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Immunity Response of Chickens (*Gallus Gallus Domesticus*) in the Early Growth Phase Post Injection Lipopolysaccharide (Lps) *E.Coli* Atcc 25922

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ABSTRACT (9)
Local Indonesian chickens are an important source of animal protein, especially in small-scale farming. However, these farms often face challenges with poor hygiene, which can lead to infections. A common bacterial culprit is *E.coli*, a Gram-negative bacterium whose outer membrane contains lipopolysaccharide (LPS). As an endotoxin, LPS triggers an immune response in the host, leading to inflammation and fever.. This study investigated the effect of LPS injection on the body temperature and leukocyte differentiation as an immuno-competence respond of native chickens. The LPS solution was extracted using a hot phenol- water method and then injected into the abdominal cavities of chickens at 14, 28, and 32 days of age. Body temperature was measured 12 hours after each injection. The immunological respond of the chicks was investigated via leukocyte differentiation using bloodsmear analysis. The results showed a significant increase in body temperature in LPS-injected chickens compared to the control group. This difference persisted across all three injections, indicating that LPS effectively stimulates the immune system. Additional support for immune stimulation came from the leukocyte differential, which revealed a notable difference in Basophil percentage in the treated chicks. Interestingly, repeated LPS stimulation also led to significant changes in overall leukocyte differentiation. These findings collectively demonstrate that LPS injection successfully enhances the immuno-competence of chicks. We realize the unique character of the immunocompetence chicks could play a role as an important in avian immunty which warrant to study in future.

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1. INTRODUCTION

Local Indonesian chickens are a common and important part of Indonesian poultry farming, used for both small and large-scale cultivation. They're a popular choice because they offer one of the most affordable sources of animal protein compared to other options like cattle(Hidayat & Asmarasari, 2015). However, the Indonesian poultry industry faces significant challenges, primarily a lack of hygiene in both feed and environment (Ismoyowati et al., 2022; Rohmadi et al., 2021). The hot and humid climate, combined with minimal animal welfare considerations, also contributes to heat stress, which continuously weakens the chickens' immunity. These environmental and feed quality issues are major contributors to diseases in local chickens. One prevalent bacterial infection is caused by *Escherichia coli*, a gram-negative bacterium commonly found as normal flora in the intestines of humans and animals.

The *E.coli* bacteria is belonging to the Enterobacteriaceae family, are also widespread in the environment. The structure of *E.coli* bacteria is coated by cell walls including the cytoplasmic inner membrane, peptidoglycan layer, periplasmic layer and outer membrane. In the outer membrane there is a main component, namely lipopolysaccharide which can be called endotoxin. Lipopolysaccharide structure is lipid A, oligosaccharide core, and O antigen unit. LPS can bind to Toll-like Receptor (TLR 4), forming a recognition receptor that initiates

the innate immune response(Hassan et al., 2020) Macrophages are the first component of innate immunity and play a role in phagocytizing antigens. In addition, macrophages release pro-inflammatory cytokines such as TNF-α and IL-1. This causes fever (hyperthermia) and inflammation (Al-Aalim et al., 2022; Reisinger et al., 2020). Hyperthermia and inflammation can occur due to *E.coli* infection that exceeds the host's tolerance limit. In addition to causing hyperthermia, LPS also causes clinical and behavioral changes after infection(Xie et al.,2000). LPS is able to stimulate the immune response and increase the response of a host by intermediating a pyrogenic response against foreign substances (Al-Aalim et al., 2022).

Our research group already investigate the effect of the LPS injection on the local Indonesian chicken from several sources of bacterial culture (Lelono et al., 2024; Lelono & Surya, 2023a), as well as those from *E.coli* strains(Noviyani et al., 2025) . However, there is a lack of information focusing on the immune response after *E.coli* LPS injection in the early growth phase with repeated (multiple) exposure to LPS stimulation. The aim of the study was to determine the effect of LPS *E.coli* administration on the immune response (*Gallus gallus domesticus*) in the early growth phase. The information of this study would contribute to the development of resistance chicken strain which are superior in their immune system, since the supreme immune traits have the potential to be passed on to their offspring.

2. RESEARCH METHOD

Animals model

The experimental animals used in this study were native chickens from farmers on Jl.Sriwijaya, Sumbersari, Jember with AKY strain (Ayam Kampung Yudhistira) aged 14 days, 18 individuals. The animals were divided into two groups, namely 9 chickens for control and 9 chickens for treatment. The control was given an injection of 2% NaCl solution while the treatment was given an injection of *E.coli* LPS. The injection was carried out from the age of 14 days, 29 days, and 43 days. One hour before LPS injection the body temperature were collected and continued each hour until 11 times. After two weeks of the injection, blood sampling was taken through the wing brachial vein followed by blood smear preparations. The body temperature and leukocytes differentiation are main data of this experiment which predicted as main factor to figure out the basic immunity profile of the chicken.

Extraction of *E.coli* LPS

The protocol used was hot phenol water. *E.coli* ATCC 25922 bacterial isolate was obtained from the Faculty of Pharmacy, University of Jember. The procedure were starting with the isolate cultured in 50 ml sterile Luria Bertani (LB) media, the culture was incubated on a shaker for 6 hours at 100 rpm. The steps were followed by transferring the culture to the tube and centrifuged at 10,000 rpm at room temperature for 5 minutes then the pellet was taken and the supernatant discarded then washed it by adding PBS solution (pH 7.2) and suspended followed by sonication for 10 minutes on ice. Proteinase-K was added with 1 ml (100 µg/ml) to the sample and then stored at 65°C as an additional hour (1-2 hours). Then added 1 µl/ml MgSO4 and 4 µl/ml Chloroform and then incubated at 37°C overnight. After this then added phenol 90% at 65-70°C equal volume and shaker at 65-70°C using a thermo-shaker for 15 minutes. The suspension was cooled on ice for 5 minutes then centrifuged at 10,000 rpm for 15 minutes at room temperature. The centrifuge results were taken supernatant and transferred to 15 ml falcon followed by the addition of 300 µl distilled water, 2 ml sodium acetate (0.5 M), and 10x the volume of 95% ethanol and store at -20°C overnight. The procedure were followed by transfer to the tube for centrifugation at 2000 Xg at 4°C for 10 minutes, then the pellet was taken and suspended with 1 ml distilled water and stored at -4°C (Modified (Rezania et al., n.d.)). The LPS for injection was dissolved with physiological salt in a ratio of 1:1 and a final concentration of 5%.

***E.coli* LPS injection**

During the experiment, 14-day-old chicks of unspecified sex were divided into two groups: a control group and a treatment group. Both groups received three intra-peritoneal injections (Wang et al., 2022) with a two-week recovery period between each injection, and the dosage varied for each administration. The control group was injected with 2% NaCl at the following doses: 0.1 ml for the first injection (at 14 days old); 0.15 ml for the second injection (at 29 days old); 0.2 ml for the third injection (at 43 days old). The treatment group followed the exact same procedure but received LPS instead of NaCl. To administer the injections, each chicken was gently stretched up and down before a 1 ml syringe containing the appropriate substance (LPS or 2% NaCl) was injected into the peritoneal space, located 0.25 cm below the tip of the sternum.

Measurement of chicken body temperature

The body temperature of the chicken was measured before LPS injection which was used as the control temperature (0th hour) of the chicken before being infected with *E.coli* LPS. Following the LPS injection, temperatures were continuously monitored every hour for 11 to 12 hours, beginning at the first hour post-injection. Measure the chicken's rectal temperature using a Thermo One thermometer. Temperature measurement begins

with stretching the chicken then inserting the tip of the thermometer that has been given a lubricant in the form of oil to reduce pain in the chicken's rectum, then waiting for the numbers that appear on the thermometer to stabilize.

Preparation of Blood Smear

Blood samples were taken from post-injection chickens in each treatment. Blood was taken from chickens at 28 days old, 42 days old, and 56 day as old. Blood was taken by puncturing the brachial vein on the chicken wing. Chicken blood droplets are taken using Hematocrit. The blood drop smears on the object glass. Then the blood smear was waited to dry for 5 minutes, the procedure were continue by fixing the blood smear by soaking in methanol solution for 5 minutes and dried. Staining involves carefully dripping 3% Giemsa onto the samples until they're submerged, followed by a 30-minute incubation. Soon after the washing was done with distilled water, samples were waited until dry and mounted using enthelan (Ardina, dan Rosalinda., 2018.). The final preparation was the observation using a binocular light microscope with a calculation of 5 fields of view.

Data Analysis

The chicken body temperature data was previously tested for normality before started the main statistical analysis. The comparison of the body temperature between treatments vs. control was tested by the independent T test and the comparison between each injection was use pair T test. Leukocyte differential data from 5 fields of view were carried out to find the average of each type of leukocyte, namely heterophils, monocytes, eosinophils basophils and lymphocytes were tested with two different methods. The comparison between treatment vs. control was used independent T test and the comparison between first, and second sequentially used by pair t test. The data of the different of each blood component from first to second injection using pair T test. To analyze the interaction between each white blood component between treatment group and sequential injection were used mixed model with ID or chicks as random factor, treatment group and injection sequence as fixed factor. The confidence level used in this study is 95% or $\alpha < 0.005$. If the sig-2 tailed value <0.05 then the hypothesis is accepted and vice versa. Analysis of statistic were using SPSS software version 22.

3. RESULT AND DISCUSSION

In this study we found that chicken were respond to stimulation of the *E.coli* lipopolysaccharide injection were indicated by the increasing of the body temperature significantly compare to the control (p value <0.001) as shown in figure 1. The temperature increases at the 3rd hour until the 11th hours were not decrease equivalent compare to the control. These indicate the immune response of the body that has not recognized foreign substances and reinforced as found by previous study (Xie et al., 2000). It also showed in broiler chickens that the maximum increase in body temperature of after injection of Salmonella LPS at the 1st hour to the 3rd hour, as well as the results of research(Møller, 2010) on avian that has been injected with LPS there was an increase in body temperature. This response comes from the induction of LPS, stimulating the innate immune system to be active, resulting in inflammation that triggers the fever(Hassan et al., 2020). In addition, LPS that enters the host body elicits a macrophage response that can produce the cytokine IL-1 and this cytokine can increase the prostaglandin hormone as a cause of fever (Mota-Rojas et al., 2021). Likewise, with the statement (Zenk et al., 2009) that LPS is the main factor in triggering acute activation by the host immune system to cause inflammation, because the LPS part of bacteria, especially lipid A, has a strong toxicity effect compared to the structure of other parts (Noviyani et al., 2025).

LPS infection is recognized by Toll Receptor Like (TLR-4) which causes myeloid 88 (MyD88) to be activated and causes the activation of the transcription factor NF- κB, then pro-inflammatory cytokines are produced including TNF-α, IFN-γ, IL-6 and IL-1β (Wang et al., 2022).TLR-4 receptor activation resulted in significant changes in the chicken leukocyte profile (Table 1). In the first injection, the percentage of heterophils, monocytes, eosinophils, and basophils was higher than in the second injection because leukocytes other than lymphocytes are the first defense against LPS by phagocytizing and degranulation (Lelono & Surya, 2023b). In contrast, the number of lymphocytes in the first injection was lower than in the second injection, because the LPS of *E.coli* bacteria induces an exaggerated cellular immune response and activates lymphocytes and produces larger lymphocytes(Zhang et al., 2018), Lymphocytes play a more role against LPS after the second exposure to pathogens and form an antibody and the development of chicken immunity, but other leukocytes are formed as helpers in fighting a foreign substance that enters the chicken's body (Susanti et al., 2020) that the number of normal lymphocyte percentages of chickens is 63-73%, while in this study the number of lymphocytes above normal limits is up to 88%

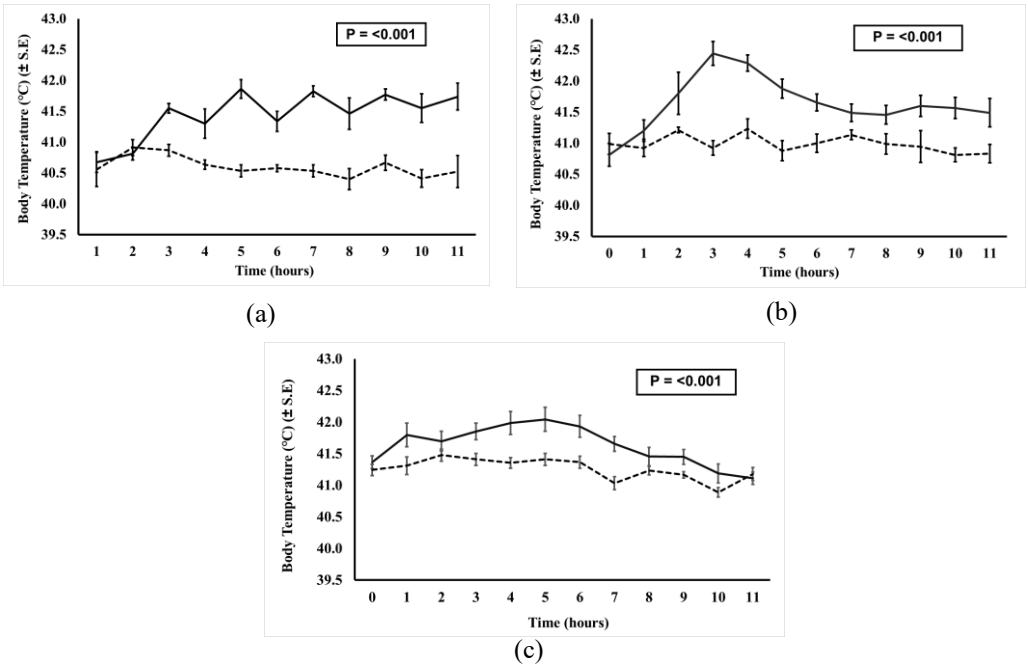

Figure 1.The immunity respond of chicks after *E.coli* LPS injection. (a) Body temperature after the first injection ; (b) Body temperature after the second injection ; (c) Body temperature after the third injection. The solid line is the treatment and dotted line is the control.

In the second injection, the difference in body temperature between control and treatment was still clear and persistence (p value<0.001) as showed in figure.1b. The increase in temperature in the treatment was pin point at the 3rd hour with the highest at 42.5℃. This result was different compare to the control which was similar to the normal temperature of 28-day-old chicks in the range between 39.5℃ to 41℃ as show in the study. by of Lelono et al (2024) that the normal temperature of native chickens at the age of 28 days ranges from 39 to 40.9. The decrease in body temperature in the treatment at 12 hours was different from the first injection. In the second injection, the body temperature at the end of the observation was close to normal temperature. This indicates that the body of chickens tolerates the presence of LPC which showed by the response and the immune system supported by the percentage of lymphocyte which increase from the first injection of 70.85 ± 4.37 to the second injection of 88.65 ± 2.15 (Table 1). Elevated of the body temperature and lymphocyte population indicate the inflammation respond. Inflammation were occurs when the innate immune system was activated and interacts with Toll-like receptor (TLR-4). This interaction will trigger inflammatory mediators in the form of cytokines IL-1β, IL-6 and TNF-α and signal the hypothalamus part of the brain (Emílio-Silva et al., 2020). In addition to inflammation that occurs in the body of native chickens, the difference between the body temperature of the first and second injection was also caused by differences in the age of individual chickens. This was supported by de Boever et al (2009) that the administration of the first and second LPS in birds of different ages, at 3 weeks and 5 weeks, showed different effects on temperature changes, where the administration of the second LPS at the age of 5 weeks could reduce the hypothermic phase of the chicken body and the duration of the fever phase decreases.

The body temperature in the third injection showed a significant difference between control and treatment (value <0.001) as showed in figure 1c. At the third injection, body temperatures were increased in the first hour after injection. This indicates that the immune system of native chickens works faster in recognizing foreign substances (*E.coli* LPS), compared to the first and second injection were the increasing period were the 3rd hour. The rapid response occurs due to the formation of memory lymphocyte cells via repeated exposure, this implied when a similar foreign substance/antigen was injected, the cell were able to recognize and works according to its function more quickly. It was also seen a fast decrease to the normal body temperature. The formation of memory lymphocytes occurs in the cellular and humoral immune systems, which form T lymphocytes and B lymphocytes(Wlaźlak et al., 2023). T lymphocytes play an active role in destroying pathogens while B cells are responsible for antibody production. Poultry adaptive responses involving T lymphocytes and B lymphocytes can be detected about one week after pathogen infection (Wigley, 2014; Withanage et al., 2005). Prior to the activation of the adaptive response in the form of T cells and memory B cells, there is activation of macrophages which become the initial defense with increased phagocytic capacity and cytokine production to fight an antigen that has been previously recognized (Sali et al., 2019; Wei et al., 2018).

We found no significant difference in leukocyte concentrations in the blood of the test animals between the treatment and control groups. The only exception was basophils after the first injection (Table 1). This could be because the chicks' physiological condition likely didn't involve significant stress or pathogen infection, which would typically alter the proportion of leukocyte components. We suspect the chicks avoided stress or pathogen infection due to relatively clean husbandry procedures. Basophils are a type of leukocyte that indicates an immune response in chicks to thermal/environmental stress and infection. These cells are rarely found in healthy chickens and are more common in sick ones (Adriaansen-Tennekes et al., 2009)

Tabel 1. Percentage of *differential leukocyte*

Differential Leukosit	First Injection		Value		Second Injection		Value	
	Control	Treatment	t	p	Control	Ttreatment	t	p
Heterofil	1,41 ± 0,64	0,78 ± 0,50	.716	0.486	0,26 ± 0,26	0,34 ± 0,14	.467	.647
Monosit	16,27 ± 2,98	19,94 ± 3,78	-.725	0.481	8,10 ± 2,01	6,96 ± 1,36	.337	.740
Eosinofil	5,22 ± 1,56	7,34 ± 2,01	-.793	0.441	3,59 ± 0,72	3,20 ± 0,90	1.206	.248
Basofil	3,64 ± 0,83	1,09 ± 0,50	2.368	0.033	1,55 ± 0,48	0,85 ± 0,31	-.680	.506
Limfosit	73,47 ± 3,71	70,85 ± 4,37	.432	0.672	86,50 ± 2,30	88,65 ± 2,15	-.265	.794

Table 2. The statistical analysis of *differential leukocyte* percentage

Differential Leukocyte	Treatment		Injection sequence		Treatment * injection sequence	
	F	p	F	p	F	p
Heterofil	0.389	0.537	3.201	0.084	0.645	0.428
Monosit	0.219	0.643	15.325	0.000	0.791	0.381
Eosinofil	0.385	0.540	4.246	0.048	0.809	0.375
Basofil	7.520	**0.010**	3.912	0.057	2.421	0.130
Limfosit	0.005	0.943	21.773	0.000	0.522	0.475

The leukocyte component concentrations changed significantly between the first and second injections, with several components showing distinct values (Table 2). However, the interaction between treatment and injection order didn't reveal differences across all components. Notably, heterophil levels remained consistent between injections. The heterophils are a key part of the chicken's innate immune system, offering a rapid, non-specific response to infection. In general, newly hatched chicks rely on this innate immunity, particularly heterophils, as their primary defense against infection and inflammation during early growth (Mehrzad et al., 2024; Wigley, 2014). In general, healthy chicks typically have low heterophil levels unless in a certain condition exposed to infection or pathogens (Hassan et al., 2020; Rawat et al., 2024) While lymphocytes, part of the adaptive immune system, significantly develop around 1-2 weeks of age (Robinson et al., 2022; Withanage et al., 2005), they do begin developing earlier. Their full activity is limited initially due to the bursa of Fabricius not being optimally developed yet. However, lymphocyte proportions can increase if the chick has been exposed to stress or pathogens (Mehrzad et al., 2024; Parmentier et al., 2004). In this study we found some obstacles such as the lack of the comparison of the similar study using Indonesian local chicken strain, another problems come from the unique character of the immunocompetence chicks which warrant to study in future.

4. CONCLUSION

LPS-injected stimulate the chickens' immune system by increasing body temperature. The differences showed persistency in the first, second and third injection. From the leukocyte differential there was supporting data where the chicks Basofil percentage showed difference in in the treatment group. Interestingly, the repeated LPS stimulation implies on significant different on the leukocyte differentiations. This research demonstrated that LPS injection is an effective stimulant of chick immune-competence. We suggest exploring the fundamental characteristics of local Indonesian chicken strains. These strains are a unique part of avian biodiversity, having adapted to various handling and rearing methods.

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