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Immunomodulator effect of Robusta Lampung coffee extract (*Coffee Canephora* Var Robusta) in layer chicken infected with *Salmonella enteritidis* bacteria

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ABSTRACT: Salmonellosis that attacks poultry, caused by *Salmonella enteritidis* is a cause of food borne disease in zoonotic humans. Treatment of this disease uses antibiotics but if used irrationally it can result in the emergence of multi-drug resistant bacteria. At present many treatments use herbs which are considered to reduce the negative effects of antibiotics and are environmentally friendly, one of which is coffee. Green coffee is considered to have many active ingredients such as chlorogenic acid (CGA), alkaloids, tannin, polyphenols and polysaccharides which are useful as antimicrobial, anti-inflammatory and enhances the body's immune system. The study used 60 heads ISA Brown day-old chicks (layer strain) chickens artificially infected with *S. enteritidis* bacteria with concentration of 10^8 CFU/ml, consisting of negative controls (healthy chickens, without coffee, positive control (chickens were given), T1, T2 and T3 was given different doses of coffee extract with 500 mg/kg BW, 1000 mg/kg BW and 1500 mg kg BW, respectively. The treatments with coffee extracts were given on days 3-15, then infected with 0.5 ml bacteria on day 16 and 60 chickens were necropsied at day 18 for histopathology. Data on relative levels of CD8 Tc cells were calculated using the flowcytometer test and analyzed quantitatively using the One Way ANOVA with a confidence level of 95% while the jejunum histopathology was observed using a microscopy and analyzed descriptively. Results showed that Robusta coffee extract from Lampung can act as an antibacterial by increasing CD8 + T cells and repairing jejunum histopathology.

Keywords: Green coffee, lymphocyte cells, salmonellosis, zoonoses

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INTRODUCTION

Salmonella enteritidis serovar enteritidis is a serotype that is often isolated from gastroenteritis due to consuming contaminated food (animal products) in humans avarages 75% caused by contaminated food products derived from beef, pork, poultry and eggs. Chicken is the largest reservoir host for *Salmonella enteritidis* (Shah *et al.,* 2012). Poultry are often infected through consumption of contaminated feed on farms or during slaughter and processing. Salmonella infection usually starts through ingestion, and is followed by colonization in the intestine.

After colonization, Salmonella is able to penetrate the mucosal epithelium which results in systemic infections (Muna *et al.*, 2016). Young chickens <2 weeks old often get gastroenteritis and systemic diseases with various mortality rates. In contrast, most adult chickens are carriers of *S. enteritidis*, without showing clinics, transmitted through feces excreted. In young broilers it can cause high mortality so it must be culling from flock (Shah *et al*., 2012).

Natural products have long been recognized as an important source of effective drug therapy. Of the 520 new drugs were approved in 1983 and 1994, 39% came from natural products and 60-80% serves as an antibacterial and anticancer drugs (Barbosa-Filho *et al*., 2006). Coffee is a mixture of over a thousand phytochemicals such as alkaloids, phenolic compounds, vitamins, carbohydrates, lipids, minerals and nitrogen compounds. Coffee has multifunctional properties as a food additive and nutraceutical namely chlorogenic acid, coffee has the effect of being anti-inflammatory, anti-oxidant, anti-dyslipidemia, anti-obesity, and cardiovascular disease, which can be used for the treatment and prevention of metabolic syndrome and related disorders.

On the other hand, as a food additive, coffee has antimicrobial activity against various microorganisms, inhibits lipid peroxidation, and can function as a prebiotic (Saeed *et al*., 2019). This research is very interesting because until now there has been no research on the use of coffee extract for chicken health performance, especially in the starter phase chicken. Poultry is the animal products as a source of high protein in humans. Disease in humans can be derived from poultry. Production of high laying hens can be monitored early so that the results of this study are expected to be a reference for further research on chickens using coffee. This study uses chicken layer strain ISA brown 1 day old and has obtained the Ethical Clearance No. 773-KEP-UB from the Bioscience Laboratory of Universitas Brawijaya.

Coffee extracts manufacture, testing Phytochemicals and LCMS (Liquid chromatography–mass spectrometry)

The coffee extraction was carried out at UPT Materia Medika, Batu, Indonesia using 90% ethanol. 414 grams of Lampung Robusta coffee soaked with 1500 ml of ethanol 90% in a jar and then tightly closed and carried out a shaker with a speed of 50 rpm. Soaking was done three times. The filtrate was evaporated using a rotatory evaporator. The extraction of liquid chocolate used for the study.

Experimental animal design

Chickens were placed in cages and given feed and drink in ad libitum. Chickens were given standard commercial feed Charoen Pokphand 511-bravo with nutritional content that is Rough Protein 21-23% - 5-8% Fat - Rough Fiber 3-5% - 4-7% Ash. Research used 5 treatments and 4 replications with three birds per replications consisting of a negative control (healthy chicken, without any coffee), positive control (chickens infected with *S. enteritidis* bacteria at concentrations of 10^8 CFU / ml), T1 (chicken given coffee a dose of 500 mg/kg bw and infected with *S. enteritidis* bacteria with a concentration of 10^8 CFU/ml), T2 (chickens were given coffee with a dose of 1000 mg/kg bw and infected with *S. enteritidis* bacteria with a

concentration of 10^8 CFU/ml) and T3 (chickens were given coffee dosage 1500 mg/kg bw and infected with *S. enteritidis* with a concentration of 10^8 CFU/ml). Supplements in chickens aged 1, 5 and 11 days were given vitamins to prevent stress, at Day 4, an ND-IB live vaccine was administered, and at Day 10, ND G7B vaccine and AI H5N1 subtype were given. Chickens were fasted for 10 hours before being given coffee extract. Coffee extract were given to chickens at days 3-16 orally.

Making bacterial suspension

S.enteritidis bacteria grown in nutrient broth media and incubated at 37°C for 24 h. Bacterial growth was measured its absorbance at a wavelength of 580 nm using a spectrophotometer and were further diluted to obtain a concentration of bacteria at 10^8 CFU/ml (Mostafa *et al.,* 2018). The bacterial suspension was given to the chickens orally with a volume of 0.5 ml per chicken. Giving a bacterial infection using a feeding tube on the 16^{th} day. A necropsy performed on the 18^{th} day.

Examination number relative of $CD8^+$ TC cells

Cells isolated from the thymus were incubated with anti-CD8 + PE chicken antibody. Samples were incubated with antibodies, added with 1 ml PBS and placed on the cuvette flowcytometer. The relative number of cells is calculated. The results obtained were processed with the BD cell quest ProT program (Kurnianingtyas *et al*., 2013).

Histopathological examination of jejunum

Chickens were killed through air embolism, then examination for jejunum intestine was done. The jejunum was washed with PBS, 10% formalin solution was added and histopathological preparations were made using hematoxylin and eosin staining (H & E) (Muna *et al.*, 2016).

Data analysis

The number of $CD8^+$ Tc cells were presented as mean ± standard deviation (S.D). Statistical analysis for animal experiment was carried out using one-way ANOVA with an error level of 0.05.

RESULTS AND DISCUSSIONS

The active ingredient of Lampung coffee extract

The active content of Lampung coffee extract using the phytochemical test shows the presence tannins and alkaloids. The LC-MS Test results show chlorogenic acid (CGA). The main polyphenol in coffee is chlorogenic acid (CGA). CGA is an ester form of cinnamic acid and quinic acid, known as 5-O-caffeoylquinic acid (5-CQA) or 3-CQA. The largest form of CGA is 5-caffeoylquinic acid (5-CQA) (Meng et al., 2013)

Calculation of weight

The results showed that the gain in weight of chicken on a negative control (healthy) higher than the positive control, which was expected since the chicken were infected with *S. enteritidis* bacteria. While the chickens fed the coffee extract at all doses showed a decrease in body weight compared to a negative chicken (unpublished data).

Commercial laying hens are genetically chosen that has the characteristics of contrafreeloading (the ability to adapt to choose the preferred feed) more active, and utilizes nutrients more efficiently so that the average metabolism of the energy produced is higher than broilers because the feed that enters the body is intended for production eggs are higher than weight gain (Buzala and Janicki, 2016). Commercial laying hens are genetically chosen that has the characteristics of contrafreeloading (the ability to adapt to choose the preferred feed) more active, and utilizes nutrients more efficiently so that the average metabolism of the energy produced is higher than broilers because the feed that enters the body is intended for production eggs are higher than weight gain (Buzala and Janicki, 2016).

CGA components have biological functions such as antibacterial, antioxidant, anti-carcinogenic, hypoglycemic, and

hypolipidemic. The results of this study are consistent with those conducted by Meng et al. (2013), CGA is claimed to modulate glucose and lipid metabolism in vivo under conditions of abnormal genetic metabolism. While CGA, caffeine, and other polyphenol compounds in green coffee bean extract act to suppress weight gain, does not cause obesity and visceral fat deposition in mice. The mechanism of CGA in reducing blood lipids is most likely related to inhibition of lipid absorption and transformation through inhibition of intestinal absorption and liver cholesterol biosynthesis (Meng et al., 2013).

Measurement of relative quantities of CD8$^+$ Tc cell

The results showed that the relative number of CD8$^+$ Tc cells in positive control (chicken infected with *S. enteritidis* bacteria) decreased, compared to the negative control group (healthy). All treatments showed comparable results compared to the negative control group. When compared with positive control, T1 showed a significant difference on the number of CD8$^+$ Tc cells closer to negative control (Figure 1). This shows that administration of coffee extract at T1 group dose has increased the activation of CD8$^+$ Tc cell effector cells.

CD8$^+$ Tc cells are effector cells of the adaptive immune system to maintain long-lasting protective immunity against intracellular bacteria, protozoa, and viruses. After reactivation by antigens, CD8 + Tc cells produce pro-inflammatory cytokines such as IFN-Y, and TNF-α for inflammatory activity (Narni-Mancinelli *et al.*, 2007).

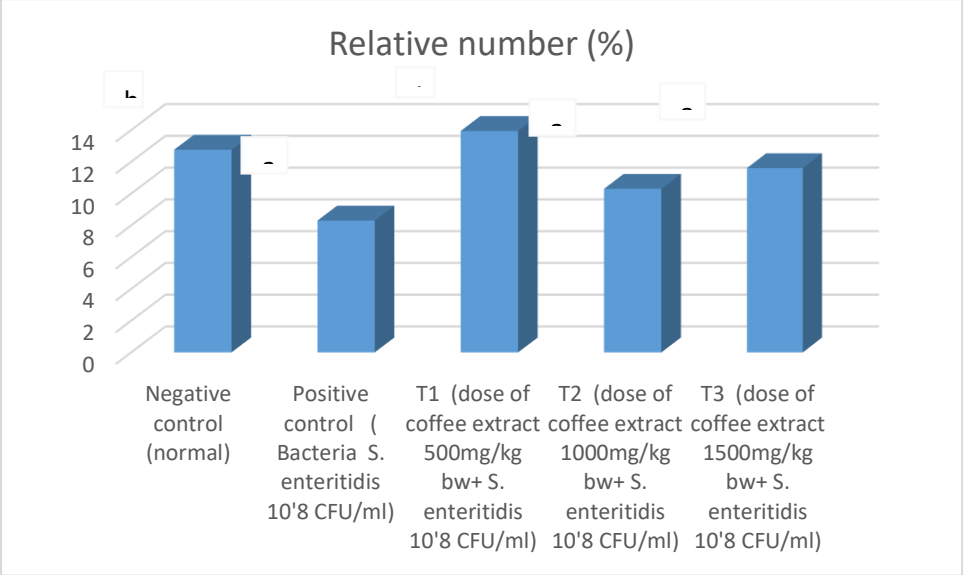

Figure 1. The number of CD8 + T cells relative shows that there are differences among the treatments (P<0,05)

During colon colonization inflammation occurs characterized by the entry of heterophils and macrophages which act as host immune systems against intracellular microbes such as Salmonella (Shah *et al.*, 2012). *S. enteritidis* bacteria have the ability to survive in intestinal cells and penetrate to the mucosal epithelium, causing spread to organs, including the spleen, liver and reproductive tract. In the digestive system of birds, including the stomach has gastric acidity (pH 2.6). Microbial killings by CD8$^+$ Tc cells via MHC-peptide bonds: T-cell receptor interactions (TCR) cause lysis of target cells via pathways involving the release of apoptotic-inducing pore proteins, perforin, and granzymes secreted by CD8$^+$ Tc cells (Jiang *et al.*, 2003).

Granulysin (in contrast to granzymes) acts as a mediator of microbial killing through disruption of membrane

permeability by damaging ionic interactions between positively charged amino acid residues and negatively charged phospholipids which result in the entry of fluids into the cytoplasm and death by osmotic lysis (Oykhman and Mody, 2010).

Histopathological Examination of jejunum

Histopathologic examination of the negative control (healthy chicken) show that the villi in the jejunum organs under normal conditions and elongated compared to the villi of the positive control (chickens infected bacteria *S. enteritidis*) appear shortening and erosion of the intestinal epithelium (Figure 2a and 2b). *S. enteritidis* bacteria can damage and make shortening of the intestinal villi. The length of villi plays a role in the expansion of the surface of nutrient absorption so that it influences weight gain. In the T1 and T2 (Figure 2c and 2d), the intestinal villi length and normal epithelium and goblet cell hyperplasia, while in T3 (Figure 2e), the intestinal villi were shorter than T1, erosion of epithelial and goblet cell hyperplasia. In the treatment of T2, the intestinal villi appear normal and elongated compared to treatment T1. Treatment T1 and T2 showed improvement of intestinal villi compared to positive control and T3. These results showed that the coffee extract at doses of 500 and 1000 mg/kg BW can improve intestinal integrity and increase the length of intestinal villi against damages of *S. enteritidis* bacteria infection.

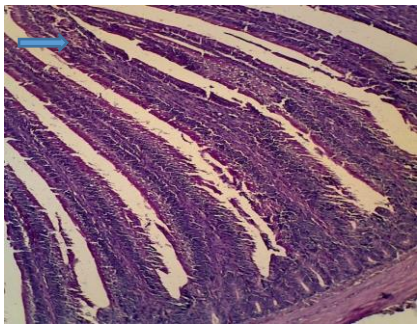

Figure 2a. Negative control. Jejunum organ. Villi appear normal, elongated, slim (blue arrow). 100x magnification.

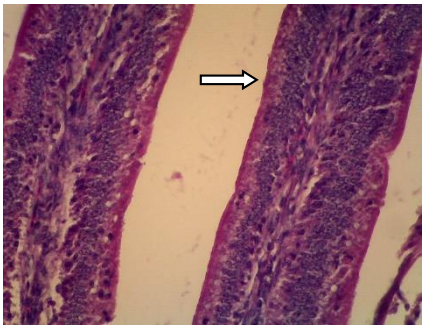

Figure 2a. Negative control. Jejunum organ. Villi appear normal, elongated, slim. Epithelium is normal (white arrow). 400x magnification.

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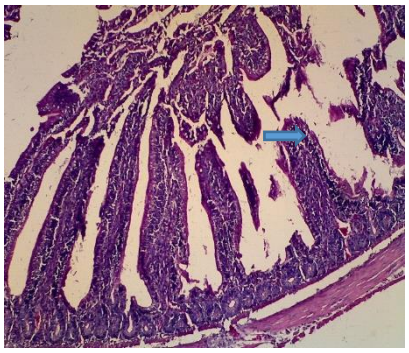

Figure 2b. Positive control. Jejunum organ. The villi are shortened and the epithelium is erosion (blue arrow). 100x magnification

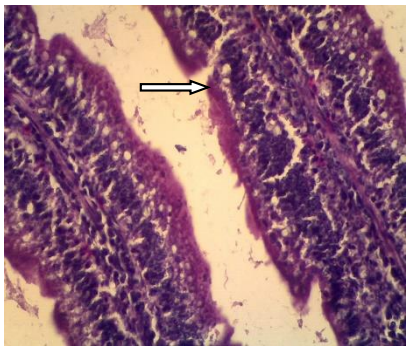

Figure 2b. Positive control. Jejunum organ. The villi are shortened and the epithelium is erosion white (white arrow). 400x magnification

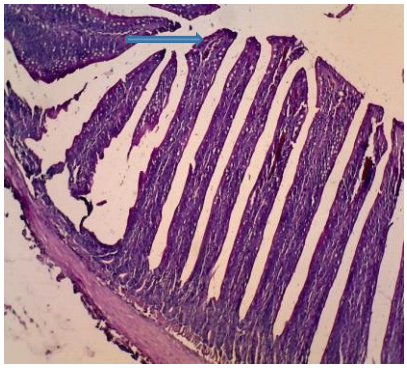

Figure 2c. T1 Treatment. Jejunum organ. Villi are elongated, partially shortened and normal (blue arrow). 100x magnification

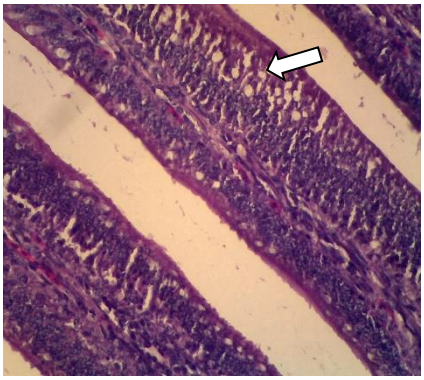

Figure 2c. T1 treatment. Jejunum organ. Epithelial and goblet hyperplasia cells (white arrow). 400x magnification

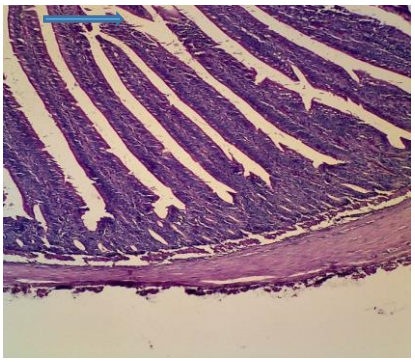

Figure 2d. T2 treatment. Jejunum organ. Elongated villi (blue arrow). 100x magnification

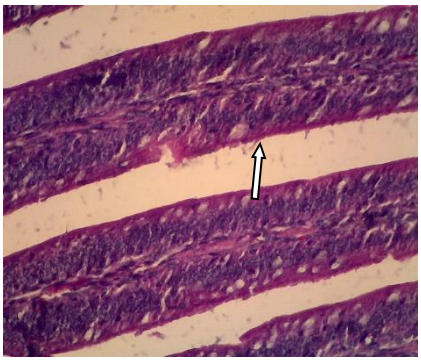

Figure 2d. T2 treatment. Jejunum organ. Epithelial and goblet hyperplasia cells (white arrow). 400x magnification

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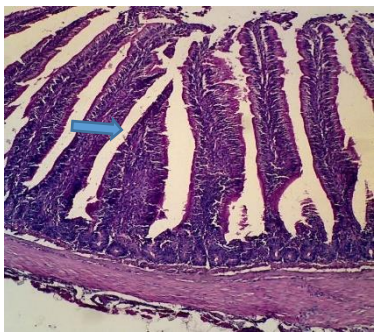

Figure 2e. T3 treatment. Jejunum organ. Villi of treatment T3 are shorter than T1 (blue arrow). 100x magnificent

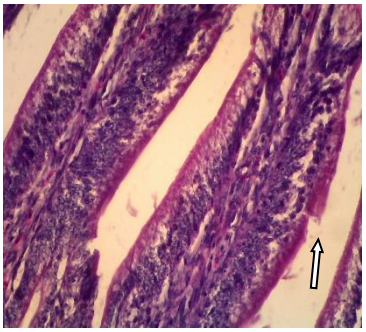

Figure 2e. T3 treatment. Jejunum organ. Epithelial erosion (white arrow) and goblet cell hyperplasia. 400x magnificent

Intestinal epithelium acts as a natural barrier to pathogenic bacteria and toxic substances in the intestinal lumen. Stressors, pathogens, and chemicals also cause interference with normal microflora or in the intestinal epithelium which can alter natural barrier permeability, facilitate invasion of pathogens and hazardous substances, modify metabolism, reduce the ability to digest and absorb nutrients, and cause chronic inflammatory processes in the mucosa intestine (Hughes, 2003; Pelicano *et al*., 2005). Jejunum chicken grows rapidly after birth. Morphologically, intestinal length increases rapidly and is accompanied by villous heights, in line with the rate of food absorption. In early life the main immunological development occurs in the intestine. Antigen and luminal nutrition encourage expansion and differentiation of intestinal epithelial and lymphoid tissue in regulating immune responses to environmental antigens (Schokker *et al*., 2010). Robusta coffee extraction contains tannin. According to a study conducted by Hughes (2003) coffee contains anti-nutritional factors such as non-starch polysaccharide (NSP), tannins, and alkaloids. Coffee drinks contain quite a lot of soluble dietary fiber, especially low substituted galactomannans and type II arabinogalactans.

NSPs on food are not digested which negatively affects animal performance. The negative effects of the NSP are related to viscosity, physiological and morphological effects on the digestive tract, interactions with epithelium, mucus and intestinal microflora. Dissolve NSP, increases intestinal transit time, delays gastric emptying and glucose absorption, increases pancreatic secretion, and absorption is slow. However, insoluble NSPs, such as pentosan (arabinoxylans and xylans), advantageously

reduce transit time, increase binding of NSPs with intestinal brush borders which can increase the thickness of the water layer that does not stick around the mucosa, causing indigestion and nutrient absorption. High viscosity also increases digestion time due to increased production of intestinal volatile fatty acid (VFA). This drastic change causes the intestinal ecosystem to reduce nutrient digestibility (Sinha *et al*., 2011).

According to Mujtaba *et al.* (2017) it was shown that CGA was able to inhibit the growth of *S. entritidis* bacteria in vitro with a diameter of 17 mm inhibition zone. Squalamine alkaloids work by damaging the integrity of the outer membrane and cytoplasmic membrane. Alkaloids work through the following mechanism: penetrate the LPS and cause depolarization of the cytoplasmic membrane so that the cytoplasmic contents leak and bacteria will die (Cuhnie *et al.*, 2014). Alkaloids can inhibit attachment, movement and production of free radicals by phagocytic cells. Alkaloids suppress antigen and mitogen-induced lymphocyte proliferation, Natural Killer cells, histamine release by mast cells, secretion of interleukin-1 (IL-1), prostaglandin and leukoetrin by monocytes (Barbosa-Filho *et al*., 2006).

Chlorogenic acid functions as an antimicrobial through the mechanism of increasing permeability of the outer membrane and plasma, resulting in loss of cell barrier function, even causing nucleotide leakage and releasing cytoplasmic macromolecules, which cause cell death (Lou *et al*., 2011; Mujtaba *et al*., 2017). Tannin can work like a siderofor to bind iron from growth media. Microorganisms that grow under aerobic conditions require iron for various functions, including reduction of ribonucleotide DNA precursors, and hem formation (Akiyama *et al*., 2001).

CONCLUSIONS

Based on the results of the study it can be concluded that Robusta coffee extract from Lampung can function as an antibacterial through increasing $CD8^+$ Tc cells and enhancing jejunum integrity. Further research is needed to obtain the optimal dose to increase the activity of $CD8^+$ Tc cells and repair intestinal histopathology.

DECLARATION OF CONFLICT OF INTEREST

There was no conflict of interest between the research team

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