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Effect of probiotics on the histomorphometry characteristics of *Mus musculus* Jejunum infected by *Salmonella gallinarum*

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Jejunum

Lactic acid bacteria

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ABSTRACT

Salmonellosis is a disease caused by *Salmonella gallinarum*, which can cause digestive tract infections. Probiotics are good microorganisms for the host because they can increase the normal bacteria flora in the digestive tract. They can maintain the intestinal mucosal barrier and prevent bacterial adhesion. This study aimed to determine the histomorphometric characteristics of the jejunum from the intestines of mice (*Mus musculus*) after being infected with *S. gallinarum*. A total of 20 mice, 4-6 weeks, were divided into four research groups: P1 (probiotics and *S. gallinarum* infection), P2 (probiotic administration), P3 (*S. gallinarum* infection), and P4 (control). The probiotics used contain microorganisms such as *Lactobacillus casei*, *Saccharomyces cerevisiae*, and *Rhodopseudomonas palustris*, dissolved in distilled water in a ratio of 1:1000. Probiotics were given orally at 0.5 ml for 7 days. *S. gallinarum* infection was given orally, with a volume of 0.5 ml (1.5×10^8 CFU/ml). The results showed that the mean score of intestinal lesions differed between groups. The width of the villi, the thickness of the mucosa, and the depth of the intestinal crypts were significantly different. The best result of histology findings was in the group of mice that were induced with probiotics (P2).

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1 Introduction

The digestive system is a channel that plays a role in food digestion, absorption of nutrients, and elimination of food waste. Pathogenic bacteria in the intestines are toxic because they can cause damage to the intestinal mucosa by adhesion, invasion, multiplication, and then releasing toxins to cause various digestive tract diseases (Ersawati et al. 2018). According to Joni and Abrar (2018), bacteria that play a role in damaging the intestinal mucosa include *Salmonella* sp., *Escherichia coli*, *Staphylococcus aureus*, *Shigella sp*., *Vibrio cholerae* and *Vibrio parahaemolyticus*.

Salmonella is a type of harmful bacteria that can be found in the gut of some hosts. Different species of *Salmonella* can attach to the mucosa of the intestine and form colonies on epithelial cells. This attachment is influenced by proteins on the surface of the bacteria, known as fimbriae and fimbrial adhesins (outer membrane proteins) (Wresdiyati et al. 2013). Once attached, *Salmonella* produces cytolethal distending toxins (CDT) that can cause inflammation, ulcers, abscesses, and even cell death in the intestinal tissue. These effects are caused by the disruption of water and electrolyte secretion and the triggering of inflammatory reactions. Therefore, it is dangerous for host cells to be infected with this bacterium (Darmawan 2017).

Increasing the intestinal mucosal barrier can help defend against pathogenic bacteria like *Salmonella*. This can be done using live beneficial microbes, also known as probiotics. Probiotic products can help balance the intestinal microflora and boost immunity. This helps restore the balance between pathogenic and non-pathogenic bacteria in the intestine (Kusuma et al. 2012). To be effective, probiotics derived from the lactic acid bacteria (LAB) group must meet certain requirements, including being non-pathogenic, active and able to grow in the digestive system, resistant to bile salts, having high viability, being anaerobic, and capable of quickly adhering to and killing or inhibiting bacterial pathogens from growing (Joni and Abrar 2018).

Several studies have shown that probiotics can increase the number of lactic acid bacteria (LAB) in the intestine and reduce the number of harmful bacteria. LAB can also reduce damage caused by pathogenic bacteria, such as *Salmonella*, by adhering to the intestinal epithelium and producing antimicrobial compounds that denature membrane proteins, change cell membrane properties and inhibit the replication of *Salmonella* (Gupta et al. 2018). Additionally, LAB can improve immunity by increasing phagocytosis and modifying the production of pro-inflammatory cytokines IFN-γ and TNF-α (Castillo et al. 2011). This study aims to evaluate the effect of probiotics on the histomorphometry characteristics of the Jejunum of Mus musculus infected by *S. gallinarum*.

2 Material and Methods

This study used 20 male mice (*Mus musculus*) aged 4-6 weeks and weighing 18-30 grams. The research steps included preparing *S. gallinarum* and probiotic suspensions, inducing them in mice, preparing histology, and analyzing the data.

2.1 Bacteria and probiotic preparation

S. gallinarum bacteria were grown on *Salmonella-Shigella Agar (SSA)* media. The *S. gallinarum* colonies were cultured in a nutrient broth medium and incubated aerobically at 37°C for 24 hours. This study used commercial probiotics containing *Lactobacillus casei*, *Rhodopseudomonas palustris*, and *Saccharomyces cerevisiae*. The probiotic solution used was 1% and was given orally to mice, with each mouse receiving 0.5 ml of the solution daily for seven days.

2.2 Induction to mice

The study involved 20 mice that were divided into four groups: P1 (probiotics and *S. gallinarum* infection), P2 (probiotic administration), P3 (*S. gallinarum* infection), and P4 (control). The probiotics were administered orally at a dose of 0.5 ml for seven consecutive days, while *S. gallinarum* was induced orally for seven days with a concentration of 1.5×10^8 CFU/ml at a dose of 0.5 ml each day. The Brawijaya University Research Ethics Commission has granted ethical permission for this study under the reference number 109-KEP-UB-2020.

2.3 Histology preparation

After the animal was euthanized by cervical dislocation, a sample was taken from the jejunum and placed in 10% neutral formalin buffer (NBF). The sample was then prepared for histopathological examination through steps, including dehydration in graded alcohol, clearing with xylene, embedding, sectioning, and staining with hematoxylin-eosin (HE).

2.4 Data Analysis

The data from the study were analyzed using lesion score, villi width, mucosal thickness, and crypt depth of the jejunum among the groups. The lesion scoring was determined based on the method of Erben et al. (2014), with some modifications required (Table 1). Statistical analysis of the data was carried out in this study using one-way ANOVA (analysis of variance) to determine the differences in each treatment of the morphometric analysis of the intestines of mice.

3 Results and Discussion

The histomorphometry of the jejunum in mice was measured using the Image-J application at a microscope magnification of 400X.

Table 1 Determination of lesion scoring of jejunum

Abnormality	Score	Description
Inflammatory cells	0	None
	1	Focal
	2	Multifocal
	3	Diffuse
Epithelial damage	0	None
	1	Epithelial cells of villi are damaged at several ends
	2	Micro erosion in some villi
	3	Severe erosion of epithelial
Mucosal edema	0	None
	1	<50% of the diameter of the intestinal wall
	2	50%-80% of the diameter of the intestinal wall
	3	>80% of the diameter of the intestinal wall

The scoring is based on the method of Erben et al. (2014)

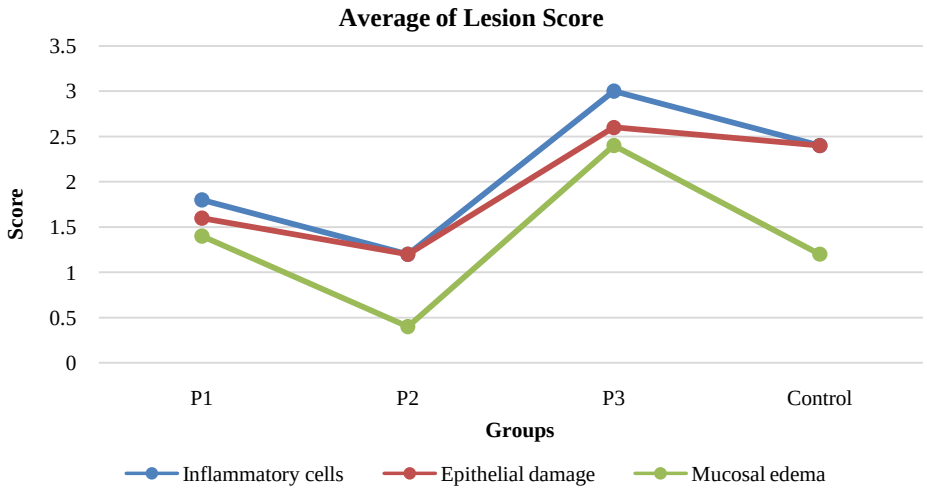

Figure 1 Average of Jejunum Lesion Score; P1 (probiotics and *S. gallinarum* infection); P2 (Only probiotic); P3 (*S. gallinarum* infection); P4 (control)

The jejunum's histomorphometric observations were conducted with four fields of view for each treatment group. The observed parameters included lesion scoring, villi width, mucosal thickness, and crypt depth. The analysis of the jejunum histopathological lesion scores results, as shown in Figure 1, revealed a significant difference in each treatment group in terms of inflammatory cells, epithelial damage, and jejunal submucosal edema of mice that were given probiotics and infected with *S. gallinarum*. Based on the average score, the induction treatment group of *S. gallinarum* had the highest levels of inflammatory cells and epithelial damage, followed by the control group, probiotics and S. gallinarum combination, and the least effective group was probiotics. The presence of inflammatory cell infiltration can be caused by infection with *S. gallinarum*, which then triggers an inflammatory reaction. The accumulation of inflammatory cells is a response to inflammation. The inflammatory reaction is a self-defence reaction in response to injury as a vascular reaction (Saraswati et al. 2015).

Epithelial damage can occur due to the penetration of *Salmonella* bacteria into the intestinal mucosa. The bacteria can stay intracellularly and proliferate before reaching the epithelium in the lamina propria. This can cause brush border degeneration. These findings support Cita's (2011) statement that *Salmonella* bacteria can penetrate the intestinal epithelial mucosa and damage the epithelial lining by multiplying in the lamina propria. Pathogenic bacterial infections can also cause submucosal edema. This

Table 2 Effect of various treatments on the mucosa thickness, crypt depth, and villi width

Groups	Average ± SD		
	Mucosal Thickness	Crypt Depth	Villi Width
P1 (probiotic + *S. gallinarum*)	347.97 ± 2.46^{ab}	114.46 ± 0.88^{c}	79.81 ± 0.72^{a}
P2 (probiotic)	369.92 ± 4.06^{a}	143.23 ± 1.99^{a}	85.13 ± 0.59^{a}
P3 (*S. gallinarum*)	280.07 ± 2.30^{b}	110.55 ± 0.76^{c}	58.46 ± 0.88^{b}
P4 (control)	295.50 ± 2.53^{b}	120.78 ± 2.22^{b}	67.20 ± 1.18^{b}

Data are mean of five replicates; ± Standard Deviation of mean; Different notations indicate significant differences between treatment groups ($p<0.05$).

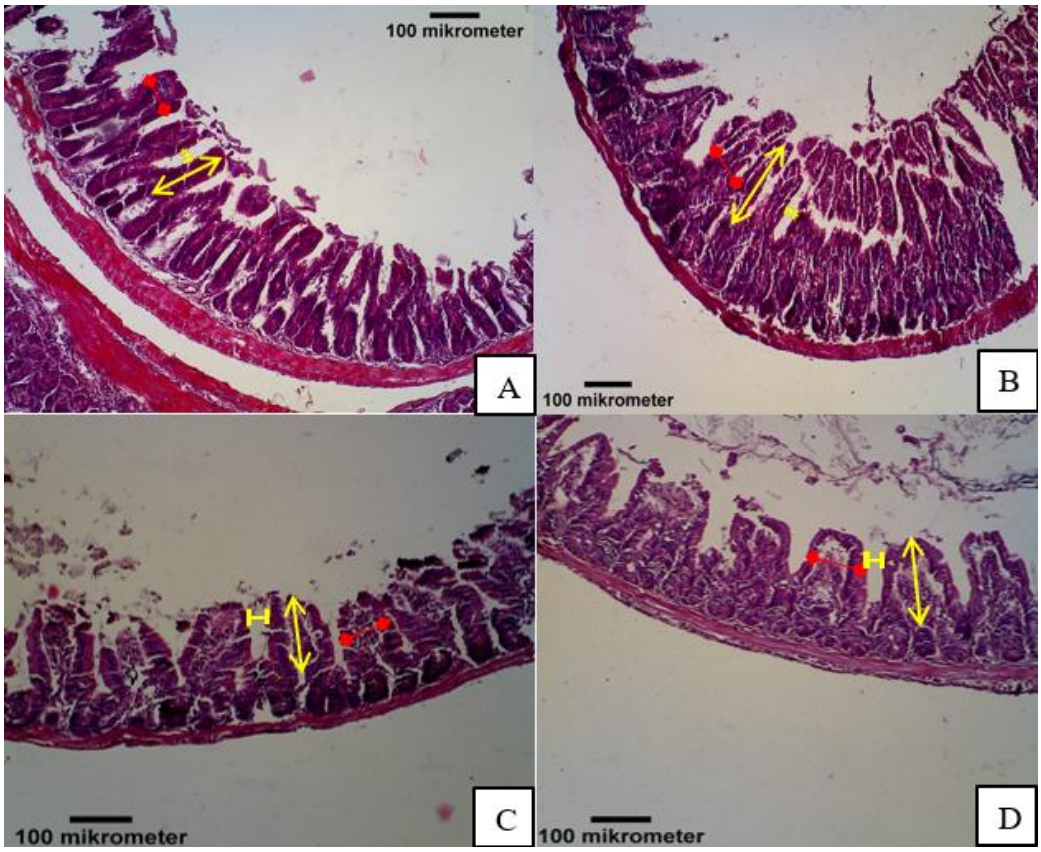

Figure 2 Histopathology of Mucosal Thickness, Crypt Depth, Villi Width (magnification of 400X); (a) probiotics and *S. gallinarum* infection; (b) Only probiotic; (c) *S. gallinarum* infection; (d) control; Yellow line represent crypt depth; red line represented villi width.

happens due to an inflammatory reaction that results in the widening of the submucosal space. Inflammatory mediators increase inflammatory proteins and substances that cause nearby blood vessels to dilate and become more permeable. This makes it easier for plasma proteins to release, causing edema (Saraswati et al. 2015).

Based on the histopathological measurements presented in Table 2 and Figure 2, it was observed that the group administered with probiotics had the highest average values for mucosal thickness, crypt depth, and villi width. The mucosal thickness of the control group was not significantly different from that of the other treatment groups. However, the group treated with probiotics differed significantly from the P1 and P3 groups. In the case of crypt depth, the group given probiotics and *S. gallinarum* was not significantly different from the other groups. However, the group given probiotics had a significant difference from the P3 and P4 groups. In contrast, no significant difference was observed in the measurement of villi width between groups P1, P2, P3, and P4. However, groups P1 and P2 significantly differed significantly from the control group and those induced by *S. gallinarum*.

An increase in the thickness of the mucosal layer and depth of the crypts in the small intestine is associated with an increase in the height of the villi. The ratio of the height of the villi to the depth of the crypts indicates a broader area for the absorption of nutrients. This is supported by Wresdiyati et al. (2013) statement, which states that the increase in the height of the villi in the jejunum is consistent with an increase in digestive and absorption functions due to the expansion of the absorption area. This is a smooth expression of the nutrient transport system throughout the body, which benefits the host. On the other hand, a decrease in mucosal thickness and crypt depth can be caused by damage to the epithelial layer due to the administration of *S. gallinarum*. According to Arya et al. (2012), *Salmonella* species can enter the small intestine by invading the intestinal tissue and persisting in intestinal cells. This can result in damage to the connecting surfaces that unite epithelial cells when penetrating the epithelial barrier.

According to research by Khan and Chousalkar (2020), providing probiotics alone as a preventive measure may not effectively inhibit the colonization of *S. gallinarum*. Administering probiotics can still cause damage to the jejunum mucosa of mice (*Mus musculus*) challenged by *S. gallinarum*. This is because probiotics' diverse and abundant microbial diversity is insufficient to reduce the number of *S. gallinarum*. Additionally, inadequate concentrations of probiotic bacteria in the intestinal tract and the short distance between the time of probiotic administration and the challenge of *S. gallinarum* could also contribute to this damage. Andino et al. (2014) also stated that the immune system may not have enough time to activate and provide sufficient protection against infection.

There was no significant difference in the width of the jejunal villi between the P1 (Probiotic+*S. gallinarum*) and P2 (Probiotic) groups. This suggests that the administration of probiotics has a significant impact on the width of the villi and that this effect was increased when probiotics were given along with *S. gallinarum*. The P1 group showed better histomorphometry than the P3 group (*S. gallinarum* only). This indicates that treatment with probiotics can reduce the damage caused by *Salmonella* by stimulating humoral and cellular immunity. The stimulation of immunity leads to an increase in the population and proliferation of lymphocytes, the maintenance of pro-inflammatory cytokines such as IFN-γ, TNF-α, and the increase of IL-12, IL-10, Immunoglobulin IgA, IgE IgG, IgM. The lactic acid bacteria found in probiotics adhere to the intestinal epithelium, stimulating macrophage activity, activating phagocytosis, and maintaining the mechanism of protection of the villi against *Salmonella*. This is achieved by maintaining the immune response (Gupta et al., 2018; Astawan et al., 2011). Probiotics in the intestines can reduce the proliferation of *Salmonella* by adhering to epithelial cells, competing with *Salmonella*, producing bacteriocin antimicrobial compounds, and interacting with the cell membrane of *Salmonella*. This results in the denaturation of proteins on the cell membrane. Probiotics also have lactic acid products that lower the pH, which disturbs the growth of *Salmonella* species (Castillo et al. 2011; Adetoye et al. 2018).

The P2 group, which was given probiotics, showed better results than the P4 group (control). The width of the villi in the P2 (Probiotic) group increased, which is influenced by short-chain fatty acids. Through fermentation, probiotic bacteria can produce short-chain fatty acids, a constituent component of intestinal epithelial cells. The more short-chain fatty acids produced, the more the multiplication of intestinal epithelial cells will be stimulated (Izzuddiyn et al. 2018). An increase in the length and width of the villi will expand the area for absorption of food and nutrients, which can improve the performance of the intestines in digesting food (Matur and Eraslan 2012). According to Dong (2019), *Salmonella* infections can significantly decrease the length and width of villi in the intestine. This is because *Salmonella* triggers inflammation, which leads to degeneration and damage of the villous epithelium. As a result, the villi become shorter and narrower, negatively affecting the intestine's ability to digest and absorb food.

Conclusions

The study's findings suggest that probiotics could repair damage to the jejunum caused by *S. gallinarum*. Moreover, the group that was given only probiotics displayed the most significant improvements in mucosal thickness, crypt depth, and villi width. They also showed minimal damage to the jejunum, as evidenced by lower histopathological lesion scores.

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