Effect of partial supplementation of sun-dried Azolla as a protein source on the immunity and antioxidant status of commercial broilers

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Abstract

Aim: The present study was conducted to evaluate the effect of partial supplementation of sun-dried Azolla as a protein source on the immunity of commercial broilers in coastal Odisha.

Materials and Methods: A 180 day-old broiler chicks were distributed in six dietary treatments viz. C1: Basal diet, C2: Basal diet + enzyme, T1: Basal diet + 5% protein from Azolla, T2: Basal diet + 5% protein from Azolla + enzyme, T3: Basal diet + 10% protein from Azolla and T4: Basal diet + 10% protein from Azolla + enzyme. Cutaneous basophile hypersensitivity (CBH) and humoral immunity response were determined at the 38th day of age. At 42nd day, the weight of lymphoid organs, an antioxidant enzyme, and lipid peroxidation activity were determined.

Results: The CBH response did not differ significantly among the treated groups, but the sheep red blood cells response was significantly higher in T4. The weight of lymphoid organs and immune organs of all the treated groups did not differ significantly (p>0.05). The erythrocyte catalase level of T4 group was found to be significantly higher than rest of the treated groups except T3.

Conclusion: It may be concluded that supplementation of Azolla at 10% of dietary protein requirement along with enzyme supplementation in an isonitrogenous diet showed a better immune response in broilers.

Keywords: antioxidant, Azolla, broiler, immune response.

Introduction

Azolla is, a protein rich aquatic plant, containing almost all essential amino acids, carotene, and several growth promoter intermediaries, minerals such as calcium, phosphorus magnesium, potassium, iron, and copper [1], and certain compounds such as carotenoids, bio-polymers, and probiotics [2]. In case of commercial broiler chickens, Azolla can be efficiently used as a feed ingredient in the form of sun-dried and ground Azolla meal [3].

Azolla meal can partially replace the dietary protein sources up to 5-10% without any adverse effect on the health and performance of the birds [4]. Inclusion of Azolla in the poultry diet helps in the economization of production cost; and thus, increasing the net profit [1]. Similar findings have been observed in the case of quails with optimum displacement level of Azolla restricted to 5% [5]. The high carotene content of Azolla is responsible for its immune-potentiating effect in poultry birds [1]. In non-ruminants, essential amino acids, linoleic acid, vitamin A, folic acid, vitamin B6, vitamin B12, vitamin C, vitamin E, zinc, copper, iron, and selenium affect one or more indices of immunity [6]. Use of Azolla as an antibacterial and antioxidant agent in complementary and alternate medicine [7] had been recommended due to its high phenolic and flavonoid content [8]. Antioxidant activity of Azolla had been successfully demonstrated in Swiss albino mice [9].

With consideration of the presence of essential nutrients in Azolla, the present experiment was planned with the objective of studying the effect of partial supplementation of sun-dried Azolla as a protein source on the immunity of commercial broilers.

Materials and Methods

Ethical approval

The experiment followed the guidelines of Institutional Animal Ethics Committee.

Study area

The experiment was conducted on commercial broilers reared in the Poultry Farm in Department of Poultry Science, College of Veterinary Sciences and...
Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar, Odisha.

**Experimental birds**

The 180 day-old broiler chicks were distributed randomly in six dietary treatments with three replications. The six dietary treatments were: C1: Basal diet, C2: Basal diet + enzyme, T1: Basal diet + 5% dietary protein from *Azolla*, T2: Basal diet + 5% dietary protein from *Azolla* + enzyme, T3: Basal diet + 10% dietary protein from *Azolla*, T4: Basal diet + 10% dietary protein from *Azolla* + enzyme. The enzyme used in the study was containing cellulase, xylanase, pectinase, and phytase. The experiment continued up to 6th week of age. The chemical composition of *Azolla* meal, ingredient composition and chemical composition of the experimental diet are provided in Tables-1, 2 and 3, respectively.

**Evaluation of immunological parameters**

At 38th day of age, two birds from each replicate in each dietary treatment were injected intradermally in the comb with 100 μg of phytohemaglutinin-P in 0.1 ml of normal saline to measure the cell-mediated immune (CMI) response by cutaneous basophilic hypersensitivity (CBH) test [10]. The thickness of comb was measured using digital caliper before inoculation and 24 h post inoculation, and CBH response was calculated as per Soni et al. [11]. At 38th day, the measure of humoral immunity was carried out as per the method described by Abdallah et al. [12]. At 6th week, three birds from each treatment were slaughtered and the weights of bursa, thymus, and spleen were recorded. About 3 ml blood, for assessing the antioxidant indices, was collected in sterilized microcentrifuge tubes containing acid citrate dextrose (citric acid 8.0 g, sodium citrate 22.0 g, and dextrose 25.0 g and volume made to 1 L in distilled water) at 0.15 ml/ml blood as anticoagulant. The blood samples were centrifuged at 3000 rpm for 10 min at 4°C and then the plasma and buffy coat were separated. The resulting erythrocyte pellet was washed thrice with phosphate buffer saline (PBS; pH 7.4; disodium hydrogen phosphate 13.65 g, sodium dihydrogen phosphate 2.43 g, and sodium chloride 10 g dissolved in 800 ml distilled water, pH adjusted to 7.4 and volume made to 1 L) as per Yagi et al. [13]. Red blood cell (RBC) diluted to 1:1 in PBS was used for the estimation of hemoglobin. For the estimation of catalase 1 ml of the 1:1 diluted RBCs in PBS were mixed with 9 ml distilled water to prepare hemolysate of 1:20 dilution. Erythrocyte catalase was assayed in erythrocytes by the spectrophotometric method as described by Bergmeyer [14]. The lipid peroxide level in the RBC hemolysate was determined by the method of Placer et al. [15].

**Statistical analysis**

The data from the experiment were subjected to statistical analysis as per the methods suggested by Snedecor and Cochran [16].

**Results**

**CBH and humoral immunity response**

The CBH response and antibody titers (log₂) against sheep RBCs (SRBC) inoculation at 6th week of age of broiler chicks is presented in Table-4. The CBH response did not differ significantly among the treated groups. However, the SRBC response was significantly higher in T4 than that of other treated groups. The SRBC response of the birds of T2 was significantly higher than that of C1 but did not differ significantly (p>0.05) from other treated groups except T4.

**Lymphoid organs**

The weights of lymphoid organs (percentage of live weight) of Vencobb broiler chicks under different dietary treatments are presented in Table-5. The average weights of lymphoid organs viz. spleen, bursa, and thymus-expressed as percentage of live weight of 6 weeks old broiler chicks ranged from 0.084±0.007 (C1) to 0.184±0.009 (T3), 0.091±0.022 (C2) to 0.194±0.058 (T4), and 0.329±0.033 (C1) to 0.577±0.152 (T4), respectively.
The relative weight of spleen of broilers in T2 was numerically higher than all the other treatments. The percent weight of bursa and thymus were found to be the highest in the birds of treatment T3. No significant (p>0.05) difference was observed on the above parameters between the treated groups. The average weight (percentage of live weight) of liver which has got some role in immunity, ranged from 1.89±0.094 (C₁) to 2.403±0.152 (T₁) and showed insignificant (p>0.05) difference between the groups.

The weight of lymphoid organs or immune organs of all the treated groups did not differ significantly (p>0.05). Supplement of dietary protein from Azolla at 5% or 10% level had no significant effect on the weight of lymphoid organs. Furthermore, enzyme supplementation had no effect on spleen, bursa, and thymus gland relative weights when compared with chick groups fed on the same diet without enzyme supplementation.

**Antioxidant status**

The antioxidant enzyme and lipid peroxidation activity in Vencobb broiler birds under different dietary treatments are presented in Table-6. Erythrocyte catalase activity (units/mg of hemoglobin) values of the birds at 6th week of age were 1.27±0.04, 1.34±0.03, 1.90±0.07, 2.05±0.20, 2.94±0.07, and 3.13±0.05 in the treatments C₁, C₂, T₁, T₂, T₃, and T₄, respectively. Erythrocyte catalase activity was significantly (p<0.05) higher in T₃ and T₄ groups compared to all other groups.

The erythrocyte malondialdehyde (MDA) levels (nmol MDA/mg of hemoglobin) of 6-week-old broiler birds under treatments C₁, C₂, T₁, T₂, T₃, and T₄ were 2.01±0.15, 1.93±0.03, 1.95±0.16, 1.93±0.12, 1.97±0.14, and 1.92±0.07, respectively. The values showed insignificant (p>0.05) difference among the treatment groups with numerically highest and lowest value represented by treatment C₁ and T₄, respectively.

### Table-3: Chemical composition (percentage of dry matter basis) of experimental diet.

| Nutrient          | Starter ration | Finisher ration | p value |
|-------------------|----------------|-----------------|---------|
|                   | C₁/C₂          | T₁/T₂           | T₃/T₄   |
| Moisture          | 13.14±0.094    | 12.90±0.039     | 13.25±0.05 |
| Crude protein     | 20.14±0.094    | 20.08±0.039     | 20.12±0.05 |
| Ether extract     | 1.57±0.039     | 1.80±0.039      | 2.01±0.05 |
| Crude fiber       | 4.03±0.039     | 4.30±0.039      | 4.86±0.05 |
| Total ash         | 6.37±0.039     | 7.21±0.039      | 7.86±0.05 |
| NFE               | 67.89±0.039    | 66.61±0.039     | 65.15±0.05 |
| Calcium           | 0.82±0.039     | 0.91±0.039      | 0.91±0.05 |
| Available phosphorus | 0.53±0.039 | 0.51±0.039       | 0.54±0.05 |
| Metabolizable energy*(Kcal/kg) | 2900.00±0.039 | 2899.78±0.039 | 2902.21±0.05 |

NFE=Nitrogen free extract

### Table-4: SRBC and CBH response of broiler chicks at 6 weeks of age.

| Parameters (unit) | Treatments | p value |
|-------------------|------------|---------|
| SRBC (log₂)       | C₁         | 0.005   |
|                    | C₂         |         |
|                    | T₁         |         |
|                    | T₂         |         |
|                    | T₃         |         |
|                    | T₄         |         |
| CBH               | 0.14       |         |

Values bearing different superscripts in a row differ significantly (p<0.05), SRBC=Sheep red blood cells, CBH=Cutaneous basophilc hypersensitivity

### Table-5: Weight of lymphoid organs (percentage of live weight) of broiler.

| Organ       | Treatments | p value |
|-------------|------------|---------|
| Spleen      | C₁         | 0.09    |
|             | C₂         |         |
|             | T₁         |         |
|             | T₂         |         |
|             | T₃         |         |
|             | T₄         |         |
| Bursa       | 0.56       |         |
|             | 0.56       |         |
|             | 0.56       |         |
|             | 0.56       |         |
|             | 0.56       |         |
| Thymus      | 0.38       |         |
|             | 0.38       |         |
|             | 0.38       |         |
|             | 0.38       |         |
|             | 0.38       |         |

### Table-6: Antioxidant enzyme and lipid peroxidation activity in broiler chicks.

| Parameters (unit) | Treatments | p value |
|-------------------|------------|---------|
| Erythrocyte catalase (units/mg of hemoglobin) | C₁         | <0.01   |
|                   | C₂         |         |
|                   | T₁         |         |
|                   | T₂         |         |
|                   | T₃         |         |
|                   | T₄         |         |
| Erythrocyte MDA (nmol MDA/mg of hemoglobin) | 0.99       |         |
|                   | 1.92±0.07  |         |
|                   | 1.97±0.14  |         |
|                   | 1.93±0.12  |         |
|                   | 1.95±0.16  |         |
|                   | 1.93±0.07  |         |
|                   | 1.90±0.07  |         |
|                   | 1.90±0.07  |         |
|                   | 2.05±0.20  |         |
|                   | 2.94±0.07  |         |
|                   | 3.13±0.05  |         |
|                   | 1.27±0.04  |         |
|                   | 1.34±0.03  |         |
|                   | 1.90±0.07  |         |
|                   | 2.05±0.20  |         |
|                   | 2.94±0.07  |         |
|                   | 3.13±0.05  |         |

Values bearing different superscripts in a row differ significantly (p<0.05), MDA=Malondialdehyde
Discussion

The CBH response and antibody titers (log₂) against SRBC inoculation at 6th week of age of broiler chicks is presented in Table-4. This implied that the CMI response in the Azolla supplemented groups was similar as that of the control groups. This is in agreement with the findings of Sujatha et al. [17]. The results implied that the SRBC response was better in groups having Azolla with enzyme supplementation. This is in agreement with the findings of Prabina and Kumar [18]. Dhumal et al. [1] reported that Azolla meal feeding in broiler improved the antibody titer values as compared to control group. This further corroborated the findings of Sujatha et al. [17] who reported higher mean hemagglutination inhibition titer against GRBC inoculation in the raw Azolla fed group than the control group. The weights of lymphoid organs (percentage of live weight) of Vencobb broiler chicks under different dietary treatments are presented in Table-5. The weight of lymphoid organs or immune organs of all the treated groups did not differ significantly (p>0.05). Supplementation of dietary protein from Azolla at 5% or 10% level with or without enzyme supplementation had no significant effect on the weight of lymphoid organs. In the available literatures, little evidence has been found regarding the CMI and humoral immune response, as well as, the weight of lymphoid organs in chicken in relation to Azolla supplementation in the diet.

The antioxidant enzyme and lipid peroxidation activity in broiler birds under different dietary treatments are presented in Table-6. The erythrocyte catalase level of T₁ group was found to be significantly higher than the rest of the treated groups except T₄. Furthermore, erythrocyte catalase levels were found to be significantly (p<0.05) higher than that of control groups. The increase in levels of erythrocyte catalase of broilers fed Azolla might be due to presence of iron and copper in Azolla as catalase is a heme-containing antioxidant enzyme, which acts sequentially to superoxide dismutase in the conversion of hydrogen peroxide to water [19]. Spears [20] reported that selenium, vitamin E, chromium, cobalt, copper, and vitamin A have immune regulatory properties in cattle. Fe³⁺ protoporphyrin is the central catalase group. Catalase activity is reduced in Cu deficiency [21]. This is because Cu is necessary to adequate Fe utilization, which is an important component of catalase. Some researchers have reported an increase [22]; others have reported a decrease in erythrocyte catalase activity to be associated with copper deficiency [23]. Erythrocyte MDA (lipid peroxidation activity) levels did not differ significantly (p>0.05) between groups. Vitamins, recognized as a potent lipid-soluble antioxidant, have been found to play a key role in the normal functioning of the immune system and protects against lipid peroxidation of the cell membrane-initiated by free radicals by arresting or entirely preventing the process [24-27]. In spite of high fiber feeding in Azolla supplemented groups, due to presence vitamins along with other essential nutrients, no significant level of lipid peroxidation activity was observed in Azolla fed groups.

Moreover, the better immune response as observed in the present study might be the due presence of essential nutrients in Azolla relating to the immunity of the birds. Azolla is rich in protein, contains almost all essential amino acids, carotene, and several growth promoter intermediaries, and minerals such as calcium, phosphorus magnesium, potassium, iron, and copper [1]. Apart from nutrients, Azolla also contains certain compounds such as carotenoids, bio-polymers, and probiotics which contribute to higher productivity and health of animals [2].

Conclusion

From this, it may be concluded that replacement of soybean with Azolla, supplying 10% of protein requirement along with enzyme supplementation in the isonitrogenous diet showed better immune response in broiler chickens.

Authors’ Contributions

This study is a component of the work towards the M. V. Sc. thesis of the first author BC. GPM, CRP, SKM & NCB: Provided guidance during the entire experiment. BC, SKM & KB: Prepared and corrected the manuscript. AD: Helped in conducting the experiment and analysis of immunological parameters. All authors have read and approved the final version of the manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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