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Salmonella typhimurium injection as an immunostimulant: Study on chickens (*Gallus gallus domesticus*)

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Abstract

Salmonella is a gram-negative bacteria commonly found in poultry-based products such as eggs and meat, which can affect quality and human health. The presence of these bacteria in poultry management can be traced to feed, water, substrate, and interactions with the environment. This study aims to investigate the immune system of the avian through injection of *Salmonella typhimurium* colonies at the age of the immune system begins to be produced independently. Salmonella culture at a density of 10^{-8} and 10^{-12} was injected into the digestive tract, and the increase in body temperature was measured immediately after injection. Two weeks later, an analysis of leukocyte differentiation was carried out. We found that Salmonella injection affected body temperature and the rate of body mass increased in all treatments and sex. This indicates that the immune system was activated even though the leukocyte differentiation indicators did not show any significant results. Chicks at the age of one month can show an immune response to bacterial infections through self-defense mechanisms. Further research needs to be carried out to understand whether leukocyte differentiation stimulates leukocyte variation with age.

Keywords: immune system, avian, body temperature, *Salmonella typhimurium*

Introduction

Digestion problems are frequently brought on by Salmonella, a gram-negative bacteria with harmful traits including *S. typhimurium* (Antunes et al. 2016, Huang et al. 2017, Shah et al. 2017). Lipopolysaccharide (LPS), the primary component of the cell membrane made up of lipid A, an oligosaccharide core, and repeated O antigen units extending from the cell surface, is present in *S. typhimurium*. LPS is a type of endotoxin that is poisonous, can activate the serum complement system, can produce biological activity such as immunogenicity, and can lead to illnesses including sepsis and typhoid fever (Hoare et al. 2006, Hughes et al. 2008, Broz and Monack 2011, Huang et al. 2017). Both people and animals, including birds, can get typhoid fever. *S. typhimurium* can cause severe systemic infections in birds that can be fatal (Parmentier et al. 2004, Tizard 2004, Vogler et al. 2021).

Infection with *S. typhimurium* in chickens can result through feed, excrement, or even eggs. Because the infection travels through the cloaca or ovaries, it is known as a vertical infection (Tizard 2004, Shah et al. 2017, Popa and Popa 2021, El-Saadony et al. 2022). *S. typhimurium* can persist in feces and cause vertical transmission to birds who have not yet been exposed to it. Salmonella germs may infiltrate the bird's cloaca, colonize its ovaries, and then move to its intestinal tract (Splichalova et al. 2019, Wessels et al. 2021).

The small intestine mucosa can be penetrated by *S. typhimurium*, which can subsequently interact with columnar epithelial cells and microfold cells (Broz and Monack 2011, Sali et al. 2019). The immune system's reaction to bacterial infections will be triggered by *S. typhimurium*'s interaction with the epithelium. Both innate and adaptive immune responses may be elicited (Broz and Monack 2011, Huang et al. 2017, Shah et al. 2017, Wei et al. 2018). Numerous macrophages and heterophils with phagocytic activity participate in the innate immune response. This is supported by research showing that the capacity of phagocytes and the production of inflammatory cytokines to kill bacteria increased significantly in heterophils, monocytes, and platelets 3 weeks after infection (Wei et al. 2018, Sali et al. 2019).

Numerous T and B lymphocytes participate in cellular and humoral responses during adaptive responses. Research has been done to support this claim. Salmonella-infected hens' intestines contain a significant amount of T cells (Broz and Monack 2011, Hill et al. 2016, Huang et al. 2017, Hafez and Attia 2020). While the chick is heavily reliant on the mother's antibodies and innate immune

response, the chicken's immune response against Salmonella will improve as it matures (Lelono et al. 2019, Pulido-Landínez 2019, Redweik et al. 2020). This study aimed to investigate the response of the chicks 4 weeks after hatching when the immune system is still developing by testing via Salmonella injection.

Materials and Methods

Experimental design

The *Gallus gallus domesticus* for this study was 1 week of age. The blood samples were taken at 6 weeks of age and at the same time, the curve of growth rates and population of *S. typhimurium* colonies were measured. To make sure the colonies are still alive and healthy, live cultures are prepared for injection three days before the application. Blood was sampled and a blood smear was made two weeks after the injection. All data collection on the chickens was made until six weeks old. The procedure of the experiment has been approved by the ethical clearance commission of the Dental Faculty, Jember University with letter number: 2289/UN25.8/KEPK/DL/2023.

Preparation of animal model

The chickens selected come from the Kuntara strain, which is known for its ability to grow quickly and function as either a meat or an egg producer. The one-week-old chicks were purchased from KAPAS FARM, which is recognized as a specialist breeder that provides this sort of chicken and is located at Curah Suko Loji Street, Rambipudji District, Jember. The chicks are then fed with a concentrated diet made for broilers and kept in cages (75 x 75 x 80 cm). Ad libitum feeding was provided, and water was prepared daily in special containers. Black Soldier Fly larvae (BSF) are a common supplement to the diet of one-week-old chicks to promote movement and boost their consumption of animal-based protein. Twenty-eight chicks were housed together in one cage, complete with food, water, and bedding. As an external heat source, a 25-watt incandescent lamp is employed. The chicks are divided into groups based on sex and treatment at a month old, with six chickens in each cage.

The biometric data from the chicks was measured as body mass using an electric scale with an accuracy of two decimals and then using a scale that is more suitable for its capacity as the chicks get bigger. A caliper was used to measure the length of the tarsus and the head, and a 30 cm ruler was used to measure the length of the wings. Repeated biometric data measures were taken every week to monitor changes in length and weight over the study.

In the Kuntara strain, sex is not easy to determine precisely, even though there are guidelines for seeing differences through indicators of wing feather arrangement patterns when they hatch. The sex differences in local strain can only be identified when they are 3 weeks old or older. Body mass, the existence of comb protrusions on the head, and the proportion of the legs and tarsus, which seem compact in male chicks, can all be used to distinguish between male and female chicks. To observe the differences in reaction between each sex, both male and female, following the injection of *S. typhimurium* colonies into the digestive tract, sex differences must be considered. The morphology, metabolism, and growth patterns of the two differ, therefore this distinction is significant.

Growth curve of *S. typhimurium* population

Bacterial culture colonies were maintained in a refrigerator at 4 °C and then cultured in an incubator at 37 °C for 24 hours. For the colony rejuvenation procedure, the culture was then inoculated on 1 slant NA media and cultured for 24 hours at 37 °C in an incubator. After that, a 24-hour-old culture of *S. typhimurium* bacteria was placed in the first test tube with 9 ml of 0.9 % physiological sodium and vortexed to start collecting data for creating growth curves. For liquid ingredients, this dilution was a 10^{-1} concentration. Using a micropipette, 0.1 ml of the suspension was then transferred into a second test tube that had 9.9 ml of 0.9 % physiological sodium. The process was then repeated until a dilution of 10^{-12} was achieved.

A spectrophotometer with a wavelength of 600 nm was used to measure the optical density (OD) in each dilution, and 0.2 ml of the solution was inoculated using a drop plate on solid NA media in a petri dish. *S. typhimurium* was injected into NA medium in petri plates, and it was then incubated at a temperature of 37 °C. A curve was created to represent the OD value every three hours. Every three hours, the number of colonies developing on the NA media in the petri dish was counted, and inspected, and a curve was drawn. The OD value and colony count curves were then compared.

Inoculum preparation

A 50 ml Erlenmeyer tube containing LB medium (Luria Bertani) was injected with a culture of *S. typhimurium* bacteria and vortexed until homogenous. This solution was then matured for six hours at room temperature on a shaker. The *S. typhimurium*-containing fluid was next added to the first test tube, which already contained 9 ml of 0.9% physiological salt, and vortexed. For liquid ingredients, this dilution is a 10^{-1} dilution. 9.9 ml of 0.9% physiological salt was used in a second test tube along with 0.1 ml of the micropipette mixture. The identical process was then carried out once more until dilutions of 10^{-8} and 10^{-12} were attained. To ensure that the culture lasts exactly six hours, the initial step was performed 24 hours before application.

***S. typhimurium* injection challenge**

Live bacterial cultures are applied when the chicken is 4 weeks old, taking into account the fact that the chick has begun to independently generate an immune system. Using the gauvage technique, 1 ml of a suspension containing *S. typhimurium* colonies was injected into each animal. It required two persons to complete this procedure, and one of them must handle the chicks while the other holds it upright and look up, aligning the beak with the ceiling and the throat with the crop. A blunt-tipped probe was used to inject the suspension into the correct inlet hole by the second person. The throat and esophagus were the two passages located at the base of the mouth. Ensuring the probe needle enters the proper location is crucial. After that, gently push the liquid in the syringe against the chicken's crop.

Body temperature were taken an hour apart after the *S. typhimurium* suspension injection using a laser thermometer (thermo gun). The chicks' wing folds and the area around the anus, where the skin is relatively thin and covered to limit heat transfer to the air, were the body sections chosen to ensure representative results as a spot of measurement.

Blood samples were collected from veins on both the right and left wings two weeks after the culture injection. In order to collect the blood, a vein is pierced with a syringe, and the dripping blood is then drawn from the capillary using a heparin-containing tube.

The blood is then put into 1.5 ml vials and labeled with the animal's code, date, and time of the sample. The 0.3 ml of blood was drawn during this collection, which was sufficient to prepare a smear plate on an object glass. After the sampling procedure, the chick is put back in its cage after pressing the puncture wound with cotton or tissue paper until all blood has stopped flowing.

Preparation of the blood smear

Blood samples from chicks were drawn from the brachial vein and put in the tube. On the object glass, the blood quickly streaks. Then, methanol was used to fix the blood sample until it sank for 5 minutes. The samples were dried by air until there were no more water puddles. The blood smear was then stained for 30 minutes with 3% Giemsa dye in methanol. After that, running distilled water is used to cleanse the alcohol. The samples were ultimately air-dried until there were no more water puddles. After the sample has been dried and cleaned, it is mounted so that a perfect area may be chosen under a microscope while also protecting the region with the leukocyte population with Enthellan.

Heterophytes, bryophytes, and eosinophils are examples of granulocytes. Lymphocytes and

monocytes are examples of agranulocytes. Leukocytes vary in shape in addition to number, and age has an impact on this as well because different types emerge and predominate at different ages. Leukocyte differentiation observations were made under a microscope, and they were then compared using manual references (Burton and Harrison 1969).

Data analysis

To ensure that the injected suspension contains healthy cultures at the growth phase, quantitative analysis of population data and growth curves was performed to observe growth trends. Data on body mass differences two weeks after injection were analyzed using a mixed model with sex and treatments as the main factors, and animals' identity as a random factor, the test then continued with an independent T test as a post hoc analysis to examine differences in further detail. The difference in body temperature after injection was analyzed using a mixed model with the treatment and sex as the main factors and animal identity as a random factor. Leukocyte differentiation data were analyzed in a mixed model with treatment and sex as the main factors, the animal identity as a random factor, and the different leukocyte categories as covariates. The SPSS 2020 software was used to perform the statistical analysis

Results

The results of determining the population curve showed that *S. typhimurium* reached the exponential growth phase at 6 hours of culture age (Figure 1), and then adjustments were made taking into account the number of colonies. This was intended so that when the injection stage was applied, the number of bacterial colonies could be measured precisely based on the previously determined method, namely 10^{-8} and 10^{-12}. Even though the highest number of bacterial colonies occurred at the 12^{th} hour of culture (Figure 2), we did not choose them. We considered that at that time the number of colonies detected could be mixed between living and dead bacteria.

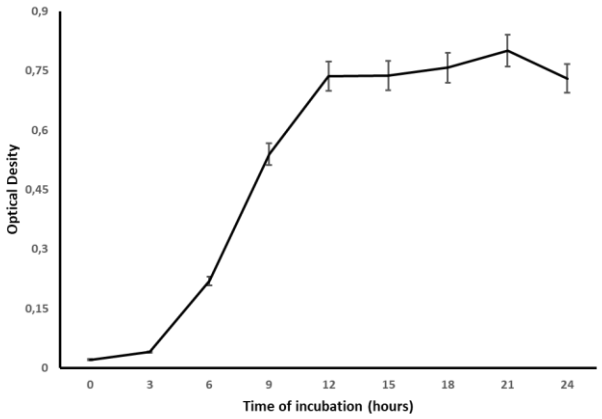

Figure 1. The growth curve of the *S. typhimurium* colony shows an exponential increase up to 12 hours of age until it slopes down.

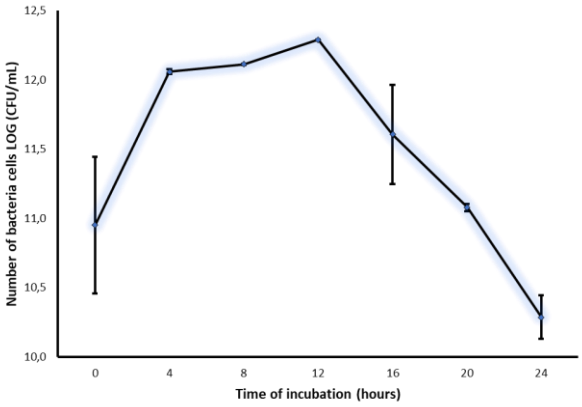

Figure 2. The growth curve of the *S. typhimurium* based on colony density showed an increase up to 12 hours of age; however, an exponential growth pattern was visible at 4 hours.

The difference in increasing body mass was indicated in the male compared to the female group. What was interesting was that the male group with low treatment (10^{-12}) could increase weight gain only in the first week. It can be seen that in the second week, there was a quite clear decline; the opposite result was seen in the group with more concentrated suspension (10^{-8}), it seemed slow at the beginning but showed an increasing trend over time. Meanwhile, when compared with females, it appears that the treatment groups for male chickens in both treatments still showed quite significant differences.

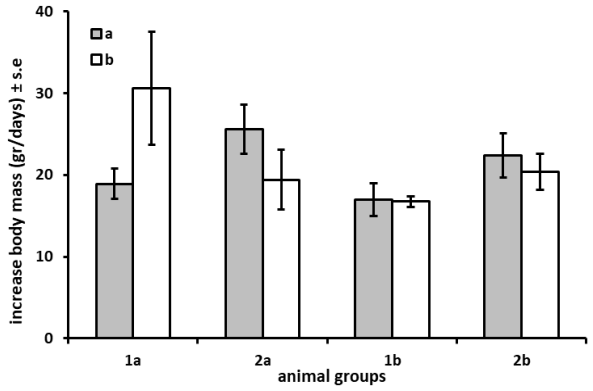

Figure 3. The rate of weight gain in the treatment group (10^{-8} and 10^{-12}) shows differences in males in the first week (1a) and the second week, while females do not show significant differences.

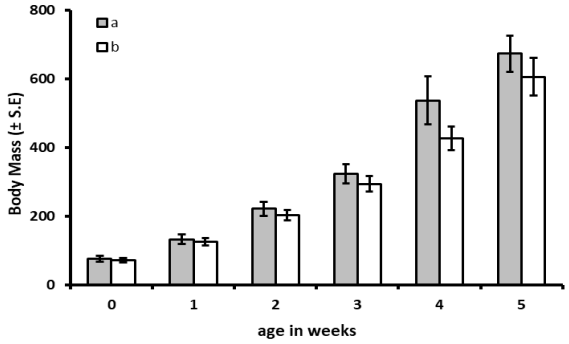

Figure 4. Addition of rooster biomass in groups in the treatment group (10^{-8} and 10^{1-2}). It appears that the first group shows a consistent increasing trend compared to the group that received thick treatment.

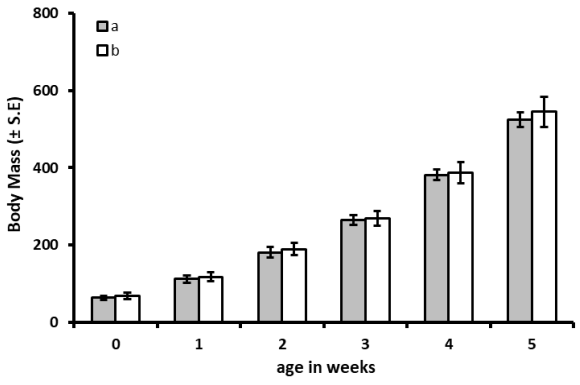

Figure 5. Increase in biomass of hens in groups in the treatment group (10^{-8} and 10^{-12}). It appears that both groups show a consistent increasing trend compared to groups without any clear differences.

Based on the statistical analysis, it is known that there is a significant effect of *S. typhimurium* injection on increasing of the body temperature (F = 3.88; p = 0.05), although there is no difference based on sex (F = 0.55; p = 0.46) or in combination between sex and the treatment (F = 1.03; p = 0.32). There was a significant difference of increase in post-injection body mass between males and females (F = 4.35; p = 0.04), There was a slight difference due to the interaction between post-injection body mass increase and also a decrease in the first and the second weeks but not statistically significant (F = 3.51; p = 0,06). The results of the leukocyte differentiation analysis showed that there were no differences in all factors, including treatment (F = 0.02; p = 0.89), sex (F = 0.21; p = 0.88), or the interaction between treatment and sex respectively (F = 1.15; p = 0.28).

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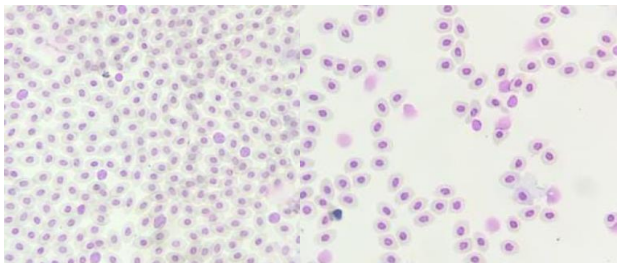

Figure 6. Leukocyte differentiation was visible after blood smear preparation. The types of leukocytes that appear are then counted and compared with the reference.

Discussion

Based on the result we found that the body temperature increased significantly after the admintration of the *S. typhimurium*. This indicated that the existence of *S. typhimurium* in the digestive system of the chicks stimulates the fever response which plays a role as a mechanism of the natural self defence. The slight increase in body temperature may have resulted from the insignificant amount of bacteria that were injected, or it may have been brought on by the structure and environment of the chicken's gastrointestinal cavities. Lactobacillus is a bacterial species that predominates in the small intestine sections.

Comparing the cecal microbiota to the small intestinal microbiota, the cecal microbiota is more diverse and rich. It is made up of yeast, *lactobacillus*, *enterococci*, and *coliforms* (Lutful Kabir 2010, Wigley 2014, Wei et al. 2018). Those circumstances might be crucial in lessening and neutralizing the harmful effects of the *S. typhimurium* infection.

S. typhimurium is widespread among bird species, we use it in our investigation as an immune challenge. Salmonella's primary reservoir is the digestive tract of animals raised for food, which easily causes contamination of a variety of foods (Finley et al. 2006, Antunes et al. 2016). By horizontal and vertical transmission at the primary production stage, some avian species, especially chickens and turkeys, are regularly colonized with Salmonella without visible symptoms (sub-clinical infections or healthy carriers) (Wigley 2014, Huang et al. 2017, Wibisono et al. 2020). Salmonella is thought to be the primary danger factor in healthy poultry animals because it makes it simple for bacteria to spread from table eggs and chicken meat to people (Antunes et al. 2016, Ijaz et al. 2021, Popa and Popa 2021).

The chicken's body temperature rose after receiving an injection of *S.tiphimurium* stimulated into its digestive tract, proving that the population of bacteria may successfully induce fever.

The capabilities of the immunity system, which is already developing on its own, may have influenced the growth of the chicks as demonstrated by the rise in body mass soon after the Salmonella assault. The intestinal epithelium of chickens is composed of a monolayer of several cell types. Tight junctions, which are found between intestinal epithelial cells, preserve the integrity of the gut barrier. Intestinal epithelial cells are held together by tight junctions, which stop enteric pathogens like Salmonella from entering the lamina propria and maintain gut homeostasis (Tizard 2004, Wigley 2014, Wei et al. 2018). Along with the gut microbiota, which prevents pathogen colonization, intestinal epithelial cells also prevent pathogen entry into the gut through their secretions. Along the length of the intestine, which is a component of the lymphoid tissues connected with the mucosa, immune cells can be found in addition to the gut epithelial cells.

Leucocyte, the primary component of the white blood cell system in avian immunity, was not distinguishable in this investigation. This could be due to the natural production of the leucocyte which depends on the age of the animals. The difference in the leucocyte group would adjust the natural mechanisms along the age and also of the development of the immune system. Generally, leucocytes can be classified into five main groups: heterophil, eosinophil, monocyte, basophil, and symposia. The avian equivalent of mammalian neutrophils, heterophils show up right away at the site of infection to get rid of invasive pathogens (Broz and Monack 2011, Wei et al. 2018, Ijaz et al. 2021). Heterophils kill the pathogens by a variety of techniques, including phagocytosis, degranulation, and oxidative burst. In the blood and gut of chickens, heterophils predominate among the granulocytes. Young chickens have a higher proportion of heterophils than adult chicks do (Wei et al. 2018, Ijaz et al. 2021). However, heterophils function less actively in young birds, as seen by lower phagocytosis, degranulation, oxidative burst, and, ultimately, bacterial death.

The chicken's immune system can be boosted in the future in a number of ways by activating the system it established on its own. The role of the intestine innate immune cells in the defense against Salmonella infections has been highlighted in numerous research (Wei et al. 2018, Adhikari et al. 2020, Ijaz et al. 2021). Numerous prebiotic and probiotic substances have been shown to boost the amount of good bacteria in the stomach.

Conclusion

The presence of a bacterial infection allows for the ability of salmonella stimulation to trigger a fever reaction. Indicators of temperature differences and changes in the rate of biomass

increase are two strategies that chickens employ to maintain their health and immunity. To understand a higher immune system response, more studies must be done using extraction steps and direct injection into the peritoneum.

Author contribution

A.S. designed the experiment, R.S. performed the experiment, collected and analysed data, and A.S. wrote the draft and presented the figure. All of the authors agree with the final version of the manuscript.

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