

Mobilization of CD4⁺, CD8⁺, and B220⁺ on Broiler Chicken Spleen with Feed Contained *Polyscias obtusa* Post Infection of *Salmonella typhimurium*

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ABSTRACT

Farmers in Indonesia used to provide feed with additional antibiotics to prevent the endemic disease in poultry such as New Castle Disease and Avian Influenza. This has a negative impact because the antibiotic residues will accumulate in meat and harmful to consumers. The aim of this research was to determine the role of simplicia *Polyscias obtusa* leaves as immunostimulants in broiler feed that had been infected with *Salmonella typhimurium*. Sixty of DOC (days old chicks) with initial weight 37 g were used in this experiment. The broilers were offered diets containing different levels of simplicia leaves of as follows; 0% with no infection (A₁), 0% (A₂), 0,08% (A₃), 0,16% (A₄), and 0,26% (A₅). At day 14th, the broiler orally infected with 10⁸ CFU/ml *Salmonella typhimurium*, 500 µl for each broiler. Treatments were allocated in a completely randomized design. The variable observed were the relative number of lymphocyte cell CD4⁺, CD8⁺, and B220⁺ of spleen analyzed by flowcytometry. The results obtained showed that additional simplicia *Polyscias obtusa* leaves in feed can significantly affect the development (relative number) of lymphocytes cell, especially T cells CD8⁺. Treatment A₄ (*P. obtusa* 2nd dose (0.16%) + *S. typhimurium* infection) had the best ability to increase the relative number of lymphocytes cell. These result strengthens *Polyscias obtusa* role as one of immunostimulatory agent, in terms of its active compounds (saponins and flavonoids).

Keywords : Broiler, immunostimulants, lymphocyte cell, *Polyscias obtusa*, spleen.

INTRODUCTION

Indonesia as a country with a wet tropical climate is one of the sites where the development of both pathogenic viruses and bacteria occurred easily. To overcome such problems, Indonesian stock farmer always use additional antibiotics in broiler chicken feed. Broiler is specified chicken for meat production. Broiler chicken could produce large quantities of meat. Each body parts of broiler chicken are in different form, the back contained more bones, more muscular thigh and tenderer breast with less fat. Broiler chicken had digestive organs in form of evolved suitable tract which directed for flying purpose. Broiler chicken has no teeth and jaw bones [1].

Sustainable feed consumption with additional antibiotics for broiler chicken leads to antibiotics residues accumulation in meat, due to its uncomplete secretion. Furthermore, supplementary continuous feeding with additional antibiotic was also triggered target bacteria or microorganism resistance.

Consequently, it is required additional food that can improve immune system of broiler chickens, so it can be an alternative to the use of antibiotics feed and safe for consumers.

Such additional feed is leaves of *Kedondong laut* (*Polyscias obtusa*), which is one of ethnomedicinal herbs with diverse savor. Choice on this herb based on its active compounds such as saponin and flavonoid. Saponin was known for its character on membrane permeability changes, as immunostimulants and anticarsinogen agent [2]. While flavonoid is one of substantial antioxidant to prevent free radical. Flavonoid was known acted as antibacteria, antiviral, antiinflammation, antialergy, antimutagenic, antitrombotic, and vasodilatation activity [3]. Flavonoids also play an important role in inflammatory reactions when infections happened [4].

Lymphocytes cell development in broiler chicken also maximized by *Salmonella typhimurium* bacteria as infection agent. *Salmonella typhimurium* is a pathogenic intracellular facultative bacteria in human and animals when it enter orally [5]. Intracellular facultative bacteria could live and reproduced in varies of cell types, including macrophage which leads prominent in immunity. Cellular immunity mechanism is the most appropriate mechanism

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to do elimination, where antibody in blood circulation unable to reach progress antigens in cells [6,7,8]. Active compounds in *Polyscias obtuse* plant were expected to enhance lymphocytes cells development which is very important in immunity function of broiler chicken, so that conventional feed usage with antibiotic contents can be reduced.

METHODS

This research was conducted on March to July 2012 in Laboratory of Microbiology and Laboratory of Animal Physiology, Department of Biology, Laboratory of Biomedic, Faculty of Medical School, and Field Laboratory of Sumber Sekar-DAU, Faculty of Animal Husbandry, Brawijaya University.

Material and Equipments

Equipments used during the study period are petri dishes, test tubes, wire, microtube, syringes, pipettes and micropipette, suction ball, graft and oose needle, objects and glass cover, vortex, incubator, chicken feed containers, and ice box.

Material for this research are NA, NB, XLD, blood agar media, KIA and LIA, H₂O₂ solution, Gram stain of A, B, C, and D, PBS solution, mill feed of BR1, and feed conversion consisted of leaves simplicia of *P. obtusa* in three different doses, DL Metionin, salt, premix, coconut oil, cassava flour, MBM, copra, fish meal, soybean meal, and yellow corn.

Research Design

Treatments in this study were divided into five treatments, with two different groups based on broiler growth phase.

Table 1. Research Design

Treatment	Group	
	Starter	Finisher
Mill Feed	A1B1	A1B2
Mill Feed + <i>Salmonella thypimurium</i>	A2B1	A2B2
<i>Polyscias obtusa</i> (0,08 %) + <i>Salmonella thypimurium</i>	A3B1	A3B2
<i>Polyscias obtusa</i> (0,16%) + <i>Salmonella thypimurium</i>	A4B1	A4B2
<i>Polyscias obtusa</i> (0,26%) + <i>Salmonella thypimurium</i>	A5B1	A5B2

Confirmation Test

XLD (Xylose Lysine Deoxycholate) test media

Pure isolates that will be tested sampled with oose and conducted streak plate on XLD media,

then incubated in 37°C temperature for 24 hours. Test will show a positive result when the streak region formed black colonies.

Catalase Test

Isolate of *S. typhimurium* bacteria from NA media subculture were taken one oose, then moved (aseptically) to object glass which etched with 3% hydrogen peroxide (H₂O₂). Positive results showed by the occurrence of air bubbles.

Gram Staining Test

One oose isolates were taken and put on object glass, then treacled by Gram A stain, left for 2 minute and washed with running water. Hereafter, Gram B stain was treacled, left for 1 minute and washed with running water. Treacle again with Gram C stain, left for 30 second and washed with running water, then Gram D treacled, left for 2 minutes and again washed with running water. Last step was observation with microscope. Result showed that *S. typhimurium* were red and classified as Gram negative bacteria.

KIA and LIA Test

Positive colonies obtained from selective test on XLD media were taken one graft, inserted into KIA (Kligler Iron Agar) and LIA (Lysine Iron Agar) media, then withdrawn and formed a line along the media. Next step is incubating in 37°C temperature for 24 hours. Formed colonies were black colored due to the generation of H₂S.

Pathogenicity Test

Isolates of *S. typhimurium* bacteria from subculture were taken one oose and inoculated (by streak) on blood agar media petri dish. Then it was incubated in 37°C for 24 hours. Formed colonies were translucent.

Standard Curves of Cells Number

Standard curves of cells number obtained by cultured the colonies of *S. typhimurium* in NB (Nutrien Broth) media. Isolates suspension with NB media with different dilution were then homogenated. Absorbance of such suspension was assessed by spectrophotometer on 600 nm wavelength and cell number was counted by haemocytometer.

Bacterial Growth Curves

Bacterial Growth Curves were made by bacteria culture wick taken from 1 oose and

grown in NB 10 ml media then incubated in 37°C for 24 hours. 6 ml culture taken and mixed with 54 ml of NB media. Then sampling performed in every 1 hour, by taking it 4 ml and added 500 µl of formalin. Absorbance of each suspension per one hour was assessed by spectrophotometer on 600 nm wavelength and the number of cell was counted with haemocytometer.

Manufacture of the Conversion Feed

Manufacture of conversion feed was by mixing some material which had varies function, with major function as nutrition sources to enhance broiler chicken growth. Conversion feed were made by mixing each composition with highest percentage (mix 3) to the lowest percentage (mix 1). Simplicia of *P. obtusa* itself was mixed together within less coconut oil and yellow corn (mix 4). Mixed feed of *P. Obtusa* divided into three different doses, i.e. 0,08%, 0,16%, and 0,26%. The order of conversion feed manufacturing are by mixed Mix 3 and Mix 2 first, continued by Mix 4 and Mix 1, till the feed completely mixed.

Oral Infection of *Salmonella typhimurium*

Salmonella typhimurium bacteria in Nutrient Broth was centrifugated in 10.000 rpm for 10 minutes with 25°C. Obtained pellet then resuspended with physiological saline solution NaCl 0,9% then taken as much 500 µl and fed orally (trough mouth) for each chicken by sterilized pipette.

Lymphocytes Cell Isolation

Spleen were crushed with wire, by adding 1 ml PBS to obtain homogenates. The homogenates then moved into propylene tubes, added PBS till 12 ml. Then it was centrifugated in 2500 rpm, 4°C for 5 minutes. Supernatant removed and obtained pellet was homogenized with 1 ml PBS, taken 50 µl, moved into microtube, and centrifugated again in 2500 rpm, 4°C for 5 minutes. Last was 100-200 µl antibody addition then homogenated in 100 µl PBS.

Flowcytometry Analysis

Isolated lymphocytes cells of spleen then added with antibody Rat anti-CD4, Rat anti-CD8 and RAT anti-CD45 conjugated to PE label for CD4 cell, Per CP for B220 cell, and FITC for CD8 cell. Conjugated results incubated for 15 minutes in ice box. The sample then added with 1 ml PBS placed inside flowcytometer cuvette. Then

choosed *acquire* and the flowcytometer calculated the total cell number and the number of cells detected by labeling antibody. Obtained results then processed by *BD cellquest Pro™* program to be analyzed with Complete Randomized Design by *SPSS 16 for Windows* program. Observed variables are relative cell T lymphocytes number of CD4⁺, CD8⁺, and B220⁺.

RESULT AND DISCUSSION

Profile Comparison of CD4⁺ and CD8⁺ Cells of Starter and Finisher Phase by Flowcytometry

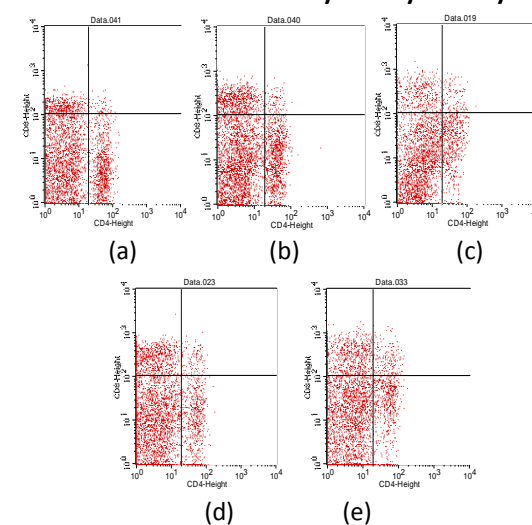


Fig 1. Profiles of CD4⁺ and CD8⁺ starter phase; (a) mill feed, (b) mill feed + *S. typhimurium*, (c) *P. obtusa* dose 0,08% + *S. typhimurium*, (d) *P. obtusa* dose 0,16% + *S. typhimurium*, (e) *P. obtusa* dose 0,26% + *S. typhimurium*

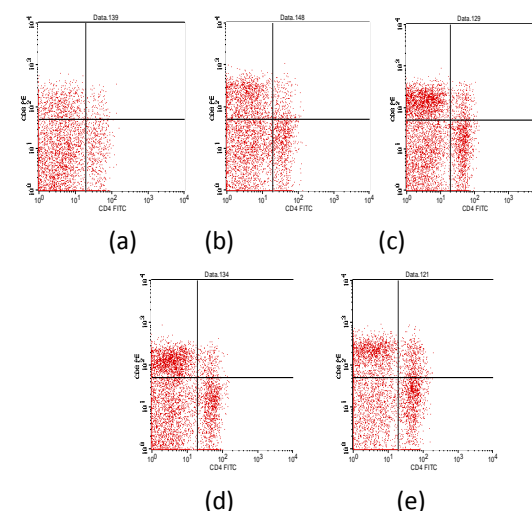


Fig 3. Profiles of CD4⁺ and CD8⁺ starter phase; (a) mill feed, (b) mill feed + *S. typhimurium*, (c) *P. obtusa* dose 0,08% + *S. typhimurium*, (d) *P. obtusa* dose 0,16% + *S. typhimurium*, (e) *P. obtusa* dose 0,26% + *S. typhimurium*

The profiles (Fig 2 and 3) showed a significance increase on relative cell number of either $CD4^+$ or $CD8^+$ based on growth of broiler chicken. Distinctive $CD8^+$ cell, different treatment (dose of *P. obtusa* leaves simplicia in feed) also affected relative cell number growth. Best treatment for increasing relative cell number of $CD8^+$ either on starter or finisher is Treatment A₄ (*P. obtusa* dose 2 (0,16%) + *S. Typhimurium* infection). The highest relative number on starter is 19,77%, whereas finisher phase is 35,69%.

Comparison of Average Cell Relative Number of $CD4^+$, $CD8^+$, and B220⁺ on Spleen Organ of Broiler Chicken in Starter and Finisher Phase

Result of analysis of variance or ANOVA showed that cell relative number of $CD4^+$ on starter and finisher phase was significantly different ($p < 0,05$). It showed that cell relative number of $CD4^+$ affected by treatment period (different growth phase), nonetheless it was not affected by different treatments. Age factor is one determinants of immunity system in avian to had optimal works, beside external factor such as dose of infected antigen virulence ability [9].

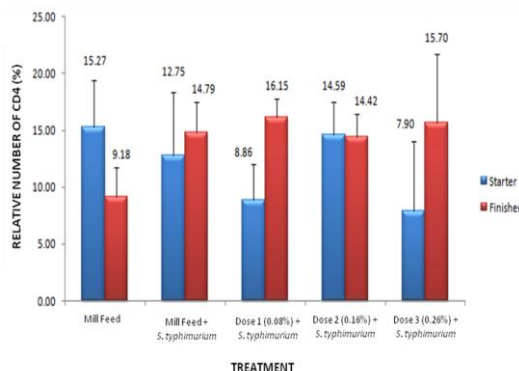


Fig 4. Relative Cell Number of T $CD4^+$ (%) Starter and Finisher Phase

Increasing of relative number of $CD4^+$ was needed to maximize important character enrolled by the cell itself. T helper $CD4^+$ Cell can secrete IL-2 cytokines which serves as stimulus (growth factor) for other lymphocytes cell such as cell B. Activated T helper $CD4$ will proliferate and differentiate into effector cells that secrete IFN- γ to activate macrophages [6,7,8].

Based on ANOVA, relative cell number of $CD8^+$ on starter and finisher phase in different treatment were significantly different ($p < 0,05$). It indicated that relative cell number of $CD8^+$ besides treatment period, were also affected by the difference of treatment (including the

different dose of *P. obtusa* simplicia in feed). The highest relative cell number of $CD8^+$ either in starter or finisher is Treatment A₄ (*P. obtusa* dose 2 (0,16%) + *S. typhimurium*), were starter phase had average value of 14,56%, and finisher phase 29,30%.

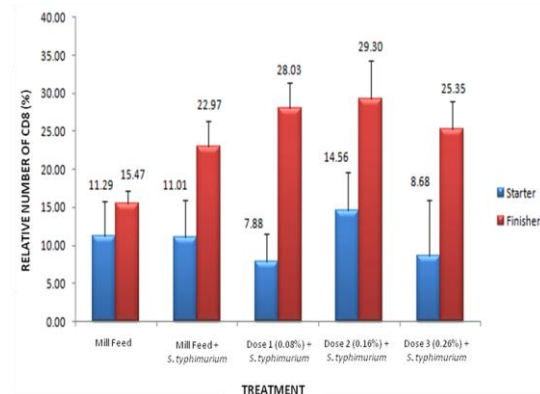


Fig 5. Relative Cell Number of T $CD8^+$ (%) Starter and Finisher Phase

Figure 5 showed that T $CD8^+$ cell had the highest relative cell number compare to the other profile lymphocytes cell. It can be assumed from the nature of *Salmonella typhimurium* which is an intracellular facultative bacterium. Facing this pathogenic bacterium, immune system majorly handled by cellular immune responds (cell mediated immunity). Activated lymphocytes cell $CD8^+$ will proliferate and differentiate into cytotoxic cell T (CTL) which can kill cell with microbes in its cytoplasm. Those microbes may be a virus which infected many types of cells, or bacteria that has been ingested by macrophage but had escaped from phagocytic vesicle and moved to cytoplasm (where it safe from phagocytic system which commonly rely on the vesicle) [6,7].

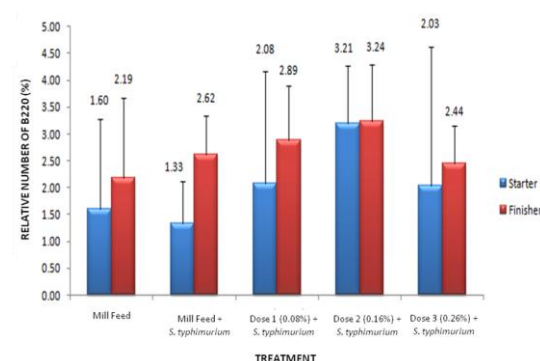


Fig 6. Relative Cell Number of B220+ (%) on Starter dan Finisher Phase

Results on ANOVA showed that relative cell number of B220⁺ neither on starter nor finisher phase were not significantly different ($p>0,05$). The highest relative cell number of B220⁺ either on starter or finisher phase also found in Treatment A₄ (*P. obtusa* dose 2 (0,16%) + Infection of *S. typhimurium*), i.e, starter phase had average range 3,21% while finisher had 3,24%.

Low relative number of B cells can be caused by the mechanism of B cells performance themselves. Cell B is the only cells that produce antibody [5,8]. Some pathogenic bacteria (including *Salmonella typhimurium*), parasite, and virus were replicated inside antibody undetected cells, because antibody only reaches the antigen within blood circulation and outside cell. Because of that, this pathogen destruction needs the role of lymphocytes T cells as cell mediated immunity (cellular immunity) [5].

These results confirm the role of *Polyscias obtusa* as one of immunostimulator agent, so that it can be an alternative to the requirement of broiler feed. Addition of immunostimulator compound increase lymphocytes respond and lead to cell proliferation [11,12,13,14]. Immunostimulation ability of this *Polyscias obtusa* can not be separated from its active compound, such as saponin and flavonoid. Flavonoid is an antioxidant compound that stimulates immune system by increasing the chelating and chemotaxis of lymphocytes [7,8,15]. While saponin itself was acted as anti fungal and anti bacteria, in avian (e.g. chicken), saponin functioned as additional compound to stimulate immunity system [16].

CONCLUSION

Feeding administration with addition of leaves simplicia of *Polyscias obtusa* in this research significantly affected the development (relative number) of lymphocytes cells, particularly on T cell CD8⁺ which had the highest relative cell number compare to the other two lymphocytes cell profile. This confirmed the role of *Polyscias obtusa* as one of immunomodulator agent, reviewed from its active compound. The best treatment performance in increasing relative number of lymphocytes cell is Treatment A₄ (*P. obtusa* dose 2 (0,16%) + infection of *S. typhimurium*). Results of ANOVA showed that T cell CD8⁺ development with the highest relative rate is not only affected by treatment period, but also affected by different treatments (including

the difference dose of *P. obtusa* simplicia in feed).

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