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Original Research Article

Effects of *Lactobacillus acidophilus* and Lampung Robusta Coffee Extract on *Shigella flexneri* Induced Balb/C Mice; A Review of Colon Histopathology and Lymphocytes

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ABSTRACT

Diarrhea is a disease that is still a health problem worldwide; one of the causes is *Shigella flexneri*. Non-dairy probiotic functional foods and beverages are gaining popularity, with researchers seeking to assuage consumer concerns regarding lactose intolerance and veganism. Coffee has anti-inflammatory and antibacterial properties. This study aims to determine the effect of a mixture of Lampung Robusta coffee extract and *Lactobacillus acidophilus* bacteria on the colon of BalbC mice that experience diarrhea after being induced by *Shigella flexneri*. Twenty 3- to 4-month-old mice weighing 35–50 grams were randomly divided into three treatment groups (125 mg/kg BW, 250 mg/kg BW, and 500 mg/kg BW) and a control group. The treatment and positive control groups were induced with *Lactobacillus acidophilus* 6x108 suspension using the sonde technique. The negative control group was not treated. Mice experienced diarrhea twice in 24 hours after induction. Each treatment group was given 0.4 ml of Lampung Robusta coffee extract and 0.5 ml of *Lactobacillus acidophilus* 1.5x108 for five days. On day 8, the colon of the mice was taken and processed histologically, and hematoxylin-eosin staining was performed. Inflammation, bleeding, congestion, and necrosis were observed. This study showed significant differences in each parameter ($P < 0.05$). Lymphocytes were only found around the inflammatory cells, with a small amount in the positive control, 125 mg/kg BW, and 500mg/kg BW treatments. However, not found in negative control and 250 mg/kg BW.

Keywords: *Lampung Robusta Coffee, Lactobacillus acidophilus, Shigellosis*

Introduction

Shigellosis is a disease that endangers public health in much of Asia, with an estimated 125 million illnesses annually and 14,000 deaths in this region alone.[1] Shigellosis is an acute intestinal infection with symptoms ranging from moderate liquid diarrhea to bacillary dysentery with severe inflammation, fever, and blood and mucus in the stool. Diarrhea is a disease still a health problem worldwide, including in Indonesia.[2] The condition is usually self-limiting but can be fatal if a person is immunocompromised or does not have access to adequate medical care. The infection is quickly resolved with a combination of oral rehydration and medication. No Shigella vaccine is available, although many vaccines are being developed and evaluated in various clinical stages.[3]

Historically, the most commonly isolated bacterium causing infections in Asia was *Shigella flexneri*. Shigella is highly contagious; the infectious dose is approximately 10-200 organisms.[4] This bacterial species is the leading cause of shigellosis in many developing countries and is the most common agent.[1] Infections caused by Shigella are generally confined to the gastrointestinal tract; invasion into the bloodstream is very rare.

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Shigella invade the colonic epithelium resulting in the induction of severe mucosal inflammation, resulting in tissue damage to the colon.[5] The pathogenesis stages of Shigella include host cell attachment, invasion, and entry into epithelial cells and spread within colonic cells. Shigella virulence factors are primarily located in the plasmid's 20-30 kb part, commonly called the "entry region".[6] Shigella has a type III secretion system (T3SS), a needle-shaped macro molecular complex that the bacterium uses to invade and escape host immune autophagy. T3SS sends effectors to the cytoplasm of host cells to manipulate cellular activity and induce apoptosis.[6] Apoptosis plays an essential role in lymphocyte development and homeostasis. Increased apoptosis can cause immunodeficiency through cell death.[7]

Research conducted by Salgado et al. showed that *Shigella flexneri* T3SS modulates the host adaptive immune response by impairing the migration pattern of CD4+ T cells independently and ceasing cell migration. These findings suggest that *Shigella flexneri* also targets T lymphocytes in vivo.[8]

The most widely used Shigella therapies are ciprofloxacin, ampicillin, doxycycline, and trimethoprim-sulfamethoxazole.[9] These therapies can reduce the severity and duration of clinical attacks of dysentery. Plasmids are essential in transmitting multi-drug resistance, and antibiotic-resistant infections are common. The misuse of antibiotics has sparked concern due to the increasing number of antibiotic-resistant bacterial strains. The persistent problem of antibiotic resistance will always inspire researchers to conduct other alternative therapies.[10]

Various plants contain bioactive compounds that can potentially overcome infectious diseases.[11, 12] The popularity and status of coffee as a major commodity crop has made world coffee consumption rise by 1.9% in 2020/2021, which will undoubtedly be a lucrative opportunity from an economic perspective. As a beverage with regular consumption patterns, coffee is an ideal base for prebiotics that require daily supplementation.[13] Several studies have also shown that coffee chlorogenic acid is antioxidant, anticancer, and antibacterial.[14]

Coffee is one of the commodities in Indonesia that is one of the foreign exchange earners from the plantation sector. Indonesia can produce around 400 tons of coffee annually, valued at 1.3 billion USD.[15] Lampung Province is the largest producer of robust coffee, accounting for 70 percent of Indonesia's coffee exports. Probiotics are live bacteria given to the host in concentrations sufficient to provide health benefits.[16] Probiotics have been used as adjunctive therapy in diseases, especially gastrointestinal disorders, both in animals and humans.[1, 7] One probiotic bacterium is *Lactobacillus acidophilus*; the probiotic role changes the microbial population and increases the host's nutritional value and disease resistance.[18] Because of its bacteriocin-rich metabolism, *Lactobacillus acidophilus* is used to treat inflammation.

Foods and beverages with non-dairy probiotic functionalities have gained prominence recently, with researchers seeking to assuage consumer concerns regarding lactose intolerance, dairy allergens, and veganism. Tremendous progress has been made in developing probiotic foods with non-dairy ingredients, but only some have utilized coffee as a beverage concept to support probiotic delivery.[13]

The polyphenol content in coffee can be used by several types of probiotic bacteria to grow.[19] Yuniarta's research shows that a dose of 250 mg/kg BW of robust coffee combined with *Lactobacillus acidophilus* (*L. acidophilus*) bacteria can be antibacterial in mice with Salmonellosis and trigger lymphocyte cell proliferation[20]. Cahyani and Setiawan's research showed that a dose of 250 mg/kg BW of robust coffee leaf extract reduced the number of necrosis cells in histopathology preparations of liver organs. [21]

Based on the above background, researchers are interested in conducting research on mice with Shigellosis in terms of colon histopathology and the presence of lymphocytes.

Methods

Study design and participant

This research activity is experimental laboratory research using the Completely Randomized Design method, a homogeneous experimental design so that the media and environment do not significantly influence. This study uses an experimental design, randomly dividing subjects into five groups, each with at least four mice. Male Balb C mice 20-30 grams. Healthy mice in the positive control. After *Shigella flexneri* induction, sick mice are characterized by small-volume and watery stool.

Sample size and data collection

The experimental animals used are Balb C strain mice (*Mus musculus*), about 2-3 months old, with a body weight of about 20-30 grams. Acclimatization is carried out for seven days to adapt to the environment; each treatment has a minimum number of replicates four times in each group, so a minimum of 20 experimental animals is needed.

Preparation of experimental animals

Mice (*Mus musculus*) Balb/C strain was acclimatized for 5-7 days in separate cages to minimize stress so animals could express their natural behavior. They were feeding and drinking ad libitum. Cells are placed in a room with maintained cleanliness and noise-free, light-dark ventilation for 12 hours a day with a temperature of 25ºC.

The extraction process of robust coffee Lampung

Robusta coffee is washed thoroughly and then dried by placing it in an oven at 40-60ºC or dried by the sun; after drying, the extraction process is carried out by pulverizing it with a blender and weighing 100 grams, soaking it with 90% ethanol solvent as much as 900 ml (1 liter) on a shaker 30 minutes and soaking one night until it settles. The top layer is taken a mixture of solvents and active substances.

The evaporation process is carried out in an evaporation flask and water bath filled with water to the brim. The water bath temperature is 90ºC, and the solvent can separate from the active substance. To obtain 1/5 of the extraction from the dry material, the solvent flow is permitted to stop dripping into the collection flask (1.5-2 hours). The results are put into plastic or glass bottles and stored in the freezer to avoid deterioration.[22]

Induction of a combination of robust coffee extract and Lactobacillus acidophilus bacteria

Treatment mice 1 (P1), 2 (P2), and 3 (P3) were given a combination of robust coffee extract and *Lactobacillus acidophilus* bacteria. Each treatment has a different dose, namely P1, giving coffee extract 150 mg/kg BW and *Lactobacillus acidophilus* $1.5x10^8$ CFU / ml, P2 giving coffee extract 200 mg/kg BW and *Lactobacillus acidophilus* 10^8 CFU / ml, and P3 giving coffee extract 250 mg/kg BW,[23] given is carried out for five days.[24] The giving of a mixture of 0.5 ml of *Lactobacillus acidophilus* with 0.2 ml of a Lampung robust coffee extract with various dose variations was carried out orally using a sonde and carried out once per day for five days.

Induction of Shigella flexneri bacteria

Induction of *Shigella flexneri* bacteria at a dose of $6x10^8$ CFU/ml as much as 0.5 ml was given to mice on day eight by giving gastric sonde fasting for 60 minutes before induction; this aims to neutralize gastric acid by sodium bicarbonate and can cause immunity to be more sensitive if there is a systemic infection.[25] Diarrhea results were shown at 2x24 hours after inoculation.

Colon organ histopathology treatment

Colon organs were taken and then cut with a size of 1x1x1 cm, then fixation was carried out using 10% formalin. After fixation, the dehydration was carried out with a solution session containing 70% alcohol, 80%, 90%, and 96% absolute alcohol, toluene, and paraffin gradually in 1 day. The next step was blocking with an embedding set poured with liquid paraffin and then cooled. The cooled block was cut using a microtome with a thickness of 4-5 microns. The last process used Harris Hematoxylin-Eosin staining and mounting media.[26]

Histopathology was observed using a Nikkon Eclipse 50i clinical microscope with a magnification of 100-400 times each in 5 fields of view. Microscopic changes were recorded based on the monitored parameters: hemorrhage, inflammation, congestion, and necrosis.

Limfosit Presence

Lymphocyte cells were detected in the colon tissue by HE staining. Histopathology was observed using a Nikkon Eclipse 50i clinical microscope with a magnification of 100-400 times

Statistical analysis

Quantitative analysis of lymphocyte count data was carried out and analyzed using the One-way analysis of variance test with a 95% confidence level to determine differences in the treatment group as a whole; if there is a significant difference ($p < 0.05$), it is continued with the Turkey test ($a = 5\%$) to determine the results of the difference between each treatment group. The histopathology column in mice data were analyzed using the Kruskal-Wallis test and continued with the Mann-Whitney test if there was a significant difference ($P < 0.05$).[27]

Ethics

The study was conducted after obtaining ethical approval with an ethical certificate No.3/EC/KEPK/FKUA/2023 from the Health Research Ethics Committee of the Faculty of Medicine, Airlangga University Surabaya.

Results and Discussion

The results of the histopathological examination and colonic lymphocytes in Balb C mice given Shigella flexneri and a mixture of robust coffee extract and probiotic bacteria Lactobacillus acidophilus orally at doses of 125, 250, and 500 mg/kg BW.

The Kruskal-Wallis test value is significantly different with a calculated *p-value* of 0.001 and a p-value of 0.05, which means there is a difference between coffee dose treatments on colonic histopathology and inflammation.

Comparing the inflammation of each treatment group against the control, the treatment group with 125 mg/kg BW showed a *p-value* of 1, indicating no significant difference after being given this dose compared to the control. The results of significant differences are shown in the 250 and 500 mg/kg BW dose treatment groups, with a *p-value* of 0.008.

The significance of a *p-value* of 0.001 means a significant difference in bleeding results between treatment groups; the results of a *p-value* of 1.000 mean there is no significant difference in bleeding in each treatment group compared to the positive control.

The test shows a significant difference in the results of the B250 group (*p-value* = 0.008) against the control; different results are shown in groups A125 and C500, which show no significant difference (*p-value* = 1.000); a significant difference in the results of the B250 and C500 groups (p = 0.008) against the control; different results are shown in the A125 group, which shows no significant difference (p = 1.000).

Based on the Kruskal-Wallis test on inflammation, bleeding, congestion, and necrosis, the *p-values* of 0.001 and 0.005 indicate significant differences in the four parameters. The Mann-Whitney posthoc test showed differences in the results of each group compared to the positive control, where the treatment dose of 250 mg/kg BW showed the most optimal results, followed by doses of 500 mg/kg BW and 125 mg/kg BW.

Lymphocyte examination reveals several lymphocyte cells around colonic inflammation in the Positive Control, A125, and C500 groups. Different results were shown in the B250 group, with no lymphocyte cells found in the histopathology preparations.

This study used BALB C mice which were divided into five groups, namely the negative control group (healthy animals), positive control (without the administration of robusta coffee extract and *L. acidophilus*), and three treatment groups, A125, B250, and C500 (given robusta coffee extract with doses of 125, 250, and 500 mg/Kg BW). Experimental animals experienced diarrhea on the second day after induction of *Shigella flexneri* with McFarland standard two, characterized by diarrhea.

The samples induced by *Shigella flexneri* showed clinical symptoms of shigellosis, starting with fever, fatigue, anorexia, malaise, and watery diarrhea, which developed into bloody diarrhea and dysentery. Dysentery is characterized by abdominal cramps and tenesmus, with an increased frequency of bowel movements consisting of blood, mucus, and pus. The severity of diarrhea is related to the extent of inflammatory lesions in the colon. This is consistent with the research.[28]

The dissection and HE painting results showed that the intestinal mucosa state in infected and treated mice varied. There are significant differences in intestinal histopathology. As shown in the table, there is a very significant difference between treatments with a meager p-value (p=0.001). Other studies also showed a significant increase in the number of bacteria in the inflammatory part of the intestine.

Of the five groups, there was a significant difference in the average number of inflammatory cells between the positive K group and B250. In contrast, the positive K group and the A125 and C500 treatments had no significant difference. This is found in the inflammatory phase, neutrophil infiltration occurs on the first day after injury due to being attracted by inflammatory cytokines produced by platelets and endothelial cells to degrade pathogens. Neutrophils then undergo apoptosis and release cytokines to attract macrophages. There is then infiltration and exacerbation of macrophages in the wound area on the second day to phagocytose.[29]

The changes are congestion, a lesion that describes circulation disorders and can also indicate tissue repair.[30] Another change is bleeding (Hemorrhage), which is the release of blood from blood vessels pathologically characterized by red blood cells outside blood vessels or in tissues.[31] Necrosis is a cell that undergoes changes that lead to cell death, caused by the presence of toxic substances that enter along with the bloodstream into the kidneys,[32] necrosis or cell damage can be characterized by cell swelling with loss of plasma membrane, changes in organelles, and nuclear changes accompanied by hypochromic. One of the causes of necrosis is the presence of chemicals that are toxins. Another visible change is inflammation. Inflammation is an important mechanism the body needs to defend itself from various hazards that disturb the balance and repair the structure and disruption of tissue function caused by these hazards.[33]

Research in a similar model (129/SvEv) showed intestinal inflammation in IL-10-/- mice decreased the number of normal intestinal flora variations and increased pathogenic bacteria. One of the causes of severe infection in the intestines of mice in this study is the toxin produced, namely the Shiga toxin.

Relationship between Robusta Coffee, Probiotic Bacteria, and Inflammation

The results showed significant results in the treatment with robusta coffee extract and *Lactobacillus acidophilus* compared to the control in reducing inflammation, which used coffee fermentation against *Shigella flexneri* bacterial infection.[34]

Robusta coffee is dominated by chlorogenic acid in coffee beans and extracts. [35] It has been reported that phenolic compounds and flavonoids were detected in Robusta coffee, responsible for bacteriostatic effects, inhibition of cell membrane function, and energy metabolism.[36] These are considered possible mechanisms of action of flavonoids against bacteria.[37,38] In addition, phenolic compounds denature bacterial cell proteins and inhibit cell multiplication.[39] Gram-positive bacteria show higher susceptibility to extracts than Gram-negative bacteria. The reason may be due to the difference in the composition of the bacterial cell envelope in Gram-negative and positive bacteria, the latter not having a low permeability barrier like the outer membrane.[40] In addition, some hydrophobic compounds, such as phenols and tannins, are difficult to be absorbed into the outer membrane (composed of phospholipids) of Gram-negative pathogens. [41] These properties are related to bioactive compounds, chlorogenic acid and its derivatives, caffeine, theophylline and theobromine, cafestol, kahweol, tocopherol, and trigonelline.

Robusta coffee extract contains twice as much caffeine as Arabica. The content varies from 3.41% per dry mass in Arabica types from Laos or Rwanda to 8.16% in Robusta coffee from Indonesia.[42,43]

It is known that the content of phenolics in green coffee beans depends on plant growth conditions, such as location, light, drainage, temperature, weather, and the process used on the beans.[44]

Table 1: Inflammation difference table

Treatment	Median (Min-Max)	*p-value*
Control Positive	2.00 (2.00 – 2.00)	0.001[a]
A125	2.00 (2.00 – 2.00)	
B250	0.00 (0.00 – 0.00)	
C500	0.00 (0.00 – 0.00)	

Table 2: Differences in inflammation of each group with control

Treatment	Control	*p-value*
A125	Control Positive	1.000[a]
B250		0.008[a]
C500		0.008[a]

Table 3: Table of bleeding differences

Treatment	Median (Min-Max)	*p-value*
Control Positive	0.00 (0.00 – 0.00)	0.001[a]
A125	1.00 (1.00 – 1.00)	
B250	1.00 (1.00 – 1.00)	
C500	1.00 (1.00 – 1.00)	

Table 4: Differences in bleeding between each group and control

Treatment	Control	*p-value*
A125	Control Positive	1.000[a]
B250		1.000[a]
C500		1.000[a]

Coffee contains chlorogenic, ferulic, caffeine, and coumaric antioxidants.[45] Melanoidin,[46] is also thought to be responsible for coffee's antioxidant and anti-inflammatory activities. The antioxidant activity of coffee depends on polyphenolic compounds, especially the presence of chlorogenic acid, which has high antioxidant activity. Caffeine, theobromine, theophylline, caffeine, and tocopherol, in addition to chlorogenic acid and its derivatives, contribute to this property of coffee.[47,48] Coffee, as an antioxidant, protects cells from damage from antioxidant stress and helps prevent several degenerative diseases.

Different results were shown in the study of Chandorkar, V., Kanoje, A., & Gomashe, A. V. 2017 conducted in-vitro by diffusion method, which stated the resistance of food bourne disease bacteria to coffee. Coffee consumption should align with recommendations. The US Department of Agriculture (USDA) and the European Food Safety Authority (EFSA) consider a daily intake of 400 mg of caffeine as a safe limit. This amounts to approximately 2-4 cups of coffee per day. However, according to the American College of Obstetricians and Gynecologists, pregnant women should limit their daily intake to a maximum of 200 mg per day. These results correspond to the test results in the C500 group, which showed less optimal results than the B250 group. Some indirect adverse effects have also been caused, such as cholecystokinin, gastrin, and motilin, whose secretion is stimulated by excess coffee consumption.[49]

Relationship between Robusta Coffee, Probiotic Bacteria, and Lymphocytes

The results showed a small number of lymphocytes infiltrating inflammatory cells in samples treated with the positive control, A125, and C500. Samples treated with B250 and negative control had no lymphocytes and showed colonic improvement. These results are by Parvez's research in 2006 which tested four types of probiotics in experimental animals, giving interesting results: L. acidophilus increased lymphocyte proliferation by 43%, while Lactobacillus casei (Yakult), Lactobacillus gasseri, and Lactobacillus thamnosis inhibited basal proliferation (14-51%) and mitogen-stimulated by the mitogen concanavalin A (43-68%) and by LPS (23-63%).

Studies using polyphenol supplementation showed increased proliferation and activation of T lymphocytes and an increased percentage of CD4+ T lymphocytes / CD8+ T lymphocytes indicating an improved immune system. Studies using extracts containing flavonoid compounds also obtained similar results. It was found that the extract stimulated T lymphocyte proliferation accompanied by an increase in the percentage of CD4+ T lymphocytes / CD8+ T lymphocytes. Research using polyphenol supplementation in tea can increase the proliferation and activation of T lymphocytes, which indicates an improvement in the immune system, the effect of flavonoids on the regulation of the immune system, found that flavonoids derived from the stems and leaves of Astragalus radix increased the proliferation of concanavalin A-induced lymphocytes, increased IL-2 expression and increased the number of T cells.[50] Other studies have also found that certain flavonoids, such as apigenin, kaempferol, luteolin, and quercetin, inhibit the secretion of proinflammatory cytokines such as IL-13, IL-2, IL-6, and IFN-γ. Other studies have suggested that epicatechin, catechin, and procyanidins, another flavonoid class, enhance TH1 response, increase T lymphocyte count in the gut, and suppress antibody response.

Prebiotic foods or compounds will stimulate the growth of probiotics and other health colonies in the gut, with particular emphasis given to Bifidobacterium and Lactobacillus spp. Indirectly, the health benefits of prebiotics are the same as probiotics, such as the production of short-chain fatty acids that lower luminal pH, stimulate the growth of beneficial gut bacteria, and suppress pathogenic bacteria. Reported data suggest that the unabsorbed portion of CGA and caffeic acid in the human gastrointestinal tract is a substrate for beneficial gut bacteria, stimulating their growth.[51,52] The cellular immune system activated by probiotic micro-organisms will increase the production of IgA (immunoglobulin A), which plays a role in the mucosal immune system. IgA synthesis depends on T cells and cytokines-activated lymphocytes produce.[53] Research reported the modulatory effect of probiotic bacteria on cytokine production by the inflamed intestinal mucosa and has great potential significance.[54] Various research findings also indicate the ability of Lactobacillus species to stimulate the production of IFNγ (interferon gamma) and IL-12, which further increases the role of the Th1-type response and improves the Th1-Th2 balance.[55] The study showed that the colon and microflora play a significant role in the absorption and metabolism of CGA from coffee, characterized by the slow appearance (48 hours) of dihydro-phenolic acid in plasma is relatively high.[56]

These results contrast previous studies that stated that certain flavonoids inhibit the proliferation of lymphocytes stimulated by phytoestrogens, such as phytohemagglutinin and concanavalin A. These results contradict those findings. Depending on their structure, some other flavonoids may inhibit the proliferation of cytotoxic T cells in mouse spleen cultures. The isoflavone genistein inhibits B cell activation and promotes the phosphorylation of tyrosine residues in some B cell proteins. Other experiments yielded comparable results. In experiments with B cell precursors stimulated by recombinant human IL-7, phosphorylation of tyrosine residues was observed, along with an increase in IP3 and inhibition of activation by the soy-derived isoflavone genistein.

Research conducted showed that lymphocytes targeted by injection of T3SS effectors did not result in cell invasion. These findings highlight the diversity of mechanisms Shigella triggers to enlarge the panel of target cells and withstand host immunity, including lymphocyte-mediated adaptive immune responses.[57] Research suggests that changes in CD4+ T cell migration patterns triggered by *Shigella flexneri* are independent of antigen-specific recognition and confirms the critical role played by *Shigella flexneri* T3SS effectors in the induction of inhibitory signals leading to changes in T cell lymphocyte dynamics.[8]

Table 5: Congestion Table

Treatment	Median (Min-Max)	*p-value*
Control Positive	1.00 (1.00 – 1.00)	
A125	1.00 (1.00 – 1.00)	0.001[a]
B250	0.00 (0.00 – 0.00)	
C500	1.00 (1.00 – 1.00)	

Table 6: Differences in congestion between each group and the control group

Treatment	Control	*p-value*
A125		1.000[a]
B250	Control Positive	0.008[a]
C500		1.000[a]

Table 7: Necrosis difference table

Treatment	Median (Min-Max)	*p-value*
Control Positive	1.00 (1.00 – 1.00)	
A125	1.00 (1.00 – 1.00)	0.001[a]
B250	0.00 (0.00 – 0.00)	
C500	1.00 (1.00 – 1.00)	

Table 8: Difference in necrosis between each group and the control group

Treatment	Control	*p-value*
A125		1.000[a]
B250	Control Positive	0.008[a]
C500		0.008[a]

Table 9: Detection of lymphocytes

Control Negative	Not Found
Control Positive	Found
A125	Found
B250	Not Found
C500	Found

Conclusion

There are differences in histopathology results in the form of a decrease in the degree of inflammation in the administration of a mixture of Lampung Robusta coffee extract and *Lactobacillus acidophilus* in the colon of mice induced by *Shigella flexneri* and There are differences in histopathology results in the form of the presence and absence of lymphocytes in the administration of a mixture of Lampung Robusta coffee extract and *Lactobacillus acidophilus* in the colon of mice induced by *Shigella flexneri*.

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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