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THE POTENTIAL OF LACTIC ACID BACTERIA ISOLATES FROM MERAWANG CHICKEN INTESTINE TYPICAL OF BANGKA BELITUNG AS PROBIOTIC CANDIDATES

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Abstract

The improvement of egg production efficiency and health remains a challenge in the cultivation of native chickens. The production of feed on farms requires significant costs, which can account for 60-70% of the total production expenses, making it essential to optimize feed efficiency in the chicken's digestive system. Furthermore, the traditional free-range farming of native chickens Merawang in Bangka Belitung, where they are allowed to roam freely in the backyard or garden, makes it difficult to control their development and health. To address these issues, efforts can be made by harnessing the potential of Lactic Acid Bacteria (LAB) present in the digestive system of native chickens to be used as probiotics. The objective of this research was to obtain potential isolates of lactic acid bacteria from native chickens Merawang as probiotic candidates. The study was carried out experimentally in four stages, namely isolation, characterization, confirmation test, and probiotic potential test. The research results indicate that there were 6 isolates with potential as probiotics, namely AM3, AM7, AM12, AM14, AM20, and AM25. These six isolates showed potential as probiotic bacteria because their lactic acid content in a medium meets the applicable standards, ranging from 0.79% to 1.84%. The isolates that performed the best as candidate probiotic bacteria were AM14, AM20, and AM25. The identified bacteria in the digestive system of native chickens belong to the Genus Lactobacillus.

Keywords: *Chicken Intestine Filigree; Lactic Acid Bacteria; Probiotics*

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INTRODUCTION

Merawang chicken is a chicken species originating from China and has long adapted to Merawang Village, Merawang District, Bangka Regency, Bangka Belitung Islands Province. Merawang chicken is a genetic resource as well as an asset for the people of the Bangka Belitung Archipelago Province since it has potential as a dual-purpose chicken that produces both eggs and meat (Nuraini et al., 2020). However, increasing production efficiency is still a problem in Merawang chicken farming since the price of chicken feed in Bangka Belitung Islands Province is relatively expensive, which can reach 60-70% of the total production cost. Relatively expensive production costs can be reduced if the efficiency of the feed given increases so that the digestive tract can work optimally in digesting and absorbing nutrients to increase chicken productivity properly. The addition of probiotic cultures can increase the daily egg production of *Hen Day Production* (HDP) (Huda et al., 2019).

In addition, the cultivation of Merawang chicken in Bangka Belitung was still carried out traditionally by being left free in the yard of the house or in the garden so that the development and health of the chicken is difficult to control.

Overcoming feed problems and disease prevention can be done one way by giving probiotics. Probiotics are additional feeds that utilize live microorganisms to improve livestock health by balancing the microflora in the digestive tract if consumed in sufficient quantities (Astini, 2014; Rahmiati & Mumpuni, 2017). The discovery of probiotic bacteria isolates taken from the intestines of broiler chicken that qualify as probiotic characteristics. The existence of probiotics can be a solution to the problem of animal feed which has a high cost value and can reduce the population of pathogenic microbes, improve the health and immunity of livestock. One group of bacteria that acts as a probiotic is Lactic Acid Bacteria (Sari et al., 2013; Fachrial & Harmileni, 2018).

Lactic Acid Bacteria (LAB) are Gram positive bacteria capable of fermenting glucose into lactic acid. The characteristics of LAB can be classified into probiotic bacteria since they are safe for consumption, able to withstand acidic pH, able to inhibit pathogenic bacteria, easy to cultivate, high viability during processing and storage, and resistant to anaerobic conditions (Lestari et al., 2018; Rahmiati et al., 2020). LAB are often found in the intestinal tract originating from the proventriculus, ventriculus, duodenum,

jejunum, ileum, and cecum (Harahap, 2015; Dewi et al., 2019). The availability of LAB isolated from various domestic sources still requires exploration to increase the collection of lactic acid bacteria isolates. One source that can be used to isolate LAB is from Merawang chicken intestine. The isolation of LAB from the intestines of Merawang chicken has never been done. Therefore, it is necessary to conduct research to obtain isolates and to identify LAB which can be used as probiotics from the intestines of Merawang chicken.

RESEARCH METHODS

This research was carried out in October 2022 - February 2023. The research location was carried out at the Microbiology Laboratory, Faculty of Agriculture, Fisheries and Biology, University of Bangka Belitung.

Intestinal Collection and Preparation

Samples were obtained from the intestines of Merawang chicken aged 3 days and 3 months, 3 of each at the Merawang Chicken Farm, Air Duren Village, Mendo Barat District, Bangka Regency. The sample of small intestine chicken that has been obtained was cleaned using sterile distilled water and then weighed as much as 1 gram for isolation.

Isolation and Characterization of LAB from Merawang Chicken Intestines

Isolation was carried out from dilutions 10-3, 10-5, 10-7, and 10-9 on MRSA + CaCO3 1% media using the scatter method. Then incubated for 48 hours at 37°C. Isolates that produce clear zones were then purified on MRSA media to characterize colonies and cells (Susilawati, 2016).

Hemolysis Test

The bacterial culture was inoculated into blood agar medium. Then incubated at 37°C for 24 hours. The clear zone that formed around the colonies on the media indicates that the microbes were pathogenic (Power & Johnson, 2009).

Physiological Test

Physiological tests were carried out on bacterial isolates, namely temperature resistance tests of 15°C, 27°C, 37°C, and 45°C, as well as pH 2.5 and 3 resistance tests and then incubated at 37°C for 48 hours (Djide & Wahyuddin, 2008).

Antimicrobial Test against S. aureus and E. coli and Antibiotic Test

Antimicrobial activity test was carried out using the McFarland standard 0.5 with the Well Diffusion Method. Each well was then added with 20 µl of BAL suspension aged 24 hours which was cultured on MRSB medium.While the antibiotic test used 10 mg of ampicillin, 30

mg of tetracycline, and 30 mg of chloramphenicol. The activity of the inhibition zone around the paper disc was measured using a vernier caliper.

Probiotic Potency Test

The probiotic potency test was carried out on MRSB liquid media and prebiotics. Preparation of LAB isolate inoculums on prebiotics and MRSB, namely 2 oses of bacterial cultures were taken and then inoculated on 25 mL of prebiotics and MRSB. Then incubated for 48 hours at 37 ℃. Bacterial cultures from MRSB media and prebiotics were carried out two tests each, namely the calculation of LAB population and lactic acid levels.

a. LAB population

The number of LAB colonies from the TPC process was diluted 10-3 - 10-7 using the Pour Plate method. The agar media that was planted was then incubated at 37℃ for 24 hours.

b. Lactic Acid Levels

A total of 5 mL of the inoculum was put into a centrifuge at 500 rpm for 10 minutes. The supernatant obtained was taken as much as 2.5 mL and added to 22.5 mL of sterile distilled water, then added 2 drops of 1% phenolphthalein indicator solution, then titrated with NaOH 0,1 N solution until a pink color was formed and the titration was stopped (Kurnia et al. , 2020).

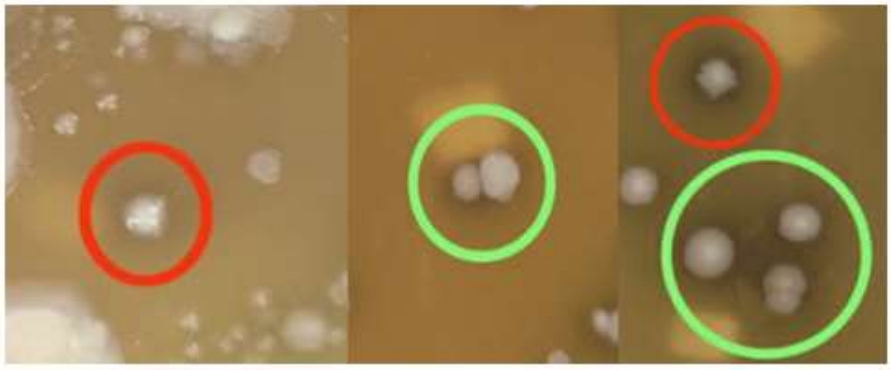
RESULTS AND DISCUSSION

Isolation and Characteristics of Lactic Acid Bacteria Isolates

Based on the isolation results, there were 6 colonies that had the potential as probiotics, marked by the presence of a clear zone around the colonies. Isolates that have a clear zone indicate that the isolate is positive for lactic acid bacteria. The selected isolates were further observed for their morphological characteristics including shape, margins, elevation, and colony pigmentation (Table 1).

Table1. Characteristics of Colony Morphology and Cells of LAB Isolates from the Intestines of Merawang Chicken

Isolate name	Colony Morphology				Cell Morphology	
	margins	Form	elevation	Color	Form	Grams
AM3	Entire	Circular	Convex	Milk white	Baccil	+
AM7	Entire	Circular	Convex	Dull white	Baccil	+
AM12	Entire	Circular	Convex	Milk white	Coccobacilli	+
AM14	Entire	Circular	Convex	Dull white	Baccil	+
AM20	Entire	Circular	Convex	Milk white	Coccobacilli	+
AM25	Entire	Circular	Convex	Milk white	Baccil	+



Fugure1. Lactic Acid Bacteria Isolate from Merawang Chicken Intestine on MRSA Media +$CaCO_3$ 1% (personal documentation)

The clear zone around the colony indicates a period of growth of LAB which produces lactic acid which reacts with $CaCO_3$ (insoluble in the media) to form calcium lactate which dissolves in the media. Calcium lactate which dissolves in the media was indicated by the presence of clear areas or zones around the growing bacterial colonies (Har & Si, 2015; Fathurrohim et al., 2022). The results of Gram staining showed that all isolates were Gram positive. The cell forms found were bacilli and coccobacilli (Table 1). The results of research conducted by M Himmah (2021) showed that the results of BAL isolates found from tofu whey were Gram positive and in the form of bacilli. Therefore, the results of Gram staining with 26 isolates were followed by Biochemical Tests.

Characteristics of LAB Isolates with Biochemical Tests

Biochemical test results (Table 2) shown by each isolate, adapted to the characteristics of lactic acid bacteria.

Table 2. Biochemical Tests of LAB Isolates from Intestines of Merawang Chicken

Isolate name	TSIA test				MR test	Motility Test	Catalase Test
	Slant	*butt*	Gas	H2S			
AM3	K	K	-	-	+	-	-
AM7	K	K	-	-	+	-	-
AM12	K	K	-	-	+	-	-
AM14	K	K	-	-	+	-	-
AM20	M	K	-	-	+	-	-
AM25	M	K	-	-	+	-	-

Description: K(Yellow), M(Red)

The results of the motility test that has been carried out, all isolates were non-motile or did not move freely in the media. The results of the Methyl Red (MR) test showed the presence of a red ring in the media after dropping the Methyl Red

reagent. The color change that formed on the media was caused by a decrease in the pH of the media by the accumulated acid products from glucose fermentation. Research conducted by Kadir (2016), related to the characteristics of isolates of lactic acid bacteria from the digestive tract of broiler Day Old Chick (DOC) also showed positive results on the MR test which was indicated by a change in the color of the media from yellow to red. The results of the catalase test on 6 isolates were catalase negative. The results of the TSIA test showed that all isolates could ferment glucose in TSIA media as indicated by a yellow discoloration on the butt (upright). However, not all isolates could ferment sucrose and lactose on TSIA media as indicated by the appearance of a red color on the slant (slant). The change in the color of the media to yellow was caused by the bacterial isolates being able to ferment sugar by breaking down the sugar into organic acids resulting in a decrease in pH to acid.

Hemolytic Characteristics and Pathogenicity

Based on the results of the study of all isolates, there were no isolates that formed clear zones around the colonies. It indicates that the bacterial isolate is gamma hemolytic (non-pathogenic). One of the important criteria for bacteria to be selected as probiotics was that they were not pathogenic or toxic to their hosts. (Mulyono & Ritonga, 2019).

Resistance of LAB Isolates to Temperature and pH

Six isolates were tested at temperatures of 15°C, 27°C, 37°C, and 45°C, and pH 2.5 and 3. Interpretation of the results was read by looking at the turbidity level of each isolate. The results of the temperature and pH resistance tests of each isolate can be seen in Table 3.

Table 3. Physiological Tests of LAB Isolates from Merawang Chicken Intestines.

Isolate name	Temperature Resistance Test				pH Resistance Test	
	15°C	27°C	37°C	45°C	2,5	3
AM3	+	+++	+++	+++	++	+++
AM7	+	+++	+++	+++	++	+++
AM12	+	+++	+++	+++	++	+++
AM14	+	+++	+++	+++	++	+++
AM20	+	+++	+++	+++	++	++
AM25	+	++	++	+++	++	++

Description: +++ = very cloudy and lots of precipitate; ++ = cloudy and quite a lot of precipitate; + = not cloudy and slightly precipitated

Based on the observation results, it can be seen that the 6 isolates were able to grow at temperatures of 15 ℃, 27 ℃, 37 ℃ and 45 ℃. However, growth was seen to be better at 37 ℃, 45 ℃ and 27 ℃ which was marked by a change in the color of the media which was more turbid and the presence of precipitate on the bottom surface of the test tube so that it can be said that LAB isolates were mesophilic and thermophilic. Data from temperature resistance testing can be used to assist applications in storing probiotic products. Based on the results of this study, the isolates can be stored at 15 ℃ (refrigerator temperature), 27 ℃ (room temperature), 37 ℃ (incubator temperature), and 45 ℃ (shipping temperature). It indicates that if the LAB isolates in this study were to be applied to a product, then incubation could use an incubator with a temperature of 37℃ and at room temperature of 27℃. The use of high temperatures of 45℃ can be used if the product was in shipping condition where the ambient temperature was slightly high. In addition, the use of low temperatures, namely 15 ℃ can be used if the product wanted to be stored for a certain period of time since in this study it showed that the activity of microorganisms was slowed down as indicated by the absence of a change in the color of the media to become cloudy but there was a slight precipitate at the bottom of the test tube. If microbes are stored below the minimum temperature, the cells of microorganisms will grow slowly because all the metabolic reactions of microorganisms are catalyzed by enzymes (Susilawati, 2016).

Based on the test results for low pH resistance with 2 different treatments, namely pH 2.5 and 3, it showed that the 6 isolates, namely AM3, AM7, AM12, AM14, AM20, and AM25, were able to survive at low pH. It proves that the 6 isolates are able to pass stomach acid so that they can be used as probiotic bacteria. It is corroborated by the statement of Garcia-Ruiz et al., (2014) stating that probiotic candidate bacteria must be able to tolerate pH 3 because that pH is the pH of the gastric mucus layer and the general pH of acids produced in the stomach normally.

Antimicrobial Activity & Antibiotic Resistance

Based on the results of antimicrobial activity (Table 4), the ability of LAB to inhibit the growth of E.coli and S. aureus bacteria is due to the presence of organic acids. Incubation carried out for 24 hours against lactic acid bacteria can produce lactic acid ranging from 16.692 – 20.620 g/L and will

increase with the length of incubation time (Rusmana et al., 2012). The high production of organic acids was able to penetrate the bacterial membrane so that weak acid anions and other antimicrobial substances accumulated in the cytoplasm. In addition, the production of lactic acid that was too high can also caused a decrease in the pH of the media. This statement is in accordance with the results of research which showed a decrease in pH caused by an increase in lactic acid levels in bacterial isolates (Table 7).

Table 4. Diameter of the clear zone against pathogenic bacteria and antibiotics

Isolate name	Clear Zone Diameter (mm) against Pathogenic Bacteria		Category	Clear Zone Diameter (mm) to Antibiotics		
	S. aureus	*E. coli*		A	Q	K
AM3	5.45	3.65	weak	15.87	-	4.69
AM7	13,43	10,21	strong	16,86	-	18.98
AM12	14.74	16,37	strong	22,26	-	5,38
AM14	16,21	17.55	strong	30,43	14.57	13.37
AM20	19	16,42	strong	27.08	8.37	20,21
AM25	15,8	15.35	strong	19.84	-	14,15

Notes: A = Ampicillin; T = Tetracycline; K = chloramphenicol

Ampicillin antibiotics were grouped into the betalactam group. This group can be inhibited by β-lactamase enzymes by degrading these compounds so that bactericidal activity can be inhibited and supports bacteria to remain resistant to these antibiotics (Sukarya et al., 2021). In addition, BAL has a cell wall composed of several thick peptidoglycan layers. The thickness of peptidoglycan is able to inhibit the entry of antibiotic compounds by reducing membrane permeability so that it can reduce the possibility of entry of antibiotic components into the cell cytoplasm (Sujadmiko, 2017).

Sukarya et al., (2021) reported that Lactobacillus plantarum isolates were resistant to tetracycline and chloramphenicol. Resistant to tetracycline due to the presence of the tet gene (M) in these bacteria which is able to assist in ribosomal protection of bacterial cells thereby increasing the chances of lactic acid bacteria cells to survive. Resistance to chloramphenicol is suspected because bacteria have a paint gene that can cause bacteria to produce chloramphenicol acetyltransferase enzymes that were capable of inactivating antibiotic compounds that enter cells before inhibition of protein synthesis occurs in bacterial cells.

Potential of LAB Isolates as Probiotics

Population of Lactic Acid Bacteria

Based on the results of the number of LAB colonies (Table 6), there was a

discrepancy between the standard number of LAB colonies where the standard was LAB in the intestine will provide an advantage for the host at least 108 CFU/g (Araya et al., 2002). Standard discrepancy was suspected since the prebiotic media contained carbohydrates available in the form of polysaccharides such as starch and fiber so that lactic acid bacteria isolates were difficult to adapt in their growth. Generally in the adaptation growth phase, bacteria tend to utilize simpler carbon sources. In this case, LAB probiotic candidates will find it easier to use available carbohydrates in the form of oligosaccharides (type III resistant starch) compared to polysaccharides (starch and fiber) as the main carbon source at the start of growth (Setiarto & Widhyastuti, 2017).

Table 6. Population of Lactic Acid Bacteria from Merawang Chicken Intestine

Isolate name	Colony Count (CFU's/mL)	
	MRSB media	Prebiotic Media
AM3	1.1 x 107	5.8 x 105
AM7	1.3 x 107	6.3 x 105
AM12	1.4 x 107	1.4 x 106
AM14	1.4 x 107	2.2 x 106
AM20	1.8 x 107	2.1 x 106
AM25	2.9 x 107	2.7 x 106

Lactic Acid Levels of LAB Isolates

The number of populations contained in each medium will affect the percentage of lactic acid levels in each medium. The more the number of populations contained in a medium, the higher the percentage of lactic acid levels so that the pH value will be lower (Table 7).

Table 7. Percentage of Lactic Acid Levels and pH Values of MRSB Media and Prebiotic Media

Isolate name	Lactic Acid Content (%)		pH value			
	MRSB media	Prebiotic Media	MRSB media		Prebiotic Media	
			Before incubation	After incubation	Before incubation	After incubation
AM3	1,12	0.79	5.95	4.87	5,12	4,19
AM7	1.33	1.26	5.95	4.85	5,12	4,21
AM12	1.37	1.33	5.95	4.85	5,12	4,17
AM14	1.66	1.44	5.95	4.88	5,12	4,17
AM20	1.77	1.66	5.95	4.51	5,12	4,12
AM25	1.84	1.73	5.95	4.63	5,12	4.09

The difference in the high percentage of lactic acid levels occurred along with a decrease in pH. Lactic acid metabolites have antimicrobial properties

against the growth of microorganisms so that they can help inhibit the growth of groups of pathogenic bacteria (Dali, 2013). The lactic acid levels produced by each LAB isolate on MRSB media ranged from 1.12–1.84, while on prebiotic media it ranged from 0.79–1.73. The range of lactic acid content in this study was in accordance with the requirements applicable to the SNI standard for probiotics, namely 0.5% -2% (National Standardization Agency, 2009).

Bacterial Isolate Identification

Growing bacterial isolates were identified manually using Bergey's Manual of Determinative Bacteriology Ninth Edition. The results of bacterial identification showed that the six bacteria were *Lactobacillus sp*. (Table 8).

Table 8. Results of Identification of Lactic Acid Bacteria Isolate from Merawang Chicken Intestine

Characteristics	Isolate Code					
	AM3	**AM7**	**AM12**	**AM14**	**AM20**	**AM25**
Macroscopic						
Color	Milk white	Dull white	Milk white	Dull white	Milk white	Milk white
Form	Circular	Circular	Circular	Circular	Circular	Circular
elevation	Convex	Convex	Convex	Convex	Convex	Convex
margins	Entire	Entire	Entire	Entire	Entire	Entire
Microscopy						
Grams	+	-	+	+	+	+
Cell Shape	Baccil	Baccil	Coccobacilli	Baccil	Coccobacilli	Baccil
Biochemistry						
Motility	-	-	-	-	-	-
Catalase	-	-	-	-	-	-
Methylene-Red	+	+	+	+	+	+
TSIA	++	++	++	++	+	+
genus	*Lactobacillus*					

Note: TSIA = ++ : fermentation of glucose, lactose, and sucrose; + : glucose fermentation

Bacteria of the genus Lactobacillus were found since these bacteria include facultative anaerobic bacteria that lived in the gastro-intestinal tract in both humans and animals. Several species of Lactobacillus have been isolated from the small intestine of animals. Some of them are *L. acidophilus, L. reuteri, L. casei, and L. fermentum* (Manin, 2010).

CONCLUSION

The results of the study on the probiotic potential of lactic acid bacteria isolates from Merawang chicken intestines can be concluded that isolates that have the potential as probiotic bacteria from the intestines of Merawang chicken are isolates AM3, AM7, AM12,

AM14, AM20, and AM25. While the isolates that have the most potential as probiotic bacteria are AM14, AM20, and AM25. This is demonstrated by its ability to survive in extreme environments with different temperature ranges and low pH. Its ability to colonize in a medium to produce lactic acid is also proven by the results of the lactic acid content test so that it can lower the pH of the environment which will affect its activity as an antimicrobial. Isolates that have the potential as probiotic bacteria from the intestines of Merawang chicken are grouped into the Genus *Lactobacillus*.

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