

Effects of Combined Red Ginger–Temulawak Herbal and Probiotic Supplementation During the Pre-Laying Period on Newcastle Disease Antibody Titers in ISA Brown Laying Hens

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Abstract. Newcastle Disease (ND) continues to pose a serious challenge to the poultry industry despite routine vaccination programs. This study aimed to evaluate the effect of combined herbal and probiotic supplementation on ND antibody titers in ISA Brown laying hens during the pre-laying period. Forty 16-week-old hens were randomly divided into two groups: a control group receiving a basal diet and a treatment group receiving a basal diet supplemented with red ginger (*Zingiber officinale* var. *rubrum*), temulawak (*Curcuma xanthorrhiza*), *Bacillus subtilis*, and *Lactobacillus acidophilus* for three months. Antibody titers were measured using the hemagglutination inhibition (HI) test at 16 weeks (pre-vaccination), 19 weeks (two weeks post-vaccination), and 23 weeks (six weeks post-vaccination). Although numerical differences in mean titers and geometric mean titers (GMT) were observed between groups, statistical analysis using the Mann–Whitney test and independent t-test showed no significant differences ($P > 0.05$). These results suggest that the combined supplementation did not significantly enhance ND antibody responses beyond vaccination alone under the conditions of this study.

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1 Introduction

Newcastle Disease (ND) is a highly contagious viral infection affecting poultry worldwide and is associated with considerable economic losses due to mortality, reduced productivity, and trade restrictions. The disease is caused by Newcastle Disease Virus (NDV), which primarily targets the respiratory, digestive, and nervous systems of chickens. Vaccination remains the main strategy for disease control; however, vaccine effectiveness can be influenced by environmental stress, management practices, and the immune status of the host [1].

In recent years, herbal feed additives have attracted attention as natural immunomodulators in poultry production. Medicinal plants such as turmeric (*Curcuma longa*) and temulawak (*Curcuma xanthorrhiza*) contain bioactive compounds with antioxidant and anti-inflammatory properties that may support immune function. Mushtaq et al. [9] reported that certain herbal supplements can enhance phagocytic activity and immune responses in poultry.

Probiotics are also widely used to improve intestinal health and immune competence. Beneficial microorganisms, including *Lactobacillus* spp. and *Bacillus* spp., help maintain gut microbial balance, enhance nutrient absorption, and stimulate immune responses [5]. Huda et al. [6] demonstrated that *Lactobacillus acidophilus* supplementation improved immune parameters in broiler chickens.

Although herbal and probiotic supplements have individually shown immunomodulatory potential, limited studies have evaluated their combined effect on ND antibody responses in laying hens. Therefore, this study was conducted to determine whether combined herbal and probiotic supplementation during the pre-laying period could enhance antibody titers following ND vaccination.

2 Material and methods

2.1 Ethical Approval

All experimental procedures were performed following the ethical guidelines authorized by the Ethics Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada (Approval No. 14/EC-FKH/Int./2025).

2.2 Study period and location

The experiment was conducted over a three-month period, from October 1 to December 31, 2024. Laying hens were reared and treated in a closed-house system at the Layer Research Facility, Poultry Research and Training Unit for Health Management (UP4MKU), Faculty of Veterinary Medicine, Universitas Gadjah Mada. Data on antibody titers analyses were carried out at the Microbiology Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada.

2.3 Experimental animals and management

A total of forty ISA Brown laying hens aged 16 weeks with uniform body weights were obtained from a commercial layer farm in Sleman, Yogyakarta, Indonesia. The birds were housed in a closed battery-cage system under controlled environmental conditions, with temperature maintained at 20–25 °C, relative humidity at 60–70%, and a photoperiod of 16 hours of light followed by 8 hours of darkness. Each cage was equipped with a manual feeder and nipple drinker, and birds had ad libitum access to drinking water. After a one-week acclimatization period, the hens were randomly allocated into two groups (n = 20 per group): a control group receiving a basal diet only and a treatment group receiving the basal diet supplemented with herbal extracts and probiotics. The supplementation consisted of red ginger (*Zingiber officinale* var. *rubrum*), temulawak (*Curcuma xanthorrhiza*), *Bacillus subtilis*, and *Lactobacillus acidophilus*, incorporated into the feed at a dosage of 3.5 kg per ton. Dietary supplementation was initiated at 16 weeks of age and continued daily until 23 weeks of age. Feed was provided at 100 g per hen per day in two portions, while water remained available ad

libitum. All birds received a pre-layer IB–ND–EDS vaccine at 17 weeks of age via intramuscular injection into the pectoral muscle.

2.4 Sample collection

Blood samples were collected at three time points to evaluate antibody responses to vaccination: at 16 weeks of age prior to vaccination (baseline), at 19 weeks of age (two weeks post-vaccination), and at 23 weeks of age (six weeks post-vaccination). At each sampling time, four hens were randomly selected from each group, resulting in eight samples per time point. Approximately 1.5–2 mL of blood was obtained from the brachial vein using sterile disposable syringes. The blood samples were allowed to clot at room temperature, and serum was separated by centrifugation before being transferred into labeled microtubes. Serum samples were stored at –20 °C until analysis of ND antibody titers using the hemagglutination inhibition (HI) test.

2.5 Preparation of red blood cell (RBC)

The preparation was carried out at the Microbiology Laboratory, Faculty of Veterinary Medicine, Universitas Gadjah Mada, using the hemagglutination (HA) and hemagglutination inhibition (HI) methods to measure antibody levels against the Newcastle Disease (ND) virus. A rapid HA test was performed prior to the HI test to ensure the quality and condition of the viral antigen to be used. Before beginning the hemagglutination test, chicken erythrocytes were first prepared. According to [8], fresh chicken blood was collected from the brachial vein and placed in tubes containing the anticoagulant Ethylenediaminetetraacetic acid (EDTA). The blood was centrifuged for 15 minutes at 2000 rpm. The supernatant was discarded, and the sedimented erythrocytes were washed three times with phosphate-buffered saline (PBS) to achieve a 100% erythrocyte concentration. A 10% erythrocyte suspension was made by mixing one drop of erythrocytes with one drop of PBS. Next, 1 ml of the erythrocyte suspension (EA) was diluted into 9 ml of PBS solution in a conical tube and homogenized. For the initial testing stage, a 1% EA suspension was used.

2.6 Preparation of 4 HAU antigen

According to [4], four hemagglutination units (HAU) represent the standard amount of virus used in the hemagglutination inhibition (HI) test, with one HAU defined as the minimum quantity of virus capable of agglutinating chicken erythrocytes. The vaccine was diluted with phosphate-buffered saline (PBS) to produce an antigen containing 4 HAU. The antigen was subjected to a slow hemagglutination (HA) test until it reached a titer of 2^2 (4 HAU), indicated by visible hemagglutination in the first and second wells.

According to the [12] guidelines for the slow HA test procedure, 25 μ L of PBS was added to wells 1–12 of a microplate. Then, 25 μ L of antigen was added to the first well and serially diluted up to the 11th well at a 2-fold rate. Subsequently, 25 μ L of 1% chicken erythrocyte (EA) suspension was added to all wells. The mixture was left at room temperature. A pinpoint pattern observed in the control well (well 12) confirmed the test's validity. The virus titer was determined from the highest dilution well that still showed complete hemagglutination against 1% EA.

2.7 Hemagglutination inhibition (HI) assay

For the slow HI test, the procedure referenced from [10] well of the microplate. Then, 25 μ L of the serum sample was added to the first well, and the mixture was serially diluted using a micropipette by transferring 25 μ L of the mixture from one well to the next up to the 11th well. After that, 4 HAU of antigen was added to each well, and the microplate was incubated for thirty minutes at room temperature. The antibody titer reading was performed by observing the last well in which the antibodies were still able to inhibit hemagglutination, indicated by the absence of erythrocyte aggregation [2].

2.8 Geometric mean titer (GMT) calculation

The geometric mean titer (GMT) was determined to reflect the average antibody level within each group at specific time points, offering a more accurate representation of the overall antibody response. Antibody titers were initially converted into \log_2 values, averaged, and subsequently transformed back to their original scale to obtain the GMT.

2.9 Statistical analysis

Data normality was assessed using the Shapiro–Wilk test, and statistical analyses were performed using SPSS version 25.0. For data that followed a normal distribution, an independent-sample t-test was applied, whereas non-normally distributed data were analyzed using the Mann–Whitney test. Results are expressed as mean \pm SEM, and statistical significance was determined at $P < 0.05$.

3 Results and discussion

3.1 Antibody response to Newcastle disease (ND)

Antibody titers against Newcastle Disease (ND) were evaluated in control and treatment groups of layer chickens at 16 weeks (before pre-layer vaccination), 19 weeks (two weeks post-vaccination), and 23 weeks (six weeks post-vaccination). The treatment group received feed supplemented with herbals and probiotics combination, while the control group was fed a basal diet. The mean (\pm SD) and geometric mean titers (GMT) of ND antibodies (\log_2) are presented in Table 1.

Table 1. Mean (\log_2) and geometric mean titer (GMT) of ND antibodies

Bird age (weeks)	Group	Mean	GMT
16	K	4.50	22.63
	HP	5.75	54.78
19	K	9.40	667.95
	HP	9.00	512.00
23	K	11.00	2048.00
	HP	10.75	1747.53

3.2 Antibody titer at 16 weeks

At 16 weeks of age, both groups exhibited comparable baseline antibody levels prior to vaccination. The treatment group showed numerically greater GMT values than the control group; however, statistical analysis revealed no significant difference ($P > 0.05$). This suggests that the initial immune status of the hens was similar between groups. Baseline ND antibody titers may reflect maternal antibodies or previous exposure, which can vary among individuals [1].

3.3 Antibody titer at 19 weeks

At age 19 weeks, antibody titers increased in both groups following vaccination. The control group showed an increase in titers with a mean value of 9.4 and a geometric mean titer (GMT) of 667.95. The treatment group also showed an increase in titers with a mean value of 9.0 and a GMT of 512. Although numerical differences were observed between groups, statistical analysis did not demonstrate a significant effect of supplementation on antibody titers.

3.4 Antibody titer at 23 weeks

At age 23 weeks, the control group maintained high and stable antibody titers (11 in all samples), with a mean of 11, a standard deviation (SD) of 0, and a geometric mean titer (GMT) of 2048. In contrast, the treatment group showed titers with a mean of 10.75 and a GMT of 1747.53. These findings indicate that although antibody titers increased following vaccination, supplementation did not result in statistically sustained enhancement by the sixth week. This may have been influenced by vaccine metabolism, a decline in immunostimulant effectiveness, or the need for continuous supplementation. These findings are supported by [11], who reported that *temulawak* supplementation enhances the immune response to ND vaccination, although its effectiveness greatly depends on the dosage and duration of administration. [6] also noted that *Lactobacillus acidophilus* can strengthen the immune system in chickens, but its benefits decrease without regular administration.

3.5 Field challenge

Newcastle Disease (ND) remains a persistent field challenge in poultry production despite the extensive use of vaccination programs. Factors such as environmental stress, management quality, and immune status can reduce vaccine effectiveness, allowing ND outbreaks to persist even in vaccinated flocks [1; 3]. In this study, the control and treatment groups both showed increased antibody titers after vaccination, but without statistically significant differences ($P > 0.05$), suggesting that vaccination alone remains effective, yet feed supplementation did not provide additional immune protection under field-like conditions. This aligns with findings by [7], who emphasized that live NDV vaccines' efficacy varies in practical applications due to host and environmental factors.

3.6 Immunomodulatory role of feed additives

The supplementation of herbal and probiotic feed additives did not demonstrate a statistically significant immunomodulatory effect by enhancing early immune responses prior to vaccination. The treatment group exhibited a numerically greater baseline geometric mean titer (GMT 54.78) compared to the control group (GMT 22.63), indicating a mild

immune-stimulating effect of active herbal compounds such as *Curcuma xanthorrhiza* and *Zingiber officinale* var. *rubrum* [11]. Similarly, probiotics such as *Bacillus subtilis* and *Lactobacillus acidophilus* support gut health and humoral immunity [6]. However, by the sixth week post-vaccination, the immune stimulation effect declined, suggesting the short-term nature of immunomodulation and the need for sustained or repeated supplementation to maintain antibody titers [11; 6].

4 Conclusion

The administration of combined herbal and probiotic feed additives during the pre-laying period did not significantly enhance antibody titers against Newcastle Disease compared to the control group receiving a basal diet alone. Statistical analyses demonstrated no significant differences in antibody titers between groups at any sampling point ($P > 0.05$). Under the conditions of this study, vaccination remained the primary determinant of humoral immune response.

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