Proceedings International Conference on Science and Technology (ICST)

e-ISSN: 2722-7375 **pp: 123-128, Vol. 1, Juni 2020**

VIABILITY OF BACTERIA USED IN PRODUCTION OF SYNBIOTICS FOR LAYING HENS

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Abstract. The study aimed to evaluate the viability of probiotics bacteria used in the production of synbiotics. This study used factorial completely randomized design 4 × 2 with 3 replications. The first treatment consisted of four bacteria isolates including B_{299} isolates, C_{610} isolates, D_{610} isolates, and E_{610} isolates. The second treatment was two prebiotics including rice bran prebiotics supplemented by egg hatching waste and rice bran prebiotics without supplemented. The suspension of probiotics bacteria (OD_{600}: 0,2) was sprayed 6 ml into 20 g prebiotics relatively and dried in an incubator with temperature 43 ºC for 24 hours. The measure of bacteria viability was carried out using the spread plate method on the first, third and fifth days. The results of this study showed that the strains of probiotics bacteria and prebiotics and interactions both did not show a significant difference (P>0,05) to the viability of probiotics bacteria; the average number of probiotics bacteria in rice bran prebiotics supplemented by egg hatching waste was 5,68 ± 1,14 log CFU/g for B_{299} isolates, 4,76 ± 1,43 log CFU/g for C_{610} isolates, 5,84 ± 1,49 log CFU/g for D_{610} isolates and 6,98 ± 1,39 log CFU/g for E_{610} isolates; the average number of probiotics bacteria in rice bran prebiotics without supplemented was 5,76 ± 1,61 log CFU/g for B_{299} isolates, 6,65 ± 1,24 log CFU/g for C_{610} isolates, 5,84 ± 1,31 log CFU/g for D_{610} isolates and 7,10 ± 0,41 log CFU/g for E_{610} isolates. In summary, the results might determine that the probiotics bacteria have grown with the same viability in synbiotics.

Keywords: synbiotics, probiotics, prebiotics, viability of probiotics bacteria

1. Background

The role of the livestock subsector was considered to provide food that is the source of animal protein for humans. Laying hens are one of the livestock that produce high protein food which is eggs. The productivity of laying hens in Indonesia can only provide 65% of the total needs [1] The productivity of laying hens can be improved by giving *feed additive* which is given in small amounts, but it can stimulate productivity and improve feed efficiency [2]. *Feed additives* used for improving the productivity were enzyme, hormone, antibiotic growth promoter, probiotics, prebiotics and synbiotics [3].

Synbiotics are a combination of probiotics and prebiotics that have many effects, especially to modulate the microbiome in the gastrointestinal tract and reduce the number of pathogen infancy [4]. Synbiotics have many proposed effects to keep the balance of the microbiome components [5]. Probiotics are viable microorganisms that can be used as feed additives that give a positive effect on poultry health [6]. While prebiotics are non-digestible carbohydrates that can stimulate the good microorganism in the gastrointestinal tract. Prebiotic ingredients, such as inulin and oligofructose, are good examples of this processed food category [7].

Synbiotics can improve the performance of intestinal and digestibility through increasing beneficial microorganisms, producing a short-chain acid in the duodenum and jejunum, and increasing the amount of intestinal villi to optimize the process of nutrient absorption [8]. The using of 2% natural synbiotics for chicken can increase the number of lactic acid bacteria in the gastrointestinal tract, reduce the number of *Escherichia coli* in ileum, heighten the intestinal villi in the duodenum, jejunum, and ileum and widen the intestinal villi in the ileum [3].

The production of synbiotics by using local feedstuff needs to be initiated to support the productivity of laying hens. The benefit of synbiotic which is from local feedstuff is not only for health, but it gives the economical benefit because synbiotics can be used as a mixture of feed ingredients to minimize feed cost in laying hens. The local feedstuff is available in large quantities and lower costs. It is the reason why the using of local feedstuff is recommended to produce synbiotics for laying hens.

Rice bran is a local feedstuff which can be obtained from by-product of grinding rice. Rice bran can be used to produce synbiotics because it is available in large quantities. The production of rice in Indonesia reaches 56,54 million tons per year which can produce 4,52 – 5,93 million tons of rice bran per year [9]. Rice bran contains oligosaccharides that are useful for probiotics bacteria, but rice bran has disadvantages; such as low digestibility, low protein content, high crude fiber, and phytic acid content. These problems can be solved by using egg hatching waste to supplement the rice bran for improving digestibility and protein content. The egg hatching waste contains 36,2% of proteins and it contains complete amino acids. The combination of rice bran and egg hatching waste can provide good nutrients for bacteria and poultry (host) [10].

Oligosaccharides from rice bran can grow *Lactobacillus sp*., *Bifidobacterium sp*. and *Bacteroides* [11]. The study aimed to evaluate the viability of probiotics bacteria used in the production of synbiotics for laying hens.

2. Materials and Methods

2.1. Preparation of LB Medium

One-hundred milliliters of LB agar consisted of 1 g tryptone powder, 0.5 mg yeast extract, 1 g NaCl, and 100 distilled water (dH2O). Then, pH was adjusted to be 7.0 with 0.1 M Sodium hydroxide (NaOH) or hydrochloric acids (HCl). 1.5 g Agar was added into the Erlenmeyer to make LB agar. Then the mixture was autoclaved and used for the transformant inoculation of bacteria.

2.2. Isolation and Identification of Probiotics Bacteria

The study was initiated by isolation and identification of bacteria which was sourced from probiotics that had been developed by Ichsan [12]. The isolation and identification were carried out through a few steps namely isolation, purification, Gram's staining, phytase test, catalase test, and endospore test. These steps were carried out until a single colony or pure bacteria was obtained. The pure bacteria that have been isolated were stored using glycerol stock at -33 ºC.

2.3. Preparation of Probiotics Bacteria

After isolated, the pure bacteria were grown in solid LB medium and inoculated into Erlenmeyer. Then the bacteria were taken in *shaker* at 37 ºC for 24 hours. The optical density of bacteria after

grown was measured by using spectrophotometer at 600 nm. The value of optical density which was needed to spray prebiotic was 0,2.

2.4. Preparation of Prebiotics

Prebiotics that was made from local feedstuff consisted of rice bran and egg hatching waste. The prebiotics consisted of 8 samples that were 4 samples of rice bran prebiotic with supplemented by egg hatching waste and 4 samples of rice bran prebiotic without supplemented. Each sample of prebiotic was made and weighed 20 g/sample. The comparison between rice bran and egg hatching waste was 1:1. Each prebiotic weighed was taken into an *aluminium foil* and sterilized by using an *autoclave*.

2.5. Preparation of Synbiotics

Synbiotics are a combination of probiotics and prebiotics. All of the prebiotics were sprayed by using 6 ml of probiotics bacteria (OD_{600} 0,2). The amount of probiotic bacteria sprayed was counted based on 30% prebiotic weight. Synbiotics were dried into an incubator at 43 ºC for 24 hours. After synbiotics dried, the viability of probiotics bacteria was counted by using the spread plate method on the first, third and fifth days.

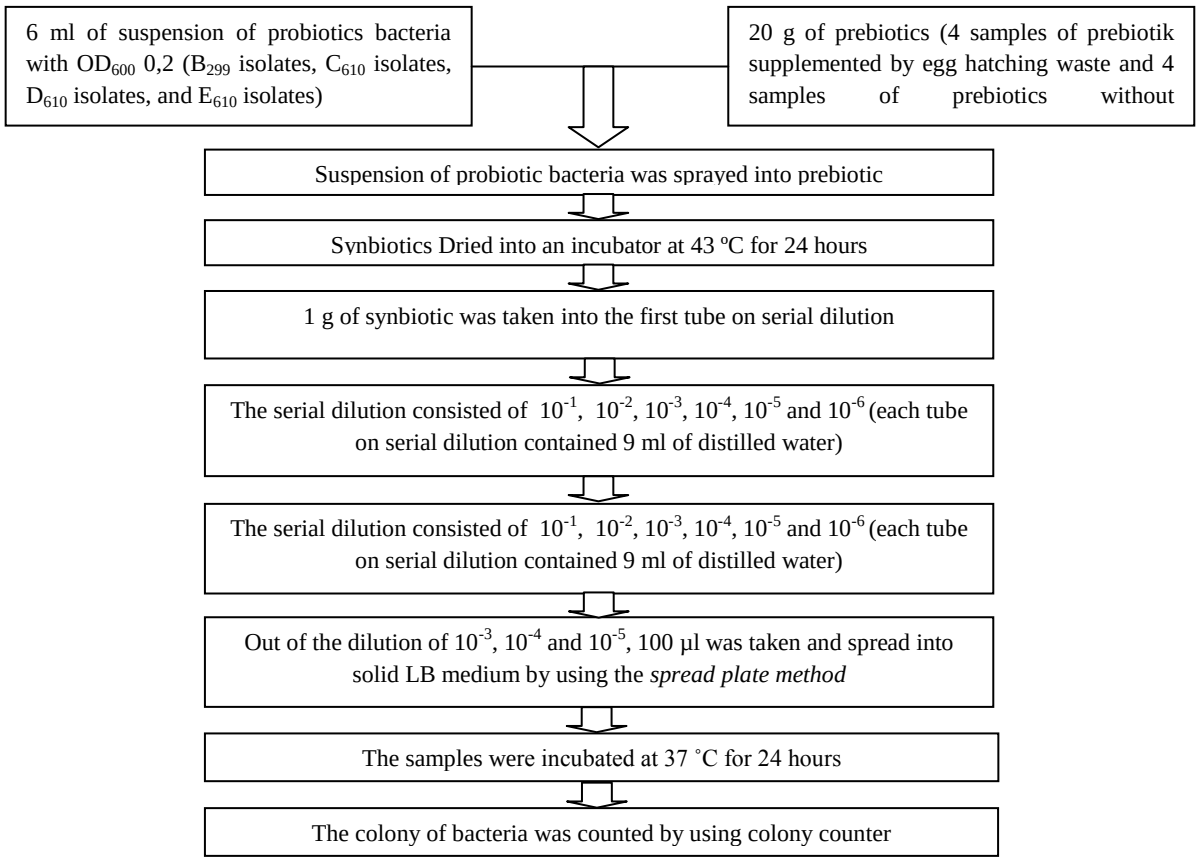

Fig. 1 Schematic representation of the evaluation of the viability of probiotics bacteria in synbiotics

2.6. The Viability Test of Probiotics Bacteria in Synbiotics

The viability test of probiotics bacteria in synbiotics was carried out by using the spread plate method, which was carried out using six times of serial dilution. The distilled water was added into each tube. Then 1 g of synbiotic was weighed and taken into the first tube on serial dilution. Each tube on serial dilution was added with 9 ml of distilled water (dH_2O). The dilution of bacteria was carried out by serial dilution 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Out of the dilution of 10^{-3}, 10^{-4} and 10^{-5}, 100 µl was

taken and spread into a solid LB medium by using the spread plate method. Then the samples were incubated in an incubator at 37 ˚C for 24 hours. Each colony of bacteria that grew was counted by using the colony counter. This process was repeated on the first, third and fifth days (see Fig. 1).

2.7. Data Analyzed

This study used factorial completely randomized design 4 × 2 with 3 replications. The first treatment consists of four bacteria isolates including B_{299} isolates, C_{610} isolates, D_{610} isolates, and E_{610} isolates. The second treatment was two prebiotics including rice bran prebiotics supplemented by egg hatching waste and rice bran prebiotics without supplemented. The data were analyzed by using software SPSS 21 (IBM). The normality of data tested by using the Kolmogrov-Smirnov test. Then the data were analyzed by using the Analysis of Variance test to determine significantly different of the treatment. If there were significantly different, the data analyzed would be continued by using Duncan's Multiple Range Test [13].

3. Results and Discussion

1.1. Isolation and Characterization of Probiotics Bacteria

To evaluate the viability of bacteria, probiotics must be separated to be a single colony. Isolation of bacteria is the most important process to obtain a single colony of bacteria. Isolation is the first step for identifying the bacteria that it is taking and moving the bacteria from their environment to artificial media [14]. This study was initiated by isolating and characterization of the bacteria which were sourced from probiotics. These probiotics had been developed by Ichsan [12].

The results of isolation were obtained four isolates of bacteria which were given the symbol B_{299} isolates, C_{610} isolates, D_{610} isolates, and E_{610} isolates. The description and characteristics of each bacterium isolate were included in Table 1.

Table 1. Description and Characteristic of Bacteria Isolates

Bacteri Isolate	Colony Shape	Colony Color	Cell Shape	Gram Stain	Catalase Test	Phytase Test	Endospore Test
B_{299}	Round	White	Cocci	+	-	+	-
C_{610}	Round	White	Bacilli	-	-	-	-
D_{610}	Round	White	Bacilli	+	+	-	+
E_{610}	Round	White	Cocci	+	+	-	-

The differentiation of morphological bacteria is one of the ways for identifying bacteria. Gram staining can show the differentiation of gram-positive and gram-negative because of the differentiation of cell wall structure. Gram-positive bacteria maintained the color from carbol gentian violet, although it was given a 96% ethanol solution. While gram-negative bacteria was red color, it was soluble by ethanol and taking red color from safranin and air fuchsin [15]. Gram-positive bacteria had the structure of the cell wall containing peptidoglycan, while gram-negative bacteria had the structure of the cell wall with high lipids [16].

The catalase test was carried out by added 3% of H_2O_2 (hydrogen peroxide). A small amount of bacterial colony was transferred to a surface of dry glass slide by using a sterile stick. 3% of H_2O_2 was dropped on the slide and mixed. The results showed that was two strains of probiotics bacteria were catalase-positive, it was D_{610} isolates and E_{610} isolates. Catalase mediated the breakdown of H_2O_2 into oxygen and water. Catalase-positive was observed by oxygen bubbles on the slide [14].

The production of phytase was tested by using sodium phytate media. The results of the phytase test showed that B_{299} isolates produced phytase that indicated by clearing zone around of bacteria colony. Phytase (Mio-inositol hexakisphosphate) is phosphomonoesterase which has the capability to hydrolyze phytic acid to be ortophosphate anorganic and phosphate esters from lower Mio-inositol [17].

The endospore is a multilayered shell that protects the bacterial genome during stress conditions. The endospore in this study was tested by boiling the colony of bacteria at 80 ºC for 5 minutes. These results showed that D_{610} isolates produced the endospores could grow after boiled at high temperatures. Moreover, there are 6 *genera* of bacteria that can produce the endospores, they are

Bacillus, Sporolactobacillus, Clostridium, Desulfotomaculum, Sporo-sarcina and *Thermo actinomycetes*. So, D_{610} isolates are identified as *Bacillus spp.* because they are gram-positive bacteria, catalase-positive and producing the endospores [18].

1.2. Viability of Probiotics Bacteria

Synbiotics are a combination of probiotics and prebiotics [4, 5]. This study aimed to evaluate the viability of each bacterium that is sourced from probiotics. The viability is the ability of bacteria to live and survive in unfavorable conditions. The viability of probiotics bacteria is the most important in the production of synbiotics because the probiotics bacteria might be capable to live and survive in synbiotics. The viability of bacteria can be determined by using two methods, which are the total plate counts and spectrophotometry [19]. This study used the spread plate methods (*total plate counts*) to determine the number of live bacteria. The viability of bacteria was presented in Table 2.

Table 2. Viability of Probiotics Bacteria (Log CFU/g)

Synbiotics	Bacteria Isolates	Time (Day)			Average (Log CFU/g)
		1	3	5	
Synbiotics (*Rice Bran Prebiotic + Egg Hatching Waste*)	B_{299}	4,48	5,81	6,75	5,68±1,14[a]
	C_{610}	3,87	6,41	4,00	4,76±1,43[a]
	D_{610}	5,60	7,44	4,48	5,84±1,49[a]
	E_{610}	5,38	7,78	7,78	6,98±1,39[a]
Synbiotics (*Rice bran prebiotic without supplemented*)	B_{299}	7,50	5,49	4,30	5,76±1,61[a]
	C_{610}	7,17	7,55	5,23	6,65±1,24[a]
	D_{610}	4,78	7,31	5,43	5,84±1,31[a]
	E_{610}	6,67	7,49	7,13	7,10±0,41[a]

The different superscripts ($_{a, b, c}$) in the column indicate statistically significant difference of bacteria viability

According to the Analysis of variance test, the strains of probiotic bacteria and prebiotics and interactions of both did not show a significant difference (P>0,05) to the viability of probiotics bacteria. The average number of probiotics bacteria in rice bran prebiotics supplemented by egg hatching waste was 5,68 ± 1,14 log CFU/g for B_{299} isolates (the improvement rate was 2,27 log), 4,76 ± 1,43 log CFU/g for C_{610} isolates (the improvement rate was 0,13 log), 5,84 ± 1,49 log CFU/g for D_{610} isolates (the reduction rate was 1,12-log) and 6,98 ± 1,39 log CFU/g for E_{610} isolates (the improvement rate was 2,4 log). The average number of probiotics bacteria in rice bran prebiotics without supplemented was 5,76 ± 1,61 log CFU/g for B_{299} isolates (the reduction rate was 3,20-log), 6,65 ± 1,24 log CFU/g for C_{610} isolates (the reduction rate was 1,24-log), 5,84 ± 1,31 log CFU/g for D_{610} isolates (the improvement rate was 0,65 log) and 7,10 ± 0,41 log CFU/g for E_{610} isolates (the improvement rate was 0,46 log).

The most interesting result was high viability by E_{610} isolates, but the low viability was presented by C_{610} isolates. The different of bacterial viability can be caused by the compatibility of probiotics bacteria and prebiotics. Each strain of bacteria has a different ability to utilize substrate from prebiotics as a nutrient source. The different of viability can be caused by the different regeneration time and cell growth for each bacterium. The low viability of probiotics was caused by processing (high temperature), storage, packaging (temperature, oxygen, humidity) and degradation in the gastrointestinal region (the low pH in the stomach and bile salt in the small intestine) [20].

In addition, the viability of probiotics can be caused by sugar concentration, nutrients and time of fermentation [21], temperature, pH, water activity and oxygen [22]. Probiotics from *Bacillus spp.* have good viability because they can produce the endospores to protect their cell in difficult conditions [18].

4. Conclusion

In summary, the results might determine that the probiotics bacteria have grown with the same viability in synbiotics. The results suggested that synbiotic can be used as a feed additive because it contained probiotics bacteria and prebiotics.

Acknowledgement

The authors are thankful to the head and technician of Laboratory of Microbology and Biotechnology, Faculty of Animal Science, University of Mataram.

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