



Immunomodulatory Effect of Synbiotics on Lymphocytes Load and Histopathology of Mice Ileum in *Salmonella typhimurium* Infection

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ABSTRACT

Salmonella enterica serovar typhimurium, a zoonotic illness spread through food and drinks (meat, milk, eggs, fish, water) and other pollutants, is the primary cause of the endemic *Salmonellosis* in Indonesia. Synbiotics, Robusta coffee extract and *Lactobacillus acidophilus* can be used instead of antibiotic therapy. In this study, mice infected with *Salmonella typhimurium* were given a symbiotic. The mice lymphocytes and Ileum's histopathology were determined. Twenty-five adult males were divided into five groups, namely, K- (Healthy), K+, P1 (Treatment 1), P2 (Treatment 2), and P3 (Treatment 3) were used for the study. The experimental animals were induced with a 10⁸ CFU/mL dose of *Salmonella typhimurium* and allowed for three days to establish infection. The treatment groups, P1-P3, were given 150, 200, and 250 mg/kg BW per oral, respectively, of the symbiotic (a combination of Lampung Robusta Coffee extracts and *L. acidophilus*). The lymphocytes at the different treatment doses and the ileum histopathology were determined using Optilab 400x magnification microscope. The results were analysed using ANOVA. The results showed that the administration of the symbiotic at a combination of Lampung Robusta Coffee extracts 200 mg/kg BW and *L. acidophilus* significantly prevented ileum damage, as seen in the number of goblet cells and mucosal atrophy, and reduced lymphocyte load. The study concluded that combining Lampung Robusta coffee (200 mg/kg BW) extract and *L. acidophilus* could be a preventive cocktail against *Salmonellosis*.

Keywords: Coffee Robusta, Gastroenteritis, Ileum histopathology, Lymphocytes, *Lactobacillus acidophilus*

Introduction

Salmonellosis caused by *Salmonella typhimurium*, an endemic and zoonotic illness in Indonesia, can be spread by food or drink (meat, milk, eggs, fish, water) and other contaminated materials. Various causes contribute to the rising incidence of *Salmonella*, including contamination during the slaughtering process, cross-infection at retail, and poor sanitation.¹ *Salmonella typhimurium* bacteria enter the intestinal area and release endotoxins that damage epithelial cells and trigger an adaptive immune response, producing lymphocyte cells so that the invading *salmonella* bacteria are eliminated by phagocytes, resulting in an acute inflammatory response in the intestinal area and the release of a thermolabile enterotoxin that stimulates adenyl cyclase activity and increase the concentration of cAMP, which inhibits Na and chloride absorption in the intestinal villi, affecting fluid production and causing diarrhoea.² Antibiotics can treat the effects of salmonellosis damage to the intestine, particularly in the ileum. However, many cases of antibiotic resistance exist, and antimicrobials (AGP) are associated with the proliferation of drug-resistant

Salmonella and the possibility of its transmission to humans.³ Synbiotic used instead of antibiotics are gaining popularity in recent times. Synbiotic are a combination of probiotics and prebiotics that support the growth of bacteria⁴, one of which is *Lactobacillus acidophilus* bacteria. *Lactobacillus acidophilus* is a lactic acid bacteria beneficial for the digestive area. It produces lactic acid, making intestinal conditions acidic and inhibiting pathogenic bacteria sensitive to acidic environments.⁵

Lactic acid bacteria secretes bacteriocins known to inhibit pathogenic bacteria cell walls,⁶ enhance the body's immune system by the induction of specific immune response to mucosal antigens forwarded by dendritic cells (DC), macrophages and produce TH1, TH2 and Th17, TH2 which will induce activation of plasma cells (effector cells) to secrete immunoglobulin on their cell surface to become Ig A antibodies.⁷ Th2 produces IL-4, IL-5, IL-10 and IL-13. These cytokines induce the formation of antibodies but also anti-inflammatory cytokines,⁸ which modulate immune responses such as lymphocytes to invade bacteria such as *Salmonella* to modulate and coordinate excessive inflammatory responses in the ileum. One example of a prebiotic is robusta green coffee which contains oligosaccharides around 0-3.5% of dry weight.⁹ It contains high chlorogenic acid and flavonoids, which function as antioxidants, antivirals, antibacterials, and antifungals, and does not cause antimicrobial resistance. Chlorogenic acid interferes with bacterial adhesion in the body.¹⁰ In the presence of flavonoids, chlorogenic acid induces proinflammatory cytokines (IL-1, IL-6 and TNF- α) to accelerate the inflammatory process by destroying infecting bacteria.¹¹ However, no studies have shown that synbiotics can prevent *Salmonellosis*. This research examines synbiotics' impact on *Salmonellosis*, the histopathology of mice ileum, and the number of lymphocytes.

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Materials and Methods

Ethical approval

This study was approved by the Faculty of Medicine Universitas Airlangga, ethical commission, with ethical certificate No.49/EC/KEPK/FKUA/2023, February 13, 2023.

Experimental animal preparation

Twenty-five male mice (*Mus musculus*) weighing an average of 30 grams were used for this experiment. They were housed under standard laboratory conditions, room temperature 17-23°C, with 12 hr light and 12 dark cycles. They had free access to BR-1® pellets and water. Mice were placed into five treatment groups with five mice each and acclimated for seven days.¹²

Extraction of Lampung Robusta coffee

Robusta coffee from the Indonesian city of Lampung was cleaned and chopped into little pieces before being heated in an oven at 40–60°C, pulverised in a blender for extraction, soaked in 90% ethanol (900 mL), agitated in a shaker for 30 minutes and allowed to stand overnight. The top layer of the extract (a combination of solvent and active substances) was removed into an evaporating flask and evaporated to dryness in a water bath set at 40°C.¹³

Infection of Experimental Animals with *Salmonella typhimurium* culture

The *Salmonella typhimurium* bacteria stock culture (turbidity of 0.5 McFarland) was obtained from the University of Surabaya Microbiology Department. The mice were induced with a 10⁸ CFU/mL *Salmonella typhimurium* and were allowed to incubate for three days to establish infection before treatment.¹⁴

Treatment of Experimental Animals with Synbiotic

The animals were treated with oral doses of the synbiotic (a combination of *Robusta coffee* extract and *Lactobacillus acidophilus*) for 12 days. The negative control (healthy, K-) group received 5 mL of physiological saline. Positive control group (K+), infected but untreated. Each treatment group 1-3 (P1, P2, and P3) received 5 mL graded doses of the synbiotic at 150 mg/kg BW (P1), 200 mg/kg BW (P2), and 250 mg/kg BW (P3) extract.¹⁵⁻¹⁶

Lymphocyte cell count

At the end of the experimental period, the animals were sacrificed under chloroform and tissues were harvested for further assays. Lymphocyte assays were performed using manual counting with Giemsa staining. Leukocyte counts were obtained by counting lymphocytes per 100 leukocytes using a CX31 microscope and averaging the resulting lymphocyte counts.¹⁷

Histopathological treatment of ileal organs

Ileal organs were washed with physiological NaCl and stored in pots with 10% formalin for at least 24 hours for organ fixation. Then dry with 50-95% alcohol for 15 minutes each time. The clarification stage uses a toluene solution for up to 120 minutes. They were then embedded by heating the paraffin to a temperature of 56-62 °C for 15 min. A blocking step was performed by blocking the organs with paraffin and hardening them for sectioning using a 6 µm thick microtome. The pieces were placed in a water bath and attached to slides with egg white. Glass articles were placed in a xylene 1,2,3 solution as a dewaxing process and rehydrated in 100%, 90%, 80%, and 70% alcohol for 5 minutes each. The slides were immersed in hematoxylin for 5–20 minutes, immersed in alcoholic acid three times under running water, washed for 10–20 minutes, and immersed in graded alcohols and xylene. Canada balm was added and covered with a coverslip.¹⁸

Observation of histopathological ileum

Histopathology of the ileum was observed using Optilab and Olympus microscopes at 100x to 400x magnification. Ileal features with lymph nodes and Peyer's patches were observed for signs of inflammation, such as villous and epithelial damage, inflammatory cell infiltration, and foveal cell hypoplasia.¹⁹

Statistical analysis

Lymphocyte count data analysis was performed quantitatively and analysed using one-way analysis of variance (ANOVA) at a 95% confidence level ($p < 0.05$) to determine differences across treatment groups, followed by Tukey's test ($\alpha = 5\%$) for differences between each treatment group.

Result and Discussion

Histopathology Ileum

The histopathological examination of the ileum was done to determine the extent of damage caused by each treatment. In the present study, histopathological ileal organs were prepared with hematoxylin-eosin (HE) staining, and five fields were observed randomly using 400x magnification to observe mucosal erosion.

Lymphocytes count

The data analysis obtained from lymphocyte count in the ileal tissue injury showed the highest mean lymphocyte results for K+ and the lowest for K-. There was a decrease in the mean lymphocyte count in the treatment groups, P1, P2, and P3, compared to K+, with the lowest mean outcome observed in P2. A plot of mean lymphocyte counts in the control and treatment groups is shown in Figure 2.

The results show that the synbiotic, *Lactobacillus acidophilus* and Lampung Robusta coffee extract significantly reduced the number of lymphocytes in Figure 2. The ileum is the organ in which most carbohydrate absorption occurs. Microscopically, the tunica mucosa is the lamina propria that protrudes into the lumen-like fingers, commonly called villi, which are bounded by epithelial tissue.⁴ The ileum histology of the K- group (negative control, healthy mice) showed no damage to the tunica mucosa of the ileum (Figure 1).

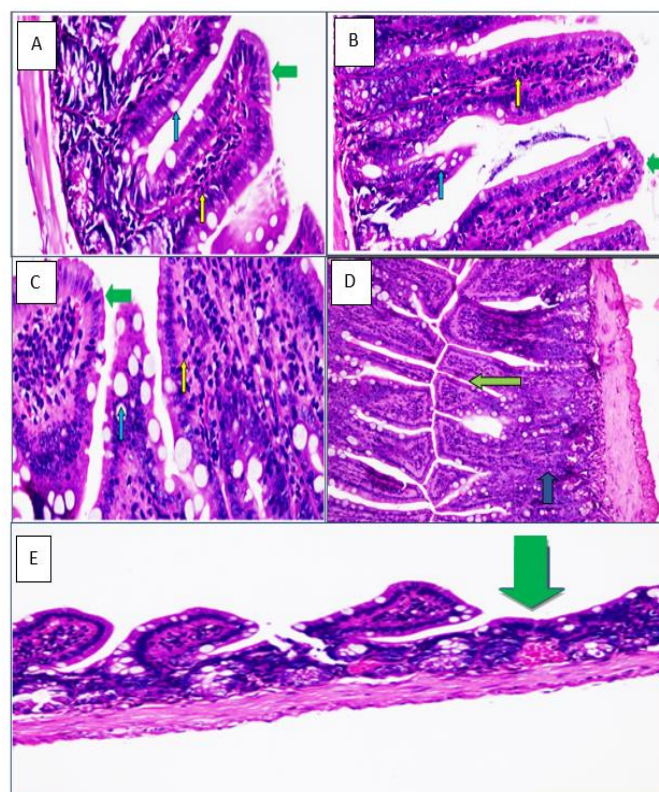


Figure 1: Result of Histopathological ileum each treatment, A) Negative control; B) positive control; C) treatment 1 (P1); D) treatment 2 (P2); E) treatment 3 (P3); Green arrow for the mucosal, yellow arrow for lymphocyte cell and blue arrow for goblet cell.

The ileum is characterised by a single layer of columnar epithelial cells that are intact and arranged regularly in a row. Among the epithelial cells, the goblet cells protect and lubricate the intestinal lining by producing mucin glycoproteins.²⁰

Based on observations on K+ preparations in which mice were induced with *Salmonella typhimurium* at a dose of 1×10^8 CFU/mL for three days, the ileal histopathology shows submucosal edema, goblet cell hypertrophy, and epithelial damage due to the invasion of *S. typhimurium* into intestinal tissue. *Salmonella sp* damages the intestinal epithelium, and the presence of endotoxins and enterotoxins released by *S. typhimurium* bacteria can also cause damage to the villi. According to²¹, mice with *S. typhimurium* infection cause submucosal edema in the streptomycin model. Submucosal edema occurs due to increased permeability resulting from the infiltration of inflammatory cells to the site of infection.²² In treatment groups P1, P2, and P3, the mice received *Lactobacillus acidophilus* mixed with coffee extract with graded doses of 150 mg/kg, 200 mg/kg, and 250 mg/kg, respectively.

The results of P1 histopathology present mucosal atrophy and epithelial erosion at an oral synbiotic dose of 150 mg/kg, and P3 at a dose of 250 mg/kg still shows mucosal atrophy. In treatment P2, with 200 mg/kg coffee extract and 10^8 CFU/ml *Lactobacillus acidophilus* bacteria, normal villi length was seen as in the negative control. There was no submucosal edema, and there was no epithelial erosion. This result may be due to the effect of giving robusta coffee extract containing chlorogenic acid, which reduces the production of histamine, bradykinin, and leukotrienes, known to reduce the increase in capillary permeability during the inflammatory phase.²³ The alkaloid content in caffeine has an anti-inflammatory effect. It inhibits the production of tumor necrosis factor-alpha (TNF- α), stimulated by LPS, and is a potent antioxidant that protects against cell damage caused by free radicals and peroxides.²³ Intake of *Lactobacillus acidophilus* probiotics can increase nutrient absorption by producing proteolytic enzymes, releasing some free amino acids, and synthesising vitamins needed for host growth.¹⁶ *Lactobacillus acidophilus* is a probiotic or good bacteria that usually live in the intestine or digestive tract. Supplements containing *Lactobacillus acidophilus* can be used to maintain the balance of these good bacteria in the digestive tract, so they can help treat diarrhea. *Lactobacillus acidophilus* supplements work by maintaining acidity in the digestive tract, thereby stopping the growth of harmful microorganisms.²⁴ Result of Lymphocyte count in this study showed that there was a significant difference ($p < 0.05$) in the mean of the negative control group (K-) to the positive control group (K+). K- has a lower average value than the treatment group because of the presence of lymphocyte cells that have not been activated in healthy mice. T lymphocyte cells proliferate when an antigen enters the body and is recognised by APC (Antigen Presenting Cell).²⁵ Some lymphocytes are detected because mice have segmented filamentous bacteria in the intestinal area that can induce an immune response in mice.²² The results of the average K+ group were higher than the K- group because continued infection with *Salmonella typhimurium* bacteria would cause tissue necrosis and complications of severe bleeding and intestinal perforation. The body's immune system will release proinflammatory cytokines, which can induce acute inflammation. Host immunity naïve T cells are activated to CD4+ and CD8+ cells in the presence of pathogenic bacteria with the induction of the body's immunological response by stimulating macrophages in the phagocytosis of bacteria. Endocytosis is used to degrade antigens into peptides that will bind to MHC II in the endoplasmic reticulum (ER) with the help of Transporter associated Ag Processing (TAP), which are exited from the cells with subsequent interactions with CD4 T lymphocytes. CD4 cells eventually develop into Th1 and Th2 cells. Th1 will create cytokines such as IFN- γ for the body's inflammatory activities.

Synbiotic is a combination of *Lactobacillus acidophilus* bacteria and Lampung robusta coffee extract. In treatments P1, P2, and P3, there was a decrease in the number of lymphocytes, which was insignificant ($p > 0.05$) at the lowest dose of 150 mg/KgBW compared to K-. The moderate reduction in the lymphocyte count may be due to the effect of giving robusta coffee extract, which contains chlorogenic acid. Chlorogenic acid also reduces the production of some proinflammatory mediators, including TNF- α , interleukin (IL) -1 β , IL-6, and interferon (IFN) - γ in macrophage cells.²⁶ The alkaloid has anti-inflammatory

abilities and inhibits the production of tumor necrosis factor-alpha (TNF- α). It also has powerful antioxidant properties that protect cells from free radicals and peroxides assault.²⁶ *Lactobacillus acidophilus* can increase nutrient absorption by producing proteolytic enzymes, releasing some free amino acids, and synthesising vitamins needed for the host's growth. *Lactobacillus acidophilus*²⁷ is a lactic acid bacteria that compete against pathogenic microorganisms in the intestinal mucosa, secretes bacteriocins to inhibit the growth of *Salmonella enteritidis* and modulate specific immune responses that produce anti-inflammatory-cytokines-such as IL-4, IL-5, IL-10 and IL-13 which work against excessive inflammatory responses resulting in a decrease in the average value of K+.²⁶ Treatment with 200 mg/KgBW oral dose of the synbiotic was shown to effectively reduce the number of lymphocyte cells in *S. typhimurium* infection in animal models.

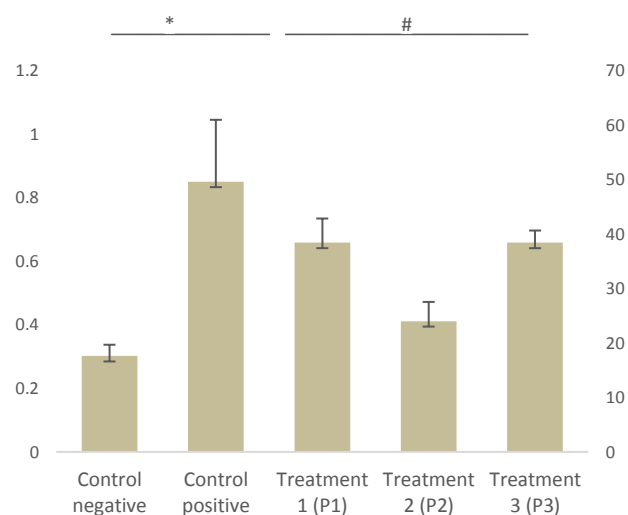


Figure 2: Showing results of mean lymphocyte count in all the groups.

Conclusion

Salmonella enters the body through the payer patch. It enters directly into the lymphoid area, where it stimulates the presence of lymphocytes and activates inflammatory cytokines, causing damage to the gut, particularly in the ileum region. While many antibiotics are used to treat salmonellosis due to its propensity to develop antimicrobial resistance (AMR), other options, such as symbiotics, are available. The symbiotic used in this study combines *Lactobacillus acidophilus* and Lampung robusta coffee extract at 150 mg/kg, 200 mg/kg, and 250 mg/kg. It was discovered that administering synbiotic *Lactobacillus acidophilus* and Lampung robusta coffee extract could prevent ileal damage and decrease lymphocyte counts in mice. The most effective dosage was Lampung Robusta Coffee Extract, 200 mg/kg BW with *Lactobacillus acidophilus* 10^8 CFU/mL.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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