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Effects of Natural Guard Liquid (an Essential Oil-Based Product) on Growth Performance, Hematological Profile, and Antibody Response to Newcastle Disease Virus in Broiler Chickens

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ABSTRACT

Natural guard liquid (NGL) is an immunomodulator consisting of an essential oil blend (lavender oil, eucalyptus oil, and pine oil) that can improve the immunity (IgG) of animals. The objective of this research was to assess the effectiveness of a mixture of NGL comprising of essential oil (eucalyptus, lavender, and pine oil) on growth performance, hematological profile, and antibody titer response to the Newcastle disease virus (NDV) in Lohman strain broiler chickens. A total of 400-day-old unsexed broiler chickens with an average weight of 42.48 ± 2.08 g were randomly distributed into four groups, each comprising two replications with 50 chicks. The control group, T0, received no essential oil, while other treatment groups, T1, T2, and T3, were administered NGL at 80, 100, and 200 ppm, respectively. The mixture was administered in drinking water for 30 days. The animals had received vaccinations at the Hatchery, including active NDV, inactive NDV, and Gumboro. Regular recordings were made for feed consumption, water intake, mortality, and body weight. Blood samples for routine hematological examination (hemoglobin, erythrocytes, packed cell volume, erythrocyte index, leucocytes, and differential leucocytes) and immune parameter (NDV antibody titer) assessment were collected at the onset as well as after 15 and 30 days of treatment. The hemoglobin levels, erythrocytes, total leukocytes, lymphocytes, and Heterophil-lymphocyte index showed significant differences in groups T1 and T2 compared to T0. While the NDV antibody titer showed a significant difference at T2 compared to T0, it was not significantly different at T1 and T3. The best results and performance was indicated in T2 (100 ppm), characterized by body weight (1,839 g), and feed conversion ratio (1.573). The hematological profile in the T2 group included hemoglobin (11.78 g/dL), total erythrocytes (2.82 $\times 10^6$ u/L), total leukocytes (52.67 $\times 10^3$ u/L]), and the NDV antibody titer (48 ± 10.20) in the fifth week. In conclusion, the optimum dose of NGL is 100 ppm, which significantly influences growth performance, hematological profile, and antibody titer against NDV.

Keywords: Antibody titer**,** Broiler chicken, Growth performance, Hematological profile, Natural Guard Liquid

INTRODUCTION

Achieving success in the poultry industry is dependent on precise decision-making in the selection of genetic resources, nutrition, management, and medication programs. Global pressure in the last decade has called for the prompt discontinuation of antibiotics as growth promoters (AGP) because of the rise in antibiotic resistance and the presence of antibiotic residues in meat, eggs, and dairy products (Jabbar and Sajjad-Ur-Rehman, 2013; Qamar et al., 2023). The ban on AGP challenges

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intestinal integrity diminishes optimal nutrient absorption and adversely affects growth in chicken broilers (Shokri et al., 2017). Consequences of abstaining from AGP include disruptions in avian gastrointestinal health, a 5-10% reduction in egg production, a 12-18% rise in disease incidents and mortality rates, and a decline in feed efficiency (Mund et al., 2017). The resultant decrease in productivity and the simultaneous increase in production costs have compelled stakeholders in the poultry industry to explore alternative replacements for AGP. Some reported alternatives include prebiotics, probiotics, synbiotics, enzymes, acidifiers, Phytogenics compounds, and essential oils (Gopi et al., 2014; Mirzaei et al., 2022).

Essential oils are highly valued for safety reasons, being categorized as "Generally Recognized as Safe" (GRAS), a designation supported by the FEMA and FDA associations in the USA and extensively used in the feed and food industry (Hallagan et al., 2020). Characterized to be aromatic, volatile compounds with a pleasant aroma derived from plants, the oil has diverse pharmacological effects, including antimicrobial, antioxidant, anti-inflammatory, antifungal, anthelmintic, immunomodulatory, and antistress (Gopi et al., 2014; Amer and Aly, 2019). Other studies have indicated that essential oil is analgesic, antipyretic, cholagogue, choleretic, cholecystokinetic, and gastrointestinal stimulant (Spiridonov, 2012; Wang et al., 2016). The usage of essential oil as feed additives for AGP substitutes has increased dramatically in recent years (Omonijo et al., 2018). The makeup of active substances, functional groups, and the cooperative relationships between their constituent parts determine the mechanism of action. Essential oils are grouped into two compounds, terpenes, and phenylpropanes, each with various derivatives. The structures of the functional groups can lead to varied efficacy for similar active compounds originating from different plants (Jugreet et al., 2020). However, the appropriate selection and composition of essential oil combinations can synergistically produce optimal positive results in reducing antibiotic usage (Langeveld et al., 2014).

Researchers have explored the impacts of essential oil usage on the performance of various livestock, such as chickens, quails, pigs, and others, showing a range of positive outcomes (Hussein et al., 2019; Su et al., 2020). Strong antimicrobial properties of oregano oil, thyme, and cinnamon, enhanced with carvacrol, thymol, and cinnamaldehyde, improve the health and growth performance of broiler chickens (Du et al., 2016; Zhang et al., 2021). Essential oils' antibacterial properties are vital for preserving intestinal health and the proper balance of normal flora, promoting growth, feed efficiency, and nutrient absorption (Shokri et al., 2017). The use of singular essential oil compounds has also shown favorable effects on poultry. For instance, eucalyptus oil, administered through drinking water, holds the potential to enhance growth performance, carcass traits, and humoral immune response and acts as an anti-stress agent (Mashayekhi et al., 2018). This particular group of essential oils primarily contains active monoterpenes (Caputo et al., 2020), having bio-insecticidal, antiviral, antibacterial, anti-inflammatory, and antioxidant activities (Limam et al., 2020). On the other hand, pine oil has been reported to positively impact breast muscle growth, immune status, and antioxidant levels in broiler chickens (Ramay and Yalçın, 2020), enhance egg production, and maintain intestinal microbial balance (Guo et al., 2022). Another review mentioned that lavender oil at 600 mg/kg of feed enhances broiler growth and maintains intestinal microbial balance, intestinal tract integrity, and antioxidant performance (Barbarestani et al., 2020). These medicinal compounds are subjected to dynamic interactions within the animal's body, affecting various physiological functions of internal organs (Sadgrove et al., 2021). Thus, the purpose of this study was to evaluate the effectiveness of the Natural Guard Liquid (NGL) a product comprising a mixture of eucalyptus, pine, and lavender essential oils in broiler chickens. The observed parameters included growth performance and health status. Performance data comprised feed and water consumption, body weight, average daily growth, and feed efficiency (Sudira et al., 2021). The health status of the chickens was evaluated based on mortality and survival rates, hematological profiles, and antibody titer response to the Newcastle Disease Virus (NDV). Hematological profiles serve as supportive laboratory diagnostics to determine the health status of animals (Haile and Chanie, 2014).

MATERIALS AND METHODS

Ethical approval

The Animal Ethics Commission of the Faculty of Veterinary Medicine, Udayana University, Indonesia, granted ethical permission for all procedures involving experimental animals. This consent is documented by approval letter B/95/UN14.2.9/PT.01.04/2023.

Experimental animals and research design

This study conducted a completely randomized design with 400 day-old unsexed broiler chicks of the Lohman strain, weighing 42.48 ± 2.08 g. The chicks were sourced

from a commercial hatchery under the brand CP-707 (PT. Charoen Pokphand Jaya Farm, Denpasar, Indonesia). Day old chicks (DOCs) were randomly assigned to four treatment groups, including a control group without essential oil and three groups treated including T1, T2, and T3 with NGL essential oil through drinking water at doses of 80, 100, and 200 ppm, respectively. Each group consisted of 100 chicks which were further subdivided into two replications, each containing 50 DOC. Natural Guard Liquid treatment commenced for 30 days, starting on day 3 to day 33. Daily observations, including feed consumption, water intake, mortality, and medication, were recorded. Blood samples for routine hematological examination and immune parameter assessment were collected upon the arrival of DOC at the farm, at 18 days of age (after 15 days of treatment), and at 33 days of age (after 30 days of treatment). The treatment duration of 15 and 30 days aimed to assess NGL efficacy during the starter and grower-finisher phases. Concurrently, body weight data were collected by weighing the chickens every week at 7, 14, 21, 28 days of age and the data were recorded in the morning before feeding.

NGL formulation (PT. Rhea Natural Sciences, Jakarta, Indonesia) comprised 2.0 mg/kg lavender oil, 0.5 mg/kg eucalyptus oil, and 3.5 mg/kg pine oil. The DOC was administered a triple vaccination at the hatchery, consisting of Gumboro Vaccine (Nobilis Gumboro 228E), inactive NDV (Nobilis ND Broiler), and active NDV (Nobilis ND Clone 30), all produced by Intervet International B.V., Boxmeer, Holland. The commercial broiler feed, for the starter and grower-finisher period used by the Hi-Pro-Vite brand (PT. Charoen Pokphand, Tbk., Surabaya, Indonesia), has the nutritional composition as in Table 1.

Table 1. Chemical composition of Hi-Pro-Vite broiler diet at starter and grower-finisher phases

Nutrient content	Starter feed	Grower-finisher
Water content	13 %	13 %
Crude protein	21-23 %	19-21 %
Crude fat	5 %	5 %
Crude fiber	5 %	5 %
Ash	7 %	7 %
Calcium	0.8 -1.1 %	0.9-1.2 %
Phospor	0.5 %	0.6 %
Aflatoxin	50 µg/Kg	50 µg/Kg
Energy metabolism	2900-3000	3000-3100 Kcal/Kg

The research was conducted in February-March 2023 at Armada Farm in Dausa Village, Kintamani Subdistrict, Bangli Regency, Bali, Indonesia. The region, situated at an altitude of 700-800 masl, experienced temperatures ranging from 20-27°C (night) to 24-30°C (day) with humidity 70-80%. Broiler chickens were reared and housed in an open pen system with a size of 8x40 meters, with controlled temperature and humidity using artificial brooding. During the brooding period, the pen temperature was maintained at 33-34°C using Gas Infrared Heaters (Gasolec B.V, Bodergraven, Netherlands), gradually decreasing by 1-2°C every 3 days until the chickens reached two weeks old, and then left at natural temperature. Subsequently, the natural temperature and humidity conditions were followed through curtain and lighting management. The lighting program was set at 23 hours of light and 1 hour of darkness, with nighttime light intensity at 20 lux during the first week and 10 lux in the subsequent weeks. The experimental pens were equipped with facilities ensuring *ad libitum* access to feed and water (Sudira et al., 2021).

Growth performance

The performance parameters observed for broilers included total feed intake (g), body weight (g), weekly body weight gain (g), average daily gain (ADG, g), and feed conversion ratio (FCR). Total feed intake (FI) was recorded daily and calculated periodically. Weekly body weight measurements were used to compute cumulative body weight, ADG, and FCR. The number of sick, dead, and culled chickens was documented to calculate morbidity, mortality, and survival rates. FCR was determined by dividing the total feed consumption by the total carcass weight at harvest (MorbosEspina and Bestil, 2016; Gharechopogh et al., 2021).

Hematological profile

Blood samples were randomly selected from each pen replication, three chickens each. A 5 ml syringe with a 22G needle was used to take 3 ml of blood aseptically from the brachial vein. Subsequently, 1 ml of blood was transferred to a vacutainer tube with EDTA anticoagulant for the hematological profile investigation, and 2 ml to a vacutainer tube without anticoagulant for serum antibody examination. Transported within three hours to the Hematology Laboratory, Veterinary Teaching Hospital, Udayana University, Indonesia, the samples were stored in a cool box with ice packs at 4 °C.

A complete blood examination comprised total erythrocytes, hemoglobin, packed cell volume (PCV) or

hematocrit, and total leukocytes using the LICARE 3-Part Vet Auto Hematology Analyzer (Licare Biomedical Limited, China). Erythrocyte indices include mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). The differential leukocyte count was performed by adopting a Giemsa-stained blood smear and counted under a microscope at 100x magnification using the straight-edge method (Buta et al., 2019). The differential leukocytes included heterophils, lymphocytes, eosinophils, monocytes, and the heterophil-lymphocyte ratio (Haile and Chanie, 2014; Buta et al., 2019).

Antibody titer against Newcastle disease virus

Specific Pathogen-free hens were used to generate a 1% erythrocyte suspension, prepared according to the procedures applied at the Veterinary Virology Laboratory, Udayana University, Indonesia. Approximately 2 ml of broiler chicken blood was drawn from the brachial vein and deposited in EDTA tubes. Following that, 5 ml of pH 7.2 Phosphate Buffered Saline (PBS) was added to the blood samples, which were centrifuged for 10 minutes at 2500 rpm. After discarding the supernatant, PBS was added before repeating the centrifugation process three times. The erythrocyte sediment was measured for concentration using a microhematocrit to determine the packed cell volume (PCV). The PCV was then diluted with PBS to a 1% concentration and ready for hemagglutination inhibition (HI) testing.

Hemagglutination inhibition testing adhered to the procedures of the Veterinary Virology Laboratory at Udayana University and the OIE (2018), and the test began with the addition of 0.025 ml of PBS to each microplate well. The first well got 0.025 ml of serum, which was serially diluted. Subsequently, 0.02 ml of 4 HAU antigen was added to each well, and the plate was kept at room temperature for 30 minutes. Each well received approximately 0.025 ml of 1% erythrocytes, and the plate was kept at room temperature for 40 minutes. Reading the HI titer comprised tilting the microplate 45º and observing the presence or absence of erythrocyte agglutination (tear-shaped). The HI antibody titer was determined by the highest serum dilution still capable of inhibiting 1% erythrocyte agglutination (OIE, 2018).

Statistical analysis

Research data were subjected to ANOVA tests and presented as mean ± SD, including feed intake, body weight, ADG, FCR, hematological profiles, and NDV antibody titers. If there is a significant difference ($p < 0.05$) between treatments, the Duncan test will be continued. The analysis was performed using IBM SPSS Statistics 26 for Windows software.

RESULTS

Growth performance

Table 2 provides a summary of growth performance data, while detailed weekly broiler performance was outlined in Tables 3, 4, and 5, along with Graph 1. Higher survival rates or lower mortality were observed in NGL treatment groups compared to control. Statistical analysis revealed that NGL supplementation in groups T1 and T2 significantly enhanced cumulative body weight, ADG, FI, and FCR when compared to group T0, but not significantly different when compared with T3 ($p < 0.05$). In the first week, there were no significant differences in growth performance between the treatment groups (Tables 3, 4, and 5, $p > 0.05$). Although group T3 outperformed the T0 group in terms of performance achievement, the difference was not statistically significant ($p > 0.05$).

Hematological profile

Table 6 presents the effect of NGL administration on the hematological profile of broiler chickens. Hemoglobin levels showed a profound increase in all NGL groups ($p < 0.05$) after 15 days of treatment compared to T0. After 30 days, a significant effect of increased hemoglobin was observed only in group T2, which did not differ from groups T1 and T3. Total erythrocytes had no effect ($p > 0.05$) after 15 days of NGL treatment, but increased significantly after 30 days in groups T1, T2, and T3 when compared to T0 ($p < 0.05$). The PCV and erythrocyte indices revealed no significant differences in all groups after 15 and 30 days of treatment ($p > 0.05$). Total leukocytes, heterophils, eosinophils, monocytes, and the heterophil-lymphocyte index were substantially lower in NGL groups compared to the control, except for monocytes, which showed no difference at 15 days ($p < 0.05$). Meanwhile, lymphocytes significantly increased in all NGL groups compared to the control group after both 15 and 30 days of treatment ($p < 0.05$).

Antibody titer against Newcastle disease virus

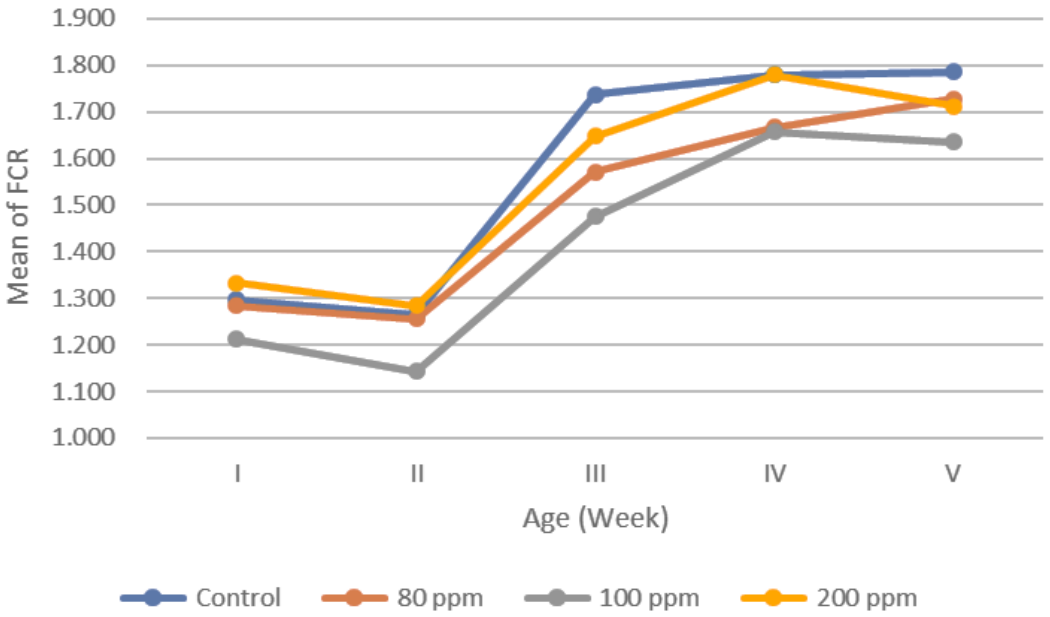
Table 7 presented the results of the Hemagglutination Inhibition test, showing antibody titer against the NDV at the time of DOC and after NGL supplementation. At the time of DOC, the titer appeared not significantly different among all groups ($p > 0.05$). Antibody titer measures against the NDV were significantly higher in group T2

compared to T0 after 15 days of treatment, with no substantial difference from groups T1 and T3 ($p < 0.05$). After 30 days of treatment, the NDV antibody titer measures were higher in group T2, compared to groups T0 and T3, with no significant difference from group T1 ($p < 0.05$).

Table 2. Performance of 33 days old broiler chickens given natural guard liquid for 30 days experiment (Mean ± SD)

Parameter \ Group treatment	T0	T1	T2	T3	P-value
Survival rate (%)	94.39	97.00	97.20	95.00	-
Depletion (%)	5.61	3.00	2.80	5.00	-
Cumulative feed intake (kg)	3.04±0.06^{a}	2.92±0.06^{b}	2.88±0.09^{b}	2.97±0.07^{ab}	0.026
Weight at 33 days (g)	1,704±69^{a}	1,807±73^{bc}	1,839±60^{c}	1,759±89^{ab}	0.001
Weekly weight gain (g)	332±13.7^{a}	353±14.5^{bc}	359±11.9^{c}	343±14.6^{ab}	0.001
Average daily gain (g/day)	51.65±2.08^{a}	54.74±2.20^{b}	55.72±1.81^{c}	53.29±2.21^{ab}	0.001
Feed Conversion Ratio	1.784±0.07^{a}	1.620±0,08^{b}	1.573±0,07^{b}	1.694±0,08^{ab}	0.013

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; ^{abc} Different superscript letters towards the row showed significant differences ($p < 0.05$).



Graph 1. The mean of feed conversion ratio of broiler chicken after 30 days of treatment with various doses of Natural Guard Liquid

Table 3. Cumulative feed intake (g) of 33 days old broiler chicken after 30 days of treatment with natural guard liquid (Mean ± SD)

Group	Week-1	Week-2	Week-3	Week-4	Week-5 (days 33)
T0	164±3.23	388±5.23^{a}	693±7.44^{a}	908±5.19^{a}	864±9.41^{a}
T1	170±3.48	363±5.98^{ab}	673±5.57^{b}	852±4.08^{b}	827±11.57^{b}
T2	162±3.25	357±5.27^{b}	640±6.72^{c}	856±5.39^{b}	820±10.09^{b}
T3	172±3.38	386±5.85^{a}	687±7.34^{ab}	883±5.17^{ab}	801±9.99^{b}

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; ^{abc} Different superscript letters towards the column showed significant differences ($p < 0.05$).

Table 4. Cumulative weekly body weight (g) of 33 days old broiler chicken after 30 days treatment of natural guard liquid (Mean ± SD)

Group	Week-1	Week-2	Week-3	Week-4	Week-5 (days 33)
T0	169±14.61	431±47.48^{a}	830±81^{a}	1,370±85^{a}	1,704±69^{a}
T1	175±19.92	484±42.71^{b}	917±93^{bc}	1,424±79^{b}	1,807±73^{bc}
T2	176±14.87	486±56.17^{b}	922±96^{c}	1,438±82^{b}	1,839±60^{c}
T3	171±14.34	471±57.95^{ab}	888±80^{ab}	1,394±97^{ab}	1,759±89^{ab}

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; ^{abc} Different superscript letters towards the column showed significant differences ($p < 0.05$).

Table 5. The average daily gain (g) of 33 days old broiler chicken after 30 days of treatment with natural guard liquid (Mean ± SD)

Group	Week-1	Week-2	Week-3	Week-4	Week-5 (days 33)
T0	18.01±2.22	40.97±6.41^{a}	57.01±14.41^{a}	69.11±17.34^{a}	72.94±33.08^{a}
T1	18.90±1.89	44.14±6.34^{b}	61.21±12.85^{b}	73.00±20.14^{b}	76.57±29.64^{bc}
T2	19.06±2.17	44.58±7.43^{b}	61.96±13.74^{b}	73.79±24.20^{b}	80.24±27.34^{c}
T3	18.43±1.90	43.90±6.86^{ab}	59.50±10.21^{ab}	70.91±14.92^{ab}	74.88±26.67^{ab}

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; ^{abc} Different superscript letters towards the column showed significant differences ($p < 0.05$).

Table 6. Hematological profile of 33 days old broiler chickens given natural guard liquid through drinking water for 30 days experiment (Mean ± SD)

Parameter	Time	T0	T1	T2	T3	P-value
Hemoglobin (g/dL)	15 days	8.08±0.86^{a}	9.78±1.08^{b}	9.85±1.16^{b}	9.43±0.99^{b}	0.026
	30 days	10.20±1.08^{a}	11.36±0.93^{ab}	11.78±0.95^{b}	11.16±1.11^{ab}	0.043
Erythrocyte (x10^{6} u/L)	15 days	2.19±0.34	2.36±0.21	2.32±0.43	2.25±0.22	0.790
	30 days	2.00±0.16^{a}	2.80±0.12^{b}	2.82±0.26^{b}	2.68±0.69^{b}	0.004
PCV (%)	15 days	24.19±1.83	26.82±2.20	26.68±1.16	25.60±1.73	0.065
	30 days	25.38±1.39	29.60±4.68	28.81±2.60	26.92±5.02	0.294
MCV fL)	15 days	113±21.54	114±11.12	119±12.82	115±15.58	0.951
	30 days	127±16.83	106±15.16	101±18.09	108±8.36	0.222
MCH (Pg)	15 days	37.50±6.74	41.75±7.02	44.88±7.67	42.30±6.62	0.665
	30 days	41.29±7.74	40.63±3.29	42.10±5.19	44.28±6.12	0.137
MCHC (%)	15 days	33.47±3.35	36.56±3.68	36.93±4.12	36.81±2.05	0.264
	30 days	40.31±5.30	39.29±7.24	42.72±5.72	42.75±9.02	0.772
Leucocyte (x10^{3} u/L)	15 days	105.03±34.69^{c}	79.07±14.58^{a}	77.33±11.08^{a}	86.37±16.59^{b}	0.042
	30 days	77.17±15.87^{b}	52.50±4.50^{a}	52.67±10.93^{a}	62.17±8.61^{a}	0.002
Heterophile (%)	15 days	22.99±2.04^{b}	16.33±2.42^{a}	15.50±4.08^{a}	18.32±3.54^{a}	0.009
	30 days	26.17±4.99^{c}	18.01±1.67^{a}	18.33±1.86^{a}	23.16±3.06^{b}	0.000
Lymphocyte (%)	15 days	62.50±3.98^{a}	71.83±2.56^{b}	74.99±5.39^{b}	69.83±5.56^{b}	0.000
	30 days	58.67±5.04^{a}	73.38±3.14^{c}	74.50±2.07^{c}	63.39±2.43^{b}	0.000
Eosinophil (%)	15 days	4.33±1.41^{b}	2.78±1.32^{a}	2.17±1.16^{a}	2.50±1.22^{a}	0.042
	30 days	7.16±2.13^{b}	4.10±1.41^{a}	3.33±1.21^{a}	4.61±3.44^{a}	0.034
Monocyte (%)	15 days	10.04±5.05	8.05±1.78	7.33±3.01	8.45±3.88	0.173
	30 days	8.12±3.03^{b}	4.50±1.04^{a}	3.83±1.17^{a}	7.83±2.78^{b}	0.005
Ratio H/L	15 days	0.36±0.03^{b}	0.23±0.04^{a}	0.21±0.04^{a}	0.26±0.06^{a}	0.000
	30 days	0.45±0.11^{b}	0.25±0.03^{a}	0.24±0.02^{a}	0.36±0.04^{b}	0.000

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; PCV: Packed cell volume/hematocrit; MCV: Mean corpuscular volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean corpuscular hemoglobin concentration, and H /L: Ratio of heterophile/lympocyte. ^{abc} Different superscript letters towards the row showed significant differences ($p < 0.05$).

Table 7. Mean antibody titers response against the Newcastle disease virus before and after natural guard liquid treatment through drinking water (Mean ± SD)

Titer HI log 2 / Group	DOC	15 days experiment	30 days experiment
T0	512±280	16±0[a]	6±4.89[a]
T1	469.33±299.27	46±0[ab]	16±10.28[ab]
T2	426.66±310.03	136±13.14[b]	48±10.20[b]
T3	448±321.27	56±9.79[ab]	6±4.89[a]
P-value	0.962	0.033	0.048

T0: Control; T1: 80 ppm; T2: 100 ppm; T3: 200 ppm; DOC: Day-old chick; HI: Hemagglutinin inhibition; [abc] Different superscript letters towards the column showed significant differences ($p < 0.05$).

DISCUSSION

The essential oils can be utilized instead of AGP since they have pharmacological effects including antibacterial, antioxidant, digestive stimulant, immunomodulatory, antifungal, antiparasitic, antiviral, and bioinsecticidal. Natural Guard Liquid, sourced from *Eucalyptus spp.*, *Lavandula spp.*, and *Pinus spp.*, comprised medicinal active ingredients. Eucalyptus oil contains oxygenated monoterpene compounds, consisting of geranyl acetate, 1,8-cineole, and trans-sabinene hydrate acetate, as well as monoterpene hydrocarbons including γ-terpinene, terpinolene, and α-pinene (Caputo et al., 2020). Lavender oil comprised linalool, α-terpineol, 1,8-cineole, camphor, terpinen-4-ol (3.08%), lavandulyl-acetate, and α-terpineol. Meanwhile, pine oil featured α,β-pinene, camphene, borneol, and phellandrene (Amer and Aly, 2019). This research showed that the achieved performance was increased because the combination of eucalyptus, lavender, and pine oil worked synergistically to produce a positive pharmacological effect on feed consumption, growth, and health.

Table 2 presented, NGL at doses of 80 and 100 ppm, the mortality rate was 2.61% and 2.81% lower, compared to the control with a 5.61% mortality rate. The most favorable outcome was observed in NGL treatment at 100 ppm, resulting in a mortality rate of 2.80% or a survival rate of 97.20%. Siaga et al. stated that high-quality broiler farms should achieve a mortality standard below 5% (Siaga et al., 2017). The mortality rate in this research was very low, suggesting that chickens treated with NGL essential oil maintained a healthy condition, positively impacting FCR and performance index. These findings were corroborated by other reviews, which demonstrated that thymol and carvacrol-containing diets have antibacterial and anti-inflammatory effects (Du et al., 2016), enhancing the integrity of the digestive system and lowering mortality in broiler chickens (Du and Guo, 2021). Similarly, black seed extract administration (*Nigella sativa*) reduced the mortality rate in broiler chickens due to infectious diseases (Kusmiyati et al., 2022). Mortality, influenced by environmental factors, stocking density, and management practices, was often more prevalent in the first stocking week and toward harvesting, attributed to factors such as heat stress (Torrey et al., 2021).

The commendable feed efficiency (Graph 1) and growth (Tables 3, 4, and 5) were attributed to the active ingredients in NGL influencing physiological functions, metabolism, and the digestive system, while also suppressing subclinical infections in the chicken gastrointestinal tract (Fu et al., 2013). The multifaceted activities of NGL essential oil, acting as an antimicrobial, anti-inflammatory, antioxidant, digestive system stimulant, and immunomodulator, contributed to these results (Amer and Aly, 2019). For instance, linalool, linalyl, and lavandulyl acetate disrupted bacterial membranes, leading to the release of cellular material. Terpenoids and phenylpropanoids, due to their lipophilicity, penetrated bacterial membranes, causing damage to bacterial cells (Adaszyńska-Skwirzyńska and Szczerbińska, 2017). Previous research showed that the addition of eucalyptus leaf powder at 1-3 g/kg of feed reduced chicken feed intake (Farhadi et al., 2017), and a dose of 0.5% in the basal diet significantly affected FCR and body weight gain (Mashayekhi et al., 2018). Lavender oil, which contains the primary active components linalool, linalyl, and lavandulyl acetate, decreased the amount of *Escherichia coli* in the ileum and increased the length of the jejunal villi in broiler chickens (Barbarestani et al., 2020). Supplementing lavender oil at a dose of 500 mg/kg of feed in quails increased the length of jejunal and ileal villi and the quantity of *Lactobacillus spp* (Özbilgin et al., 2023). Pine essential oil supplementation at 100 mg/kg enhanced microbial composition and production performance in laying hens

(Guo et al., 2022). The inhibitory properties against pathogenic bacteria contributed to balancing the microbial population in the poultry intestine (Barbarestani et al., 2020).

Essential oil, when supplemented in both feed and drinking water, stimulates the digestive system (Gopi et al., 2014). The oil enhanced choleretic activity, promoting the secretion of bile and pancreatic juice (Wang et al., 2016), as well as cholekinetic activity, facilitating bile flow from the liver to the intestines (Spiridonov, 2012). Bile and pancreatic juice, rich in endogenous enzymes such as amylase, lipase, and protease, played a critical part in the hydrolysis of complicated bonds in nutrients, thereby increasing digestibility. Proteolytic activity maximized protein digestibility, reducing digesta viscosity, and facilitating broader transport of digesta into crypts through the villi of the small intestine. The cholekinetic activity of essential oil reduced intestinal peristaltic movement, ensuring optimal nutrient absorption (Alagawany and Abd El-Hack, 2020).

As shown in Table 6, red blood cells and hemoglobin increased, while PCV and the erythrocyte index remained within the normal range without significant differences from the control group. The standard range for broilers was reported as 2.2-4.5 (x106 cells/µL) for total red blood cells, 8.0-13.6 (g/dL) for hemoglobin, and 22-45% for PCV (Buta et al., 2019). Erythrocytes and hemoglobin play a crucial role in oxygen transport, carbon dioxide removal, nutrient transport, hormone circulation, metabolite movement, heat regulation, and immune product carriage. The significant increase observed in this research suggested a potential role of NGL bioantioxidants in influencing erythropoiesis and capturing free radicals in erythrocytes. The increased number of cells enhanced nutrient and substance transport, supporting the results in broiler chickens receiving essential oil formulations (Farhadi et al., 2017; Heydarian et al., 2020).

The total leukocytes and leukocyte differentials in NGL-treated broilers were significantly lower than the control, except for lymphocytes, which had higher values (Table 5). Elevated leukocyte counts have been linked to infectious agents, such as mycobacteria, viruses, fungi, and parasites. It was observed that broilers were highly susceptible to infections and subclinical inflammation (Torrey et al., 2021). The active compounds in NGL, acting as preventatives against subclinical infections, contributed to lower leukocyte counts. Similar results were observed in broilers treated with eucalyptus oil, lavender, clove, cinnamon, and *Aloe vera*. Moreover, the heterophil-to-lymphocyte (H/L) index was significantly lower in NGL group than in the control. That ratio represented an indicator of chickens adapting to environmental stressors, both internal and external. Studies have suggested that the essential oil in NGL mixtures contained antioxidants functioning as anti-stress agents (Wu et al., 2015; Limam et al., 2020).

Table 7 presented the protective maternal antibody titer against the NDV and the homogeneity in DOC. Antibody titer was considered protective when there was inhibition at a serum dilution of 1:16 (2^4 or $\log_2$ 4) (OIE, 2018). Maternal antibodies decreased and became non-protective by 10 days (HI titer < $\log_2$ 4, Gharaibeh and Mahmoud, 2013). By day 18, the titer decreased but remained protective in all groups, with only NGL 80 and 100 ppm groups maintaining protective titer by day 33. The most robust immune response statistically was observed at the 100 ppm NGL dose, maintaining high and protective levels into the fifth week. The antibody formation process comprised antigens overcoming non-specific defenses (Dahlia et al., 2019), leading to B lymphocyte-mediated humoral immune responses and plasma cell development (ShresthaSadeyen and Iqbal, 2018). In the control group, the consistent decline in antibody titer, still detectable until the fifth week, suggested no trigger by field infections. Chickens relied on maternal antibodies and triple hatchery vaccination for immunity, with essential oil NGL at 80 and 100 ppm influencing the immune system simulation by slowing down the decline in the titer and maintaining protection against the NDV in the fifth week. This proved that NGL essential oil possessed immunomodulatory capabilities, prolonging protective antibody titer compared to the control group. Researchers state essential oil immunomodulation acted to be an immunostimulator and immunosuppressant (Gopi et al., 2014), immunoregulator (Gandhi et al., 2020), immunostimulation and immunorestorative (Ogbue et al., 20220). Essential oil, applied as feed additives in poultry and piglet farming, enhanced immunity. The analysis results were supported by several publications showing increased immune responses in chickens with dietary administration of eucalyptus oil at 250 mg/kg (Farhadi et al., 2017), the addition of 0.5% eucalyptus leaf powder to basal feed (Mashayekhi et al., 2018), and pine extract at 100 mg/kg (Guo et al., 2022).

CONCLUSION

In conclusion, the supplementation of Natural Guard Liquid essential oil through drinking water improved growth performance and had a positive impact on the hematological profile and NDV antibody titer. The most

significant improvements were observed at a dose of 100 ppm, positioning natural guard liquid as an alternative reference for feed additives in poultry production. Therefore, more studies need to evaluate NGL on immune response against other infectious diseases.

DECLARATIONS

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Availability of data and materials

The author confirms that the data supplied are currently available for justifiable reasons.

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Authors' contributions

I Made Merdana, Ida Bagus Komang Ardana, Yousef Haig Setrak Babikian, and Rubiyanto Widodo Haliman designed the research. Luh Made Sudimartini, Kadek Nanda Maharanthi, Ni Luh Eka Setiasih and I Ketut Sumadi carried out the research, data collection, and laboratory examinations. Haig Yousef Babikian evaluated the molecular biology. Theng In Yen, Kristina, and Hendi Yanto Effendy traced the essential oil and conducted data analysis. Hendri Lainman as trial coordinator and organizer. All authors participated in writing and revising the manuscript and approved the final edition of the manuscript.

Competing interests

There were no conflicts of interest among the writers of this paper.

Ethical considerations

The authors have checked the ethical issues, including plagiarism, consent to publish, misconduct, double publication and/or submission, and redundancy.

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