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THE EFFECT OF CHLOROGENIC ACID IN ROBUSTA LAMPUNG GREEN COFFEE EXTRACT (*Coffee canephora var Robusta*) AS AN ANTIOXIDANT IN LAYER CHICKEN INFECTED WITH *Salmonella enteritidis* BACTERIA

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Bacteria

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Antioxidant

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ABSTRACT

The poultry industry is crucial in meeting the population's nutritional needs worldwide. However, various bacterial infections have been reported in poultry chickens, decreasing their production rate. Farmers have been using synthetic antibiotic agents to manage these infections, which are costly and have several side effects. One of the most commonly reported bacterial pathogens is *Salmonella enteritidis*, which causes high mortality and low poultry production. Mortality and illness rates are associated with gastrointestinal diseases caused by suboptimal absorption. This study was conducted to evaluate the anti-inflammatory and antioxidant effect of Chlorogenic acid (CGA) from robusta lampung green coffee extract on *S. enteritidis* infected chickens. ISA brown day-old layer chickens were used in this study. A total of sixty chickens were divided into five groups, with each group having 12 replications. The formulated groups were C+ (chicken infected by *S. enteritidis* 10^8 CFU/ml), C- (healthy chicken), T1 (chicken infected by *S. enteritidis* 10^8 CFU/ml and 500 mg/kg BW (bodyweight) coffee extract), T2 (chicken infected by *S. enteritidis* 10^8 CFU/ml and 1000 mg/kg BW coffee extract), T3 (chicken infected by *S. enteritidis* 10^8 CFU/ml and 1500 mg/kg BW coffee extract). The Nrf2, HO-1, and SOD levels were measured using BD cell quest ProT programmed through the flow cytometry method. Data of total cells were calculated for their average value and obtained data were statistically analyzed by One Way ANOVA ($p<0,5$). The results of the study showed that giving coffee extract at a dosage of 500 mg/kg BW to *S. enteritidis* infected chickens increased the Nrf2 and SOD levels but decreased HO levels. This research showed that Lampung robusta coffee extract could potentially be an anti-inflammatory compound and antioxidant for poultry industries.

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1 Introduction

The demand for poultry products increases yearly due to human population growth, and the poultry industry plays a crucial role in meeting the need for animal protein (Nataamijaya 2017). However, the industry faces various challenges, including controlling diseases that affect the chickens (Regar et al. 2013). In particular, two *Salmonella* species, *S. enteritidis* and *S. typhimurium*, can cause higher infection and mortality in young chickens (Wiryawan et al., 2005). *Salmonella* infected chickens may also spread the bacteria to healthy ones, leading to rapid outbreaks. Infection of *Salmonella* may also cause diarrhea due to damage to the intestinal microflora (Wiryawan et al. 2005). The bacteria can also survive for a long time in the environment, including poultry cages (Mshelbwala et al. 2017).

Salmonella infection is associated with oxidoreduction in infected chickens. Nuclear factor erythroid-derived 2-related factor-2 (Nrf2) is an elevated antioxidant and antitoxic gene that protects against oxidative stress by activating detoxification and antioxidant genes (Dong et al. 2008). The activation of Nrf2 triggers the formation of HO-1 (heme oxygenase-1) and SOD antioxidants, which help in reducing inflammation in body cells (Habtemariam 2019).

Indonesia has many plants that could be used as food or feed supplements with significant medicinal properties. Coffee (*Coffea canephora*) is one of them, and it contains several bioactive ingredients, including Kahweol and cafestol, which have antioxidant effects (Tribudi et al. 2020). These compounds activate HO-1 via the Nrf2 pathway, promoting cytoprotection against oxidative injury (Hwang and Jeong 2008). Coffee extract also contains other antioxidant compounds, such as phenol or phenolic acid, one of which is Chlorogenic acid (CGA). CGA has an immunomodulatory effect by inhibiting inflammation and the production of prostaglandins (PGE2) (Johnston et al. 2003). It also inhibits the synthesis of other mediators, such as IL-1β, interferon-γ, monocyte chemotactic protein-1, and macrophages inflammatory protein-1α (Bagdas et al. 2020). CGA is a flavonoid that can increase endogenous antioxidants by stimulating the expression of SOD (Liang and Kitts 2015). This enzyme defends tissues from oxidative stress by scavenging superoxide anions, one of the primary reactive oxygen species (ROS) produced from molecular oxygen in cells. Excessive accumulation of ROS can cause oxidative damage to major cellular macromolecules (proteins, lipids, and DNA) and cause necrosis (Hwang et al. 2020; Mazur-Bialy and Pocheć 2021).

SOD catalyzes the dismutation of anion O_2, reactive oxygen into hydrogen peroxide (H_2O_2), and oxygen (O_2) inside mitochondria. This reaction can block inflammatory cytokines and stimulate anti-inflammatory cytokines like IL-10 to neutralize oxidative reactions caused by *Salmonella* bacteria (Hutabarat et al. 2020). The poultry industry uses many feed additives, such as antibiotics, prebiotics, probiotics, synbiotics, short-chain fatty acids, and vaccines, to lower the mortality of livestock caused by *Salmonella*. However, disease outbreaks can still occur even after using these feed additives (Van Immerseel et al. 2002). Therefore, this study aims to determine the effect of Lampung robusta coffee extract as an anti-inflammation supplement to increase the chicken's immune system against *S. enteritidis* infection by assessing Nrf2, HO-1, and SOD levels.

2 Materials and Methods

This study used ISA Brown day-old chickens as an animal model and obtained ethical approval from Brawijaya University with the number 1142-KEP-UB.

2.1 Lampung Robusta Coffee Extraction

Creating Lampung robusta coffee extract involves dissolving 414 grams of Lampung robusta coffee in a bottle of 1500 ml of 90% ethanol. Next, the solution is homogenized using a centrifuge at 50 rpm. The solution is then filtered and evaporated at 60°C, using a rotatory evaporator until all the ethanol has evaporated (Bahrin et al. 2018). The final result is a brown-colored liquid (Qosimah et al. 2020).

2.2 Preparation of Animal Model

This study utilized a type of chicken feed called Charoen Pokphand 511-bravo (PT. Charoen Pokphan Indonesia Tbk), which contains approximately 21-23% rough protein, 5% fat, 3-5% rough fiber, and 4-7% ash (Qosimah et al. 2020). The research involved ISA Brown layer chickens divided into five treatment groups with 12 replications. The treatment groups were as follows: C- group (healthy chickens), C+ group (chickens infected with *S. enteritidis* bacteria at a concentration of 10^8 CFU/ml), T1 group (chickens infected with 10^8 CFU/ml *S. enteritidis* and given 500 mg/kg BW coffee extract), T2 group (chickens infected with 10^8 CFU/ml *S. enteritidis* and given 1000 mg/kg BW coffee extract), and T3 group (chickens infected with 10^8 CFU/ml *S. enteritidis* and given 1500 mg/kg BW coffee extract). The coffee extract was administered as a preventative measure before the chickens were infected with *S. enteritidis*. On the first day of the research, the chickens were fed with vita stress, which contains vitamins and electrolytes (Medion, Indonesia), to prevent stress. On the fourth day, the chickens were injected with an inactivated emulsion ND (Newcastle disease)-IB (Infectious bronchitis) vaccine (Medion, Indonesia). In contrast, ND G7B vaccine (Medion, Bandung, Indonesia) and AI (Avian influenza) H5N1 vaccine (Medion, Bandung, Indonesia) were administered simultaneously on day 10 to prevent virus infection (Qosimah et al., 2020. From day 3 to day 16, Lampung robusta coffee extract was given orally at 1 ml per chicken.

2.3 Preparation of Bacterial Suspension

The *S. enteritidis* bacteria used in the study were obtained from the Microbiology and Immunology Laboratory at the Faculty of Veterinary Medicine, Universitas Brawijaya in Indonesia. The bacterial testing was conducted using Rappaport Vasiliadis Medium, Xylose Lysine Deoxycholate Agar (XLD agar), Triple sugar Iron Agar (TSIA), and LIA (Lysine Iron Agar), and supported by The Microbact ™ Gram-negative system from Oxoid. The bacteria were cultured in nutrient broth and incubated at 37°C for 24 hours. Bacterial growth was observed and stained using Gram staining and then analyzed using the biochemistry method to confirm the characteristics of the bacteria. The bacteria concentration was estimated by observing the spectrophotometer at an absorption wavelength of 580 nm to be 10^8 CFU/ml (optical density = 0.6). Each chicken was given 0.5 ml of bacterial suspension per oral, resulting in every chicken receiving a $5x10^7$ CFU/ml (Qosimah et al. 2020).

2.4 The Analysis of HO-1, Nrf2, SOD-1

A chicken necropsy was conducted on day 18, and the spleen was collected. The spleen was washed with PBS (phosphate buffer saline) and homogenized in a petri dish using a syringe. Cell suspension obtained from the spleen was washed with PBS, then mashed by a syringe and put inside a polypropylene bottle. 10 mL PBS was added to the tubes and centrifuged at 2500 rpm at 10°C for 5 minutes. The supernatant was removed from the pellets, and the pellets were resuspended in 1 ml of sterile PBS. From this, 50 µL splenic cell suspensions were separated into a 1.5 mL microtube containing 400 µL of PBS and centrifuged as previously mentioned (2500 rpm, 10°C, 5 minutes). Afterwards, 10 µL of anti-HO-1 FITC, anti-Nrf2 FITC, and anti-SOD-1 reagents (BioLegend, USA) were added, and the mixture was incubated for 30 minutes. After incubation, the mixture was rinsed with the wash-perm solution and centrifuged again as per the initial condition (2500 rpm, 10°C, 5 minutes), then incubated for 20 minutes and resuspended with PBS. After that, the sample was analyzed using a flow cytometer (BD FACS Calibur, USA) (Hermanto et al. 2020).

2.5 Statistical Analysis

The HO-1, Nrf2, and SOD-1 levels were analyzed using the BD CellQuest ProT program on the FACS Calibur TM device from BD Biosciences, San Diego, CA, USA. The statistical analysis was performed using One-way ANOVA ($p<0.5$). If a significant difference was observed between the treatments, the analysis proceeded to the post-hoc Tukey test using SPSS Statistic 21 software.

3 Results

3.1 The Nrf2

There is a significant difference in the Nrf2 level between the positive control group (C+) and the negative control group (C-). The Nrf2 level in the negative control group (11.49%) is higher than that of the positive control group (7.36%). The normal control group (C-) did not show any significant difference from the treatment groups T1 and T2 but a significant difference from treatment group T3. The cell counts of the negative control (11.49%), T1 (10.54%), and T2 (11.2%) are lower than those of T3 (15.23%). The T3 group has a higher cell count than the entire treatment group (Figure 1).

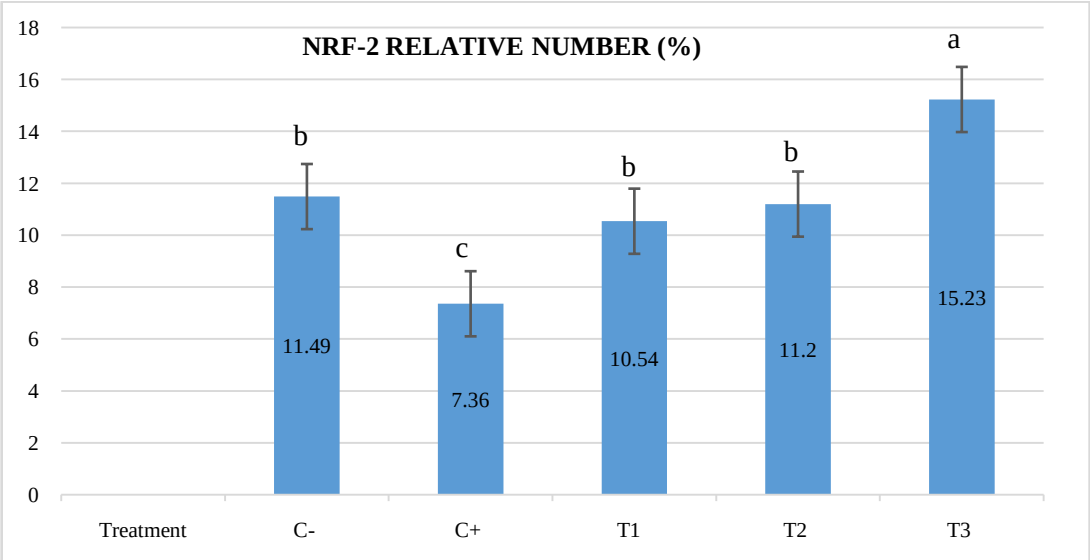

Figure 1 Nrf-2 level in chicken receiving treatments; Data are mean of twelve replicates; Columns without common letters differ significantly at LSD P≤0.05)

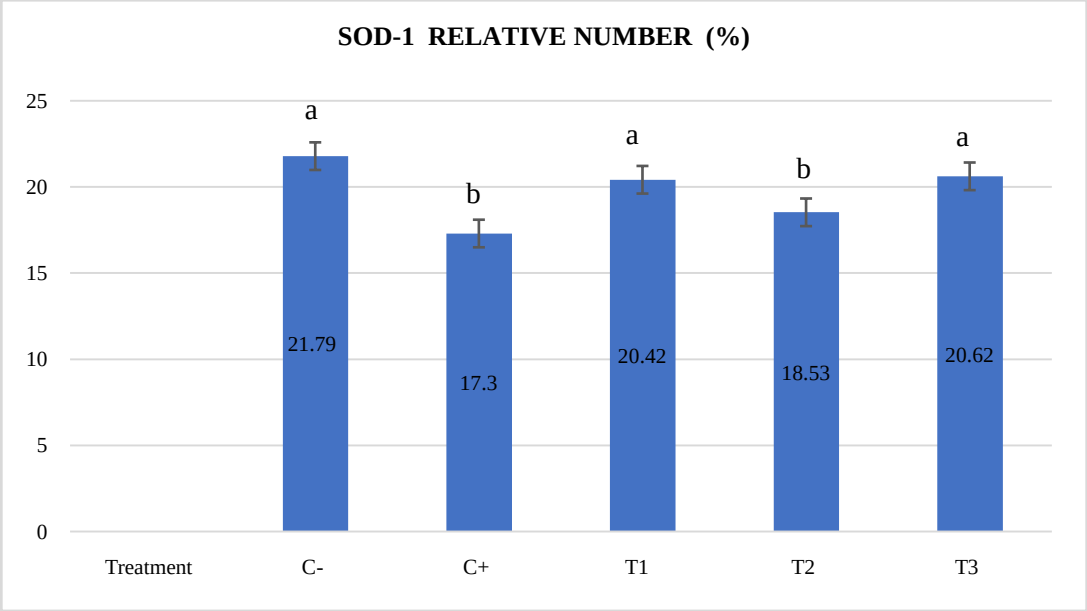

Figure 2 SOD-1 level in chicken receiving treatment; Data are mean of twelve replicates; Columns without common letters differ significantly at LSD P≤0.05)

3.2 The SOD-1 level

The research conducted showed that the level of SOD-1 varied significantly between the positive (C+) and negative (C-) control group. Among the tested treatments, the negative control had the highest SOD-1 level at 21.79%, followed by treatment groups T1 (20.42%), T3 (20.62%), and T2 (18.53%). These values were significantly higher than the positive controls (17.3%), as shown in Figure 2.

3.3 The HO-1 level

The HO-1 level was tested alongside two other parameters and significantly differed between the groups. The highest HO-1 level was observed in the positive control C+ at 26.56%. The levels were followed by group C- (20.36%) and group T1 (19.16%). The treatment groups T2 and T3 had even lower levels at 17.6% and 15.85%, respectively. This indicates that Lampung robusta coffee significantly affects HO-1 levels, as shown in Figure 3.

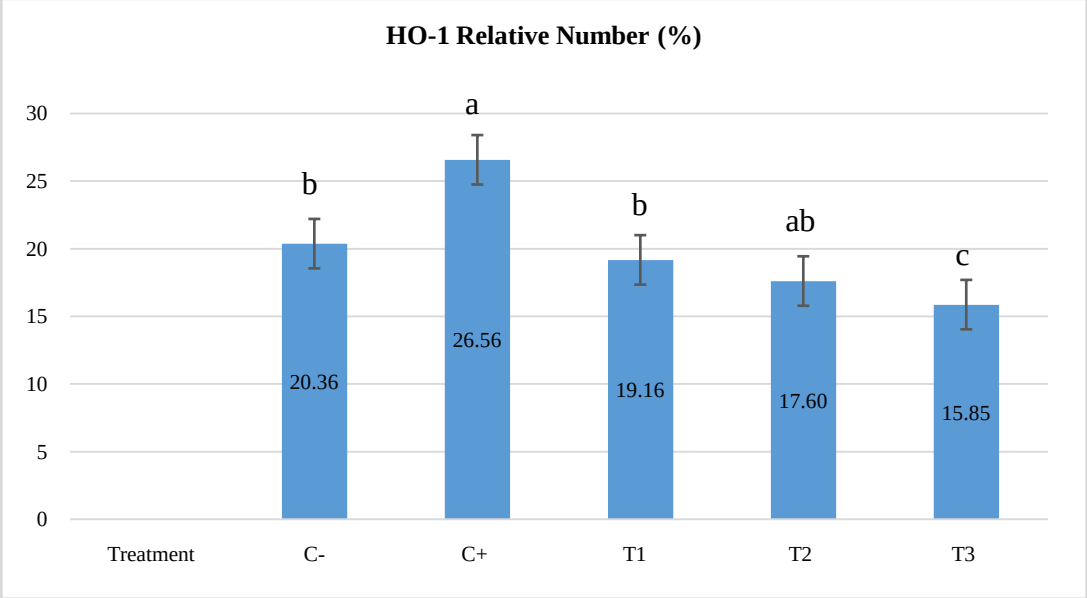

Figure 3 HO-1 levels in chickens receiving treatment; Data are mean of twelve replicates; Columns without common letters differ significantly at LSD P≤0.05)

4 Discussions and Conclusion

Various chemical compounds, including caffeine, chlorogenic acid, trigonelline, carbohydrates, volatile flavor, and minerals, have been found in coffee. Chlorogenic acid (CGA), which protects coffee from microorganisms and insects, also exhibits antioxidant, immunostimulatory, anti-inflammatory, antibacterial, and hepatoprotective effects. Robusta coffee beans contain higher levels of chlorogenic acid (6.1-11.3 mg/gram) than other coffee beans (Farhaty and Muchtaridi 2016; Qosimah et al. 2020). Nrf2 is responsible for regulating many antioxidant genes or proteins, such as glutamate-cysteine ligase, glutathione peroxidase 1 (GPX1), thioredoxin reductase 1 (Txnrd1), NAD (P), H-quinone oxidoreductase 1 (NQO1), glutathione-S-transferase (GST), SOD, catalase, peroxiredoxin (PRDX1), ferritin, and oxygenase-1 (HMOX1, HO-1). Additionally, the Nrf2 protein can stimulate HO-1 (Habtemariam 2019). Results of the study suggest that the Nrf2 level is higher in the negative group (C-) than in the positive group (C+), which can induce the SOD level. Under healthy conditions, a higher level of endogenous antioxidant SOD is present in the body, which neutralizes free radicals. When *Salmonella* bacteria are orally administered, they penetrate and invade the intestinal mucosa to proliferate and replicate intracellularly in chickens. Upon lysis or death of host intestinal cells, *Salmonella* releases LPS endotoxin inside the intestine, which can enter the lymph and blood circulation to spread into all organs and cause septicemia. This allows the bacteria to interact with phagocyte cells, macrophage, and monocyte receptors, which could stimulate the expression of IL-1, TNF, and other inflammatory cytokines (Frost et al. 2002), leading to an inflammatory response. Chickens infected with *S. enteritidis* bacteria (positive control) showed clinical symptoms of diarrhea.

The inflammation caused by *S. enteritidis* infection binds cytokine receptors to the surface of the intestine, leading to the downregulation of nuclear factor-2 (Nrf2) and SOD levels (Mshelbwala et al. 2017). SOD, or superoxide dismutase, is an antioxidant enzyme that comes in three forms: SOD1, SOD2, and SOD3, and among these, SOD1 makes up about 70% of SOD expression (Hwang et al. 2020). The mechanism of antioxidant activity involves decreasing the production of reactive oxygen species (ROS) and scavenging them (Mazur-Bialy and Pocheć 2021). SOD-1 can protect the mucosa, balance the immune system, and inhibit inflammation by blocking the entry of immune cells by regulating the intestinal immune response (Hwang et al. 2020). Antioxidants play a role in decreasing inflammation caused by *S. enteritidis* infection. Additionally, SOD catalyzes the conversion of anion superoxide radical into hydrogen peroxide (H_2O_2), which is toxic to bacteria (Hwang et al. 2020). A high concentration of *S. enteritidis* causes an increased inflammation response, but coffee can induce SOD to neutralize free radicals. All the treatment groups have chlorogenic acid (CGA) as an active compound of coffee, which is why these groups significantly increase SOD-1 levels compared to the positive control (Liang and Kitts 2015). SOD-1 blocks macrophage activation and inflammatory molecules by stopping excessive ROS generation. ROS is a product produced by macrophages and is essential in removing pathogens. However, excessive accumulation of ROS can cause oxidative damage to major cellular macromolecules such as proteins, lipids, and DNA, leading to cell necrosis (Mazur-Bialy and Pocheć 2021).

The study observed higher HO-1 molecules in the positive control group than in the other treatment groups. This may be due to the oxidative stress caused by *Salmonella* infection that upregulated the antioxidant HO-1. The expression of HO-1 is mainly found in the spleen and chicken tissue. Apart from the spleen, the expression of HO-1 is more profound in other reticuloendothelial systems, such as the liver and bone marrow (Ryter et al. 2006). HO-1 antioxidant expression responds to oxidative stress caused by intracellular bacteria. HO-1 is an enzyme that catalyzes heme degradation, which produces biliverdin, iron ions, and carbon monoxide. The degradation of heme releases Fe2+, CO, and biliverdin. Intracellular pathogens use heme as a source of iron for replicating, which directly increases their survival and growth in their host cells, including phagocytes. Due to tissue damage, CO (carbon monoxide) and biliverdin can activate the immune response to control pathogen replication and pathogenesis regulation (Costa et al. 2020). Biliverdin reductase releases bilirubin, the primary intracellular source of iron and carbon monoxide (Habtemariam 2019). The antioxidant effect of HO-1 is related to its ability to produce bilirubin, which eliminates ROS, including OH, singlet oxygen, and O2. The emergence of HO-1 also protects cells from oxidative disorders by regulating the degradation of heme and increasing biliverdin, which has strong antioxidant properties. CO and other heme products function as anti-apoptosis (Turkseven et al. 2005; Ryter et al. 2006). Other treatments (T1, T2, and T3) also produced antioxidant HO-1, but the level is lower than the positive control. Those treatment groups showed the dynamic content of coffee extracts can bind free radicals and lipopolysaccharides to achieve similar conditions with the negative control (Kim et al. 2017). HO-1 expression levels in healthy individuals under normal conditions act as natural, free radical prevention and have very low HO-1 expression levels but still respond to various transcriptional chemical activation and physical stimulation (Ryter et al. 2006). The active compound of coffee is enough to trigger HO-1, but its concentration is very low. Other compounds in coffee, such as tannin, alkaloid, and saponin, also have antibacterial properties (Pratita 2017). Saponin and tannin disturb bacteria's permeability and metabolism, inhibiting bacterial activity and growth. Alkaloids disrupt the formation of peptidoglycan in the cell (Khan et al. 2018). No difference was observed in chicken performance between all groups. Weight gain

in chicken is not significantly different between all treatment groups. However, a high dose of coffee extract might irritate the gut and trigger the increased expression of Nrf2 and SOD-1 but not HO-1. In this case, although Nrf2 was known to trigger the formation of HO-1, the level of HO-1 still decreased. The green coffee extract could reduce inflammation by increasing the level of the antioxidant enzyme SOD but not the HO-1 level. The Lampung robusta green coffee extract could potentially be used as an antioxidant against *S. enteritidis* infection in the 500 mg/kg BW dosage, as shown in the treatment group T1, by increasing the Nrf2 and SOD but decreasing the HO-1 level.

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