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EFFECT OF POWDER PROBIOTIC ON THE LEUKOCYTE, HETEROPHIL AND LYMPHOCYTE LEVEL ON LAYING HENS

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Abstract

Powder probiotic has a good effect on the digestive tract which improvement of the immune system. This study to know the impact of powder probiotics on the immune system which includes levels of leukocytes, heterophils, lymphocytes, and heterophils to lymphocytes ratio in laying hens age 90 weeks. This study was conducted from February to March 2021 at Laying Hens Farm in Sukarapih Village, Sukasari, Sumedang, Jawa Barat. The object study were 40 laying hens aged 90 weeks. Completely Randomized Design (CRD) was applied which consists of four treatments and five replications. The treatments are basal ration without powder probiotic (T0); basal ration + 2 % powder probiotic (T1); basal ration + 3% powder probiotic(T2); and basal ration + 4% powder probiotic (T3). Statically this study showed no significant difference in levels of leukocyte, heterophil, lymphocyte, and heterophil to lymphocyte ratio.However, the administration of powder probiotic 4 % has improved the levels of, heterophil,lymphocyte, and heterophil to lymphocyte ratio on laying hens aged 90 weeks near rejected.

Key words: *heterophil, laying hen, leukocyte, lymphocyte, probiotic.*

INTRODUCTION

The productivity of laying hens is influenced by the immune system. A strong immune system can increase productivity. In addition, the productivity of laying hens is also influencedby age. In the first year productivity will be optimal, but laying hens with high productivity can produce up to 2-3 years. Laying hens at a productive age of 22-72 weeks can produce as many as 260 eggs/year. Laying hens with the age of 72 weeks will decrease productivity, thus the chickens will be rejected laying hens. According to Salang (2015), the production of laying hens aged 82 weeks is below 50%. Because of the normal rate of egg production decrease in laying hens is 0.4-0.5% per week. One of the efforts to increase productivity uses probiotics as feed additives in the ration, which improve digestibility and increase immunity.

Probiotics are microorganisms that play a role in improving the ecosystem of intestinal flora, which impacts improving health. Some probiotics can reach the colon, which decreases of total pathogenic bacteria in the colon. Giving probiotics from an early period can improvesthe balance of intestinal microflora (Adriani, 2005; Adriani et al., 2019). Administration of probiotics in recent years is beneficial in modulating the immune system by enhancing intestinal barrier function. The gastrointestinal tract is in contact directly with various antigens from the environment, microbes, and pathogens. *Gut-associated lymphoid* tissue includes two main components, respectively intestinal microflora and the local immune system that interact (Kusumo, 2010). The composition of the intestinal tract consists of various defenses. They are physical defense from mucus and epithelial cell layers of the non-specific immune system, innate immunity (macrophages, invariant T cells, and defensins), and specific defense systems(production of antibodies and T cells). The intestine is an organ with the largest immune system in the body. The cells that make up the intestines are coats with a mucous barrier constantly undergoing a process of regeneration. Probiotics can strengthen immunity because of mucus. This mucus provides an advantage for probiotic bacteria, which is a medium for

attaching probiotics to the intestinal wall. According to Perdigon et al. (1991), the attachment of probiotics to mucus occurs because of the mucus-binding-protein substances that probioticshave. Mucus-binding-proteins can recognize antibody proteins and several types of probioticsso that they increase specific immune responses as immunomodulators. Resistance to enteric pathogens is influenced by balance interactions between the gut microbiota, epithelium, and the immune system (Patterson & Burkholder, 2003). Lactic Acid Bacteria (LAB) uses common probiotics are *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. However, these two bacteria are reduced in the colon, so they are not reliable probiotics. Meanwhile, bacteria that live along the digestive tract and obtain in large numbers on the small intestine are *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. They are reduced pathogenic bacteria in the colon (Adriani & Lengkey, 2010). Adding beneficial microorganisms to the gut could prevent or delay some diseases by improving the immune response or by producing bioactive metabolites (Lesmana et al, 2021). *Lactobacilli* treatment leads to higher antibody production in chickens. *Lactobacillus acidophilus* will inhibit the growth of pathogenic bacteria, which can damage the permeability of blood cell membranes and will end with leak or damage to blood cell walls (Latipudin et al., 2018). Administration of probiotics in powder form is easier to feed livestock than in liquid form (Adriani et al., 2020).

Leukocytes are divided into two groups, they are granulocytes (heterophils, eosinophils, and basophils) and agranulocytes (monocytes and lymphocytes). Granulocytes and monocytes defense the body from pathogens by phagocytosis, while main function of lymphocytes relates to the immune system. The addition of live microorganisms to the ration has been found to stimulate the immune system (Toms & Powrie, 2001; Koenen et al., 2004)and strengthen non-specific immunity (Placha et al., 2010). Probiotics will stimulate lymphocyte or immunocompetent cells to maintain immunity through the response of lymphoid tissue. Lymphoid tissue is the tissue production and maturation of lymphocytes. Thelymphoid tissue will trigger plasma cells to produce antibodies (Dewi & Herlisa, 2015).

In a previous study, probiotics consisted of *Lactobacillus fermentum, Lactobacillus plantarum,* and *Pediococcus pentosaceus* were given 0%, 1%, 2%, and 3% to Peking ducks. The results of that study showed administration of probiotics as much as 2% was quite effectivein reducing level of basophils, eosinophils, H/L ratio, and increasing lymphocyte level. Leukocyte levels according to treatment (0%, 1%, 2% and 3%) were 23.38 x 10^3, 20.26 x 10^3, 21.85 x 10^3, and 22,34 x 10^3 cells/mm^3, while lymphocyte levels were 35.7%, 46.8%, 51%, and 45.2% (Wulandari, 2014). Another study showed probiotic *L. acidophilus* given as much as 0,1 x 10^9, 2 x 10^9; and 3 x 10^9 CFU/kg, resulted leukocyte levels were 43.96 x 10^3, 47.76 x10^3, 50.78 x 10^3, and 52.88 x 10^3. The administration of *L. acidophilus* in this study was not significant effect, but it showed that the higher probiotic level given can increase leukocyte components in the blood turn to increase in lymphocytes, so stimulate the immune response in chickens (Alaqil et al., 2020). Several studies were conducted by Asmara et al. (2019) which showed the administration of probiotics had a very significant effect on the total leukocytes, heterophils, and lymphocytes of broiler chickens. Another study from Februansyah (2018) wasfound probiotic Bacillus plus vitamin and mineral at 0.1%, 0.5%, and 1% increase the immunesystem of broiler chickens as seen from leukocyte level and differential leukocytes levels, especially heterophils. eosinophils, and lymphocytes levels.

In addition, a study by Gunawan & Sundari (2003) used *Lactobacillus acidophilus* as much as 2% and 4% in laying hens rations can increase 5-11% egg production and suppress feed conversion ratio. The study conducted by Lutfiana et al. (2015) stated the administration of probiotics 2% and 3% was able to increase hemoglobin total in laying hens compared to 0% and 1% treatments. Recent study conducted by Kumalasari et al. (2020), the administration of dry probiotic as much as 2% of the total broiler chicken ration were increased growth performance, body weight gain and giblets. In addition, dry probiotic decreased abdominal fat, and lipid profiles of blood and meat.

This research to know effect of powder probiotics on the immune system which includes levels of leukocytes, heterophils, lymphocytes, and heterophils to lymphocytes ratio in laying hens age 90 weeks. The main novelty in this research is using yoghurt based on probiotics as animal feed, the microbiota consortium described above and powdered by spray drying method.

MATERIALS AND METHODS

Materials: Fresh cow's milk, Lactic Acid Bacteria culture i.e., *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidhophilus*, *Bifidobacterium bifidum,* skimmed milk and maltodextrin DE 10-12 as probiotic encapsulate, Plate Count Agar (PCA).
Bird and Ration: A total of 40 laying hens strain Lohman Brown 90 weeks old with Hen Day Production (HDP) between 50-60 %. The experiment was conducted for 30 days from February to March 2021 at Laying Hens Farm, Sukarapih Village, Sukasari, Sumedang, Jawa Barat. The basal ration used mixture of corn, bran, concentrate, top mix, and macro minerals. The concentrate used consisted of corn gluten, pollard, meat and bone meal, soybean meal, oil, calcium phosphate, calcium carbonate, sodium chloride, amino acids, vitamins, trace minerals, and antioxidants. The basal ration contains a metabolic energy of 2700 kcal/kg and 16.5% crude protein.
Processing of Powder Probiotic: The probiotics were used 5% (v/v) *Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus acidophilus, Bifidobacterium bifidum* inoculated into 250 ml De Man Rogosa and Sharpe (MRS) medium and then incubated at 45ºC for 14 hours. Fresh cow's milk was pasteurized and cooled to 45ºC before added 5% of the consortium bacteria, then homogenized. The fermentation process was carried out for 14 hoursat room temperature. The process making probiotic powder was liquid fermented milk added with skimmed milk and maltodextrin as encapsulated materials, sterile distilled water (1/2 of the total volume of the solution), and homogenized. After homogenized, the mixture was dried used a spray dryer with an inlet temperature of 160^0C and outlet 65-70ºC (Juniawati et al., 2019). Administration powder probiotics are 2, 3, and 4 % of total ration. This dose based a reference in the study of Kumalasari et.al. (2020) and Adriani et.al. (2020) by administration 2% probiotics in broiler chicken rations showed significant results on blood biochemistry and broiler chicken production.

Statistical Analysis

This experiment was used Completely Randomized Design with 4 treatments i.e., T0 = basal ration without powder probiotic; T1 = basal ration + 2 % powder probiotic; T2 = basal ration + 3% powder probiotic; T3 = basal ration + 4% powder probiotic. Under each treatment 5 replicates were considered for each parameter such as leukocyte level, lymphocyte level, heterophil level, and heterophil to lymphocyte ratio. Data analysis were used analysis of variance (ANOVA) and orthogonal polynomials to test significance between treatments. Significance was considered at $P \leq 0.05$ levels.

RESULTS AND DISCUSSIONS

Results

Leukocyte Levels

The effect of ration administration of powder probiotic on leukocyte level of layer hens is presented in Table 1.

Table 1. Immune Response of Laying Hens Supplemented Powder Probiotics

Variable	Treatments			
	T0	T1	T2	T3
Leukocyte (cells/mm^3)	43,980 [a]	48,320 [a]	41,060 [a]	43,160 [a]
Heterophil (%)	2.8 [a]	3.2 [a]	3.8 [a]	1.8 [a]
Lymphocyte (%)	90.8 [a]	90.4 [a]	89.8 [a]	92.2 [a]
H/L Ratio (%)	0.031 [a]	0.035 [a]	0.042 [a]	0.020 [a]

Note:
T0 = Basal ration without powder probiotic (Control)
T1 = Basal ration + 2 % powder probiotic
T2 = Basal ration + 3% powder probiotic
T3 = Basal ration + 4% powder probiotic

The average leukocyte level was highest at T1 (48,320 cells/mm^3) and lowest at T2, which was 41,060 cells/mm^3. In T1 was increased in leukocyte levels by 9.87%, while other treatments decreased by 1.86% (T3) and 6.64% (T2). Leukocyte levels were not significantly different (P>0.05).

Heterophil Levels

The effect of ration administration of powder probiotic on heterophil levels of layer hens is

presented in Table 1. The average heterophil level was highest at T2 (3.8%) and lowest at T3, which was 1.8%. In T3 was decreased in heterophil levels by 9.87%, while other treatments increased by 1.86% (T1) and 6,64% (T2). Heterophil levels were not significantly different (P>0.05)

Lymphocyte Levels

The effect of ration administration of powder probiotic on lymphocyte level of layer hens is presented in Table 1. The average lymphocyte level was highest at T3 (92.2%) and lowest at T2, which was 89.8%. In T3 was increased in lymphocyte levels by 1.54%, while other treatments decreased by 0.22% (T1) and 1.10% (T2). Lymphocyte levels were not significantly different (P>0.05).

Heterophil to Lymphocyte Ratio

The effect of ration administration of powder probiotic on heterophil to lymphocyte ratio (ratio H/L) of layer hens is presented in Table 1. The average ratio H/L was highest at T2 (0.042) and lowest at T3, which was 0.020. In T3 was decreased in ratio H/L by 35.48%, while other treatments increased by 12.9% (T1) and 35.48% (T2). Ratio H/L were not significantly different (P>0.05).

Discussions

The Effect of Powder Probiotic on Leukocyte Levels Laying Hens

Leukocytes are the body's immune system (Mushawwir et al., 2020) in cellular and humoral defense against foreign substances at the site of damage (Indah, 2016). Leukocyte observation is used to diagnose the health condition of livestock. Leukocytes consist of heterophils, eosinophils, basophils, lymphocytes, and monocytes.

Normal levels of leukocytes in chickens range from 8-20 x 10^3 cells/mm^3 (Soeharsono et al., 2010). According to Jannah et al. (2017), the range of 225.20 – 487.40 x 10^3 cells/mm^3 is still in normal condition. Leukocytes levels in all treatments showed in the normal range. Leukocytes levels that deviate from normal conditions correlates with the health condition of the animal (Suriansyah et al., 2016). However, the high production of leukocytes cannot be assumed that laying hens are sick. An increase in leukocytes level describes a humoral and cellular response against pathogenic agents that cause disease in the body. This high leukocyte level because laying hens were pathogenic infections or immune system disorders, so that was an increase in the body's defense ability, while the decrease in leukocytes level can also be assumed that there is no infection or disruption of pathogenic bacteria attack the body (Soeharsono et al., 2010). Therefore, it is necessary to measure differential leukocyte levels to determine the health of the laying hens under study.

Factors that affect leukocyte levels include stress, age, environment, biological activity, hormones, and ultraviolet light or radiation. Environmental stress causes physiological processes to become abnormal, which affects the hormonal balance in the chicken body whichcan cause an increase in leukocyte levels. Environmental stress will increase the production of corticosteroids and glucocorticoids, which causes a decrease body's defense system. The increase corticosterone hormone levels in poultry that will trigger cell damage including blood cells due to reduced body oxygen intake. The mechanism of the body's response to the pathogen invasion can be seen from the increase and decrease in total leukocyte (Saputro et al., 2013; Latipudin et al., 2018; Falahudin et al., 2016).

The highest average leukocyte level at T1 with 2% supplemeted powder probiotic was increased by 9.87% compared control treatment. This showed that the administration of 2% probiotic powder of thetotal ration can improve the body's immune system, so that chickens can produce more leukocytes. LAB in probiotic powder can reduce pathogenic microbes in the digestive tract by increase beneficial microbes. High leukocyte levels are caused by a response to disease, both infections and foreign substances. This process will form antibodies. According to Hartoyo et al. (2015), the function of leukocytes is protect the body from pathogens by phagocytosis and produce antibodies. It is also proven by the higher egg productivity on T1 at the time of the study with an average of 80 items for 4 weeks. Probiotics can improve the balance of intestinal microflora, so that can improve immunity and egg productivity (Tang et al., 2017).

The average leukocytes levels were lower at T2 (3% powder probiotic) and T3 (4% powder probiotic) than control, with each decrease at 6.64% (T2) and 1.86% (T3). This decrease in leukocyte levels was related to intestinal microflora balance, which will result in reduced infections in the body. According to Sjofjan et al. (2020), the increase and decrease in the leukocyte levels is a response mechanism against invading pathogens. Based on this, decrease in pathogens in the digestive tract will reduce infection and decrease leukocytes levels.

The Effect of Powder Probiotic on Heterophil Levels Laying Hens

Heterophils include the group of granulocytes in leukocytes. They are the forefront (first line) which acts rapidly as a nonspecific immune response and earliest in defense againstpathogens infection (Hewajuli & Dharmayanti, 2015). Heterophils function is the body's line of defense against pathogens, especially bacteria. It is phagocytic with engulfs microflora andremains of dead cells and can enter the infected tissue.

The normal range of heterophil levels in poultry are 20%-40% or 4 – 8 x $10^3/mm^3$ (Soeharsono et al., 2010). Meanwhile, the heterophil levels of laying hens in the study were below the normal range. This can be caused age factor of laying hens on the study being old orheading to reject, so they are can not produce heterophil optimally. According to Devi et al. (2019), the factors that influence differential leukocyte levels include environmental conditions, age, and nutritional content of the feed. According to Nasrullah et.al. (2020) and Sukmana (2019), the average heterophil level of broiler chicken aged 40 weeks was 12.8% and the age of 85 weeks was 4.6%. Whereas in this research, 90 weeks of heterophil levels were 2.8%. The average heterophil levels was bigger at T1 (2% powder probiotic) and T2 (3% powder probiotic) compared control, with each increase at 1,8% (T1) and 6,64% (T2). Theincrease in heterophil level occurs due to a physiological stress response. Pathological conditions that cause neutrophilia include acute infection, inflamemation, tissue damage, andmetabolic disorders (Riswanto, 2013). This increase is an effort from leukocytes to fight pathogens by heterophils return the livestock body to normal condition (Mushawwir et al., 2017).

The lowest average leukocyte level at T3 with 4% supplemented powder probiotic was decreased by 1.8% compared other treatments. heterophil can attack pathogen by migrating to attack areas, penetrating vessel walls, and destructing pathogen (Hutasoit, 2010). When there is no infection, it does not affect the increase in heterophil level (Wulandari et al., 2014), because the increase in the heterophils levels is caused by pathogen infection (Sugiharto et al., 2014). The administration of probiotic powder reduces the heterophil levels laying hens in the study. According to Adriani (2010), probiotic plays a role in suppressing the growth of microflora pathogen that cause digestive tract diseases. This is because LAB produces antimicrobials include bacteriocin, hydrogen peroxide, and various natural antibiotics. Antimicrobials produce by probiotic in T3 can help alleviate the work of heterophils in inhibit the growth of pathogenic bacteria, so that can reduce heterophil in phagocytosis and decrease compare to other treatments.

The Effect of Powder Probiotic on Lymphocyte Levels Laying Hens

Lymphocytes are a part of leukocytes that do not have agranulocytes or nuclei. Lymphocytes are cells capable of recognizing and destroying antigenic. Lymphocytes consist of B lymphocytes or cells B (naive and active) and T lymphocytes (T cells). When an antigen is detected, T cells and B cells in the bone marrow will enter secondary lymphoid organs such as lymph nodes and spleen to activate these antigens into effector cells and memory cells. Active cells will then migrate to peripheral tissues where infection occurs (Sukmayadi et al., 2014).

The range normal of lymphocyte levels on poultry is 30-70%. However, the lymphocyte levels in this study were all above normal levels. This is caused the old age laying hens. The old age of chicken makes the cortex in the follicles thicker and more lymphocyte cells. According to Nasrullah et al. (2020) and Sukmana (2019), the average lymphocytes levels of broiler chicken aged 40 weeks was 80.2% and the age of 85 weeks was 82.9%. Whereas in this research, 90 weeks of heterophil levels were 90.8%.

The highest average lymphocyte level at T3 with 4% supplemented powder probiotic was increased by 1.54% compared other treatments. This caused powder probiotics can increase LAB in the digestive tract. LAB improves intestinal microbial balance, adheres to the gut, secretes active metabolites, and competes with pathogenic bacteria (Boostani et al., 2013; Trela et al., 2020). According to Saki et.al. (2018), the humoral immune response can be stimulated because of high levels of probiotics so that the leukocyte component in the peripheral blood leads to an increase in the lymphocyte population. The parts of LAB that can stimulate immunity are endotoxic lipopolysaccharide, peptidoglycan, and lipoteichoic acid. Lipoteichoic acid from *Lactobacillus* sp. and *Bifidobacteria* sp. has a high affinity for epithelial membranes, acts as a carrier for other antigens, and binds them to target tissues for an immune reaction to occur. LAB attach to intestinal epithelial cells can activate macrophages. The attachment of microflora to mucosal epithelial cells is the result of a special binding process between surface adhesives on microflora and mucosal receptors on the cell membrane. (Gusils et al., 2002; Surono, 2004).
In addition, probiotics activate dendritic cells in Peyer's patch that stimulates the mucosa circulating pool of T-lymphocytes generated from within the Peyer's patch. In this waythese T-cells might also exert their immune modulation at distant mucosal sites (Clancy, 2003). Probiotic powder modulates the production of cytokines as antibody-producing metabolites from monocyte macrophages, mitogens, and antigens that promote lymphocyte proliferation (Rohyati, 2012). T lymphocytes will release interferon that plays a role in activating macrophages and B cell differentiation. Meanwhile, B lymphocytes will produce antibodies that play a role in humoral-specific immunity (Galdiano et al., 2007).

The Effect of Powder Probiotic on Heterophil to Lymphocyte Ratio Levels (Ratio H/L) Laying Hens

The ratio heterophil to lymphocyte (ratio H/L) uses as an indicator of stress in chickens, stress conditions will be seen if the value is above the normal range (Sugito & Delima, 2009). Factors that affect ratio H/L are feed, light, age, and environmental temperature (Mashaly et al., 2004).
The level of body resistance on poultry to environment ranges from ratio H/L around 0.2 – 0.8 with a normal value of 0.5 (Emadi & Kermanshahi, 2007). Meanwhile, the percentage ratio H/L in this study showed from 0.020 to 0.042. In this study, laying hens with all treatments supplement powder probiotic did not stress, which showed by ratio H/L below the normal range. According to Kusnadi (2008), the higher stress level makes the ratio heterophil to lymphocyte increase.

CONCLUSIONS

The administration of powder probiotic overall was not significant. Although not significant, the administration of 4% probiotic can improve total lymphocyte, decrease heterophil and H/L ratio. This research show that probiotic can increase the immunity of laying hens aged 90 weeks/ rejected chicken

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