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Effect of *Bacillus amyloliquefaciens* as probiotic on total colonies of bacteria, pH and cellulase activity in the small intestine of domestic chicken

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Abstract

This study aimed to determine the effect of the addition of *Bacillus amyloliquefaciens* into drinking water on the total colonies of *Bacillus amyloliquefaciens, Lactobacillus* sp, pH, and cellulase activity in the small intestine of domestic chicken in starter period. This study was used 200 days old of domestic chicken CP 808 kept for 8 weeks. Type of cage used 20 units cage measuring 85 cm x 70 cm x 30 cm which each unit contained 10 chickens. The study used the Completely Randomized Design (CRD) with 4 treatments in 5 replications. Treatment was the addition of *B. amyloliquefaciens* into drinking water (without *B. amyloliquefaciens*, 45 x 10^9 CFU/ml, 65x10^{10} CFU/ml, and 43 x 10^{12} CFU/ml). The variables observed were the total colonies of *B. amyloliquefaciens, Lactobacillus* sp, pH, and cellulase activity in the small intestine of domestic chicken. The results showed that addition of *B. amyloliquefaciens* in drinking water of domestic chicken increased the total colonies of *B. amyloliquefaciens*, *Lactobacillus* sp, and cellulase activity in the small intestine, but did not affect the pH. The highest total colonies (*B. amyloliquefaciens* and *Lactobacillus* sp) and cellulase activity were found on the addition of *B. amyloliquefaciens* at a level of 43 x 10^{12} CFU/gram. Meanwhile, the pH intestine added *B. amyloliquefaciens* tended to be lower than without *B. amyloliquefaciens*.

Keywords: Cellulase activity, Domestic chicken, pH, Total colonies, Waretha probiotic

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Introduction

Livestock is progressing rapidly in Indonesia due to the increasing public understanding of animals need as a source of protein. One livestock that provides animal protein for humans is poultry, such as domestic chicken farmed in rural areas. So far, the potential of domestic chicken has not been utilized optimally, it can be identified by several factors, such as the traditional maintenance system, the high mortality rate, badly housing system, and inadequate feeding. According to Rasyaf (1998), traditional maintenance caused the population and productivity of domestic chicken to be smaller than actual biological abilities.

The increasing demand for domestic chicken has led to increase attention of farmers to improve the quality of livestock and maintain the chicken to stay healthy by improving the feed quality. One popular method is

to add antibiotics compound. Antibiotic compounds are useful for promoting livestock growth, increasing feed efficiency and reducing mortality. But, according to Kompiang (2002), the use of antibiotics has begun to be prohibited currently because it left residues in the carcass when consumed by humans. Therefore, it is necessary to look for other alternatives to replace antibiotic compounds in livestock products, such as probiotic. According to Riaz et al. (2015), probiotic generally recognized as safe for consumption. Nahrowi (2006) stated that the probiotic is an alternative source of antibiotic substitutes.

Probiotic contains microbial non-pathogenic (bacteria, yeast, fungi) and can be used to increase growth, ration conversion efficiency, and chicken health (Stark and Wilkinson, 1989). Ray (1996) stated that probiotic is useful in preventing the reaction of pathogenic bacteria, stimulating intestinal peristaltic activity, detoxifying poisons in food, and providing enzymes to help digest some foodstuffs. The type of microbes used as probiotics depend on the chemical and physical properties of the digestive. Some digestive organs of chicken (crop, proventriculus and gizzard) have high acidity, therefore microbes used must be suitable for acid condition.

Bacillus sp is not commonly found in the digestive tract of chickens but it has ability to control pathogenic bacteria (Barrow, 1992). According to Jin et al. (1996), *Bacillus* sp included in feed or as a probiotic can increase the *Lactobacillus* sp number in the small intestine due to it is able to colonize the gastrointestinal wall and increases the natural *Lactobacillus* sp number, thereby suppressing undesirable microorganisms, such as *Escherichia coli*, and *Salmonella* sp. The addition of probiotic into drinking water serves to maintain the balance of the microflora in the digestive tract and provide the enzymes needed to digest crude fiber, protein, and fat (Soeharsono, 1999).

Waretha probiotic is cellulolytic and can reduce crude fiber because it produces extracellular cellulase and hemicellulase. Waretha probiotic contains *B. amyloliquefaciens* which is a sub species of *Bacillus subtilis* and serves to stimulate the body's immunity (Wizna et al., 2007). It lives in association with broiler intestinal wall with a population of 6 x 10^6 CFU/gram and produces cellulase activity 7,681 units/ml in the small intestine, and also can increase *Lactobacillus* sp and suppress *Escherichia coli* population. *B. amyloliquefaciens* has cellulase activity Cx and C1 of 0.873 and 0.259 units/ml higher than *Trichoderma harzianum* i.e 0.655 and 0.307 units/ml (Wizna et al., 2007). Moreover, *B. amyloliquefaciens* also produces enzymes such as *alpha-amylase, alpha-acetolactate decarboxylase, beta-glucanase, hemicellulase, maltogenic amylase, urease, protease, xylanase* and *chitinase* (Luizmeira, 2005).

The oral suspension of *B. amyloliquefaciens* (6x10^6 CFU/ml) of broilers when day-old chicks (DOC) on the pathogenic test was obtained 19.58 x 10^{10} CFU/ml in fresh intestine at 5 weeks old (Wizna, 2006). The addition of *B. amyloliquefaciens* when day-old chicks is only needed once during the maintenance period (10^{12} CFU/ml/individual) for broilers and three weeks for laying hens (10^{12} CFU/ml). Zurmiati et al. (2017) found that the addition of probiotic containing *B. amyloliquefaciens* through drinking water to 3,000 ppm (43x10^{12} CFU/gram) for 6 weeks increased the total colonies of *Bacillus* sp and reduced pH in the small intestine. It is necessary to study more the effect of Waretha® probiotic on total colonies of *B. amyloliquefaciens*, *Lactobacillus* sp, pH and cellulase activity in the small intestine domestic chicken.

Material and Methods

The study was conducted at Poultry Farms and Laboratory, Faculty of Animal Science Universitas Andalas for 8 weeks of chicken breeding research in the cage and continued to laboratory. The study used *B. amyloliquefaciens* (Waretha probiotic) which was supplemented into drinking water for periods of domestic chicken starter. There were 200 strains of unsex one-day-old domestic chicken of CP 808 used in 20 cage units. Each treatment consisted of 10 chickens placed into cage units, each size of 85 cm x 70 cm x 30 cm. The cage unit was equipped with a dining room, drinking area, and 60 watt incandescent lamp for 3 weeks old or until the feathers grew. After that, the incandescent lamp was turned on at night only and equipped with dining and drinking areas, scale, and chicken dropping. The treatment ration material consisted of 52% Br 511 commercial rations, 33% rice bran and 15% corn (Table 1 and Table 2).

Table-1. Ingredient and nutrient content of the diets for domestic chicken

Ingredient	Nutrient content					
	Crude protein (%)	Crude Fat (%)	Crude Fiber (%)	Calcium (%)	P available (%)	Metabolizable Energy (Kcal/kg)
Br 511[a]	21.50	5.00	5.00	0.90	0.60	3025
Rice bran[b]	11.00	6.29	12.5	0.10	0.21	1630
Corn[c]	8.4	2.66	4.57	0.37	0.1	3370

[a]Label of Commercial ration CP Br 511, [b]Adha (2015), [c]Scott et al. (1982)

Table-2: Nutrient content of the basal diets for domestic chicken

Nutrient content	Basal diet (%)*
Crude protein (%)	16.07
Crude fat (%)	5.07
Crude fiber (%)	7.41
Calcium (%)	0.55
P available (%)	0.39
Metabolizable energy (Kcal/kg)	2616.40

*Calculated based on analyzed ingredient composition

Treatment

The study was designed by using Completely Randomized Design (CDR) with 4 treatments and 5 replications. Each experimental unit consisted of 10 chickens. The treatments were 4 levels of Waretha probiotics through drinking water: W0 (without Waretha probiotic); W1,1 gram/liter (45 x 10^9 CFU/ml); W2, 2 gram/liter (65 x 10^{10} CFU/ml); W3-3 gram/liter (43 x 10^{12} CFU/ml).

Making Waretha probiotic from *B. amyloliquefaciens*

Waretha probiotic was made from 100 grams of rice bran, sterilized using an autoclave for 15 minutes at 120°C 1 atm, then cooled at room (24°C). Ten ml of distilled water were put into a petridish overgrown with pure culture of *B. amyloliquefaciens*, then the petridish was shaken slowly until mixed and then put into an enlemeyer tube containing distilled water as much as 190 ml. Sterile rice was mixed with 200 ml suspension of *B. amyloliquefaciens*, incubated for 24 hours at 40°C and then dried in an oven at 60°C. The powder was used as an inoculum with a *B. amyloliquefaciens* population of 1,011 CFU/g.

The addition of Waretha probiotic in starter chickens was done by dissolving 1 gram of probiotic concentrate 45x10^9 CFU/g on one liter of water, then stir well. It was given to 10 chickens that have been served for two hours before 11 am to 1 pm. Then the water was poured into one gallon of drinking water to be given to starter chickens.

$$\text{Dilution probiotic} = \frac{45 \times 10^9}{1000} = \frac{45000 \cdot 10^6}{1000} = 45 \times 10^6 \text{ CFU/ml}$$

On the first day of chicken arrival, sugar water was provided to restore chicken stamina, providing a ground floor with newspapers and heating lamps that have been turned on. Waretha probiotics were given on 4th day, 4th week and 8th week according to treatments.

The Waretha probiotic calculated as follows: the cage was given the number and the treatment was placed randomly inside the cage. Placement was done by taking 10 randomly and then weighed and searched average weight as a benchmark, then taken 2 levels below and 2 levels above the weight of the benchmark. The Chickens were cut after 8 weeks, and the small intestines were taken to be analyzed in the laboratory.

Total colonies of Bacteria on small intestine

The calculation of *B. amyloliquefaciens* and *Lactobacillus* sp population used a dilution method and total plate count (Cappucino and Sherman, 1987; Hadioetomo, 1991). The small intestine was cut as much as 1 gram, mashed and diluted from 10^{-1} to 10^{-7}. One ml of suspension was inserted into a test tube 1 containing 9 ml distilled water, then shaken until homogeneous using vortex, hence a dilution of 10^{-1} was formed. One ml of 10 dilutions was transferred with a sterile micro pipette into the 2nd test tube which also contained 9 ml of distilled water, then shaken until homogeneous and 10 dilutions were formed became 1 ml of each dilution 10^{-3}, 10^{-5}, and 10^{-7} samples were put into a petridish filled with selective Bacillus media and selective medium *de Man Rogosa Sharpe* (MRS) broth for *Lactobacillus* sp, then incubated at room (± 28°C) for 24 hours for *Bacillus* sp and 48 hours for *Lactobacillus* sp. Then the colonies grown in petridish were calculated using a colony counter, assumed that one colony came from

one spore.
The formula to determine the total bacterial colony (CFU/g) as follows:

$$= \text{total colony} \times \frac{1}{\text{dilution}} \times \frac{1}{\text{sample (g)}}$$

pH Intestine
Determination of pH intestine was carried out using the electrometric method. PH measurement was done by cutting the intestine and taking as much as 1 gram of intestinal fluid, then inserted into a measuring cup containing 10 ml of distilled water, then homogenized and left for 30 minutes and then measured using a pH meter.

Cellulase enzyme activity
Samples were taken from 8 weeks old chicken to see the cellulase activity curve. The small intestine of each group was collected and homogenized by 1 ml by adding 75 ml of 0.05 M phosphate buffer (pH 7), shaken and filtered. It was put into a 250 ml Erlenmeyer which had been placed in a 500 ml glass beaker containing ice cubes. During the filter, the filtrate was stirred at low speed and the temperature was kept cold (4°C). The filtrate was centrifuged 4,500 rpm for 30 minutes in that cold condition, then the crude filtrate extract enzyme was obtained.
Cellulase activity test was determined by Somogyi-Nelson (Nelson, 1944). The substrate of CMC 1% of 0.5 ml and 0.5 ml enzyme filtrate were inserted into a test tube, incubated for 30 minutes at 40°C in the shaking water bath, then added 1 ml of Nelson AB solution, heated in boiling water for 20 minutes. After cold, 1 ml of phofatmolibdat and 7 ml of distilled water were added, at a wavelength of 575 nm. The magnitude of cellulase activity used the following formula:

$$\text{Enzyme Activity (U/ml)} = \frac{X \times P \times 1000}{T \times BM}$$

X = Result of conversion standard curve
P = Dilution
T = Time
BM = Molecular weight of glucose

Data analysis
The variables observed were total colonies of *B. amyloliquefaciens*, *Lactobacillus* sp, pH, and cellulase activity in the small intestine. Data were analyzed statistically using analysis of variance (ANOVA) and Duncan's New Multiple Range Test (DNMRT) at a significant level of 5% (Steel and Torrie, 1980).

Results and Discussion

Total colonies of B. amyloliquefaciens
The addition of *B. amyloliquefaciens* (Waretha probiotic) in drinking water for domestic chickens has increased total colonies of *B. amyloliquefaciens* and *Bacillus* sp in small intestine of the starter period. The highest colonies was found in W3 treatment (78.8 x 10^7 CFU/g). The increase of total colonies *B. amyloliquefaciens* occurred along with the increased dose of Waretha® probiotic given in each treatment (Table 3).

Table-3: The colonies of *Bacillus* sp and *B. amyloliquefaciens* on domestic chickens

Treatment	Total colonies of *Bacillus* sp + *B. amyloliquefaciens* (CFU/g)	Total colonies *B. amyloliquefaciens* (CFU/g)
W0 (Non-Waretha® probiotic)	39.8 x 10^5 d	0
W1 (45 x 10^9CFU/ml)	22.4 x 10^7 c	22.0 x 10^7
W2 (65 x 10^{10}CFU/ml)	35.4 x 10^7 b	35.0 x 10^7
W3 (43 x 10^{12}CFU/ml)	79.2 x 10^7 a	78.8 x 10^7
Error standard	0.06	

The total increase in *B. amyloliquefaciens* colonies in the small intestine occurred in each treatment due to the level given to each treatment also increases, so that the total colony also increased in the small intestine. Waretha® probiotics could grow well in the digestive system of chicken because the conditions of the intestinal system was suitable to life needs of bacteria, such as availability of nutrients, pH, temperature, and humidity. The pH range for monogastric animals is usually reported at 5.59 - 6.62 (Ao et al., 2008). *B. amyloliquefaciens* is called as probiotic because it can live in environmental conditions with a pH of 4-6, humidity of 50-90% and temperature of 25-33% (Sutedjo et al., 1991).
In W0 treatment (control), there was still *Bacillus* sp in the small intestine, which was 39.8 x 10^5 CFU/gram, because the bacteria was natural bacteria contained in the small intestine. This result was lower than that obtained by Sjofjan (2003) which obtained total colonies of *Bacillus* sp in the small intestine as much as 8.2 x 10^9 CFU/g. The lower of total colonies of

Bacillus sp in the small intestine in this study compared to the Sjofjan (2003) was caused by differences in livestock species, age and environment. According to Gabriel et al. (2006), factors that affected the total colonies of microflora in the intestine included internal factors, age of livestock, environment, and feed consumption.

Sumardi et al. (2012) said that *Bacillus* sp is a natural microflora of chicken intestine. According to Barbosa et al. (2005), the species of *Bacillus* sp existed in chicken intestines, including *B. subtilis, B. pumilus, B. licheniformis, B. clausii, B. gaterium, B. gaterium, B. gaterium, B. firmus,* and *B. cereus*. Therefore, the total bacterial colonies that can be obtained in the small intestine were a mixture of all Bacillus species, which was dominated by *B. amyloliquefaciens*, because the addition of Waretha® probiotics to level 43 x 10^{12} CFU/g. Budiansyah (2004) explained that the mechanism of probiotic is to stick and colonize the digestive tract and survive to get food and produce antimicrobial substances.

The Total colonies of *B. amyloliquefaciens* in this study ranged from 0 to 78.8 x 10^7 CFU/g. This result was lower than Wizna (2006) who obtained *B. amyloliquefaciens* (6 x10^6 CFU/g) in broilers when day-old chicks from pathogenic tests obtained 19.58 x 10^{10} CFU/g of fresh intestine at chicken in 5-weeks-old. The lower of total colonies of B. *amyloliquefaciens* in this study compared to the Wizna (2006) was caused by differences in livestock species and age of livestock during the study.

Total colonies of Lactobacillus sp

The addition of *B. amyloliquefaciens* (Waretha probiotic) in drinking water for domestic chicken has increased total colonies of *Lactobacillus* sp in small intestine of the starter period. The highest colonies was found in W3 treatment (34.8 x 10^7 CFU/g). The increase of total colonies *Lactobacillus* sp occurred along with the increased dose of *B. amyloliquefaciens* given in each treatment (Table 4). The results showed that the *B. amyloliquefaciens* colonize with *Lactobacillus* sp in the small intestine of domestic chickens. Jin et al. (1996) stated that *Bacillus* sp will colonize in the small intestinal wall and help to increase *lactobacillus* in the gastrointestinal. [Table 4] The total colonies of *Lactobacillus* sp obtained in the study ranged from 26.6 x 10^5 to 34.8 x 10^7 CFU/gram. This result was higher than the research conducted by Tang et al. (2017) which obtained total colonies of *Lactobacillus* sp ranged from 7.46 to 7.80 log 10 (CFU/g) in the digestive tract of laying hens.

Table-4: Total colonies of *Lactobacillus* sp in small intestine of domestic chickens

Treatment	Total Colonies (CFU/g)
W0 (Non- Waretha Probiotic)	26.6 x 10^5 d
W1 (45 x 10^9CFU/ml)	11.2 x 10^7 c
W2 (65 x 10^{10}CFU/ml)	21.4 x 10^7 b
W3 (43 x 10^{12}CFU/ml)	34.8 x 10^7 a
Error standard	0.06

Number followed by same letter on different row indicates no significant differences with DMRT 5%

Lactobacillus sp also found in W0 treatment without Waretha® probiotic (26.6 x 10^5 CFU/g) due to *Lactobacillus* sp is natural microflora that contained in chicken intestine. According to Daud et al. (2007), microbes can grow and develop in the chicken intestine, among other types of Lactic Acid Bacteria (LAB), *Bacillus* sp and *Lactobacillus* sp. Probiotics as living microbes or spores can live and grow in the intestines and benefit the host either directly or indirectly from their metabolites so that beneficial microbes can develop (Kompiang, 2009). Giving beneficial microbes that produce microbial colonization in the digestive tract of its host causing faster growth, more resistance to certain diseases, and an increase in metabolism of food substances (Hooper et al., 2001; Stappenbeck et al., 2002).

pH intestine

The addition of *B. amyloliquefaciens* (Waretha probiotic) in drinking water to 3000 ppm (43x10^{12} CFU/g) did not affected the pH intestine of domestic chicken significantly in starter period. The highest pH was found in the W0 treatment (6.16) and the lowest was in the W3 treatment (5.7) (Table 5). It was because lactic acid produced by natural microflora in the small intestine was a little, along with the total colonies produced not too high up to 10^7 CFU/g. Manin et al. (2007) found natural *Lactobacillus* sp in the small intestine was 11-19 x 10^7 CFU/ml, it was lower than this study of 34.8 x 10^7 CFU/g.

The pH of the small intestine of domestic chicken obtained in the study ranged from 6.16 to 5.7. The decrease of pH in the small intestine was not much different with Zurmiati et al. (2017) who found the pH of the small intestine from 6.14 to 5.53 in the duck's

small intestine given Waretha® probiotic.

Table-5: The pH of small intestine of domestic chicken

Treatment	pH of small intestine
W0 (Without Waretha® probiotic)	6.16^{ns}
W1 (45 x 10^9CFU/ml)	5.88 ^{ns}
W2 (65 x 10^{10}CFU/ml)	5.84 ^{ns}
W3 (43 x 10^{12}CFU/ml)	5.70 ^{ns}
Standard error	0.11

ns = non significant

According to Lopez (2002), giving probiotic bacteria will produce acid that can reduce pH in the digestive tract. Wu et al. (2011) also stated that *B. amyloliquefaciens* produces lactic acid and bacteriocin which can decrease the pH of the intestine and play an important role in *Lactobacillus* colonization and suppress growth *E. coli*.

Cellulase activity

The addition of *B. amyloliquefaciens* (Waretha probiotic) in drinking water for domestic chicken has increased cellulose activity in small intestine of the starter period. The highest colonies was found in W2 and W3 treatment (34.8 x 10^7 CFU/g). The higher the dose of Waretha® probiotics, the more cellulase activity in the small intestine of domestic chickens increased (Table 6). It was due to the *B. amyloliquefaciens* can reduce crude fibers and produce extracellular enzymes cellulase and hemicellulase to help digest the nutrients needed for growth by chickens (Wizna et al., 2007). According to Cowan and Still (1973) and Alexander (1997), *Bacillus* sp can produce various enzymes such as cellulase, hemicellulase, proteases, alpha-amylase, urease, xylanase, and chitinase.

Table-6: Cellulase activity in the small intestine of domestic chickens

Treatment	Cellulase Activity (Unit/ml)
WO (Without Waretha® probiotic)	0.335 c
W1 (45 x 10^9 CFU/ml)	0.731 ab
W2 (65 x 10^{10} CFU/ml)	0.950 a
W3 (43 x 10^{12} CFU/ml)	1.085 a
Standard error	0.110

Number followed by same letter on different row indicates no significant differences with DMRT 5%.

The cellulase activity was obtained in this study ranged from 0.335 to 1.085 units/ ml. This result was lower than that found by Wizna et al. (2007) which was 6 x 10^6 CFU/ g of *B. amyloliquefaciens* which lived on the broiler intestinal wall and produced cellulase activity of 7,681 units/ml in the small intestine. The lower activity of cellulase enzymes in this study compared to the Wizna (2007) was caused by differences in livestock species, and age of livestock during the study.

In the treatment without *B. amyloliquefaciens* (W0) there was still cellulase activity due to microbes in the digestive tract produce enzymes needed for digestion process, such as cellulose. According to Rizal (2006), in the digestive tract of chicken, there is various kinds of beneficial bacteria such as cellulolytic bacteria, which dominate the digestive tract of chicken in addition to bacteria from the amylolytic group. These bacteria can degrade cellulose into substances that are more easily absorbed by the body.

Conclusion

The addition of *B. amyloliquefaciens* in drinking water of domestic chicken increased the total colonies of *B. amyloliquefaciens*, *Lactobacillus* sp, and cellulase activity in the small intestine, but did not affect the pH intestine. The highest total colonies both *B. amyloliquefaciens* (78.8 x 10^7 CFU/g) and *Lactobacillus* sp (34.8 x 10^7 CFU/g) and cellulase activity (1.085 unit/ml) were found in the addition of *B. amyloliquefaciens* at a level of 43 x 10^{12} CFU/ml. Meanwhile, the pH of the small intestine given *B. amyloliquefaciens* ranged from 5.70 - 5.88, it tended to be lower than without *B. amyloliquefaciens*.

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Contribution of Authors

Wizna: Conceived idea, conducted experiment and write up of article

Yanti EP: Helped in experiment, compilation of results and statistical analysis
Amizar R: Helped in experiment and article write up

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