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Effect of the combined application of Lampung Robusta Coffee Extract and *Lactobacillus acidophilus* on the Ileum and Caecum Histopathology in *Salmonella enterica* infected Balb/C Mice

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Ileum

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Histopathology

ABSTRACT

Salmonella enterica is a gram-negative bacterium that can cause Salmonellosis and gastroenteritis in humans and animals. Further, this bacterial infection is also associated with the reactive oxygen species (ROS) by lipid peroxidase that can destroy the intestinal cell's membrane. This study aimed to evaluate the preventive effect of the combined application of Lampung Robusta coffee extract and *Lactobacillus acidophilus* on the Ileum and Caecum Histopathology in *Salmonella enterica* infected Mice. In this study, male Balb-c mice aged between 8-10 weeks and weight 20-25 grams were used, these experimental animals were divided into six experimental groups namely K^- (Negative control without any infection), K^+ (Positive control with *S. enterica*), KL (Only *L. acidophilus* treated mice), P1, P2, and P3 were given a preventive extract of coffee with a concentration of 250 mg/kg BW, 500 mg/kg BW, and 750 mg/kg BW respectively and *L. acidophilus* to *S.enterica* infected mice and arrange in completely Randomized Design. Descriptive histopathological analyses were carried out after HE staining and villi's length and width for ileum's histopathology and counting goblet cells for caecum's histopathology was scored. The results of the study revealed that administration of Robusta Coffee extract @ 250 mg/ kg BW and *L. acidophilus* has a preventive effect on the ileum and caecum damage caused by salmonellosis.

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1 Introduction

Salmonella enterica belongs to the family Enterobacteriaceae and is associated with food-borne gastroenteritis infection in humans. *S. enterica* serovars *enteritidis* is one of the serovars associated with human salmonellosis and increases the cases of salmonellosis in humans. It infects the human body through contaminated food or drinks and causes gastroenteritis with diarrhea, stomach cramps, and bacteremia (Asminarti 2014). Furthermore, *S.enteritidis* infection also triggers inflammation in the intestine region, especially in the ileum and cecum. This inflammatory process will increase the number of free radicals (ROS) due to bacterial phagocytosis. In this manner, ROS will damage the cell components and the cell will lose its integrity and, if it occurs continuously, will cause tissue damage and oxidative stress. At higher stress, the epithelial cells also damage villi and disrupted the process of absorption of nutrients.

A wide range of antibiotics has been used for the treatment of *S. enterica* infection. However, their use is limited because, in some cases, antibiotic administration can cause local hypersensitivity to the skin, allergic reactions, and toxic reactions, as well as bacterial resistance to antibiotics that arise due to the excess and inappropriate use of antibiotics and this is one of the reasons of antibiotic treatment failure (Asminarti 2014). Natural sources of antibiotics can be used as an alternative to the synthetic antibiotic. According to Muhammad and dan Mumun (2015), coffee is a rich source of caffeine and chlorogenic acid, and among these, caffeine can damage bacterial cell amino acids and cause lysis, while chlorogenic acid of coffee can damage the polarity of the bacterial cell walls, in this manner coffee, can serve as an alternative natural antibiotic. Further, chlorogenic acid as an antioxidant can cut the chain oxidation reaction from free radicals so that free radicals will not react with other cells, and damage organs (Wulandari 2016).

Higher consumption of coffee increases the caffeine content in the body and this enhances the level of HCl production and indigestion. High HCl levels will irritate the intestinal mucosa, so excessive coffee consumption has a risk of intestinal ulcers (Rizkiani 2009). For this reason, it is difficult for coffee to be used singly so in this study, Lampung Robusta Coffee is combined with probiotics to avoid the drawbacks of excess coffee use.

Probiotics compete with pathogenic bacteria on the intestinal mucosa and have various beneficial effects on the host (Anggarini 2011). *Lactobacillus acidophilus* is one of the lactic acid bacteria that can be used as Probiotics. This bacterium has anti-microbial properties and can be inhibited the growth of pathogenic bacteria such as *Salmonella.* It can produce lactase, and vitamin K, which inhibited the adhesion or growth of bacteria (Senditya 2011). *L. acidophilus* also functions as an immunomodulator (Galdeano et al. 2019). Robusta coffee also has various oligosaccharides that serve as a source of nutrition for *L. acidophilus* bacteria (Grace 2017). Therefore, this study aimed to determine the effect of the combined application of Lampung Robusta coffee extract with *L. acidophilus* on the histopathology of the ileum and cecum of mice infected by *S. enterica*.

2 Materials and Methods

2.1 Preparation of Lampung Robusta Coffee Extracts

Lampung Green Robusta coffee extract was prepared at the Pharmacology Laboratory of the Faculty of Medicine, Universitas Brawijaya, Indonesia. For this, 100g of coffee powder was taken into the Erlenmeyer flask and mixed with 900ml of 90% ethanol. The prepared solution is shaken for 30 minutes and soaked overnight, this solution was taken into the evaporator flask and evaporated for 1.5-2 hours at 90°C in a water bath. The obtained extract was roasted and weighed to obtain a fixed extract weight (Juliantari 2018).

2.2 Animal Models

As an animal model, 24 male Balb-c mice aged between 8-10 weeks and weight 20-25 grams were used, these animals were divided into 6 cages and acclimatized for 7 days. Mice that have met BB standards are given a BR-1 feed and drink which is replaced every day.

2.3 Culturing of *Lactobacillus acidophilus* bacteria

Pure culture of *L. acidophilus* bacteria was obtained from the Faculty of Medicine, Universitas Brawijaya, Indonesia, and cultured on MRSA media with 1% $CaCO_3$. The cultured bacterial strain was confirmed by Gram staining and catalase test and bacterium that has been confirmed as *L. acidophilus* are multiplied on the Nutrient broth media. The turbidity level was confirmed using Mc Farland 0.5×10^8 to obtain bacteria with 10^8 CFU / ml concentration.

2.4 *Salmonella enteric* serovars *enteritidis infection in Balb-C mice*

Pure culture of *S. enterica* serovars *enteritidis* has been obtained from the Great Hall of Veterinary Wates, Yogyakarta, cultured on the BSA media, and culture plates were incubated at 37°C. Blackish brown colonies on the nutrient media revealed the presence of the *S. enteric*, which was further confirmed by the Gram staining. Identified *S. enterica* serovars *enteritidis* has been multiplied on the Nutrient broth. The bacteria were then tested using Mc Farland 0.5×10^8 to obtain bacteria with a concentration of 10^8 CFU/ml. Before infecting with *S. enterica* mice were kept on fasting and this was followed by giving 0.5 ml of drink with *S. enterica* culture using gastric sonde for 2 days.

2.5 Giving combinations to mice

Mice showing *S. enterica* infection symptoms were identified and used for coffee extracts and *L. acidophilus* treatment. These experimental animals were divided into six experimental groups namely K^- (Negative control without any infection), K^+ (Positive control with *S. enterica*), KL (Only *L. acidophilus* treated mice), P1, P2, and P3 were given a preventive extract of coffee with a concentration of 250 mg/kg BW, 500 mg/kg BW, and 750 mg/kg BW respectively and *L. acidophilus* and arrange in completely Randomized Design. The coffee extracts solution and *L. acidophilus* was taken in a 1 ml syringe and given with gastric sonde. This combination was given every day for 15 days. The mice's weight was calculated every 7 days, and their doses were recalculated to match the weight of the mice.

2.6 Necropsy and preparation of ileum and cecum

Before necropsy, mice were weighed first, and this was followed by cervical dislocation. These mice were taken for a necropsy by following standard procedure and ileum and cecum are taken out for histopathological observation.

2.7 Preparation of Ileum and cecum

The excised organs were cleaned with NaCl and stored in an organ pot containing 10% formalin for fixation. This was followed by the dehydration with 50-95% alcohol series, for each alcohol percent, 15 minutes were assigned. After this, organs were taken for clearing alcohol with the help of toluol fluid for 120 minutes. Then the organs were fixed in the paraffin wax and 6µm thin sections were prepared with the help of a microtome. Paraffin wax was removed in a water bath, rehydrated with 100%, 90%, 80%, and 70% alcohol for every 5 minutes, and fixed with xylol followed by Canada balsam.

2.8 Histopathology Observation

Histopathology was observed using optilab and Olympus microscopes at 100-400 magnification. Observations were made to observe the signs of inflammation such as villi and epithelial damage, inflammatory cell infiltration, and goblet cell hypoplasia.

2.9 Data Processing

Histopathology of ileum and cecum was analyzed by qualitative descriptive method with a light microscope type CX31RBSFA and photographed using optilab at 100-400x magnification. Several observations were made on the histopathology of the ileal organs, including inflammation cells, villi length, width, and the number of goblet cells. According to Harimurti and Rahayu (2009), measurements of villi length, villi width, and crypt depth are minimum of using three visual fields per slide with the help of the Imageraster application. The length of the villi is measured from the apex of the villi to the basal villi. The villi width was measured on the basal villi, and the crypt depth was measured starting from the basal villi to the basement membrane. The unit used to measure villi length and width is a micrometer (µm). In the histopathology of the caecum, the number of goblet cells is calculated by taking histopathic photographs using optilab at 400x magnification in as many as 5 fields of view/repetition, the total number obtained later in the average and scoring (Stecher et al. 2005).

3 Results and Discussion

3.1 Ileum histopathology

The Ileum histopathology examination aims to determine the degree of damage in each treatment. In this study, observations were taken using the HE staining method and 5 random fields in one preparation using a magnification of 100x and 400x. Measurement of villi length, width, and crypt depth using 100x magnification while observation of villi erosion, goblet cells, and inflammatory cells was taken at a 400x magnification.

The measurement of villi length and width are presented in Table 1. Measurement of villi length and width aims to support the scoring data of villi observations. According to Erben et al. (2014), villi scoring starts from the shortening of the light villi, which is the ratio of the length of the villi and the crypts to 2: 1 - 3:1 with a score of 1-3. In the shortening of villi, the ratio between the length of villi and crypt becomes 1: 1 to 2: 1 with a score of 2-4, and if there is villous atrophy score was recorded between 3-5 (Table 1).

Observations in mice of the negative control group (K^-) with a magnification of 400x (Figure 1) did not show any significant changes in villi with mild scoring (3). This is supported by the villi length and width measurements in Table 1. In negative controls, villi size looks uniform even though they have a shorter length when compared to the positive control group. In addition, no erosion or rupture was found in the villi, so it can be said that the villi are in good condition. Good villi conditions can improve the process of absorption of nutrients. These results are in line with the results of Putra and dan Kusdiyantini (2018), where feces of mice in negative control showed normal conditions. An indicator of inflammation is the infiltration of inflammatory cells in the intestinal villi and it can be seen with the widening of the intestinal villi.

Based on the changes in villi, the positive control group (K^+) experienced a shortening of villi size with moderate scoring (4), which means that the ratio between villi length and crypts is approximately 1: 1. Further, *Salmonella* sp. infection can cause intestinal epithelial damage. When it invading to intestinal tissue, it damages the intestinal epithelium. Based on observations, positive

Table 1 Effect of Lampung Robusta coffee extract and *L. acidophilus* applications on the various parameters of *S. enterica* infected mice intestine

Treatment Group	Villi's Length and Width		Villous blunting Score	Fecal Characteristics		Goblet's cells and predefined criteria	
	Length (µm)	Width (µm)		Fecal Characteristics	Observation	Average Cells/Field	Scoring
K^+	257.4	93.7	Moderate (4)	Oval forms; Black and Hard	Normal	29.2	0
K^-	228.1	66.2	Mild (3)	Not oval shape; Brownish; Flaccid, and little bit slimy	Diarrhea (4)	22.25	1
KL	347.4	84.3	Mild (1)	Not oval shape; Brownish, and little bit slimy	Diarrhea (3)	28.00	1
P1	305.3	73.2	Mild (2)	Oval forms; Hard and Brownish	Normal	26.73	1
P2	268	66.8	Mild (2)	Oval forms; Black, and little bit slimy	Diarrhea (3)	24.05	1
P3	249.9	74.9	Moderate (1)	Oval forms; Brownish and little bit slimy	Diarrhea (3)	24.06	1

control group mice ileum showed epithelial erosion and hypertrophy in goblet cells, infiltration of inflammatory cells consisting of PMN (Polymorphonuclear), and MN (Mononuclear). The type of PMN that can be observed is neutrophils. According to Rosales (2018), *Salmonella* sp. infection first disturbed the morphology of neutrophils. Infiltration of inflammatory cells indicated the presence of antigens that enter the body, one of which is pathogenic bacteria such as *Salmonella* sp. As the number of inflammatory cells in the tissue increases, the villi will look more expansive.

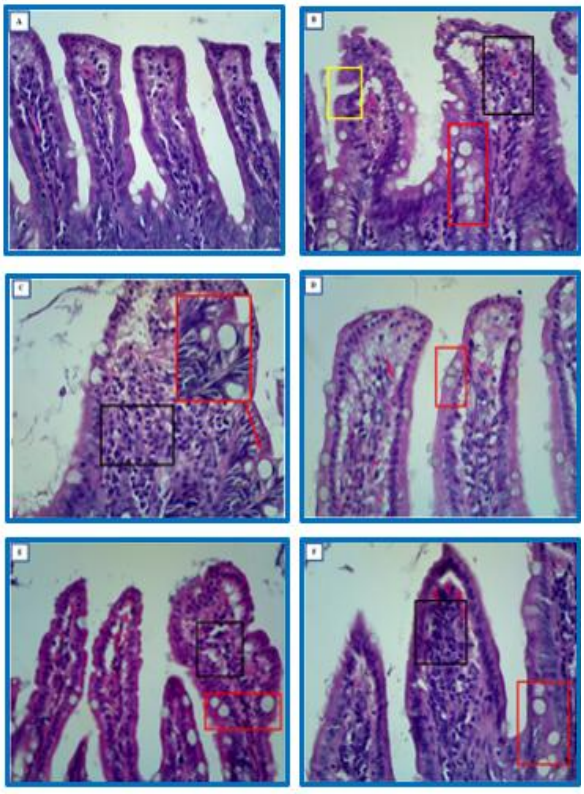

Figure 1 Microscopic observation of mice's Ileum at 400x magnification, (A) negative control, (B) positive control (C) *L. acidophilus* control, (D) Treatment P1, (E) Treatment P2, and (F) Treatment P3

Further, in the positive control, an increase in the activity of goblet cells can be seen. This is related to the body's form of compensation for the presence of antigens that can cause damage to organs. The body tries to eliminate antigens that enter the body by increasing the activity of goblet cells in mucus secretion. Increased mucus secretion aimed to protect epithelial cells from damage. Increased mucous secretion and villous damage in positive control mice can be seen in Table 1 (Balqis et al. 2015).

In the *L. acidophilus* control group (KL), goblet cell hypertrophy increases mucous production. *Salmonella* sp. can trigger cell inflammation from the blood vessels into the tissues (Figure 1C). However, *L. acidophilus* control showed no epithelial erosion compared to positive control. Lactic acid bacteria can protect the mucosal lining by colonizing the intestinal mucosal surface, making pathogenic bacteria unable to adhere. Results presented in Table 1 revealed that in *L. acidophilus* control villi length increased which might be associated with the probiotics administered. Uptake of probiotics stimulates epithelial cell proliferation and increased the production of the fatty acids which positively affect the length of villi.

In treatment groups P1, P2, and P3, several abnormalities were reported between goblet cell hypertrophy and inflammatory cell infiltration into the tissues. Entry of the *Salmonella* sp., into the body, triggers the release of various inflammatory mediators including histamine. Histamine can cause vasodilation of blood vessels. At the time of vasodilation of blood vessels, blood plasma comes out and causes edema. In the current study, the villi length of P1 (Figure 1D) has increased compared to the P2 (Figure 1E)

and P3. Further, the most severe damage has been reported in the P3 group (Figure 1F), which had the most petite villi length in all three treatment groups. This might be due to the high dose of Lampung Robusta coffee extract. According to Selviana (2015), the caffeine content of the coffee triggers excessive gastric acid secretion and damages the gastric and intestinal mucosa. Further, in mice group P2 the types of inflammatory cells were polymorphonuclear and mononuclear (Figure 1E). According to Adenin (2019), injured tissue activates the release of several cells, including leukocytes, erythrocytes, and platelets. In bacterial infections, leukocyte cells that play an important role are neutrophils. Neutrophils play a significant role in the first 24-48 hours of infection. Monocytes will assist neutrophils, later turning into macrophages after exiting the blood vessels. Among the tested three doses, the most severe level of organ damage was reported in P3 (histopathology of the ileum; Figure 1F), and this might be due to an inflammation that can elicit intestinal motility and affect the absorption of nutrients and fluids (Keraru 2017).

3.2 Histopathological Examination of the cecum

Histopathological results of a healthy negative control group without any treatment (K^-) had a normal cecum where the epithelial cells (columnar layer) were visible without any damage and normal goblet cells and did not show hypoplasia (Figure 2A). Both width and length of villi look the same without any widening due to infiltration of inflammatory cells. The histopathology of this group will be compared with other treatment groups.

The histopathology of the positive control group (K^+) showed that due to severe erosion or rupture, villi lost their original shape. Along with this, no epithelial compartment cells appeared, goblet cells were almost absent or hypoplastic, and the size of submucosal edema and villous were also reduced (Figure 2B). All these symptoms are associated with the *S. enterica* infection, which has LPS, Vi, H, and O antigens that disturbed the appearance of inflammatory cells. Further, Macrophages also produced ROS as a defense mechanism and this ROS destroyed the infection-causing bacteria. Increased ROS, an oxidant, can continuously damage the composition of the epithelial lipid membrane which damage the structure of the villi. According to Marchelletta et al. (2014), *S. enterica* infection in mice will cause submucosal edema, caecal edema, goblet cell hypoplasia, and damage to the caecal epithelium. Submucosal edema occurs due to increased permeability caused by the infiltration of inflammatory cells to the site of infection. Similarly, goblet cell hypoplasia is also caused by the infection of *S. enterica*, and in the intestine, these cells respond by removing mucus continuously and these cells became empty, shrink, and disappear in the HE coloring (Songhet et al. 2011).

The *L. acidophilus* control group (KL) showed poor results and various damage including erosion in some epithelium, villi fusion, and submucosal edema in villi. However, an increase in the number of goblet cells or hyperplasia was reported which represented better villous conditions as compared to the positive control (Figure 2C). Goblet cell hyperplasia seen in histopathy is

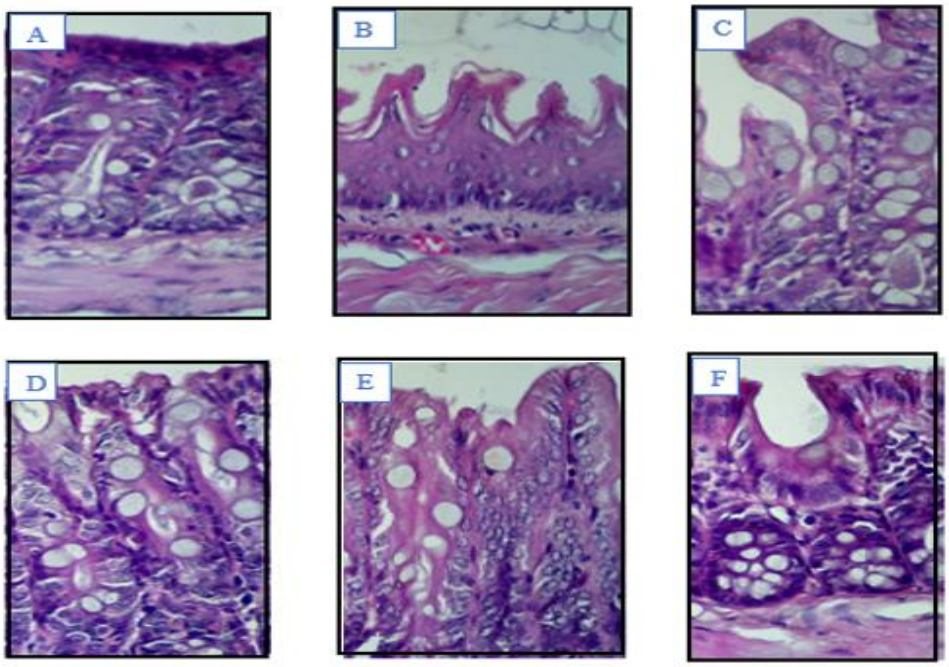

Figure 2 Microscopic observation of mice's caecum at 400x magnification; (A) negative control, (B) positive control (C) *L. acidophilus* control, (D) Treatment P1, (E) Treatment P2, and (F) Treatment P3

caused by the induction of *L. acidophilus* as a preventive function known as immunomodulators, especially IgA which increases mucous production. According to the Galdeano et al. (2019) mucus acts as the first-line defense against the antigens by binding with Ig A and forming "immune exclusion," then capturing and killing bacteria so that bacteria cannot affect adhesion to villous intestines. Arguello et al. (2017) state that administering high *Salmonella* inflammation reduced the expression of genes that encode bile acid transport. It interferes with the absorption of bile salts in the intestine and high bile salts in the intestine can reduce the amount of *L. acidophilus* in the intestine.

Experimental animal groups P1, P2, and P3 were treated with a combination of coffee extract and *L. acidophilus*. These three groups did not show any significant difference among themself and histopathological examination also did not show damage in villi as reported in the positive or *L. acidophilus* control. Here combined application of coffee extract and probiotic *L. acidophilus* play a significant role in mitigating the harmful effect of the *S. enteric* infection. Results are in agreement with the findings of Galdeano et al. (2019), those who reported the immunomodulatory role of *L. acidophilus* and competition between *L. acidophilus* and *S. enteric* which will reduce the chance of *S. enterica* infection and villi deformation.

Lampung Robusta coffee extract is a reach source of various alkaloids, including caffeine, chlorogenic acid, flavonoids, tannins, and saponins which not only have immunomodulatory properties but also antibacterial properties that work to reduce *S. enterica* infection. Chlorogenic acid also has antioxidant properties which can neutralize the ROS and prevented villous damage (Wulandari 2016). Cecum histopathology of treatment P1 is not much different from the negative control histopathology where no forms of damage such as erosion or rupture of villi, change in goblet cells were reported and the mucosa also appeared intact with the shape of a thumb (Figure 2D). Histopathology of the treatment group P2 (Figure 2E) and P3 (Figure 2F) also looked good and did not show any erosion and rupture in the epithelial cells. However, in treatment group P2, submucosal edema appeared with irregular villous form, whereas in treatment group P3, more visible submucosal edema and monocyte infiltration was reported. Further, results of the histopathological cross-section revealed that the combination of P1, namely Lampung Robusta coffee extract @ 250 mg/kg BW and L. acidophilus has a significant effect in preventing the cecum damage because its histopathic appearance approaches negative control.

Goblet cells are secretory cells found in the intestine and the number of these cells increases in the intestine crassum, especially in the distal colon and rectum. These cells are essential for mucus synthesis and secretion to the intestine lumen. This mucus lubricates the lumen and provides a defense against pathogenic bacteria (Bergstrom et al. 2008). Based on goblet cells number, Stecher et al. (2005) suggested 4 important categories i.e. 0 (the average number of goblet cells is more than 28 cells/field of view), 1 (the average number of goblet cells is 11-28 cells/field of view), 2 (the average number of goblet cells 1-10 cells/field of view), and 3 (the average number of cells is less than 1 cell/field of view).

Results of the study revealed that the average number of goblet cells in the negative control group (K^-) was 29.2 and it was included in the scoring 0 (Table 1). This result is in agreement with the findings of Stecher et al. (2005), who said that the average number of normal goblet cells in SPF mice was 28 cells/field of view. Further, the number of goblet cells in the positive control (K^+) has the lowest number, although it scored 1, this insignificant decrease in goblet cells is associated with the *Salmonella* infection, which focuses on attacking Payer patches which are dominant in the ileum. As per Santos et al. (2003) cecum does not have Payer patches, that's why in the current study less damage was recorded to the goblet cells and they scored 1. Further, Mansson et al. (2012) also suggested that *S. enterica* infection can cause epithelial damage and proliferation, continuing to deplete or reduce goblet cells. According to Songhet et al. (2011), *S. enteric* infection enhances the mucous discharge from goblet cells, and with time the goblet cells became empty and shrink and finally these cells disappear in HE staining. In this manner, the results of this study are in line with the previous findings.

The *L. acidophilus* treatment group also showed a score of 1, but the number of goblet cells was recorded 28 cells/field of view and this number was equivalent to the normal SPF mice as per the criteria of Stecher et al. (2005). This result is significantly different from the positive control and this might be due to the immunomodulatory role of *L. acidophilus* which will bind to mucus and induce goblet cell hyperplasia (Galdeano et al. 2019). Further, Liu et al. (2019) also stated that giving a mixture of *Lactobacillus* and *Bacillus* to pigs infected with *S. infantis* can increase the number of goblet cells because a probiotic can improve the integrity of the bacteria in the intestine barrier and protect the intestine from damage caused by *Salmonella* invasion, in this manner, results of the previous studies corroborate with the findings of the current study.

The treatment groups P1, P2, and P3 also had the scoring 1 with the number of goblet cells 26.73, 24.05, and 24.06 cells/field of view respectively. Here also a higher number of goblet cells was recorded as compared to the positive control treatment group. The coffee extract contains various alkaloids such as caffeine, chlorogenic acid, tannin, and antibacterial flavonoids which inhibit the entrance and multiplication of *S.enterica* bacteria. The combination of coffee extracts and *L. acidophilus* can reduce the

damage of goblet cells due to *S. enterica* invasion, and amongst the tested doses, combinations of Lampung Robusta coffee extract @ 250 mg/kg BW and *L. acidophilus* is the most effective in preventing the goblet cell hypoplasia.

Conclusions

Providing a combination of Lampung Robusta Coffee (Coffea canephora) and Lactobacillus acidophilus extracts can prevent ileum and cecum damage in mice induced by *S. enterica* with the best combination dose of Lampung Robusta Coffee extract @ 250 mg/kg and *L. acidophilus*.

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