Jurnal Sain Peternakan Indonesia
Available at https://ejournal.unib.ac.id/index.php/jspi/index
DOI: https://doi.org/10.31186/jspi.id.19.2.59-67

P-ISSN 1978-3000
E-ISSN 2528-7109
Volume 19 Issue 2 April-June 2024

Supplementation of *Azolla microphylla* Flour in Broiler Feed Increases Antibody Titers against *Avian Influenza* and *Newcastle Disease*

R. Ermawati[1], M. M. P. Sirat[2]*, M. Hartono[2], F. Fathul[2], R. Hanafi[1], and T. A. Fauzi[2]

[1]Study Program of Animal Husbandry, Department of Animal Husbandry, Faculty of Agriculture, University of Lampung
[2]Study Program of Animal Nutrition and Feed Technology, Department of Animal Husbandry, Faculty of Agriculture, University of Lampung
Jl. Soemantri Brodjonegoro 1, Bandar Lampung, Lampung, Indonesia 35145
**Corresponding Author*: m.mirandy@fp.unila.ac.id
Revised: 2024-05-24, Accepted: 2024-05-27, Publish: 2024-06-29

ABSTRACT

This study aimed to determine the level of protection provided by supplementation of *Azolla microphylla* flour in broiler feed against the antibody titers profile of Avian Influenza and Newcastle Disease. The research was conducted over two months (July-August 2021) at the broiler unit of the Integrated Field Laboratory, Faculty of Agriculture, University of Lampung. The experimental design was a Completely Randomized Design with four treatments and five replications. Each replication consisted of five Cobb CP707 strain broilers, resulting in 100 broilers. *Azolla microphylla* flour supplementation in the feed was administered at different doses: P0=100% commercial feed (control); P1=97.5% commercial feed + 2.5% *Azolla microphylla* flour; P2=95% commercial feed + 5% *Azolla microphylla* flour; P3=92.5% commercial feed + 7.5% *Azolla microphylla* flour. Serum samples were collected from 30-day-old broilers, with one sample taken from each replication, totalling 20 samples. The samples were analyzed for Avian Influenza and Newcastle Disease antibody titers at AGRILab Vaksindo Laboratory using the Hemagglutination Inhibition test. Each treatment's antibody titer data for Avian Influenza and Newcastle Disease were tabulated and presented in histograms to facilitate descriptive analysis. Antibody titers ($\log_2$ X) of Avian Influenza 4.00±0.71 and Newcastle Disease 4.60±2.07 from P2 were included in the protection level based on the WOAH Standard. The study concluded that supplementing 5% *Azolla microphylla* flour in broiler feed increases the antibody titers against Avian Influenza and Newcastle Disease to protective levels.

Keywords: Antibody titer, Avian influenza, *Azolla microphylla*, Newcastle disease, Protection level

INTRODUCTION

The poultry farming industry is expected to expand alongside the increasing population of Indonesia, indicating a rising demand for domestic animal protein sources. Tangendjaja, (2007) asserts that to support the self-sufficiency of poultry farming enterprises, it is crucial to strengthen the self-sufficiency of feed provision. Therefore, feed technology innovation, including exploring conventional feed sources, needs to be emphasized (Tangendjaja, 2007). Azolla is one of the unconventional feed ingredients that can fill a niche as an alternative feed ingredient for poultry, including chickens (Subudhi & Singh, 1978). *Azolla microphylla* demonstrates significant potential as a livestock supplement due to its high-quality nutritional content and rich amino acid composition. According to Chatterjee et al. (2013), chemical analysis of *Azolla microphylla* consists of organic matter 80.53%, crude protein 24.06%, crude fibre 13.44%, crude fat 3.27%, ash 19.47%, and total nitrogen 37.71%. Naghshi et al. (2014) state that using 5% Azolla meal in Cobb broiler feed during the starter and finisher phases significantly enhances body weight gain among treatments and reduces feed efficiency.

According to Murthy et al. (2013), incorporating Azolla into the feed can reduce feed costs by 16.6 to 18.5%. Azolla can substitute forage and protein sources for ruminants (Bhatt et al., 2021) and serve as a protein and mineral source for pigs and poultry (Wulandari et al., 2019). The use of Azolla in dairy cows can increase milk production (Yanshi & Rahal, 2019). Other benefits of Azolla include enhancing poultry egg production (Rai et al., 2012). Based on a study by Ermawati et al. (2023), supplementation of 5% *Azolla microphylla* flour in the broiler feed were effectively enhances body weight gain, maintains total plasma protein within normal ranges, and

keeps the total microbial contamination of broiler carcasses within safe limits for consumption.

Genetic traits and environmental conditions influence broiler superiority, including food, temperature, and management practices. However, broilers are susceptible to viral diseases such as Avian Influenza and Newcastle Disease. Avian Influenza and Newcastle Disease are two significant poultry diseases capable of causing recurring outbreaks, often leading to similar clinical symptoms and pathological lesions in birds with high morbidity and mortality rates, thus resulting in significant economic losses in the poultry industry (Ekaningtias et al., 2017).

Diseases caused by viruses can be prevented by increasing antibody titers in broilers. Antibodies are proteins formed in response to antigens entering the body. Enhancement of antigen response is achieved through increased antibody titers. Antibody titers represent the number of antibody units per unit volume of serum (Subowo, 2009). *Azolla pinnata* exhibits significant immunomodulatory potential in commercial broilers by replacing 5.5% of the basal diet with *Azolla pinnata* meal on a dry matter basis may enhance immunity. It is observed that the mercaptoethanol-resistant (IgG) antibody titer (log 2) increases in response to sheep red blood cells (SRBC) (Bhattacharyya et al., 2016). This study aimed to determine the effect of *Azolla microphylla* flour supplementation in broiler feed against antibody titers of Avian Influenza and Newcastle Disease in broilers to aid farmers in preventing these diseases, provide alternative supplementary feed sources for their poultry, and establish the optimal Azolla flour dosage, thereby contributing to the improvement of farmer welfare through enhanced broiler health and productivity.

MATERIALS AND METHODS

Study Area

The research was conducted over 2 months (July–August 2021) at the broiler unit of the Integrated Field Laboratory, Faculty of Agriculture, University of Lampung. The study employed an experimental design with supplementation of *Azolla microphylla* flour in broiler diets using a Completely Randomized Design (CRD) with four treatments and five replications. Each replication consisted of five Cobb CP 707 broiler chickens, resulting in 100 broilers utilized in the study. The treatment design was as follows:

P0 : 100% commercial feed (control)
P1 : 97.5% commercial feed + 2.5% *Azolla microphylla* flour
P2 : 95% commercial feed + 5% *Azolla microphylla* flour
P3 : 92.5% commercial feed + 7.5% *Azolla microphylla* flour

Material

The equipment used included tools for the collection, drying, and milling of *Azolla microphylla*, such as mesh sieves, tarps, 1 unit of a digital hanging scale with a capacity of 50 kg with an accuracy level of ±10 grams, 1 unit of a digital scale with a capacity of 3 kg with an accuracy of ±0.1 grams, one grinding machine, one package of plastic bags sized 1 kg, and writing utensils. Equipment for the proximate analysis of the nutritional value of the treatment diets included an analytical balance, an oven set at 135°C, an electric furnace at 600°C, porcelain crucibles, Erlenmeyer flasks, Whatman ashless filter paper, spray bottles, desiccators, glass funnels, Crude Fiber Apparatus, Soxhlet Apparatus, Kjeldahl tubes, electric stoves, and linen cloth. Broiler maintenance equipment included one open house coop, one fogging device, one sprayer for coop disinfection, one hand spray bottle for personnel hygiene, brooding area dividers, 20 units of 60 Watt light bulbs, 20 units of baby chick feeders, 20 units of chicken waterers, one digital scale, and 1 thermohygrometer. Equipment for blood serum collection included 20 units of 5 ml disposable syringes, 20 units of 5 ml non-EDTA tubes, 20 units of Eppendorf tubes, and 1 cooler box for storing blood serum.

The materials used in this study included 100 Cobb CP 707 broiler day-old chicks with an average body weight of 37-38g/chick, raised for 30 days, and BR1 commercial feed produced by PT. Japfa Comfeed Tbk., *Azolla microphylla* meal, killed ND and AI vaccines (Medivac ND-AI®), live ND vaccine (Medivac ND Clone®), and materials for proximate analysis such as Azolla microphylla meal, 0.25 N H2SO4, 0.313 N NaOH, acetone, distilled water, Whatman ashless filter paper No.41, litmus paper, 1% H3BO3, HCl, and chloroform.

Method

Proximate Analysis

The research activity commenced with a pre-research phase involving the production of *Azolla microphylla* flour. *Azolla microphylla* plants were sourced from the paddy fields of Pekon Bumi Rejo, Pagelaran District, Pringsewu Regency. The *Azolla microphylla* plants obtained underwent enrichment and separation of impurities, followed by sun-drying and finely grinding into flour. Proximate analysis was conducted at the Animal Nutrition Laboratory, Department of Animal Husbandry, Faculty of Agriculture, University of Lampung. Proximate analysis results of nutritional value of the treatment diets are presented in Table 1.

Table 1. Proximate analysis results of nutritional value of the treatment diets

Nutritional Value (%)	Treatment			
	P0*	P1**	P2**	P3**
Protein	21.00	21.01	21.03	21.04
Lipid	5.00	4.93	4.86	4.97
Water	12.00	11.94	11.94	11.91
Ash	1.00	1.57	2.15	2.72
Crude Fiber	5.00	5.32	5.64	5.96
BETN	56.00	55.19	54.38	53.57

Note: Proximate Analysis Results from the Laboratory of Animal Nutrition and Feed, Department of Animal Husbandry, Faculty of Agriculture, University of Lampung, 2021. (*)Treatment diet without supplementation of *Azolla microphylla* flour; (**)Treatment diet with supplementation of *Azolla microphylla* flour.

Broiler maintenance and treatment examination

The research activity involved placing day-old chicks (DOC) broilers into the brooding area for seven days. The DOC was given water mixed with sugar solution as electrolytes upon arrival. Feed was provided *ad-libitum*. Treatment feed examination commenced on the 8th day after the arrival of DOC until the chickens reached 30 days of age. Weighing three broiler samples per cage was conducted at 07:00 AM local time to obtain body weight data, which was the basis for calculating the amount of *Azolla microphylla* flour supplementation according to the treatment. Broilers were supplemented with *Azolla microphylla* flour based on the calculated percentage dose. The percentage dose calculation was based on the dry matter content of *Azolla microphylla* in the daily broiler feed requirement according to the age of the broiler. Broiler feeding was divided into the starter phase (0-4 weeks) and the finisher phase (4-6 weeks). The quantity of starter feed was divided into four groups: the first week (1-7 days of age) at 17 grams/day/bird, the second week (8-14 days of age) at 43 grams/day/bird, the third week (15-21 days of age) at 66 grams/day/bird, and the fourth week (22-29 days of age) at 91 grams/day/bird. Feed mixing according to the treatment was performed once a week, then packed into 1 kg plastic bags and labelled using a permanent marker according to the treatment. Broilers were fed according to their needs and provided ad-libitum water at 08:00 AM local time. Lighting was turned on from 17:30 to 06:00 AM. Temperature and humidity measurements in the cages were taken daily at 07:00, 12:00, and 17:00 local time using a thermohygrometer placed in the middle of the cage and hung on the cage wall.

Vaccination of Avian Influenza and Newcastle Disease

The vaccination activities included administering ND and AI-killed vaccines (Medivac ND-AI®) via subcutaneous injection and ND live vaccine (Medivac ND Clone®) administered via conjunctival instillation when the broilers were seven days old. Booster vaccination with ND live vaccine (Medivac ND Clone®) was administered via conjunctival instillation at 21 days of age.

Serum Collection

Blood sampling was conducted when the broilers reached 30 days old, with one broiler taken from each experimental plot, resulting in 20 samples. Blood was collected using a 5 ml disposable syringe via the brachial vein, drawing 3 ml of blood. Serum samples were prepared by allowing the blood samples to stand in tubes without EDTA for approximately 1–2 hours at room temperature until separation occurred between the blood cells and the yellowish serum.

The serum was then transferred into micro tubes and labelled according to the treatment sample code (Syukron et al., 2013). These samples were sent to the AGRILab Vaksindo Laboratory to analyse AI and ND antibody titers using the Hemagglutination Inhibition (HI) or Agglutination Inhibition tests. The Hemagglutination Inhibition (HI) test at the AGRILab Vaksindo Laboratory utilized the Indirect method.

Data Analysis

The antibody titers of Avian Influenza and Newcastle Disease for each treatment were organized in tabular and histogram forms to provide data for descriptive analysis.

RESULTS AND DISCUSSION

Antibody Titers of Avian Influenza

The mean and standard deviation of the antibody titers against Avian Influenza (AI) test results are presented in Table 2 and visualized in the histogram in Figure 1. The descriptive analysis results indicated that supplementation treatment of 5% *Azolla microphylla* flour in the broiler feed (P2) resulted in the highest increase in antibody titers against AI.

Table 2. The results of the Hemagglutination Inhibition test for antibody titers against Avian Influenza.

Repetition	Treatment			
	P0	P1	P2	P3
	---------- $\log_2$ X ----------			
R1	2	4	4	2
R2	3	3	5	4
R3	5	2	3	4
R4	2	4	4	4
R5	4	5	4	5
Mean±SD	3.20±1.30	3.60±1.14	4.00±0.71	3.80±1.10

Note: P0=100% commercial feed (control); 97.5% commercial feed + 2.5% *Azolla microphylla* flour; 95% commercial feed + 5% *Azolla microphylla* flour; 92.5% commercial feed + 7.5% *Azolla microphylla* flour. Antibody titer testing conducted at AGRILab Vaksindo Laboratory.

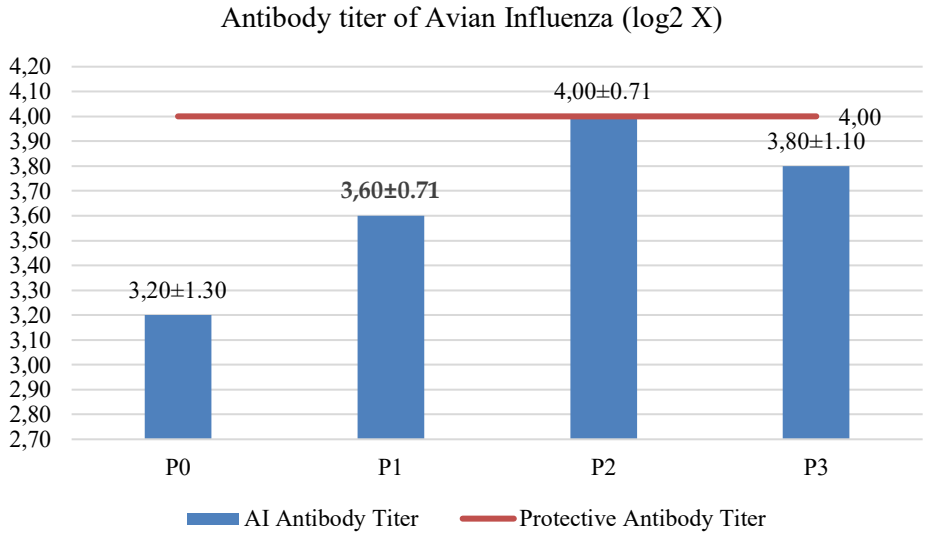

Figure 1. Protection level of *Azolla microphylla* flour supplementation in feed against mean antibody titers of Avian Influenza.

Antibody Titers of Newcastle Disease

The mean and standard deviation of the Antibody Titers against Newcastle Disease (ND) test results are presented in Table 3 and visualized in the histogram in Figure 2. Based on the descriptive analysis results, it is indicated that supplementation treatment of 5% *Azolla microphylla* flour in the broiler feed (P2) resulted in the highest increase in antibody titers against ND.

Table 3. The results of the Hemagglutination Inhibition test for antibody titers against Newcastle Disease.

Repetition	Treatment			
	P0	P1	P2	P3
	---------- $\log_2$ X ----------			
R1	4	6	2	5
R2	4	5	6	4
R3	4	4	7	3
R4	4	4	5	4
R5	5	4	3	4
Mean±SD	4.20±0.45	4.60±0.89	4.60±2.07	4.00±0.71

Note: P0=100% commercial feed (control); 97.5% commercial feed + 2.5% *Azolla microphylla* flour; 95% commercial feed + 5% *Azolla microphylla* flour; 92.5% commercial feed + 7.5% *Azolla microphylla* flour. Antibody titer testing was conducted at AGRILab Vaksindo Laboratory.

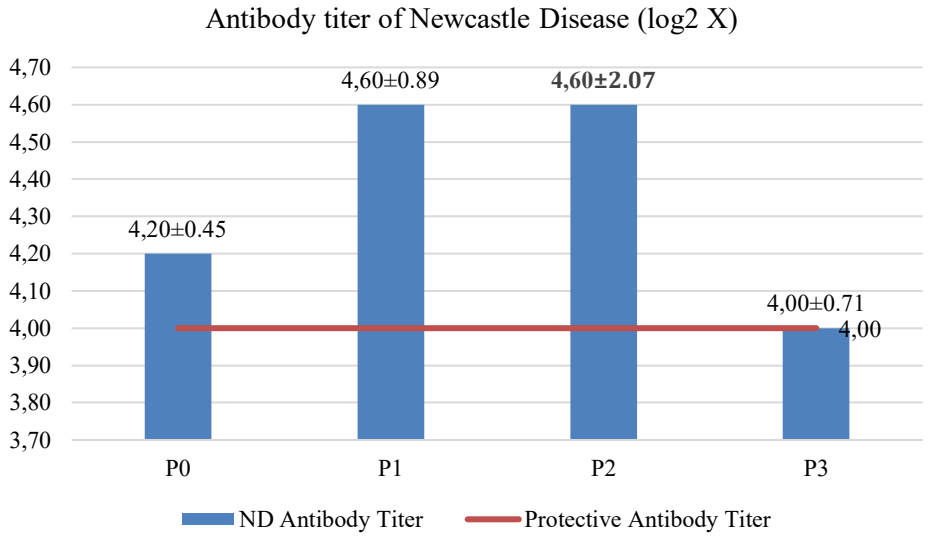

Figure 2. Protection level of *Azolla microphylla* flour supplementation in feed against mean antibody titers of Avian Influenza.

Discussion

Avian influenza has emerged as a significant pathogen affecting poultry populations. This viral infection targets avian species, leading to bird mortality and posing a zoonotic threat to human health. The zoonotic potential of avian influenza has garnered considerable scientific scrutiny, stimulating research efforts to mitigate its impact. Avian influenza, characterized by its viral etiology, manifests as asymptomatic infections in diverse host species. However, it precipitates acute and often fatal disease states in chickens, turkeys, and numerous other avian species (Nguyen et al., 2005).

Antibody titers are immune substances produced by white blood cells to detect antigens or disease agents using blood serum as a sample in testing techniques. Broiler antibody titer examination is a blood test to determine the serum protein's ability in blood-containing antibodies to neutralize antigens entering the body. Antibody titer examinations in this study were conducted using the Hemagglutination Inhibition (HI) test. The HI test is primarily used to determine if antibodies indicating influenza A virus infections are subtyped as H5 and H7 or other H subtypes (H1-4, H6, H8-16). HI titres may be regarded as positive if there is inhibition at a serum dilution of 1/16 (2^4 or $\log_2$ 4 when expressed as the reciprocal) or more against 4 HAU of antigen (WOAH, 2021a). The principle of the hemagglutination inhibition (HI) test involves the inhibition of hemagglutination between the antigen and red blood cells by antibodies present in the post-vaccination test serum (Khodijah, 2023).

The criteria for examination results were that the serum tested was declared protective against Avian Influenza and Newcastle Disease if

the result obtained is >$\log_2$ 4 or >log 16 (WOAH, 2021a; WOAH, 2021b). The protection level based on the standards of AGRILab Vaksindo Laboratory also refers to these WOAH standards. Based on the research results presented in Tables 2 and 3, supplementation of 5% *Azolla microphylla* flour in broiler feed resulted in the highest increase in antibody titers of Avian Influenza ($\log_2$ X) was 4.00±0.71 and Newcastle Disease ($\log_2$ X) was 4.60±2.07 as visualized in Figure 1 and Figure 2. The AI and ND antibody titers in treatment P2 with 5% *Azolla microphylla* flour supplementation in broiler feed were included in the protection level against AI and ND with protection levels above $\log_2$ 4 according to WOAH standards, according to Bhattacharyya et al. (2016, replacing 5.5% of basal diet with *Azolla pinnata* meal on a dry matter basis may elicit higher immunity in 42 days old commercial broilers.

These results also indicate a positive influence of the provided feed. High AI and ND titers produced from broiler feed supplemented with *Azolla microphylla* flour may be due to the 15.99% protein content in *Azolla microphylla* flour used in this study, as determined by proximate analysis. The high protein content in broiler feed will enhance livestock immunity. Antibodies are proteins formed in response to antigens entering the body. Increased response to antigens is achieved through increased antibody titers. (Nugrahawaty, 2002) their study suggested that the higher the protein level in the feed, the higher the antibody titer obtained after ND vaccination.

The vaccination activities included administering ND and AI-killed vaccines (Medivac ND-AI®) via subcutaneous injection when the broilers were seven days old. Inactivated (killed) vaccines present a substantially higher cost than live vaccines, necessitating individual handling and injection of birds. These vaccines are formulated from allantoic fluid and inactivated by adding formaldehyde or beta-propiolactone (BPL), rendering them non-infective. The inactivated fluid is subsequently emulsified with mineral or vegetable oil and administered via intramuscular or subcutaneous routes. Subcutaneous administration offers the advantage of preventing virus dissemination and mitigating adverse respiratory reactions. (WOAH, 2021b).

ND live vaccine (Medivac ND Clone®) was administered via conjunctival instillation when the broilers were seven days old. Booster vaccination with ND live vaccine (Medivac ND Clone®) was administered via conjunctival instillation at 21 days of age based on WOAH (2021b) that revaccination is then carried out 2–4 weeks later According to (WOAH, 2021b), live virus vaccines may be administered to birds by incorporation in the drinking water, delivered as a coarse spray (aerosol), or by intranasal or conjunctival instillation. Vaccines have been constructed to give optimum results through application by specific routes.

The protein content in *Azolla microphylla* flour is the foundation for antibody formation within the broiler's body. It follows Lehninger (1997) statement that proteins are classified based on their biological functions into 1) defence proteins (immunoglobulins/ antibodies); 2) catalytic proteins (enzymes); 3) transport proteins (haemoglobin, lipoproteins, etc.); 4) mineral carrier proteins (ovalbumin, casein, and ferritin); 5) contractile proteins (actin and myosin); 6) structural proteins (collagen); and 7) regulatory proteins (hormones). Antibodies are specific proteins the immune system produces to identify and neutralize foreign invaders, such as viruses and bacteria. The role of antibody titer tests is to qualitatively (presence) and quantitatively (amount) determine antibodies in the blood. Research conducted by Nugrahawaty (2002) indicates that the higher the protein level in the feed, the higher the antibodies obtained after vaccination. The presence of protein from *Azolla microphylla* in the study has increased broiler antibody titers against AI and ND. Sanotharan & Peramunagama (2023) also stated that Azolla could be used as an ideal feed source for providing an alternate protein source without any adverse effect on the production status of the animals.

Exposure of antigens into the body of chicken hosts will result in specific antibodies against the injected antigen. Based on (Wibawan, 2008), there are three important types of antibodies: IgY, IgA, and IgM. IgM is formed in the highest quantity at the onset of infection (antigen exposure), followed by a significant increase in IgY production after subsequent boosters (second and subsequent exposures). IgA plays a crucial role in mucosal surface defence. The production of IgY antibodies in avian species can be achieved through vaccination techniques involving subcutaneous, intramuscular, or oral administration of antigens and adjuvants at specified intervals (Carlander, 2002; Hammond, 2009).

Wibawan (2008) stated that antibodies could be detected in the bloodstream one-week post-vaccination at sufficiently high levels. In this study, revaccination with ND live vaccine (Medivac ND Clone®) was administered via conjunctival instillation 14 days following the first vaccination. Revaccination can elicit a secondary immune response characterized by a shorter lag phase and the activation of memory cells within the immune system, thereby leading to a more rapid and robust increase in antibody titers, consequently resulting in elevated levels of antibodies. IgM levels peaked about seven days after antigen exposure. IgY could be detected in the serum 6 to 7 days after exposure. The IgM concentration decreased before IgY levels peaked 0 to 14 days after antigen exposure (Wibawan & Soejoedono, 2014).

According to Balqis et al. (2011), the commercial vaccine (H5N1) is protective as it could induce the formation of humoral responses in chickens, evidenced by an increase in serum antibody titers post-vaccination. This study demonstrates that supplementing 5% *Azolla microphylla* flour in the broiler feed could enhance antibody titers against Avian Influenza and Newcastle Disease to protective levels in broilers vaccinated with the commercial vaccine.

In this study, there is a suspicion that the higher ND antibody titer (Table 3) compared to AI antibody titer (Table 2) was due to the administration of live ND vaccine. Inactivated vaccines contain oil adjuvants thus the antigen release process is slower. Besides slowing down antigen release, oil adjuvants can also enhance vaccine immunogenicity. The relatively long time to trigger maximum antibody formation, however, the immune response can last longer in the chicken's body compared to the use of live/active vaccines, which can trigger maximum antibody formation in a short period (Aiyer Harini et al., 2013).

CONCLUSION

Supplementation of 5% *Azolla microphylla* flour in broiler feed increases the antibody titers against Avian Influenza and Newcastle Disease to protective levels.

ACKNOWLEDGEMENT

The authors thank the Institute for Research and Community Service of the University of Lampung for funding this research through the University of Lampung Junior Lecturer Research Grant Scheme in 2021 with contract number 1596/UN26.21/PN/2021.

REFERENCES

Aiyer Harini, P., Ashok Kumar, H. G., Praveen Kumar, G., & Shivakumar, N. (2013). An overview of immunologic adjuvants - A review. *Journal of Vaccines and Vaccination*, *4*(1), 167. https://doi.org/10.4172/2157-7560.1000167

Balqis, U., Hambal, M., Mulyadi, Samadi, & Darmawi. (2011). Peningkatan Titer Antibodi Terhadap Avian Influenza Dalam Serum Ayam Petelur yang Divaksin Dengan Vaksin Komersial. *Agripet*, *11*(1), 5–9. https://doi.org/10.17969/agripet.v11i1.647

Bhatt, N., Tyagi, N., Chandra, R., Meena, D. C., & Prasad, C. K. (2021). Growth performance and nutrient digestibility of azolla pinnata feeding in sahiwal calves (bos indicus) by replacing protein content of concentrate with azolla pinnata during winter season. *Indian Journal of Animal Research*, *55*(6), 663–668. https://doi.org/10.18805/ijar.B-4004

Bhattacharyya, A., Shukla, P. K., Roy, D., & Shukla, M. (2016). Effect of Azolla Supplementation on Growth, Immunocompetence and Carcass Characteristics of Commercial Broilers. *Journal of Animal Research*, *6*(5), 941–945. https://doi.org/10.5958/2277-940x.2016.00122.4

Carlander, David. (2002). *Avian IgY antibody : in vitro and in vivo* [Uppsala University, Medicinska vetenskapsområdet, Faculty of Medicine,]. https://www.diva-portal.org/smash/record.jsf?pid=diva2%3A161296&dswid=-157

Ekaningtias, M., Wuryastuty, H., & Wasito, W. (2017). Pendekatan Diagnosis Avian Influenza Virus dan Newcastle Disease Virus pada Kasus Lapangan Ayam Petelur: Imunopatologis Streptavidin Biotin. *Jurnal Sain Veteriner*, *35*(1), 118–126. https://doi.org/10.22146/jsv.29299

Ermawati, R., Sirat, M. M. P., Hartono, M., Fathul, F., & Farda, F. T. (2023). Efektivitas Suplementasi Tepung Azolla

microphylla dalam Ransum Terhadap Bobot Badan, Total Protein Plasma Darah dan Total Cemaran Mikroba Karkas Broiler. *Jurnal Ilmiah Peternakan Terpadu*, *11*(1), 65–81. https://doi.org/10.23960/jipt. v11i1.p65-81

Hammond, Edward. (2009). *Some intellectual property issues related to H5N1 Influenza viruses, research and vaccines*. Third World Network. https://www.twn.my/title2/IPR/pdf/ipr12.pdf

Khodijah, N. N. (2023). *Deteksi Antibodi terhadap Virus Newcastle Disease pada Ayam Pedaging (Gallus domesticus Linnaeus, 1758) di Peternakan SMS Bekasi dengan Uji HA-HI* [Fakultas Sains dan Teknologi Universitas Islam Negeri Syarif Hidayatullah]. https://repository.uinjkt.ac.id/dspace/bitstream/123456789/66775/1/NADINI%20NUR%20KHODIJAH-FST.pdf

Lehninger, A. L. (1997). *Dasar-Dasar Biokimia* (M. Thenawidjaja, Ed.; 1st ed.). Penerbit Erlangga.

Murthy, T. N. K., Ashok, M., Thirumalesh, T., Umesh, B. U., & Nataraju, O. R. (2013). Effect of partial replacement of Azolla for concentrate supplement on lactating crossbred cows. *Environment & Ecology*, *31*(2), 415–417.

Naghshi, H., Khojasteh, S., & Jafari, M. (2014). Investigation the effect of different levels of Azolla (Azolla pinnata) on performance and carcass characteristics of cobb broiler chicks. *International Journal of Farming and Allied Sciences*, *3*(1), 45–49. https://ijfas.com/2014-3-1/

Nguyen, D. C., Uyeki, T. M., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., Smith, C., Rowe, T., Lu, X., Hall, H., Xu, X., Balish, A., Klimov, A., Tumpey, T. M., Swayne, D. E., Huynh, L. P. T., Nghiem, H. K., … Katz, J. M. (2005). Isolation and Characterization of Avian Influenza Viruses, Including Highly Pathogenic H5N1, from Poultry in Live Bird Markets in Hanoi, Vietnam, in 2001. *Journal of Virology*, *79*(7), 4201–4212. https://doi.org/10.1128/jvi.79.7.4201-4212.2005

Nugrahawaty, T. (2002). *Efek Berbagai Tingkat Protein Pakan Terhadap Titer Antibodi Ayam Petelur Jantan yang Divaksin ND (Newcastle Disease)* [Skripsi, Universitas Airlangga]. https://repository.unair.ac.id/131194/

Rai, R. B., Dhama, K., Damodaran, T., Ali, H., Rai, S., Singh, B., & Bhatt, P. (2012). Evaluation of Azolla (*Azolla Pinnata*) as a poultry feed and its role in poverty alleviation among landless people in northern plains of India. *Veterinary Practitioner*, *13*(2), 250–254. https://www.vetpract.in/contents/vp-13-2-dec-2012

Sanotharan, N., & Peramunagama, S. S. M. (2023, December 10). Use of Azolla as a sustainable protein and mineral supplement for livestock feed. *Proceedings of the 79th Annual Sessions* . https://doi.org/1391-023X

Subowo. (2009). *Imunobiologi Edisi 2*. Sagung Seto. https://sagungseto.co.id/product/kedokteran/immunology/imunobiologi-ed-2/

Subudhi, B. P. R., & Singh, P. K. (1978). Nutritive Value of the Water Fern Azolla pinnata for Chicks. *Poultry Science*, *57*(2), 378–380. https://doi.org/10.3382/ps.0570378

Tangendjaja, B. (2007). Inovasi Teknologi Pakan Menuju Kemandirian Usaha Ternak Unggas. *Wartazoa*, *17*(1), 12–20. https://doi.org/10.14334/wartazoa.v17i1.886

Wibawan, I. W. T. (2008). Pemanfaatan Telur Ayam sebagai Pabrik Biologis (Kajian Pustaka). *Majalah Ilmiah Peternakan*, *11*(1), 36–41. https://ojs.unud.ac.id/index.php/mip/article/view/1763

Wibawan, I. W. T., & Soejoedono, R. D. (2014). *Intisari Imunologi Medis*. PT. Penerbit IPB Press.

WOAH. (2021a). *WOAH Terrestrial Manual 2021 Chapter 3.3.4: Avian influenza (including infection with high pathogenicity avian influenza viruses)*. World Organisation for Animal Health. https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.03.04_AI.pdf

WOAH. (2021b). *WOAH Terrestrial Manual 2021 Chapter 3.3.14: Newcastle Disease (Infection with Newcastle Disease Virus)*. https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.03.14_NEWCASTLE_DIS.pdf

Wulandari, E. C., Wahyuni, H. I., & Suthama, N. (2019). Kualitas Fisik Cangkang Telur Ayam Arab dengan Ransum Kombinasi Azolla microphylla dan Sumber Mineral Kalsium Berbeda. *Jurnal Wahana Peternakan*, *3*(1), 21–25. https://doi.org/10.37090/jwputb.v3i1.113

Yanshi, & Rahal, A. (2019). Azolla-Emerging Animal Feed. *International Research Journal of Natural and Applied Sciences ISSN*, *6*(1), 1–12. https://aarf.asia/pages/applied2.php?p=Volume6, Issue1, January, 2019