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Effect of *Bacillus subtilis* and *Bacillus coagulans* Liquid Probiotics on Duodenum Histomorphology in Broiler

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Abstract

Introduction: Probiotics can nourish the digestive tract and increase nutrient digestibility so that nutrient intake is met for livestock. *Bacillus subtilis* and *Bacillus coagulans* as a prophylaxis against bacterial diseases by balancing intestinal microbes, increasing digestibility, and can secrete protease, lipase, and amylase enzymes. The purpose of this study was to determine the effect of probiotics *Bacillus subtilis* and *Bacillus coagulans* on duodenal histomorphology.

Objective: Twenty-four broilers were randomly divided into 4 groups. The control group was not given probiotics, groups P1, P2, and P3 were given probiotics of 2 ml, 4 ml, and 6 ml. Probiotics were given for 14 days. One way ANOVA showed a significant difference in each groups ($P<0.05$) and continued with the Duncan test.

Results: The average length of the duodenal villi in each treatment showed that the control group was significantly different from the P1, P2, and P3 groups. The P3 group had the highest average length of duodenal villi.

Conclusion: The conclusion from the research that has been done is that the use of *Bacillus subtilis* and *Bacillus coagulans* as probiotics has an effect on the broiler duodenal villi which is characterized by an increase in the average length of the duodenal villi according to the increase in the probiotic dose.

Keywords: *Bacillus subtilis; Bacillus coagulans*; Probiotics; Broiler; Duodenal; Healthcare

1. Introduction

Broilers are a type of chicken that is in demand in Indonesia. Many chicken breeders prefer to develop broiler chicken businesses, this is because market demand for broilers increases every year. Broilers have an important role as a source of animal protein with their relatively cheap price and tender meat so they are preferred by the public [1]. Broilers are very popular because they have advantages including very fast growth with a high body weight in a relatively short time, low feed conversion, and produce quality soft fibrous meat. Rapid growth can be balanced by providing high protein feed without compromising adequate energy and protein (E/P) balance and sufficient minerals for maximum growth. Protein feed ingredients, fish meal and soybean meal, are processed to microparticle size. Microparticles are feed particles with sizes ranging from 0.2 - 5000 μm [2].

Intensive broiler maintenance requires the availability of feed ingredients that are able to meet nutritional needs. The use of imported feed has an impact on high production costs so its use is replaced with local feed ingredients. Local feed

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ingredients have several disadvantages such as low quality and uncertain availability. Broiler productivity is influenced by the health of the livestock, broiler body weight can increase if the livestock is in a healthy condition and is not attacked by disease. To prevent disease in broilers, farmers use antibiotics. The use of antibiotics can cause residues in livestock which can result in bacterial resistance in humans. To minimize the use of antibiotics, farmers can replace them with probiotics [3].

Probiotics can play a role in replacing the function of antibiotics in the digestive tract, so that they function to maintain the health of the digestive tract of livestock, especially poultry [4]. Providing probiotics to poultry can reduce or prevent pathogenic microbial contamination which can affect production results. Providing probiotics in feed can increase the number of beneficial microbes for livestock in terms of feed efficiency. Thus, giving probiotics to poultry is expected to improve appearance. Probiotics can nourish the digestive tract and increase nutrient digestibility so that nutrient intake is met for livestock [5]. The probiotics that are widely used are lactic acid bacteria which can increase the ability of non-specific immunity. In addition, lactic acid bacteria can increase the efficiency of digestion and absorption of nutrients. Broilers require high quality feed to support their growth. Providing additional food in feed has been proven to increase feed efficiency thereby providing benefits to breeders [5].

Bacillus subtilis and *Bacillus coagulans* are two types of useful bacteria to maintain the health of the digestive tract and is found in probiotics. *Bacillus subtilis* and *Bacillus coagulans* is an example of bacteria from the non-pathogenic genus Becillus. *Bacillus coagulans* is one of the lactic acid bacteria (LAB) which produces lactic acid as its final product. *Bacillus subtilis* as a probiotic it can produce 66 antimicrobial compounds such as polymyxin, bacitracin, and subtilin which can control the development of microorganisms in the digestive tract. *Bacillus subtilis* functions as additional feed for animals, increases the immune response, can act as a prophylaxis against bacterial diseases by balancing intestinal microbes, increases digestibility, and can secrete protease, lipase and amylase enzymes [6].

The small intestine is the main organ where digestion and absorption of digestive products takes place. Based on its anatomy, the small intestine can be divided into three parts, namely the duodenum, jejunum and ileum. A healthy broiler chicken digestive tract is characterized by the development of weight and length of the digestive tract as well as optimal development of intestinal villi so that it can optimize nutrient absorption [7]. Based on this background, further research can be carried out regarding the effect of administering liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* on the histomorphology of the duodenum of broiler chickens.

2. Material and methods

This research was carried out in October-December 2021. The implementation of adaptation, induction and therapy treatment on experimental animals of Lohmann strain broiler chickens was carried out in the Experimental Animal Cage, Faculty of Veterinary Medicine, Universitas Airlangga. The experimental animals were adapted for 7 days before being given treatment. Histology preparations were made at the Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, as well as observations of duodenal histomorphology and data analysis were carried out at the Pathology Laboratory, Faculty of Veterinary Medicine, Wijaya Kusuma University. Histological preparations of the duodenum are made using staining Hematoxillin-Eosin (HE).

2.1. Materials

The research materials used were 24 Lohmann strain broilers aged 20 days, probiotics Bacillus subtilis code FNCC 0059 then *Bacillus coagulans* concentration 1x107 CFU / ml, chicken drinking water, and broiler feed Hi-pro-vite CP511B Part 2: Early Period (Starter). The research equipment used was a plastic chicken battery cage measuring 120 cm x 56 cm x 35 cm along with a place to eat and drink, plastic bottles, masks, gloves, paper labels, shovels, trash bags, syringes, digital scales, cooler boxes, plastic bags, and disinfectant.

2.2. Animals

The chickens used were 24 broilers aged 20 days with a body weight ranging from 1.5 kg/head. The cage used is a battery cage made of plastic measuring 120 cm long; width 56 cm; and 35 cm high. Each cage room is equipped with a feeder and drinking water. 1 cage room contains 1 chicken. The cage is cleaned first before the chicken is put in, then sprayed with disinfectant. Feeding and drinking areas must be cleaned. Chickens were randomly assigned to 4 treatments and 1 week of adaptation period before being given treatment.

2.3. Methods

Research using liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* and experimental animals in the form of 24 Lohmann strain broilers. The experimental animals were divided randomly into 4 groups as follows: Control group: Chickens were only given food and drink without being treated with liquid probiotics Group 1: Chickens were given liquid probiotics at a dose of 2 ml/head/day orally Group 2: Chickens were given liquid probiotics at a dose of 4 ml/head/day orally Group 3: Chickens were given liquid probiotics at a dose of 6 ml/head/day orally.

Probiotics were given to chickens once a day in the afternoon for 14 days. On the 41st day, the chickens were euthanized. Next, surgery is performed on the broiler 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. Organ cuts the duodenum taken is stored in a container containing 10% formalin liquid. 10% formalin-fixed duodenal sections were embedded in paraffin and stained with Hematoxylin eosin (HE). Histological preparations that were ready on a glass object were examined using a microscope at 40x magnification. The duodenal organ that has been removed is then placed in a 10% formalin solution and then made a histology preparation for examination.

2.4. Examination of histology preparations

Histology preparations are made using a method consisting of tissue fixation, incubation, and staining. Histomorphological examination of the duodenum using a microscope equipped with a digital camera, the parameter observed was the length of the duodenal villi by measuring from the base of the duodenal villi to the top end, at a magnification of 40 times 5 field of view [8]. Measurements were made using the computer image raster 3.0 program to measure the length of the duodenal villi. For measurements, a randomly selected cross-sectional section of the duodenum with 5 fields of view was used. The criteria for selecting villi are based on the intact lamina propria layer measured at 40x objective magnification. The length of the villi was measured along the apex to the junction of the villi crypts [9].

2.5. Data analysis

The data obtained will be statistically analyzed using Analysis of Variance (ANOVA). If significantly different results are obtained then yes followed by Duncan's Multiple Distance Test. Statistical analysis using the SPSS 21.0 for Windows program.

3. Results

Research data on the average length of villi in the duodenum of broilers given liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* can be seen with the help of a light microscope with 40x magnification, then photographed with a flat screen computer (Opti Lab) and measurements were carried out using Image Raster software. The research results are presented in the form of photos and measurement results (quantitative). Quantitative data consisting of the length of the duodenal villi were analyzed statistically using the ANOVA method. Data continues with Duncan test to compare each treatment group. Statistical analysis was carried out using the SPSS for Windows computer program.

Histological preparations of duodenal villi were stained with HE staining. HE staining is a general stain to see general tissue morphology. Observation of the preparations using a microscope will show different villi lengths in each treatment. The difference in villi length can be seen in Figure 1 which is the result of measuring the length of broiler duodenal villi observed under a light microscope with 40x magnification. 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 where they have a function important for absorption [10].

Based on the results of observations of histological preparations carried out under a microscope, the histomorphological differences in the length of the duodenal villi were quite significant in each treatment. There was an increase in the length of the duodenal villi in each treatment group compared to the control group but it was still within the normal range (Figure 1). Based on the results of the ANOVA test, a p value <0.05 was obtained, indicating that there was a significant difference in the length of the duodenal villi produced by the treatment group compared to the length of the duodenal villi produced by the control group, then continued with the test.

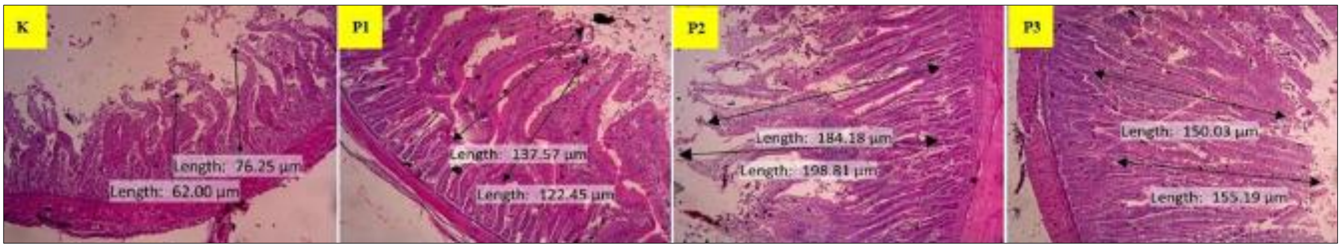

Figure 1 Microscopic image of duodenal villi length (black arrow). Information: P0: The control group was not given liquid probiotics *Bacillus subtilis* and *Bacillus coagulans*. Treatment group 1 was given liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* as much as 2 ml/head/day (P1), 4 ml/head/day (P2) and 6 ml/head/day (P3)

Table 1 Average Length of Duodenal Villi

Group	Villi Length (µm) (Mean ± SD)
K	$68.25^{a} \pm 8.17$
P1	$126.09^{b} \pm 4.14$
P1	$158.72^{c} \pm 2.83$
P3	$199.42^{d} \pm 10.72$

Note: Different superscripts in the same column indicate a real difference. Information: P0: The control group was not given liquid probiotics *Bacillus subtilis* and *Bacillus coagulans*. Treatment group 1 was given liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* as much as 2 ml/head/day (P1), 4 ml/head/day (P2) and 6 ml/head/day (P3).

Advanced test results (test Duncan) in Table 1 shows that there is a significant difference in the length of the duodenal villi produced by the control group. Based on the description above, it can be seen that liquid probiotics are given *Bacillus subtilis* and *Bacillus coagulans* able to increase the length of broiler duodenal villi, and P3 with a dose of 6 ml/head/day had the largest average value of duodenal villi length. The difference in the average length of duodenal villi in the four treatment groups showed a significant influence on the histomorphology of the broiler duodenum. Figure 2 shows a graph of the average length of broiler duodenal villi. Groups P1, P2, and P3 have villi lengths that are significantly different from group K. Group K had the lowest average length of duodenal villi. Group P1 had the lowest average of 68.25 µm between P2 and P3. The P2 treatment group had a higher average than P1 of 126.09 µm. The P3 group had the highest average duodenal villi length of 199.42 µm of all treatments, which shows that the P3 group had a big influence on the presence of probiotics *Bacillus subtilis* and *Bacillus coagulans*.

4. Discussion

The results of this study indicate that giving liquid probiotics Bacillus subtilis And Bacillus coagulans can increase the length of broiler duodenal villi. Based on the results of this study, the P3 group that was given probiotics at 6 ml/head/day had the highest average length of duodenal villi among all research groups. Meanwhile, the lowest average length of duodenal villi was shown in the control group, followed by the P1 group.

Broilers given liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* 6 ml/head/day had the highest duodenal villi length compared to broiler chickens given probiotics of 2 and 4 ml/bird/day orally. At the highest dose of P3, namely 6 ml, there was a significant difference in the length of the duodenal villi compared to P1 and P2. The P3 group has the highest average villi length because on the surface of the villi there are microvilli as cytoplasmic extensions which can increase absorption efficiency. The greater the surface area of the duodenal villi, the greater the opportunity for absorption from the digestive tract [11]. Leeson et al. [12] stated that in birds given Bacillus sp. will experience better development of duodenal villi compared to birds without treatment Bacillus sp. Bacillus sp. has a role in the growth performance of broiler duodenal villi. Lenhardt and Mozes [13] stated that the longer the duodenal villi, the greater the effectiveness absorption of food essences through the duodenal epithelium. Increasing the height and width of the villi can provide a greater surface area for nutrient absorption and lead to better intestinal performance [11, 14]. The ratio of villi height and crypt depth is an indication of the wider absorption area in the digestive system.

Bacillus coagulans can grow well in the chicken's digestive tract because the condition of the chicken's digestive tract is in accordance with the living requirements of these two bacteria, such as nutrient availability, pH, temperature and humidity. The pH range of the small intestine is 5.59-6.62. *Bacillus coagulans* It is known as a producer of lactic acid which has the characteristics of being resistant to high temperatures, so that it creates an acidic atmosphere and grows well in the small intestine, can balance intestinal flora, is antagonistic to pathogenic bacteria, helps digestion, produces anti-several disease compounds, produces several vitamins and so on [6].

Bacillus subtilis is a gram-positive bacteria that is useful for killing pathogenic bacteria because it can produce bacteriocins. Broilers given probiotics Bacillus subtilis will have a good effect on health. Probiotics *Bacillus subtilis* can change the pH of the intestine to become acidic and produce compounds that are similar to antibiotics. Probiotics from bacteria *Bacillus subtilis* serves as additional feed in animals which can act as a prophylaxis against bacterial diseases by balancing intestinal microbes, and increasing digestibility, increasing immune response [15, 16].

The larger the dose of liquid probiotics *Bacillus subtilis* and *Bacillus coagulans.* The longer the duodenal villi are because the probiotics given can help digestion, produce substances or vitamins which result in increased appetite resulting in increased body weight, as well as an increase in the immune system. Another cause that causes an increase in the length of the duodenal villi is probiotics *Bacillus subtilis* and *Bacillus* coagulans in the small intestine produces several enzymes that help digest food substances needed for chicken growth [17]

Effect of probiotics *Bacillus coagulans* and *Bacillus subtilis* greatly affects the broiler duodenum, especially the duodenal villi. Duodenal villi influence the absorption process. The morphological integrity of the intestine can be used as a basis for evaluating the normal physiology of the organ. Intestinal epithelial cells originating from the base of the crypts migrate upward and move along the surface of the villi to the tips of the villi to replace exfoliated epithelial cells. Longer villi can increase nutrient absorption in the small intestine due to the greater surface area available [18].

The reduced length of duodenal villi is due to probiotics *Bacillus subtilis* and *Bacillus coagulans* not yet given. This is due to the increase in duodenal villi which are bacteria *Bacillus coagulans* and *Bacillus subtilis* in the small intestine produces several enzymes to help digest food substances needed for growth by chickens, in accordance with the opinion Soesanto [15] that bacteria *Bacillus sp* can produce various types of enzymes such as *Bacillus sp*. can inhibit the growth of several pathogenic microbes, and can form a symbiotic relationship with other beneficial microbes in the poultry digestive tract. The decomposition of complex components of rations into simpler components by digestive enzymes can be increased by adding enzymes produced by microbes to the rations given to chickens [19].

The difference in length of duodenal villi in each treatment was caused by probiotics *Bacillus coagulans* able to improve the microbial balance of broiler intestines, especially the duodenum so that it can stimulate broiler appetite. Fuller [20] added that intestinal microbial balance is achieved when beneficial microorganisms can suppress harmful microorganisms. Harmful microorganisms are pushed out of the digestive tract ecosystem by normal digestive tract microbes or beneficial microbes. Meanwhile probiotics *Bacillus subtilis* able to increase digestibility and has the function of being able to secrete protease, lipase and amylase enzymes, so there is a possibility that it plays a role in feed digestion. The role of the protease enzyme is of course to increase the absorption of amino acids because the protease enzyme can optimize the breakdown of protein into amino acids in the small intestine. *Bacillus subtilis* in broiler chickens it has been proven to be able to reduce broiler pathogenic microbes, improve feed conversion, body weight, mortality and survival ability of broilers.

Absorption of food that has been completely digested takes place in the small intestine through two channels, namely blood capillaries and lymph channels in the villi [21]. 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, 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 [22]. 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 [21].

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 absorptive cells of the villi and transported by Blood goes to the liver through 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 [22].

The ability to digest and absorb food substances can be influenced by the surface area of the intestinal epithelium, the number of folds and the number of villi and microvilli which expand the area of absorption and are also influenced by the height and surface area of the villi, duodenum, jejunum and ileum. The surface area of the small intestine, such as the height of the villi, describes the area for absorption of nutrients. Villi are small finger-like or leaf-like projections found on the mucous membrane, 0.5-1.5 mm long and only found in the small intestine [23]. 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 greatest [24]. Absorption in the duodenum is further expanded by the presence of these villi.

One parameter that can be used to measure the quality of growth is the morphological structure of the intestine. The small intestine is composed of four layers, namely the tunica mucosa, tunica submucosa, muscularis externa, and tunica adventitia. On the tunica mucosa there are projections or what are also called villi which function as absorption areas. The higher the protrusions or villi, the wider the absorption area and the smoother expression of the nutrient transportation system throughout the body [25]. The aim of providing probiotics is to control the ecosystem and maintain the health of the digestive tract so that the absorption process can be maximized [2]. This study used samples in the form of broiler duodenum that had been given probiotics *Bacillus subtilis* and *Bacillus coagulans* as much as 2 ml, 4 ml, and 6 ml. Probiotics were given orally for 14 days before organ harvesting.

5. Conclusion

Giving liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* with graded doses was able to increase the length of duodenal villi in broilers, especially the P3 group with a dose of 6 ml/head/day. Group P3 had a significant difference in average length of duodenal villi from groups P1 and P2. P3 had the highest average value of 199.42 μm with liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* with a dose of 6 ml/head/day compared with groups P1 and P2, it can be concluded that liquid probiotics were given *Bacillus subtilis* and *Bacillus coagulans* has a good impact on the duodenum so that the absorption of food substances is maximized. Healthy villi growth can increase nutrient absorption so that it has a good impact on intestinal performance and increase in broiler body weight. Suggestions that can be conveyed in this research are liquid probiotics *Bacillus subtilis* and *Bacillus coagulans* can be applied in additional feed so that it can improve broiler digestion.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The study was approved by the Faculty of Veterinary Medicine Animal Ethics Committee of Universitas Airlangga. All variables were considered in accordance with the Ethics Committee related to the animal handling to ensure no discomfort or pain was caused to the animals during sampling (certificate registration number: 1.KE.118.10.2021.

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