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Effect of *Spirulina* on growth, immunity, gut bacterial load and histopathology of broiler birds

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ABSTRACT

This study was carried out to evaluate the effects of *Spirulina* on growth, immunity, gut microbiome, and important organ histopathology in Vencobb broilers. 160 day-old chicks were randomly assigned to four diets. T_0: basal diet, T_1: basal diet + *Spirulina* (0.50% of basal diet), T_2: basal diet + *Spirulina* (1.0% of basal diet), T_3: basal diet + *Spirulina* (1.50% of basal diet). The experimental period was 35 days. Body weight change was measured at weekly intervals. Humoral immunity, cellular immunity, lymphoid organ mass, histopathology, intestinal bacterial load and intestinal morphology were measured on the 35thday of the experimental feeding period. Body weights were significantly higher in all treated birds than in control. Lymphatic organ weights, HI titers and CBH responses in the various treatment groups were significantly higher than those in the control group. The total bacteria (log_{10} cfu/ml), *Escherichia coli* and *Salmonella* counts in the intestinal contents were significantly lower in the *Spirulina* treated group than in the control group, and the intestinal *Lactobacillus* count was higher in the treated birds than in the control group. The villi length and width of the treated birds were significantly greater than those of the control group. Histopathology studies have shown that Spirulina @ 1.5% of the basal diet have no detrimental effect on the bird's liver and kidneys. It can be concluded that adding 1.50% *Spirulina* to the diet of Vencobb broilers improves growth, immunity and reduces gut microbial load.

Keywords: Broilers, Growth, Gut microbes, Histopathology, Immunity, *Spirulina*

In poultry rearing, use of antibiotics as growth promoters to enhance the growth and feed efficiency is a common practice. But frequent use of antibiotics leads to antibiotic resistance causing a potential threat to public health. Now-a-days, the demand for non-conventional growth promoters for livestock and poultry has increased. *Spirulina platensis,* a filamentous cyanobacteria alga used as a growth promoter has increased significantly in poultry industry (Ibrahim *et al.* 2018). It contains high amount of protein, carbohydrate, several poly-unsaturated fatty acids (PUFA), minerals, vitamins, pigments like chlorophyll, high concentration of beta-carotenoids, xanthophylls and phycocyanin (Herath *et al.* 2023). *Spirulina* have anti-oxidant, anti-inflammatory, anti-cancerous effect (Pestana *et al.* 2020). Carbohydrate extracted from the *Spirulina platensis* known as spirulin popularly acts as prebiotics for the beneficiary microbiota present inside the body of the organism (Sugiharto *et al.* 2018). It inhibits several bacteria like *Staphylococcus faecalis*, *Staphylococcus epidermitis*, *Candida albicans*, *E.coli* and promotes the probiotic effect enhancing lactic acid bacteria (El- Dayem *et al.* 2021). During recent research, it was found that prebiotic effects of *Spirulina* ultimately affect the immunity of broilers (Moustafa *et al*. 2021). Due to the presence of different nutritionally beneficiary bioactive compounds and growth promoting property of *Spirulina*, it may become a potential poultry feed additive for commercial purpose. It also improves mineral absorption, prevents diarrhea and optimizes nutrient digestion (Zahir *et al.* 2019). Studies on supplementation of *Spirulina* and its effect in broiler chicken is relatively scanty and largely unknown; therefore, the present study was designed to examine the effects of *Spirulina* supplementation in distinct characteristics like growth, immunity, gut microbial load and histopathology of Vencobb broiler chicken.

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MATERIALS AND METHODS

The present study was carried out as per the guidelines laid down by the Institutional Animal Ethics Committee, College of Veterinary Science and Animal Husbandry, Odisha University of Agriculture and Technology, Bhubaneswar. A total of 160 day-old Vencobb broiler chicks were randomly assigned to four dietary treatments. Each treatment had four replicas, each with 10 chicks. In T_0 group, birds were fed with the regular basal diet considering it as control. In T_1, T_2 and T_3, birds were fed with basal diet as similar to that of the control in addition with *Spirulina* powder at 0.5%, 1.0% and 1.50% of basal diet, respectively. The experimental diets were made in accordance with BIS (2007). A deep litter system was used to rear the chicks. Before experiment, the pen, feeders, and waterers were properly cleaned with water followed by disinfection with Germex (Vetneeds lab). Birds were given the RD (Lasota strain) vaccination on the seventh day, the IBD (Intermediate strain) vaccine on the 14th day, and a booster dose of RD on the 22nd day.

Preparation of Spirulina powder: Spirulina was cultivated in the month of July, 2022 in a modified Zarrouk's medium (NaCl 1.0 g/L; $CaCl_2.2H_2O$ 0.04 g/L; KNO_3 2.5 g/L; $FeSO_4.7H_2O$ 0.01 g/L; EDTA (Na) 0.08 g/L; K_2SO_4 1.0 g/L; $MgSO_4.7H_2O$ 0.2 g/L; $NaHCO_3$ 16.8 g/L and K_2HPO_4 0.5 g/L). Algae were incubated in an artificial pond at mean temperature of 30°C. Harvesting was performed after 12-14 days. It was dried at 40°C and the composition of *Spirulina* powder was measured by AOAC (1995).

Body weight gain: Individual body weights of the Vencobb birds were measured by using electric pan balance at different weeks of the experiments. The initial body weight was subtracted from final to get the BW gain.

Estimation of bacterial load: For collection of caecal content, four birds from each treatment (total 16 birds) were sacrificed at 35th day. The caeca were separated, and the contents were aseptically collected in sterile bottles filled with ordinary saline and wrapped in aluminium foil. In a sterile ependymal tube, the inoculums were serially diluted, using a 1 ml suspension with 9 ml of normal saline. Caecal samples were serially diluted 10 times to a dilution level of 10^{-6}. Then, 0.1 ml of the dilution was pipetted out and incubated for *Salmonella, E. coli,* and *Lactobacillus count*, in McConkey's agar, Eosin-Methylene blue agar, and *Lactobacillus* MRS agar, respectively. The samples were applied to the agar surface using a sterilised glass spreader while simultaneously rotating the petridish beneath. For the total bacterial count, nutrient broth is employed. The petridish were incubated at 37°C for 24 h and individual colonies were counted with a colony counter and quantified as CFU/ml = (No. of colonies ×dilution factor) / volume of culture plate.

Immunity study: The cellular immunity was assessed at the age of five weeks (Edelman *et al.* 1986) by injecting PHA-P intradermally in the foot web of two birds from each replicate (eight birds in each treatment). The humoral immunity was determined after fifth week of the experiment as per method of Khalifeh *et al*. (2009).

Immunological organs: At the fifth week after the start of the feeding regimen, four birds from each treatment were randomly selected and killed by modified Kosher procedure (Panda and Mohapatra 1989). The thymus, bursa of fabricius, and spleen were collected and weighed in an electronic top pan balance.

Intestinal morphometry and histopathology of vital organs: At the end of 5th week of the experiment, representative portions of the duodenum, jejunum, ileum and vital organs (liver and kidney) were taken from two birds of each replicate (total 8 birds per treatment) and stored in 10% normal saline. After processing the tissues, it was fixed with 1% formalin. The formalin fixed tissues were first rinsed with water, then dehydrated in alcohol, and then cleaned in xylene. Sections were cut from prepared paraffin blocks at a thickness of 5 microns and stained using the haematoxylin and eosin procedure. For histological analysis, the stained slides were inspected under Leica DM500 light microscope.

RESULTS AND DISCUSSION

Chemical composition of basal feed: The broiler pre-starter, starter, and finisher rations had crude protein contents (%) of 22.38, 21.14 and 19.40, respectively (Table 1). The protein and energy needs were in line with those outlined in BIS (2007). *Spirulina* powder contains 46.85% CP and 7.90% EE. Similar to our result, Becker (2007) reported 46-63% crude protein and 4.0-9.0% ether extract in dry *Spirulina.* Ali (2017) found that the lipid and ash contents of *Spirulina platensis* were 5.3% and 6.9%, respectively, which is lower than our results, while the protein and fibre contents were 61.8% and 9.5%, respectively, which is higher than our results. The differences could be explained by either the growth environment or the difference in the climate during the time of cultivation.

Body weight gain: The weight of broiler chicks at several week of their life during the course of experimentation

Table 1. Ingredient and proximate composition of the basal diet

Particular	Pre-starter (%) (1-14th day)	Starter (%) (15-21st day)	Finisher (%) (22-35th day)
Maize	51.50	53.50	56.50
Soyabean meal	43.00	39.00	35.00
Vegetable oil	2.00	4.00	5.00
Dicalcium phosphate	1.83	1.83	1.83
Limestone	0.93	0.93	0.93
DL-Methionine	0.05	0.05	0.05
L-Lysine	0.03	0.03	0.03
Vitamin and mineral premix	0.15	0.15	0.15
Common salt	0.51	0.51	0.51
Total	100	100	100
Proximate composition (% DM)			
Crude protein	22.38	21.14	19.40
Ether extract	2.92	4.98	5.80
Crude fibre	4.80	4.63	5.10
Total ash	9.30	8.37	8.92
Nitrogen free extract*	60.60	60.88	60.78
Calcium	1.24	1.18	0.89
Phosphorus	0.57	0.62	0.51
Metabolisable energy (Kcal/kg) *	2995	3140	3225

*Calculated value.

Table 2. Body weight changes (g) in experimental birds

BW (g)(week)	Treatments				P-value
	T_0	T_1	T_2	T_3	
Initial	49.79±0.72	48.84±0.79	48.97±1.18	46.77±0.55	0.750
1st	171.08±3.28	167.90±3.46	172.95±2.92	174.40±3.36	0.470
2nd	502.35±9.17	503.21±12.02	501.5±7.21	502.85±10.30	0.990
3rd	790.65^{a}±19.29	837.81^{b}±4.37	863.60^{bc}±10.47	878.90^{c}±10.47	<0.01
4th	1253.80^{a}±23.22	1299.40^{ab}±27.69	1379.60^{b}±29.06	1386.00^{b}±31.27	0.030
5th	1661.50^{a}±28.87	1806.50^{b}±20.41	1775.20^{b}±21.84	1883.50^{c}±21.84	<0.01

^{abc}Values bearing different superscripts in a row differ significantly (P<0.05).

period showed a significant difference in body weight change in supplemented groups than control (Table 2). The day-old live body weight of experimental broiler chicks was almost alike indicating the well randomization of distributing chicks between the experimental treatments. It was seen that the body weight of different dietary groups remains to be similar in the 1st and 2nd week. From the 3rd week the average weekly body weight of chicks in all the dietary groups were significantly (P<0.05) higher than control birds. This implies that supplementation of *Spirulina* above 0.5% in the ration of Vencobb broilers had a significant effect on body weight gain of birds. At the end of the experiment, it was found that supplementation of *Spirulina* @ 1.5% had significantly (P<0.05) improved the body weight gain as compared to others.

Similar to our results, Jamil *et al.* (2015) observed significant increase in body weight of broiler birds supplemented with 2 g, 4 g and 8 g *Spirulina* per kg of diet for 4 weeks. Mirzaie *et al.* (2018) observed significant increase in body weight gain of heat stressed birds supplemented with 0.5% to 2% *Spirulina*. Pandav and Puranik (2015) observed significant improvement in growth rate in Indbro fast white broiler birds supplemented with *Spirulina* enriched with zinc and iron. Selim *et al.* (2017) observed that *Spirulina* at a concentration of 0.3% is enough to increase the growth rate and feed intake of Norfa laying hens. Park *et al.* (2018) observed significant increase in growth rate of broiler birds supplemented with 0.25, 0.5, 0.75, 1.0% *Spirulina* for 5 weeks. Ibrahim *et al.* (2018) observed significant improvement in growth rate of Japanese quail supplemented with 0.5, 1, 2 g *Spirulina* extracts. *Spirulina* supplementation at 2% body weight significantly (P<0.05) improved body weight gain, feed intake and feed conversion efficiency (Khan *et al.* 2021). El-Dayem *et al.* (2021) observed a significant (P<0.05) increase in body weight gain and decreased feed conversion ratio, when *Spirulina* was added at doses of 1 and 2 g/kg to quail diet. The increase in body weight in birds supplemented with *Spirulina platensis* may be due to improved feed utilisation efficiency, vitamin and minerals absorption and optimizing the nutrient digestion process (Gruzauskas *et al.* 2004).

Contrary to this, when *Spirulina* was provided to the broiler diet as 10-20% replacement of soyabean meal, it showed gradual decrease in growth rate (Toyomizu *et al.* 2001). The decreased body weight may be due to different strains, high dose rate and environmental condition compared to present experiment.

Intestinal bacterial load: On the 35th day of the experiment, the total plate count ($\log_{10}$ cfu/ml) of cecal contents was significantly higher (P<0.05) in the T_0 group than in the *Spirulina* treated groups (Table 3). Similarly in this experiment it was revealed that the Log_{10} values of intestinal *E. coli* and *Salmonella* count was found to decrease significantly (P<0.05) in all the treated birds than control. Earlier studies have suggested that *Spirulina* and its extract is an effective antimicrobial agent against bacteria, fungi, and viruses (Shanmugapriya *et al.* 2015). Intestinal *Lactobacillus* count was found to be significantly (P<0.05) higher in all *Spirulina* treated groups than control birds. Similarly, Park *et al.* (2018) observed that supplementation of 1% *Spirulina* significantly increased the *Lactobacillus* count in ceacal content of birds. Fathi *et al.* (2018) observed that supplementation of *Spirulina* @ 0.7 and 0.9 g/kg diet showed decreased microbial load and increased *Lactobacillus* count in the intestinal content. Shakoori *et al.* (2018) observed significant increase in *Lactobacillus* count and decrease in coli form count in broiler birds supplemented with 0.66% and 1% of micro encapsulated *Spirulina*. The non-digestible part of the

Table 3. Log_{10} (cfu/ml) values of intestinal bacterial load of experimental birds at 35th days

Parameter	Treatments				P-value
	T_0	T_1	T_2	T_3	
Total plate count	8.90^{c}±0.02	8.10^{b}±0.02	7.80^{a}±0.04	7.66^{a}±0.06	0.037
E. coli count	5.70^{b}±0.10	3.11^{a}±0.43	3.75^{a}±0.35	3.07^{a}±0.11	<0.01
Salmonella count	3.45 ^{c}±0.10	2.99^{b}±0.10	2.45^{ab}±0.21	2.14^{a}±0.17	<0.01
Lactobacillus count	3.10^{a}±0.15	4.50^{b}±0.10	5.60^{c}±0.16	6.10^{cd}±0.10	<0.01

^{abcd}Values bearing different superscripts in a row differ significantly (P<0.05).

Table 4. Immune status and immune organ weight of experimental birds

Attribute	Treatment				P-value
	T_0	T_1	T_2	T_3	
Immune status					
CBH (%)	116.43^{a}±2.71	124.40^{ab}±9.10	131.31^{ab}±5.21	147.47^{b}±7.80	0.044
HI titre (Log_2)	4.40a±0.66	5.30^{ab}±0.33	6.42^{bc}±0.57	7.00^{bc}±0.66	0.010
Immune organ weight (% BW)					
Spleen	0.18^{a}±0.01	0.22^{a}±0.02	0.28^{b}±0.01	0.29^{b}±0.02	0.024
Thymus	0.40^{a}±0.09	0.42^{a}±0.08	0.51^{b}±0.09	0.64^{c}±0.06	<0.01
Bursa	0.09^{a}±0.01	0.10^{a}±0.02	0.12^{a}±0.02	0.19^{b}±0.01	0.031

^{abc}Values bearing different superscripts in a row differ significantly (P<0.05).

cell wall of *Spirulina* and chlorophyll present in it act as a source of prebiotics, so help in the growth of *Lactobacillus*.

Immunity and weight of lymphoid organs: Spirulina supplementation enhanced the humoral and cellular immunity in Vencobb birds (Table 4). Supplementation of *Spirulina* @ 1% in the diet significantly (P<0.05) enhanced the immune system of chicken through enhanced antigen processing, T cell activity and microbe destroying capacity (Qureshi *et al.*1996). Lokapirnasari *et al.* (2016) observed significant increase in immunity in chicken infected with Avian Influenza H5N1 virus followed by supplementation with 10% and 20% *Spirulina*. *Spirulina* enhances the function of macrophage and increased the potency of the mononuclear phagocyte system. Active principles like β-glucan and phycocyanin present in *Spirulina* enhanced the production and maturation of antibodies in birds (Seyidoglu *et al.* 2017). Supplementation of *Spirulina platensis* to K strain chicks @ 0, 10, 100, 1,000 and 10,000 ppm improved the IgG and total anti-SRBC titers (P<0.05) over the controls. Similarly, birds supplemented with 10,000 ppm *Spirulina* in the diet had a higher PHA-P-mediated lympho proliferative response (Qureshi *et al.* 1996). Supplementation of *Spirulina* significantly reduces the expression of cytokines like IL-1β, IL-6 and TNF-α; C-phycocyanins and β-carotene present in *Spirulina* blocks the activity of NF-κB and suppresses TNF-α. Similarly, β-carotene present in *Spirulina* also inhibits proinflammatory cytokines like IL-1β and IL-6 (El- Dayem *et al.* 2021).

In view of immunity status of the broilers, the weight of immune organs was measured. Supplementation of *Spirulina* at different concentration in the diet of Vencobb broiler chicken significantly (P<0.05) increased the weight of immune organs like spleen, thymus and bursa, which supports the finding that it has got immune enhanced effect in broiler chicken (Table 4). Similar to our findings, Fathi *et al.* (2018) observed that birds fed with *Spirulina* @ 0.7 and 0.9 g/kg of diet have increased immune organ weights (Bursa, thymus and spleen). Similarly, supplementation of *Spirulina platensis* to K strain chicks @ 0, 10, 100, 1,000 and 10,000 ppm increased thymus weight compared to the control birds, whereas no changes in the weight of bursa and spleen of birds were observed in supplemented birds (Qureshi *et al.*1996).

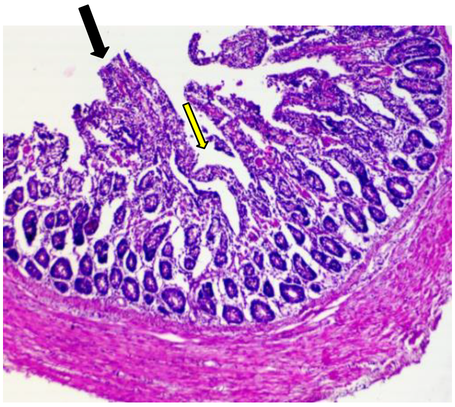

Fig. 1. Duodenum of birds of T_3 showing wider villi (Black arrow) and crypt (Yellow arrow).

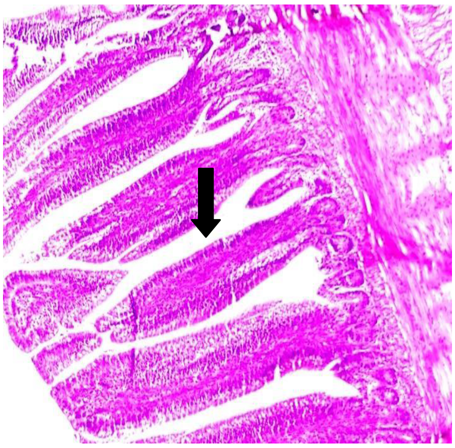

Fig. 2. Photomicrograph of jejunum showing marked increase in villi height in T_3(Arrow).

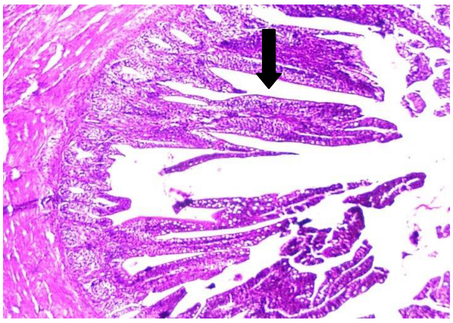

Fig. 3. Photomicrograph of ileum showing marked increase in villi height in T_3 (Arrow).

Intestinal morphometry studies: Photographs of histological sections of the intestine are shown in the Figs. 1-3. It can be seen in the figure that the height of the villi is higher in the T_3 group fed 1.50% *Spirulina*. The data mentioned in Supplementary Table 1 represents the histological analysis of villi length, villi width and crypt depth in duodenum, jejunum and ileum parts of intestine. In the current study, the villi length (µm) and villi width at middle (µm) of duodenum, jejunum and ileum in the small intestine of broilers of different treated groups differed significantly ($P<0.05$) compared to control birds. The crypt depth was significantly higher ($P<0.05$) in treated birds than control in duodenal part of the intestine. Similar to our study, inclusion of *Spirulina* @ 10 g/kg diet significantly increased the villi length and decreased the pathogenic bacteria in intestine of broiler birds (Shanmugapriya *et al.* 2015). *Spirulina* supplementation improved villous length and increased goblet cell numbers in chicken fed 1, 1.5, and 2 g *Spirulina*/kg diet compared to control birds (Khan *et al.* 2021). Pestana *et al.* (2020) observed that when *Spirulina* was added @ 15% in the basal diet it significantly ($P<0.05$) increased in length of intestinal parts compared to that of the control group. Khan *et al.* (2020) stated that broiler chicken supplemented with 1, 1.5, 2 g *Spirulina* per kg basal diet have increased crypt villi, villi length and goblet cell. The increased villi length, villi width and crypt may be due to pre and probiotics effect of *Spirulina* in birds. Due to increase in villi and width, the absorption of various nutrients was significantly increased leading to better performance of birds.

Histopathological studies of vital organ: Histological studies of vital organ (liver and kidney) in *Spirulina* supplemented broilers showed no deformities/ changes in liver and kidney tissues indicating no detrimental effects of *Spirulina* on vital organ of broilers (Supplementary Figs. 1-4). Park *et al.* (2018) observed that dietary supplementation of 0.25, 0.5, 0.75, 1.0% *Spirulina* in broiler birds has no significant ($P<0.05$) effect on liver, gizzard weight of birds. Similarly, Fathi *et al.* (2018) observed that birds fed with *Spirulina* @ 0.7 and 0.9 g/kg of diet for 38 days had no adverse effect on liver, heart and gizzard histology of the birds.

From this experiment, it may be concluded that supplementation of *Spirulina* @ 1.5% in Vencobb broiler ration enhanced the body weight gain, immunity and reduced the gut microbial load without any adverse effect on the vital organs of chicken.

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