51 ± 0.02	1.45 ± 0.07	1.42 ± 0.05	1.41 ± 0.06	1.37 ± 0.05
Globulin (g/dl) *	$1.30^{b} \pm 0.04$	$1.46^{ab} \pm 0.08$	$1.48^{ab} \pm 0.05$	$1.46^{ab} \pm 0.07$	$1.58^{a} \pm 0.15$
A/G ratio	1.17 ± 0.03	1.02 ± 0.09	0.97 ± 0.06	0.99 ± 0.09	0.94 ± 0.12
Glucose (mg/dl) *	$290.12^{a} \pm 5.74$	$279.25^{ab} \pm 4.73$	$272.75^{bc} \pm 3.85$	$266.62^{bc} \pm 2.10$	$262.12^{c} \pm 1.28$
Urea (mg/dl) *	$7.22^{a} \pm 0.60$	$6.18^{ab} \pm 0.33$	$5.77^{ab} \pm 1.27$	$5.16^{ab} \pm 0.39$	$4.87^{b} \pm 0.45$
ALT (U/l)	50.12 ± 2.67	49.18 ± 1.76	48.08 ± 3.53	49.87 ± 2.41	49.01 ± 2.89
AST (U/l) *	$552.50^{a} \pm 9.49$	$502.75^{b} \pm 7.72$	$511.78^{b} \pm 8.70$	$479.22^{b} \pm 7.66$	$495.54^{b} \pm 4.38$
Serum lipid profile					
Triglycerides	122.25 ± 5.58	113.87 ± 4.09	110.75 ± 5.04	106.75 ± 9.65	108.62 ± 8.74
Total Cholesterol*	$118.37^{a} \pm 4.02$	$112.12^{ab} \pm 4.11$	$108.50^{ab} \pm 3.75$	$110.50^{ab} \pm 4.49$	$100.75^{b} \pm 6.50$
HDL Cholesterol	33.87 ± 1.65	35.37 ± 2.19	35.62 ± 1.97	34.25 ± 1.56	34.37 ± 1.63
LDL Cholesterol*	$60.05^{a} \pm 3.53$	$53.97^{ab} \pm 2.14$	$50.72^{ab} \pm 2.19$	$54.90^{ab} \pm 3.71$	$44.65^{b} \pm 4.93$
VLDL Cholesterol	24.45 ± 1.11	22.77 ± 0.81	22.15 ± 1.00	21.35 ± 1.93	21.72 ± 1.74

Values are expressed as Mean ± SE. Different superscripts (a, b, c, d) within a row indicate significant differences among treatments ($P < 0.05$). Parameters marked with (*) showed significant treatment effects. Treatment groups: T1 = Basal diet (Control); T2 = Basal diet + 1% Spirulina; T3 = Basal diet + 1% Spirulina + Protease enzyme; T4 = Basal diet + 2% Spirulina; T5 = Basal diet + 2% Spirulina + Protease enzyme.

Table 2: Effect of supplementation of Spirulina and exogenous Protease enzyme on duodenal morphometry (µm) in broilers

Treatments	Villus height (µm)*	Crypt depth (µm)*
T_1	$1920.10^{c} \pm 7.26$	$319.80^{bc} \pm 4.33$
T_2	$1941.71^{b} \pm 7.85$	$336.42^{ab} \pm 4.84$
T_3	$1963.34^{a} \pm 7.59$	$353.67^{a} \pm 9.88$
T_4	$1910.97^{c} \pm 5.89$	$307.85^{c} \pm 9.11$
T_5	$1914.58^{c} \pm 5.70$	$313.20^{bc} \pm 8.09$

^{abc} Values bearing different superscripts in a column differ significantly * ($P < 0.05$)

Duodenal morphometry

The villi height and crypt depth (µm) were increased gradually as the level of spirulina increased from 0 to 1% and there after it was decreased at 2% level (Table 2).

Discussion

Oxidative stress and Antioxidant status

MDA, an indicator of lipid peroxidation, decreased significantly with increasing levels of Spirulina and was further reduced by protease supplementation. This reduction might be attributed to the antioxidant compounds present in Spirulina, such as beta-carotene, zeaxanthin, phycocyanin and allophycocyanin (Mirzaie *et al.*, 2018). Further, enzymes supplementation improved the availability of nutrients in spirulina leading to decrease in MDA values. The results are in agreement with the fundings of Moustafa *et al.* (2021) in heat stressed broilers and Long *et al.* (2020) in broilers. El-Bahr *et al.* (2020) reported significant decrease in MDA values in broilers fed with different types of microalgae *viz.*, *Spirulina*, *Chlorella* and *Amphora* sps. Dietary supplementation of Spirulina (up to 2 %) and exogenous protease significantly increased ($p<0.05$) Superoxide dismutase activity (U/mg protein) compared to control, indicating enhanced antioxidant defence against reactive oxygen species. The improvement is consistent with findings reported by Park et al. (2018), Mirzaie et al. (2018) and Moustafa et al. (2021).

Glutathione peroxidase (U/mg protein) activity increased significantly in Spirulina-supplemented groups and was further enhanced by protease addition. This response may be due to improved availability of bioactive antioxidant compounds from Spirulina. Similar increases in GPx activity have been reported by Mirzaie et al. (2018) and Moustafa et al. (2021). Broilers receiving Spirulina showed significantly higher TAC values than the control, suggesting improved overall antioxidant capacity. The increase may be attributed to antioxidant constituents such as phycocyanin, carotenoids and phenolic compounds present in Spirulina. Comparable findings have been reported by Long et al. (2020) and Moustafa et al. (2021). The increased concentration of TAC indicates the improvement of the non-enzymatic antioxidant defence system in broilers.

The significant increase in GPx, SOD activity and TAC observed in Spirulina-supplemented broilers indicates there was a great correlation between addition of microalgae and improvement of antioxidant capacity. This effect may be attributed to the presence of antioxidant compounds in Spirulina, including phycocyanin, carotenoids, phenolic compounds and bioactive peptides (Barkia et al., 2019). In particular, C-phycocyanin and β-carotene are known to scavenge free radicals and protect cells against oxidative damage (Khan et al., 2005).

Serum biochemical profile

Serum total protein (g/dl), albumin (g/dl) and A/G ratio were not significantly affected by dietary treatments, whereas serum globulin (g/dl) levels were significantly higher ($p<0.05$) in spirulina-supplemented groups compared to control. Similar findings were reported by Fathi et al. (2018), whereas Sugiharto et al. (2018) did not find significant difference in serum globulin also.

Spirulina supplementation up to 2% significantly reduced serum glucose and urea levels, with further reductions observed following protease enzyme supplementation. These results agree with the reports of Pestana et al. (2020), Moustafa et al. (2021) and Long et al. (2020). Low serum urea levels observed suggest efficient nitrogen utilization and protein metabolism, which might have been enhanced by microalgae and protease supplementation thus contributing to a better balance between body protein synthesis and body protein degradation (Metayer et al., 2008).

Regarding liver function, spirulina supplementation significantly decreased ALT and AST activities, corroborating findings by Fathi et al. (2018), Jamil et al. (2015), Moustafa et al. (2021), and Selim et al. (2018). Liver, being the main metabolic organ of the body, its damage releases the higher amount of liver enzymes *i.e.,* ALT and AST. Reduced levels of liver enzymes in the present study indicates the hepatoprotective activities of spirulina as a result of antioxidant and anti-inflammatory properties as reported by Deng *et al.* (2010).

For serum lipid profile, spirulina had no significant effect ($p>0.05$) on serum triglycerides (mg/dl), HDL cholesterol (mg/dl) and VLDL cholesterol (mg/dl). However, total cholesterol and LDL cholesterol levels were significantly reduced ($p<0.05$) compared to the control group, consistent with reports by Moustafa et al. (2021), Fathi et al. (2018), and Mirzaie et al. (2018).

The hypolipidemic effect of spirulina might be attributed to C-phycocyanin-mediated inhibition of pancreatic lipase activity (Deng *et al*. 2010) or due to reduced intestinal cholesterol absorption by dietary fibre in microalgae which was evidenced by non-significant decrease in the ether extract retention in spirulina supplemented groups.

Duodenal morphometry

Duodenal morphometry showed significantly higher ($p<0.05$) villus height and crypt depth in broilers supplemented with 1% spirulina and protease, followed by 1% spirulina alone, compared to the control and 2% spirulina groups. Similar findings were reported by Khan et al. (2020) and Shanmugapriya et al. (2015), who observed improved villus height with spirulina supplementation up to 1%, followed by a decline at higher inclusion levels. The enhancement in intestinal morphology may be attributed to the high-quality protein and essential amino acid content of spirulina, while the reduced villus height and crypt depth at 2% supplementation could be associated with increased digesta viscosity, limiting the absorption of substrates.

Conclusion

From the present study, it was concluded that, spirulina supplementation, combined with exogenous protease, enhanced the antioxidant status of broilers and significantly reduced serum total and LDL cholesterol levels. Duodenal morphometry of broilers was improved at 1% level of spirulina with protease. However, further research needs to be carried out to optimize spirulina inclusion levels and enzyme combinations for efficient use in broiler and layer diets for profitable poultry farming.

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All experimental procedures were conducted following ethical guidelines approved by the Institutional Animal Ethics Committee.

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