Characteristics Isolate Bacteria Lactic Acid of Origin Digestive Tract of Broiler as Probiotic Candidate for Poultry

Muh. Nur Hidayat, Ratmawati Malaka, Laily Agustina, Wempie Pakiding

Abstract: The purpose of this study was to isolate and characterize lactic acid bacteria (LAB) which is a probiotic from broiler age 3 days old. Isolation of lactic acid bacteria using MRSA media added 1% $CaCO_3$ in a petri dish, then incubated at 37^0C for 48 hours. Colonies of LAB may be associated with the formation of clear zones around the colony. Selected pure isolates were then combined with morphology, Gram staining, test methyl red, motility test, catalase test, and fermentation type test. Observation of identification of genotype characteristics of LAB using Bacteriological Manual of Bergey Determinative. The probiotic test was performed, ie pH endurance test, bile salt resistance test, and antimicrobial activity test. In this study, four isolates were produced (H2, H3, H5 and H7). Based on the results of the test, the characteristics of isolates of lactic acid isolates consisting of *Enterococcus* (H2) and *Lactobacillus* (H3, H5 and H7). The probiotic test showed that isolate H7 has acid resistance (pH 2, 3, 4, 5, and 6), resistance to bile salt 0.5% and has high antimicrobial activity against pathogenic bacteria *Staphylococcus aureus* and *Salmonella thypii*. Isolate H7 has the potential to be developed as a probiotic identified as *Lactobacillus* sp.

Key Words: Lactic acid bacteria, probiotics, broilers, isolates

1. INTRODUCTION

Utilization of lactic acid bacteria as probiotics is an approach to reduce the use of antibiotics growth-promoting (AGP) in the poultry industry. Micro flora can be used as an alternative to reduce or eliminate contamination of enteric disease which is of particular concern to the poultry industry as it can decrease productivity, increase mortality, and contamination associated with poultry products for human consumption [1].

Lactic acid bacteria group has been developed as a probiotic for livestock because it is considered relatively safe. There are many reports showing potential isolates of lactic acid bacteria isolated from various sources to be developed as probiotics. The selection of probiotic strains must satisfy non-pathogenic, acid-resistant and bile-resistant criteria, capable of producing antimicrobial substances, able to modulate the immune response and be able to influence intestinal metabolism [2].

The poultry digestive tract is a site for the development of various micro flora, both beneficial and disadvantageous. The adverse microbes are classified as pathogens, while those favorable as probiotics. One of the microbial groups that appeal to the poultry digestive tract, namely lactic acid bacteria. The bacterium is a normal micro flora of the human digestive tract and livestock. Therefore, lactic acid

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bacteria (LAB) is one of the normal microbial populations that are important in poultry. In the gastrointestinal tract of chicken, microbes exist almost along the length of the intestine, the main microorganisms found in the crop, small intestine and ceca are groups of Lactobacilli bacteria which specifically produce lactic acid and acetic acid.

One of the requirements that must be considered in the search for probiotic microbes, which is a normal micro flora of livestock digestive system that will become the object of application of the probiotic. One group of microbes that have colonized the broiler's digestive system from day one, namely lactic acid bacteria (LAB). According to [3] lactic acid bacteria, Lactobacillus strain has a high ability to stick to the intestinal epithelium. Based on this, then in this study will utilize the small intestine of broiler aged three days as a source to isolate lactic acid bacteria potentially as probiotics for poultry

2 MATERIALS AND METHODS

2.1 Candidate Isolation LAB

Three-day-old broiler chicken is used as a sample. After the cut, the contents of the internal organs will be removed. The small intestine samples taken were then fed into the Erlenmeyer and dissolved with aquades and homogenized with a stomacher for 30 seconds. Broiler intestine then put in a reaction tube containing sterile aquades for dilution. The result of sampling that has been made is then inoculated into the petri dish containing MRSA media which has been added $CaCO_3$ 1%, then incubated at 37^0C for 48 hours. LAB colonies can be identified with the formation of clear zones around the colony.

The selected colony was then taken with a sterile ole needle by touching on the surface of the bacterial colony and then inoculated the surface of MRSA media that had been given 1% $CaCO_3$ in a petri dish with a scratch method to obtain a separate colony. Then it was incubated at 37^0C for 48 hours. Purification is done with repeatable casting on the same medium and condition to obtain a single colony. Selected pure isolates were then identified based on LAB characteristics.

2.2 Morphological Identification with Gram staining

The object glass is cleaned with 96% alcohol then fixed above the spirit light, then the active isolate is taken aseptically and placed on top of the object glass and then flattened. Re-deflected above the spirit light. After a cold drop of violet crystals 2-3 drops for 1 minute, then washed with water running and dried in the air. After that iodine drops for 1` minute, washed with water running and dried in the air. Then dropped with 96% alcohol for 30 seconds, then washed with running water and dried in air. Finally spilled with Safranin for 45 seconds, then washed with running water and excess water removed with absorbent paper. This observation is done by looking at the shape and color of the cell under a microscope with a certain enlargement.

2.3 Biochemical Test

2.3.1 MR test (Methyl Red)

A total of 1 ose bacterial isolates were extracted from the culture stock and inoculated on a liquid Methyl Red-Voges Proskauer (MR-VP) medium in the test tube. Furthermore, it is incubated for 5 x 24 hours at 37^0C. A total of 5 drops of MR-VP were added over a bacterial isolate preparation. Positive results when the complex formed a pink

2.3.2 Motility Test

The bacterial isolates were taken and then inoculated vertically until mid-medium Sulfid Indol Motility (SIM), then incubated at 37^0C for 24 hours. Motility (positive outcome) of bacteria is shown when there is growth on the surface of the medium and non-motile bacteria (negative results) grows along the puncture.

2.3.3 Catalase Test

Bacterial isolates were taken 1 ose on the MRSA medium and placed on a clean object glass. The sample was then sterilized with 3% H_2O_2 reagent and left for a while. The positive test is characterized by bubble formation, and negative test if no bubbles are formed.

2.3.4 Test type of fermentation

Bacterial isolates were taken as 1 ose to be

inserted into a Hungate tube containing 9 ml of MRSB and tube Durham, then it was incubated for 24 hours and 48 hours at 37^0C anaerobic temperature. Next observations are made into the Durham tube to see the appearance of air bubbles. If the air bubbles form, then the fermentation test is heterofermentative (positive), whereas if not formed air bubbles, the fermentation test is homofermentative (negative).

2.4 Probiotic Test

2.4.1 Test of resistance to acid pH conditions

Cultures that have been grown on MRSB media that has been adjusted to the pH treatment then performed multilevel retailers. The last two retailers were taken 1 ml for planting (Duplo) on MRSA media and incubated for 24 hours. The difference between the number of control colonies (pH 7) and treatment pH (pH 2, 3, 4, 5, and 6) is an indicator of isolation resistance against acidic pH [4][5].

2.4.2 Bile salt resistance test

The rejuvenated culture was then inoculated on MRSB (control) and MRSB + 0.5% (b / v) bile salts. Subsequently incubated at 37 ° C for 24, and 48. The last two retailers were taken 1 ml to be grown (Duplo) on MRSA medium and incubated at 37 ° C for 24 and 48 hours. The difference between the number of colonies grown on MRSA (control) media with MRSA + 0.5% bile salts is an indicator of isolate resistance to bile salts [6].

2.4.2 Test antimicrobial activity.

Cultures of pathogenic bacteria Staphylococcus aureus and Salmonella thypi were included as much as 0.2 ml 1789rlenmeyer containing 100 ml of dilute NA medium. NA media containing pathogenic bacteria is poured into a 20 ml petri dish previously inserted in a ring. Furthermore, the rings that have been planted into the petri dish are removed so that the hole (well) is formed. BAL insulation is then inserted into a 50 µl hole. Petri dishes were incubated at 37 ° C for 24 and 48 hours. The clear zone formed on LAB isolates was measured by a ruler [7].

3 RESULTS AND DISCUSSION

3.1 Isolation and Characteristics of Isolate BAL

In this research have been isolated four isolates of LAB from broiler digestive channel age one day. All of the isolates obtained were Gram-positive, negative catalase, non-motile, homofermentative fermentation type. Identification of LAB isolates from broiler age one-day digestive tract based on observation on morphology (characteristic of Gram), physiology and biochemical characteristic (catalase test, methyl red test, motility test, and fermentation test) are presented in Table 1.

3.2 Resistance of isolate to various pH conditions

Table 1. Morphology, physiological characteristics Isolate BAL Origin of Gastrointestinal Tract Broiler age three days.

Isolate Code	Characteristics of Isolates					
	Gram staining	Morphology	Catalase Test	Test MR	Test Motility	Test Fermentation
H2	Positive	Coccus	-	+	non-motile	homofermentative
H3	Positive	bacilli	-	+	non-motile	homofermentative
H5	Positive	bacilli	-	+	non-motile	homofermentative
H7	Positive	bacilli	-	+	non-motile	homofermentative

The pH conditions of the gastrointestinal tract vary greatly in each section. It becomes one of the requirements for microorganisms to survive in the digestive tract of poultry. The bacteria to be used as probiotics should have resistance to various conditions of the pH. The lowest pH value in the poultry digestive tract on the ventricular part, which is about 2-3.5.

The growth of LAB isolates under various conditions of treatment pH can be used as an indicator of resistance to acidic conditions. A small log value indicates that the isolates have strong resistance to the acid. In Figure. 1 shows no isolates that fall less than one log (<1 log) at pH 2, pH3, pH 4 and pH 5, except Isolate H7 which decreases less than one log (<1 log) at pH 6. Log values of pH 3, pH 4, pH 5, and pH 6 on all isolates (H2, H3, H5, and H7) in this study were categorized as resistant since they had a population decline between 1-3.5 logs. At the pH 2 treatment, resistant isolates were only isolates H2 and H7, whereas isolates H3 and H5 were not resistant because they had a population decline of more than 3.5 logs. Categorization of resistance level of bacteria to stomach acid based on [8] statement, that ability of LAB resistance to stomach acid can be grouped into three categories, that is considered highly resistant if population decline <1 log, resistant 1,5-3,5 log and not resistant if decrease> 3.5 logs.

The acidity test was performed on LAB isolate to see the ability of the isolates to grow in various conditions of acidity of the digestive tract of the broiler. The ability of bacteria to survive the condition of the acidity of the ventriculus (stomach) is an important thing to consider for a bacteria to be used as a probiotic candidate in poultry. Bacteria that enter into the ventriculus will decrease the population, due to the influence of hydrochloric acid (HCl) on the ventriculus, which is about pH 2-3.5. HCl is a strong acid and a major component of gastric acid.

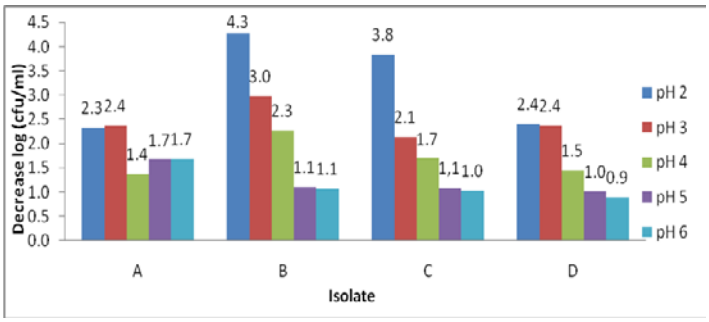

Figure 1. Resistance of isolate to various pH conditions

In this study, all isolates tested possessed varying growth abilities especially at low pH (2 and 3). According to [9], the ability of BAL to survive at low pH, because the intracellular pH can adjust to the decrease in extracellular pH, so as not to cause a large proton gradient. Further [10], states, that in addition to LAB experiencing slow growth at low pH, LAB cells can also be damaged due to acid and loss of viability. Each strain has different resistance to acid or low pH. According to [11], there are several possible mechanisms by which bacteria regulate the internal pH, but the most important is the proton translocation by the ATP-ase enzyme.

3.3 Resistance to Bile Salts

Bacteria that successfully pass various variations of acidity of the gastrointestinal tract of the broiler before the small intestine, then into the early part of the small intestine (duodenum). In this section according to [10], the bacteria will face the availability of oxygen (O_2) low, bile salts and competition with other microbes found in the intestine to obtain energy sources. Therefore one of the requirements that also must be considered for testing probiotics candidate bacteria, which has resistance to bile salts.

Bile salt resistance test is the next test on four isolates of LAB which have been tested for acid resistance (pH) to be used as probiotic candidate. The concentration of bile salts used in this study, 0.5% (w/v). This is based on the statement of , that the concentration of bile salts in the duodenum is equal to 0.5%.

The bile salts present in the intestine are synthesized in the liver by conjugating the heterocyclic steroids derived from cholesterol and channeled to the intestine via the twelve fingers. The

bile salts will then be reabsorbed from the lower ileum and back to the liver to be secreted again into the bile. The duration of bacteria in the intestine is about 4-6 hours. Bacteria that have passed through bile salts should be able to colonize the lower intestinal tract to be able to say probiotic bacteria [10].

Figure 2 shows that all LAB isolates obtained from DOC broiler's gastrointestinal tract have varying resistance capability to bile salt treatment. In a row the decrease of colony count from lowest to highest was 1.9 log/ml (isolate H3), 2.2 log/ml units (isolate H5 and isolate H7), and 2.4 log/ml units (Isolate H2). Although there is a decrease in the number of colonies in all isolates, basically all isolates can still have resistance to bile salts, because it has a population decline between 1-3.4 log.

The decrease of LAB isolate population in this study was caused by the ability of bile salts to damage LAB cells. According to [12], in addition to the biological function of bile salts as detergents that emulsify and dissolve lipids, bile salts also have antimicrobial properties by destroying bacterial cell membranes. The activity of bile salts as antimicrobials works by disrupting the function of cell walls and bacterial cell membranes. One of the compounds that make up the cell wall and the bacterial cell membranes, namely lipids and derivatives. Bile salts have the ability to work on these lipid compounds, thereby causing damage to cell walls and cell membranes. According to [13] [14], that bile salts are as active compounds on the cell surface, so they can penetrate and react with the cytoplasmic membrane side which further leads to changes and destruction of the membrane structure. The diversity of fatty acid structures in bacterial cell membranes causes a difference in permeability and is thought to affect bacterial resistance to bile salts.

LAB that is resistant to bile salts according to [15], because they have Bile Salt Hydrolase (BSH) enzymes that are regulated by the BSH gene. This enzyme is able to physically change the chemistry of bile salts, so it is not toxic to LAB. BSH enzyme activity against bile salts has been studied by [12], which demonstrated the ability of BSH enzymes to decompose conjugated bile acids into unconjugated bile acids and release glycine or taurine amino acids.

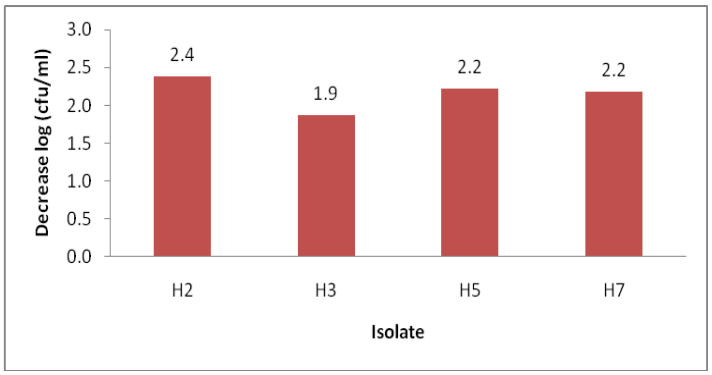

Figure 2. Resistance of bile salts to LAB isolates

3.4 Antimicrobial Activity

One of the microbial conditions can be used as probiotics, which have antimicrobial activity against pathogens (*Pseudomonas aeroginosa, E. coli, Streptococcus mutans, Clostridium dificile, Enterococcus hirea, Salmonella enterica, Staphylococcus aureus*) tested in vitro [16]. In Table 3 it was shown that the antimicrobial activity of isolate H2 could not inhibit Salmonella thypii growth after 24 hours incubation, but after 48 hours incubation, there was a 5.6 mm inhibition. Isolate H2 may inhibit the growth of Staphylococcus aureus after 24 hours incubation by 8.6 mm, but after 48 hours incubation, the inhibitory capacity decreased to 5.5. In isolate B the inhibitory ability of Salmonella thypi after incubation 24 hours, ie 7.3 mm and slightly increased after 48 hours incubation (9.0). Isolates that are unable to maintain their inhibitory ability against pathogen after 48 hours incubation can be classified as having bacteriostatic antimicrobial activity.

In the antimicrobial activity test against Gram-negative bacteria Salmonella thypii, the largest inhibitory diameter after 24 hours incubation, ie isolate H7 (8.4 mm) and after incubation 48 hours to 11.7 mm. The smallest inhibitory diameter of Salmonella thypii pathogen bacteria after 24 hours incubation was found in isolate H5 (5.4 mm) and after 48 hours incubation was found in isolate H3 (9.0 mm).

The antimicrobial activity of isolates against Gram-positive pathogen bacteria Staphylococcus aureus has variable inhibitory power. the largest inhibitory diameter after 24 hours incubation, ie isolate H2 (8.6 mm) and after 48 hours incubation in isolate H3 (9.2 mm). The smallest inhibitory diameter against pathogenic bacteria Staphylococcus aureus

Table 3. Antimicrobial Activity of LAB isolate

Type Microbes	Diameter of antimicrobial activity (mm) isolate							
	H2		H3		H5		H7	
	24 Hours	48 Hours	24 Hours	48 Hours	24 Hours	48 Hours	24 Hours	48 Hours
Salmonella thypii	5.6	11.3	7.3	9.0	5.4	10.6	8.4	11.7
Staphylococcus aureus	8.6	6.0	4.5	8.7	5.5	9.2	5.7	8.3

after 24 hours incubation was found in isolate H2 (4.5 mm) as well after 48 hours of incubation was found in isolate H2 (6.0 mm).

All of the isolates (H2, H3, H5, and H7) obtained in this study had antimicrobial activity in inhibiting the growth of Gram-negative and Gram-positive bacteria tested. An antimicrobial activity of isolates against pathogens can be observed in the formation of clear zones around the holes (ring) that have been filled with existing isolates. The resulting clear zone is used as an indicator of no pathogenic bacteria growing in the area. According to [17] the diameter of the inhibitory zone against pathogenic bacteria can be grouped according to three categories, ie 0-3 mm inhibitory zone diameter indicates low antimicrobial activity,> 3-6 mm mean medium antimicrobial activity and inhibitory zone diameter> 6 mm indicates high antimicrobial activity.

The antimicrobial activity of LAB isolates in this study is generally greater in Gram-negative bacteria than Gram-positive bacteria. Differences in the structure of both Gram-negative bacteria and Gram-positive bacteria may be one of the reasons for differences in bacterial growth ability of antimicrobial compounds produced by lactic acid bacteria isolates in this study. This is in line with the study of [18], using isolates of lactic acid bacteria from the gastrointestinal tract of Thai local chickens showed that the antimicrobial activity of the isolates was generally stronger against Gram-negative bacteria (*E. coli* and *S.thypii*) than the Gram bacteria positive (*S.aureus*). Gram-positive bacteria resistance to antimicrobial compound activity caused by thicker cell wall than Gram-negative. According to [19], Gram-positive bacterial cell walls are thicker because they contain 90% peptidoglycan and a thin layer of teichoic acid, whereas Gram-negative cell wall cells contain only 5-20% of peptidoglycan and other layers composed of proteins, lipolytic and lipoproteins. Furthermore, according to [20], the peptidoglycan layer on Gram-positive bacterial cell wall has a thickness of 20-80 nm with the largest composition of Teichoic, Teichuronic acid and various Polysaccharides. Polysaccharides and amino acids on the Peptidoglycan sheet are so polar that in this type of bacteria have thick cell walls that can withstand the activity of bile fluid in the intestine, Peptidoglycan sheets are susceptible to lysozyme that can be damaged by bactericidal compounds. While on the cell wall of Gram-negative bacteria has a layer of Peptidoglycan thickness of 5-10 nm with the main composition of Lipoprotein, outer membrane, and Lipopolysaccharide.

The peptidoglycan layer possessed by Gram-positive bacteria also affects bacterial resistance to acids. Peptidoglycan is a large molecule composed of sugar and amino acids. The two constituent sugars are N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). A single peptidoglycan layer binds to another layer through the amino-acid chain part, thereby forming a strong cross-linking the entire cell. The entry of acid into the cell can be through several ways, among others, through the te acidic acid found only in the cell wall and cell wall membranes from Gram-positive. The acetic acid is known to have a negative charge that can limit the kind of substance to be bonded and transmitted in the cell. In addition, it can be through adsorption that affects the permeability and porosity of the cell wall causing disruption of peptidoglycan synthesis so that the cell formation is not perfect because it does not contain peptidoglycan and its cell wall only includes cell membrane. This situation causes the bacterial cells to be susceptible to lysis, especially Gram negative bacteria whose peptidoglycan content is less when compared with

Gram positive bacteria, which in turn will result in cell death [10].

Several studies have shown that lactic acid bacteria can produce antimicrobials that can inhibit other microbes. The ability of isolates in this study to inhibit the growth of pathogenic bacteria showed that the lactic acid bacteria obtained had antimicrobial compounds. During its growth, lactic acid bacteria can produce secondary metabolite compounds that have antimicrobial activity. According to [21], the metabolites produced by LAB are organic acids, hydrogen peroxide, bacteriocin, and other components. Bacteriocin is an antimicrobial peptide which during growth grows exponentially. Insufficient quantities can kill bacteria (bactericidal) or other bacteria (bacteriostatic) of competing barriers in the same ecology.

The mechanism of inhibition of lactic acid against bacterial cells because the lactic acid has a hydrophobic nature so as to facilitate diffusion in the form of protons into the cell through the cell membrane. As a result, intracellular pH is higher than that of extracellular pH. Furthermore, within the cells, lactic acid dissociates and decreases intracellular pH by releasing protons [22]. The release of protons/hydrogen ions can disrupt metabolic functions such as substrate translocation and oxidative phosphorylation, causing the bacterial cells to be inhibited by their growth [23]. The inhibitory activity of antimicrobial compounds against pathogenic microbial cells can be seen from the formation of clear zones around the isolates tested. In general, the mechanism of action of an antimicrobial compound can be done by disrupting or destroying the cell wall, reacting with the cell membrane leading to increased cellular permeability, the inactivation of essential enzymes, and the destruction or inactivation of function from the genetic material [24].

4 CONCLUSION

Based on various probiotic test results including pH test, bile salt test and antimicrobial activity test against Gram-negative bacteria and Gram-positive bacteria, the characteristic of isolate that can be used as a probiotic candidate is commonly found in isolate H7.

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