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Effect of supplementing humic substance on immune parameters in broilers

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Abstract
This study looked at how broilers immune parameters measurements were affected by humic substance supplementation. One hundred twenty-day-old broiler chicks were divided into four groups: the control group (T_1) was fed a standard diet prepared in accordance with the Bureau of Indian Standards (2007), while the three experimental groups were fed diets containing 0.02% humic substance (T_2), 0.04% humic substance (T_3), and 0.02% bacitracin methylene disalicylate (T_4). On the 42nd day of the trial, there was no discernible improvement in the immune response against Newcastle disease and infectious bursal disease, nor was there any significant difference in the immune organ weight between all the groups and the control.

Keywords: Humic substance, Immune parameters, antibiotic growth promoter, broilers

1. Introduction
The poultry business has seen substantial increase in recent years, which may be attributed to several factors such as the introduction of new strains, disease control, and scientific management approaches. The world's supply of premium proteins, such meat and eggs, has expanded dramatically as a result of this development, resulting in an increase in farmers' revenue. However, there is a dubious practice of using subtherapeutic antibiotics in chicken farming alongside this accomplishment. In the past, this technique aimed to enhance chicken development and health by lowering pathogens, modifying immunology, and decreasing inflammation (Niewold, 2007) [12]. Scientific evidence alarmingly demonstrates that the careless use of antibiotics is contributing to the evolution of bacterial resistance (Apata, 2009) [4]. Numerous ecosystems have been home to these resistant bacteria, which might be harmful to human health (Zhang *et al*., 2020) [18]. The European Union introduced a comprehensive ban on the use of growth-promoting antibiotics in animal production in 2006 in response to the growing concern over antibiotic resistance. The United States then acknowledged the potential risks to public health associated with antibiotic resistance in 2017 (Salim *et al*., 2018) [14]. This dual scenario highlights the need for a careful balance between expansion aspirations and public health concerns by posing significant questions regarding the sustainability and long-term effects of antibiotic usage in the chicken business.
Broiler chicken diets are starting to use humic materials as an option that is gaining popularity in poultry nutrition. The special properties of humic compounds, which are produced when organic waste breaks down, can help poultry grow, digest nutrients more efficiently, and generally feel better. When organic matter decomposes, long molecular chains and high molecular weight organic molecules are created. These chemicals are known as humic substances. The three primary fractions of humic substances, which comprise most soil organic matter, are fulvic acids (an acid-insoluble and alkali-soluble fraction), humin (an acid-insoluble and alkali-soluble fraction), and humic acids (HA), an acid-insoluble fraction (Abd El-Hack, 2016) [1].
Humic substances are essential for poultry productivity because they contain several important characteristics, such as proteins, water solubility, antibacterial qualities, and immune-stimulating substances. Schepetkin *et al*. (2003) [15] shown that they may modify the intestinal microbiota by increasing the quantity of beneficial bacteria. Furthermore, research by Taklimi *et al*. (2012) [17] suggests that the villi height and crypt depth of the broiler's jejunum may be

impacted by humic acids (HA). Furthermore, studies have demonstrated the anti-inflammatory, adsorbent, antitoxic, and antibacterial properties of humic materials, which safeguard the mucosa of the stomach. Because of their diverse structures and functional groups, humic substances have a significant adsorption capacity that influences their spectral, electrochemical, colloidal, and ion exchange characteristics.

Koksal and Kucukersan (2012) [9] found no significant impact of humates in broiler diets on serum antibody levels for Newcastle Disease ($p>0.05$). Nagaraju *et al*. (2014) [11] observed numerically higher but statistically non-significant ($p>0.05$) HI titers for Newcastle Disease with humic acid (HA) supplementation, while IBD titers were significantly improved ($p<0.05$) with HA compared to antibiotic treatments. Simakova *et al*. (2021) [16] reported that different concentrations of humic substances enhanced lysozyme and bactericidal activity in broiler blood serum, improving nonspecific resistance. Akaichi *et al*. (2022) [3] discovered that on day 16, broilers fed a diet with both humic acid and organic acids had significantly higher antibody titers against Newcastle Disease Virus (NDV) and Infectious Bronchitis Virus (IBV) compared to those receiving only organic acids ($p<0.05$). Additionally, humic acid alone increased antibody titers against IBV by day 26 ($p<0.05$).

Koksal and Kucukersan (2012) [9] investigated the effects of incorporating humates into broiler diets on the weights of immune organs, finding no significant differences ($p>0.05$) in the weights of the spleen and bursa fabricius among treatment groups. Nagaraju *et al*. (2014) [11] examined a humic acid-based product as an antibiotic substitute in broilers, reporting no significant differences in the relative weights of the thymus, spleen, and bursa among different treatments. Disetlhe *et al*. (2017) [6] supplemented humic acid and enzymes in canola-based broiler diets, finding improved distribution and density of lymphoid tissue in birds fed humic acid. This suggests that humic acid supports the growth of immune organs like the thymus and bursa of Fabricius, crucial for avian immune systems.

2. Materials and Methods

Venkateshwara Hatcheries Pvt. Ltd. provided 120 day-old commercial broiler chicks for this investigation, and Novel Links, Srigandada Kaval, Bengaluru provided the humic substance. The chicks were first evaluated on the basis of their weight upon acquisition, and then they were split into four experimental groups at random. There were three duplicates in per group, and each replicate included 10 chicks. Based on the guidelines provided by the Bureau of Indian Standards (BIS) in 2007, the basal diet (T_1) was created. For Treatment 2 (T_2), 0.02% humic material was added to the baseline diet. For Treatment 3 (T_3), 0.04% humic material was added to the baseline diet. Additionally, 0.02 percent antibiotic BMD (bacitracin methylene disalicylate) was added to the baseline diet for Treatment 4 (T_4).

Up to the age of six weeks, the chicks were kept under regular management procedures and grown in a deep litter system. The birds were vaccinated according to a standard vaccination schedule. Water and food were given to the animals whenever they needed them during the trial. The KVAFSU Institutional Animal Ethics Committee in Bidar, Karnataka, gave its approval to the study.

2.1 Immunological response

2.1.1 Antibody titers against Newcastle and Infectious bursal disease

During the study, blood samples were obtained from two birds from each replication on the 42nd day. Subsequently, serum was separated from these blood samples, and the antibody titer against the Newcastle disease virus was determined through hemagglutination (HA) and hemagglutination inhibition (HI) tests. Additionally, the antibody titer against the Infectious bursal disease virus was assessed using the enzyme-linked immunosorbent assay (ELISA) test.

At the conclusion of the experiment, the levels of antibodies against Newcastle disease and Infectious bursal disease were determined in different treatment groups. For Newcastle disease, the HA and HI tests, as described by Allan and Gough in 1974, were utilized. Meanwhile, for Infectious bursal disease, an ELISA kit was employed to assess antibody levels.

2.1.2 Lymphoid organs weight

At the conclusion of the experiment, two birds from each replication within each treatment group were euthanized for the purpose of assessing the weights of lymphoid organs, specifically the spleen, thymus, and bursa of Fabricius. These recorded organ weights were then expressed as a percentage of the weight of the live bird prior to slaughter. This methodology allowed for the evaluation of the relative size and development of these important immune organs in response to the experimental treatments.

General formula for calculation of per cent relative weight of immune organ is asfollows:

$$\text{Lymphoid organ weight (\%)} = \frac{\text{Lymphoid organ weight (g)}}{\text{Pre slaughter live weight (g)}} \times 100$$

2.1.3 Serum biochemistry

On the 42nd day of the study, blood samples were collected from two birds from each replicate using a sterile 2 ml syringe. The blood was collected into plain vacutainer tubes without anticoagulant, following standard procedures as outlined by Calnek *et al*. (1991) [19]. The collected blood samples were then left to stand for 8 to 10 hours to allow for the separation of serum.

After the separation period, the serum was carefully collected and stored at -20 °C until subsequent analysis was conducted. For analysis, the serum samples were thawed, and various biochemical parameters including triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and total cholesterol were assessed. This analysis was performed using a serum biochemical analyzer with commercially available kits, ensuring accurate and reliable measurement of these lipid-related parameters.

3. Results

3.1 Immunological response

3.1.2 Antibody titers against Newcastle disease and Infectious Bursal Disease

The antibody titers against Newcastle disease ($\log_{10}$ HI titer) in groups T_1, T_2, T_3 and T_4 were 1.304, 1.405, 1.455 and 1.455, respectively. Statistical analysis revealed no significant ($p>0.05$) difference in antibody titers against Newcastle disease among the various treatment groups compared to control.

At the end of 42nd day, the antibody titers against Infectious bursal disease (ELISA) in groups T_1, T_2, T_3 and T_4 were 2551.5, 2600.33, 2652.50 and 2615.83, respectively. Statistical analysis revealed no significant ($p>0.05$) difference in antibody titers against Infectious bursal disease among the various treatment groups compared to control.

3.1.3 Immune organ weights (% of live weight)

The relative weight of spleen, weight of thymus and weight of bursa of fabricius on 42nd day of the experiment in groups T_1, T_2, T_3 and T_4 revealed no significant (p>0.05) difference between the treatments and control groups.

4. Discussion

4.1 Immunological response

4.1.1 Effect of humic substance on immune parameters

There was no significant difference (p>0.05) in immunological response of birds in the groups fed with humic substance compared to the control group at the end of the experiment.

The current findings are consistent with those of Nagaraju *et al*. (2014) [11] who investigated the efficacy of a humic acid based product as a substitute for antibiotics in broilers and concluded that the mean HI titer values for Newcastle disease were statistically (p>0.05) non significant among different treatments groups compared to control.

In contrast Ahfeethah *et al.* (2023) [2] concluded that on supplementation of humic acid and probiotics in broiler chickens resulted in significant increase (p<0.05) in antibodies titer against Newcastle disease virus in chickens which was supplemented humic acid daily for 42 days compared to the control.

4.1.2 Effect of humic substance on immune organs

There was no significant difference (p>0.05) in immune organs weights of the birds in the groups fed with humic substance compared to the control group at the end of the experiment.

The current findings are consistent with those of Nagaraju *et al*. (2014) [11] who investigated the efficacy of a humic acid based product as a substitute for antibiotics in broilers and concluded that there were no significant differences (p>0.05) observed in the relative weights of the thymus, spleen and bursa among the different treatment groups.

In contrast Simakova *et al*. (2021) [16], conducted the micromorphometric analysis of the immune system organs of broiler chickens at the age of 41 days. In the spleen of humates supplemented poultry groups, the average number and diameter of follicles were found to exceed those then the control group. In the bursa of Fabricius, all experimental chickens exhibited a higher number and relative area of follicles. The relative areas of the cortex and medulla of the follicles also exceeded those in the control group. In the thymus of experimental chickens, the relative area of the lobules of the cortical and medullary layers was greater compared to control.

5. Conclusion

The addition of humic substance and bacitracin methylene disalicylate (BMD) to the basal diet did not result in significant improvement in the immunological response against Newcastle disease and Infectious bursal disease on the 42nd day of the experiment. Additionally, there was no significant difference (p>0.05) observed in the immune organ weight of birds fed with humic substance and BMD compared to the control group at the end of the experiment (42nd day).

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