

Original Article

Histological Features of the Duodenum of Mice (*Mus musculus*) Treated with Liquid Probiotic Isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*

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ABSTRACT

This study aims to determine the histological description of the height and width of the duodenal villi of mice (*Mus musculus*) treated with liquid probiotic isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*. Experimental mice were divided into four groups with ten replications. Group P0 was given aquadest, P1 was given probiotic isolate at a dose of 0.09 mL/kg BW, P2 was given probiotic isolate at a dose of 0.27 mL/kg BW and P3 was given probiotic isolate at a dose of 0.81 mL/kg BW for 24 hours. Histological examination of the duodenum of mice was carried out by making histological preparations from the height and width of the duodenal villi which were observed under a microscope with 100x magnification. Statistical analysis in this study used One-Way ANOVA and Duncan's test. The results showed that the control group and the treatment group had significantly different height and width of the duodenal villi ($p < 0.05$). The conclusion of this study is that the administration of isolates *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* can increase the height and width of the duodenal villi of mice and the P2 group with a dose of 0.27 mL/kg BW has the greatest average value for the height and width of the duodenal villi with an average height of 360.80 μm and an average width of 152.30 μm .

Keywords: *Bacillus coagulans*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, duodenal villi height, duodenal villi width

Introduction

Antibiotics are a revolution in the field of medicine. This is based on increasing life expectancy, and quality of life, and decreasing death rates after using antibiotics. Over time, antibiotics are also used to increase livestock growth or are often called Antibiotic Growth Promoters (AGP). The way growth-promoting antibiotics work is by controlling the population of normal flora in the digestive tract (Ramirez *et al.*,

2020). The main drawback of antibiotics is that their effect is that they also kill beneficial bacteria as well as pathogenic bacteria so that the body's ecosystem is disturbed, which ultimately causes bad effects on the body such as superinfection and drug resistance (Patangia *et al.*, 2022). During the 1990s, a ban on the use of antibiotics as growth promoters in the European Union (the ban came into effect in 2006) began to be implemented due to concerns about the presence of residues in livestock products such as meat, eggs and milk (Castanon, 2007).

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As an alternative to AGP is probiotic supplementation. Probiotics is a term that refers to microorganisms that provide benefits to humans and animals. These microorganisms play a role in the balance of intestinal microbes and also play an important role in maintaining health (Wang *et al.*, 2021). Probiotic microorganisms mostly come from the genera *Lactobacillus* and *Bifidobacterium* but can also come from the genera *Bacillus*, *Pediococcus*, and some yeasts (Fijan, 2014).

Probiotic microorganisms can be from the *Bacillus* genus such as *Bacillus coagulans* and *Bacillus subtilis*, as well as from the yeast group such as *Saccharomyces cerevisiae*. *Bacillus* bacteria are able to control pathogenic bacteria (as competitive exclusion) in the digestive tract (Bahaddad *et al.*, 2023). The genus *Saccharomyces* is able to reduce the number of pathogenic bacteria and increase the number of beneficial aerobic and anaerobic bacteria in the intestine. A decrease in the number of pathogenic bacteria in the intestine will affect changes in the intestinal villi, resulting in increased nutrient absorption in the intestine.

A number of probiotics have an influence in regulating the physiological characteristics of the digestive pathway, including intestinal permeability and the immune system in the intestinal mucosa (Mazziotta *et al.*, 2023). Apart from that, probiotics have the function of improving digestion, fighting harmful bacteria in the intestines, preventing inflammation in the intestines and helping the process of absorbing nutrients in the intestines. The process of absorbing nutrients from food is part of the function of the small intestine. One part of the small intestine is the duodenum. The duodenum is the initial part of the small intestine and is the entrance for food into the intestine. Duodenal villi are protrusions of the epithelium in the intestine which function to increase the surface area for nutrient absorption (Kai, 2021).

The combination of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* isolates will be implemented in animal feed, so safety testing needs to be carried out using the acute toxicity test method. Acute toxicity test is a test of the effects of a compound that occurs within a short time after administration in a single dose. Several sensitive main indicators used to observe the toxicity effects of a toxic substance are mortality, body weight and organ weight of experimental animals (Guo *et al.*, 2022). The level of safety of a material can also be evaluated by looking at the picture of changes in body organs,

such as the heart, lungs, liver, intestines, kidneys and spleen (Wani *et al.*, 2015).

This test is carried out by giving the substance to be tested once to experimental animals within a 24 hour period (Erhirhie *et al.*, 2018). The purpose of acute toxicity is to detect the toxicity of a substance, determine target organs and their sensitivity, obtain data on the danger after acute administration of a compound and to obtain initial information that can be used to determine the dose level required for subsequent toxicity tests (Borgert *et al.*, 2021). Animals commonly used for acute toxicity tests are mice (Nath and Yadav, 2015).

Based on the background above, research on liquid probiotics combining isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* on the histological picture of the height and width of the duodenal villi of mice (*Mus musculus*) has never been reported, so there is a need for research on the histological picture of the height and width of the mouse duodenal villi, which were given liquid probiotic isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*.

Materials and methods

Research design

The research will be carried out from October 2021 to March 2022. The administration of liquid probiotic isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* and the duodenum organs of mice were taken at the Biochemistry Laboratory, Faculty of Medicine, Airlangga University, Surabaya. Organ preparations were made at the Pathology Laboratory, Faculty of Veterinary Medicine, Airlangga University. Examination and observation of organ preparations was carried out at the Pathology Laboratory, Faculty of Veterinary Medicine, Airlangga University, Surabaya.

The number of research samples amounting to 40 individuals was obtained from the Federer formula calculation $[(t-1)(n-1) \geq 15]$ where t is the number of treatments to be given and n is the number of samples per group to be searched (Charan and Kantharia, 2013). The calculation results showed that the number of repetitions was at least 6 repetitions for each treatment. In this study, 10 replications were used per treatment with a total sample size of 40 individuals.

Preparation of experimental animals

The experimental animals used were 40 mice (*Mus musculus*) with a body weight of 20-30 g. Mice

were divided randomly into eight cages. Each cage was filled with 5 randomly selected mice. So in one treatment group, 10 mice were divided into two cages due to limited cage space. All mice were first adapted for seven days in a cage at room temperature. Mice were fed animal pellets and given drinking water *ad libitum* and received the same treatment.

Preparation of 10% BNF solution

The materials needed to make a 10% BNF solution are 100 ml of 40% formaldehyde, 900 ml of distilled water, 4 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, and 6.5 g of Na_2HPO_4 . All ingredients are mixed evenly then stored in a plastic bottle and labeled.

Research procedure

After the mice had been adapted for seven days, 40 mice were randomly divided into four groups consisting of the control group, treatment group one, treatment group two, and treatment group three (Table 1).

Table 1. Division of treatment groups

Treatment group	Treatment
Control group (P0)	Mice were given distilled water as a control
Treatment group one (P1)	Mice were given liquid probiotic isolates of <i>Bacillus coagulans</i> , <i>Bacillus subtilis</i> , and <i>Saccharomyces cerevisiae</i> at 0.09 mL/kg BW
Treatment group two (P2)	Mice were given liquid probiotic isolates of <i>Bacillus coagulans</i> , <i>Bacillus subtilis</i> , and <i>Saccharomyces cerevisiae</i> at 0.27 mL/kg BW
Treatment group three (P3)	Mice were given liquid probiotic isolates of <i>Bacillus coagulans</i> , <i>Bacillus subtilis</i> , and <i>Saccharomyces cerevisiae</i> at 0.81 mL/kg BW

Administration of liquid probiotic isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* to mice was carried out orally using a probe. After 24 hours, the mice were anesthetized with ketamine and xylazine and then the duodenal organs were removed for histological examination of the height and width of the duodenal villi. The abdomen of the mice was dissected using surgical equipment to remove the duodenum organ. The duodenum organ is cut horizontally. The duodenum organ was cut 1 cm from the stomach organ. The slice of the duodenum organ is in the form of a tube 2 cm long. All organs are cut to the same size and in the same place. The pieces of the duodenum organ that were taken were stored in a container

containing 10% BNF fluid. BNF-fixed duodenal sections were embedded in paraffin and stained with Hematoxylin-eosin. Histological preparations that were ready on a glass object were examined using a microscope at 100x magnification.

Preparation reading

The process of observing the preparations used a Nikon brand light microscope with a magnification of 100x (10x objective lens and 10x eyepiece lens). The organ observed was the small intestine, duodenum. Observations on the duodenum consist of villi height and villi width. The results of these parameter calculations can be used as an indicator of the effect of administering liquid probiotics containing isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* on the histological picture of the height and width of the duodenal villi of mice.

Calculation of the height and width of the villi

The steps for measuring the height and width of the villi were first determined using a Nikon microscope set at 100x magnification. The histology image appears on the monitor screen. Once the desired image is found, photographs are taken of all the preparations to be measured. Minimum measurements were carried out three times per slide made for each parameter, then measurements of the height and width of the villi were carried out using a flat-screen computer with Image Raster software. Initially, the standard size μm is determined first with the help of a computer, that is, the magnification value used or desired is converted into units of length (μm). The μm units obtained are then used as a standard for measuring the height and width of the villi displayed on the monitor screen. Histological Sample Measurements based on Iji *et al.* (2001), namely measuring the height of the villi (μm), measuring the highest distance from the villi, while measuring the width of the villi (μm): measuring the apical width, middle width and basal width of the villi and then averaging them (Figure 1).

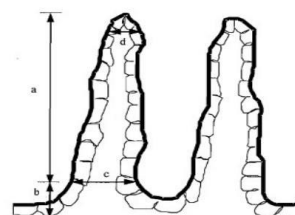


Figure 1. Measurement of the height and width of the villi. a: villi height; b: crypt depth; c: basal width; d: apical width

Data analysis

The results of this research are presented in the form of photos and calculated figures (quantitative). Quantitative data consisting of the height and width of the duodenal villi were analyzed using the ANOVA method. The data was continued with the Duncan test to compare each treatment group. Statistical analysis was carried out using the SPSS for Windows computer program.

Result

Based on the results of observations of histological preparations carried out under a microscope showed significant differences in the histological appearance of the height and width of the duodenal villi in each treatment group. There was an increase in the height and width of the duodenal villi in each treatment group compared to the control group but it was still within the normal range (Figure 2).

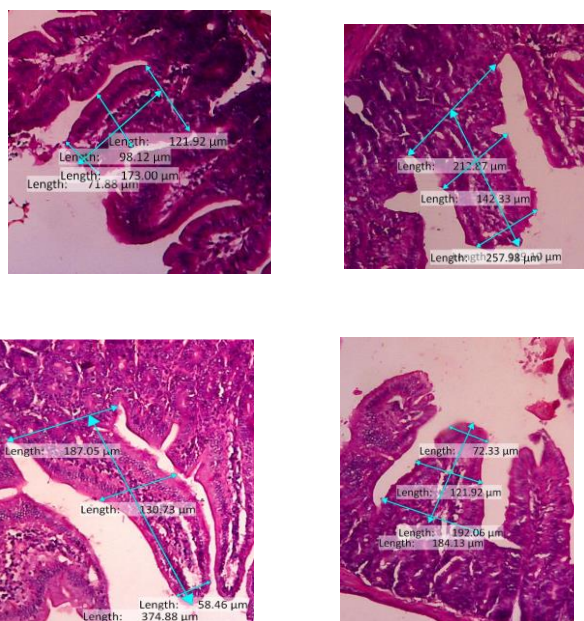


Figure 2. Comparison of the histological images of the height and width of the duodenal villi of each group using HE staining and 100x microscope magnification

Based on the results of the ANOVA test, a p-value <0.05 was obtained, indicating that there was a significant difference in the height and width of the duodenal villi produced by the treatment group compared to the height and width of the duodenal villi produced by the control group, then continued with the Duncan test. The mean and standard deviation

(SD) of the height and width of the duodenal villi can be seen in Table 2.

Table 2. Mean and standard deviation (SD) of height and width of duodenal villi

Treatment	Mean ± SD height of duodenal villi	Mean ± SD width of duodenal villi
P0	201.30 ^a ± 29.37	94.00 ^a ± 8.05
P1	275.80 ^b ± 42.76	136.10 ^b ± 20.30
P2	360.80 ^c ± 67.98	152.30 ^c ± 24.24
P3	199.30 ^a ± 29.88	106.00 ^a ± 13.26

Note: Different superscripts in the same column indicate significant differences

The results of the follow-up test (Duncan's test) in Table 2 show that there is a significant difference in the height and width of the duodenal villi produced by the treatment group compared to the height and width of the duodenal villi produced by the control group. Based on the description above, it can be seen that the administration of liquid probiotic isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* was able to increase the height and width of the duodenal villi of mice, and at P2 with a dose of 0.27 mL/kg BW had the highest mean height and width of the duodenal villi. The increase in the height and width of the mouse duodenal villi is presented in Figure 3 in graphical form.

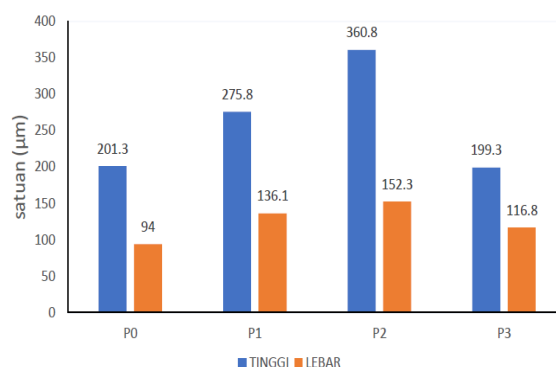


Figure 3. Graph of the average values for the height and width of the mouse duodenal villi

Group P1 and group P2 had villi height and width that were significantly different from group P0, whereas group P3 had villi height and width that were almost the same as group P0.

The mean values for the height and width of the duodenal villi of mice at P1 and P2 were not much different, namely with a mean height of 275.80 μm and a mean width of 136.10 μm at P1 and a mean value of 360.80 μm for height and width of 152.30 μm at P2. while the average values for the height and width of the duodenal villi at P0 and P3 are almost the same, namely with an average height of 201.30 μm and an

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average width of 94 μm at P0 and an average height of 199.30 μm and an average width of 106 μm at P3. The P2 group with an isolated dose of 0.27 mL/kg BW was the group that had the highest mean value for the height and width of the duodenal villi, while the P3 group with an isolated dose of 0.81 mL/kg BW was the group that had the highest mean value. and the smallest width of mouse duodenal villi.

Discussion

Height and width of mouse duodenal villi

The aim of calculating the height and width of the villi of the duodenum of mice is to determine the comparison between each treatment group and the control group and to determine the most effective dose to be given to mice, which can then be converted to the dose given to probiotic isolates to livestock. Nolte *et al.* (2016) explained that the height of villi in small rodents, especially rats and mice, ranges from 200-500 μm . The width of the villi of the duodenum of normal mice is 92-300 μm (Chen *et al.*, 2009). The results of the analysis of the average height and width of the duodenal villi of mice (Table 2) show that the average height and width of the duodenal villi of the test animals in all treatment groups were still within the normal range. The higher and wider the villi, the more microvilli there will be. Reducing the number of pathogenic bacteria will reduce villous damage so that villous growth can be maximized. This can be seen by the addition of villi and microvilli which enable the duodenum to absorb better. Increased villous growth can expand the surface area of the plasma membrane so that more nutrient molecules can pass through the plasma membrane. Microvilli are extensions of the thin plasma membrane on the surface of the villi and each microvilli can only be seen using an electron microscope. Microvilli are found in the intestines and other places and have an important function for absorption (McConnell *et al.*, 2009).

Based on the research results, P2 showed the largest mean height and width of duodenal villi with a mean height of 360.80 μm and a mean width of 152.30 μm . The increase in the height and width of the duodenal villi in P2 was due to isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* at a dose of 0.27 mL/kg BW, which was the most effective dose for reducing the number of pathogenic bacteria in the intestine. In the P3 group, the average height and width of the duodenal villi decreased but were still within the normal range. The cause of this decrease was that the volume of isolate

given was too much, which caused an atmosphere that was too acidic in the duodenum and resulted in several duodenal villi rupturing.

Absorption in the small intestine

Absorption of food that has been completely digested takes place in the small intestine through two channels, namely the blood capillaries and the lymph channels in the villi (Montoro-Huguet *et al.*, 2021). Depending on the type of nutrition, transport across epithelial cells can be passive or active. Passive transport means that the process of transporting molecules is simple, just ordinary diffusion, and does not require energy. Molecules that can be absorbed by passive transport are water, small molecules, and inorganic molecules. Active transport means the transportation of these molecules requires energy (ATP) and often requires carrier molecules and co-transport molecules for the absorption of glucose from the intestinal lumen to the enterocytes (intestinal epithelium). Without the presence of these two molecules, glucose cannot enter the intestinal epithelium. The molecules absorbed in this way are glucose and amino acids (Gromova *et al.*, 2021). Fructose sugar moves by facilitated diffusion down the concentration gradient from the lumen of the small intestine towards the epithelial cells, then fructose leaves the basal surface and is absorbed into the microscopic blood vessels or capillaries in the center of each villus. Other nutrients, such as amino acids, vitamins, small peptides, and most glucose molecules, will be pumped against a concentration gradient by villus epithelial cells (Kiela and Ghishan, 2016).

The process of nutrient absorption occurs in the intestine and is carried out by cylindrical columnar epithelial cells found in the villi. Apart from absorption cells, inside these villi there are blood vessels, lymph vessels and goblet cells. Goblet cells are located tucked between the absorption cells, they are fewer in number and increase in the duodenum. Goblet cells produce acidic glycoproteins which function to protect and lubricate the mucosa lining the small intestine. Amino acids and glucose are absorbed by the absorption cells in the villi and transported by the blood to the liver via the hepatic portal vein system. The fatty acids react first with bile salts to form a fat emulsion. The fat emulsion together with glycerol is absorbed by the cells in the villi. From within the villi, fatty acids are released, then the fatty acids bind to glycerin and form fat again. The fat that is formed then enters the lymph vessels located in the

center of the villi. Through the lymph vessels to the veins, the fat emulsion process occurs, while the bile salts enter the blood to the liver and are formed again into bile (di Gregorio *et al.*, 2021).

In the mucosa there are villi, crypts and Liberkun's glands. On the surface of the villi of the small intestine there are a row of cylindrical epithelial cells, apart from that there are also mucus-producing goblet cells and lysozyme-producing Panet's cells. Crypts move every 10-14 hours to replace loose epithelial cells. The time required for epithelial cells to move from the crypts to reach the tips of the villi is approximately 48 hours. The number of villi in the duodenum is the largest (Rossi *et al.*, 2022). Absorption in the duodenum is further expanded by the presence of these villi. Giving isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* can help reduce the number of pathogenic bacteria in the duodenum so that when the number of pathogenic bacteria in the duodenum decreases, the duodenal villi will grow higher and wider, which can result in better absorption by the duodenum.

Conclusion

Based on the research that has been carried out, it can be concluded that administering liquid probiotic isolates of *Bacillus coagulans*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* at doses of 0.09 mL/kg BW and 0.27 mL/kg BW can increase the height and width of the duodenum villi of mice (*Mus musculus*). Apart from that, the P2 group with a dose of 0.27 mL/kg BW had the largest mean height and width of duodenal villi, namely with a mean height of 360.80 μm and width of 152.30 μm and it can be concluded that this dose was the highest dose. effective in reducing the number of pathogenic bacteria in the intestine.

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