Phenotypic Identification of Lactobacillus from Breast Milk Based on Their Ability to Ferment Sugars

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Abstract. The purpose of this study was to identify the species of Lactobacillus which has the potency as superior probiotic based on their ability to ferment sugars. The study was conducted in two steps, the first was a confirmation test of the five lactic acid bacteria (LAB) isolates of breast milk A1, A6, A8, B8, and B10b. All of the LAB isolates were Gram positive rod and negative catalase. This shows that all of the isolates were of the genus of lactic acid bacteria and readily for the second step testing. The second step was to determine the LAB isolates ability to ferment 50 sugars (carbohydrates) using API 50 CHL kit. Results from the API 50 CHL kit test showed that the 5 LAB isolates were of indigenous LAB with the identification level of 88,7% - 99,9%. The species found were three spesies of Lactobacillus plantarum-1 (Lactobacillus plantarum-1 A1, Lactobacillus plantarum-1 A8, Lactobacillus plantarum-1 B8),and 2 spesies of Lactobacillus rhamnosus (Lactobacillus rhamnosus A6, and Lactobacillus rhamnosus B10b), respectively.

Keywords: **breast milk, lactobacillus, probiotic, species, API 50 CHL kit**

I. INTRODUCTION

Changes in food consumption patterns in society today raises negative health problems leading to the new emergence diseases, especially which affected the digestive tract. Such problems arise because of changes in lifestyle, behavior, and experience in choosing what foods to be consumed [1]. To overcome these problems, the population of good bacteria or beneficial bacteria must be dominant to the harmful bacteria. One effort that can be done to balance the number of good bacteria and bad bacteria is in the normal state which is the number of good bacteria should be more than the bad bacteria is through sufficient consumption of probiotics [2].

Probiotics are live bacteria that if consumed in sufficient quantities will have some good effects to the human health. Probiotics can derived from Gram positive bacteria, Gram negative bacteria, yeast or fungi. Many of the probiotic drinks and food mainly produced using the lactic acid bacteria which is a Gram-positive, catalase-negative that produce lactic acid by way of fermenting carbohydrates [3]. The usual LAB known as probiotics are of the genus Lactobacillus and Bifidobacterium which is normally found in the gastro intestinal tract [4]. Lactic acid bacteria provide a big contribution in the food industry and human health particularly the digestive system. Lactic acid bacteria has a role in maintaining the balance of normal flora in human guts and improving the human immune system. Generally, the probiotic candidate LAB is isolated from clinical samples with the assumption that clinical isolates can survive in the human gastro intestinal environment. One of the best clinical sample for good probiotic is the breast milk.

Puspawati and Nocianitri (2012) isolated 28 lactic acid bacteria of breast milk where five of the isolates have potential properties as superior probiotic bacteria, however the species or strains of breast milk isolates were still unknown [5]. Therefore, in order to assure that the 5 isolates were a potential probiotics it is necessary to further identify the strains based on their ability to ferment sugars.

II. RESEARCH METHOD

The materials used in this study were: LAB isolates isolated from breast milk (A1, A6, A8, B8, and B10b), MRSA Broth, MRSA Agar, NaCl, crystal violet, lugol, 95% ethanol, safranin, immersion oil, 70% alcohol, API 50 CHL kit, cotton, aluminum foil, heat-resistant plastic, spirit, and

tissue paper. The procedures of identifying strains of Lactobacillus with the API 50 CHL kit:

Resuscitate Isolates

Take an Eppendorf tube containing LAB with the code Lactobacillus sp. from the freezer (temperature of -20ºC). From each tube take a loop-full of culture, then inoculate to each tube containing 5 ml MRS broth media. Both test tubes are then incubated for 24-48 hours, positive results is shown by the appearance of turbidity in the tube. From the positive tubes, take a loop-full then streak in quadrants on a petri dish containing MRS agar. Both petri dishes were then incubated for 24-48 hours, take single colonies that is grown on the petri after incubation using a sterile loop and then inoculate in test tubes, each containing MRS broth media as much as 5 ml. Both of these tubes are then incubated for 24-48 hours in which a positive result is indicated by turbidity in the tube. The tubes with positive results containing Lactobacillus will be used as a culture to be identified.

Preparation of the strip and inoculum

Add about ± 1 ml sterile distilled water into each well reaction or plastic placemat in API 50 CHL kit container to create high humidity. Move 2 strips (consisting of micro tube No. 0-19 and 20-39) from its storage box and then divide into 4 strips smaller (micro-tube 0-9, 10-19, 20-29 and 30-39) and all four strips are placed in incubation box. Take last strip (micro-tube number 40-49) from its storage box and then place it after another strip to complete the entire strip in the incubation box. Harvest bacteria from tube containing isolates of Lactobacillus sp. by centrifugation, and then about 100 µl of cell suspension is transferred into ampoules which have been given medium or suspension of API 50 CHL and then it is homogenized.

Inoculation suspension on strip

Take 100 µl bacterial suspension using sterile micropipette, then inoculate in 50 micro tubes API 50 CHL containing carbohydrate test and close the top with 1 ml of sterile liquid paraffin. While inoculating, avoid the formation of bubbles by placing suspension on the pipette tip opposite to the side of the cupule (inoculation hole). All the micro-tube were incubated at 37ºC for 24-48 hours in an anaerobic state. A color change from blue to green to yellow or black is recognized as a positive [3].

Interpretation of data

In identifying Lactobacillus or LAB using API 50 CHL kit, positive results in each of the carbohydrates tested is indicated by the color change of the indicator bromocresol purple to yellow as a sign of the formation of lactic acid fermentation. Specific for micro-tube no.25, color change is from purple to black. Observations were carried out twice: after incubation for 24 and 48 hours. The observed data at each micro-tube is then recorded on observation sheets to match the identification types available.

III. RESULTS AND ANALYSIS

Confirmation test

The survey results revealed that the five isolates of LAB isolated from breast milk had the characteristics of lactic acid bacteria. This indicates that these isolates are still in pure condition and is not contaminated during storage. The confirmation data test results can be seen in Table 1, while the image of isolate A1 with Gram stain can be seen in Figure 2. Lactic acid bacteria genus based on the observation of morphological and physiological properties can be matched based on the tables of different characteristics of lactic acid bacteria [6].

TABLE 1.
CONFIRMATION TEST RESULTS OF LAB ISOLATES ISOLATED FROM BREAST MILK.

No	Isolate Code	Confirmation Test		
		Catalase Test	Gram Stain Test	Cell Form
1	A1	Negative (-)	Positive (+)	Rod
2	A6	Negative (-)	Positive (+)	Rod
3	A8	Negative (-)	Positive (+)	Rod
4	B8	Negative (-)	Positive (+)	Rod
5	B10b	Negative (-)	Positive (+)	Rod

From the results of confirmation test, it is known that 5 isolates of LAB isolated from breast milk A1, A6, A8, B8 and B10b are isolates that negative catalase (-), positive Gram (+) and rod cell morphology. This shows that all of these isolates are of lactic acid bacteria genus and the results are consistent with data from previous studies conducted by Puspawati and Nocianitri, 2012.

Inoculation suspension on strip

Based on the results in Table 1, it can be seen that all breast milk isolates used are of negative catalase (-). Catalase testing of LAB isolates from breast milk can be seen in Fig. 1.

Catalase test is a test to determine the ability of bacteria to degrade hydrogen peroxide compound (H2O2) into H2O and O2 by catalase enzyme. Catalase enzyme has an important role in the growth of aerobic bacteria because H2O2 formed with the help of various respiratory enzymes can be toxic to microbial cells. H2O2 component is one of the results of bacterial aerobic respiration where the results of the respiration can actually inhibit the growth of bacteria because it is toxic to the bacteria itself. Therefore, these components must be divided in order not to be toxic. Generally, lactic acid bacteria are negative catalase (-).

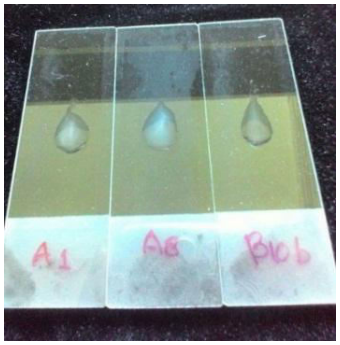

Fig 1. Results of catalase test from LAB isolates from breast milk (A1, A8, dan B10b).

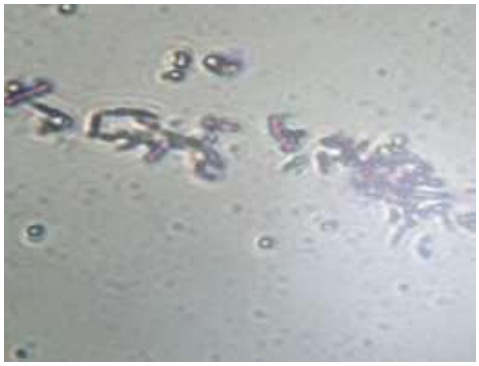

Fig. 2. Morphology Form Isolate A1 (rod) with 1000x magnification

Gram Staining

Based on the results in Table 1, all of the breast milk isolates used were Gram positive (+). According to Widyastuti and Sofarinawati (1999), in general characteristics of LAB are Gram positive and negative catalase [7]. LAB are found in two distinct phyla, namely Firmicutes and Actinobacteria. Within the Firmicutes, LAB belong to the order Lactobacillus and include the following genera: Aerococcus, Alloiococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconoctoc, Oenococcus, Pediococcus, Streptococcus, Symbiobacterium, Tetragenococcus, Vagococcus, and Weissella, which are all low guanine-cytosine content organisms (31-49%) [8]. LAB in Actinobacteria phylum only includes species of Bifidobacterium genus. All of the LAB isolates used were Gram-positive bacteria. Based on the composition of the cell wall, bacteria are divided into two groups: Gram positive and Gram negative bacteria. Gram-positive bacteria consists of 90% cell wall peptidoglycan layer, while the other is a thin layer of teichoic acid. Gram-negative bacteria consists only of 5-20% of cell wall peptidoglycan layer, while the other layer is composed of proteins, lipopolysaccharides, and lipoproteins. Differences in the composition of the cell wall of Gram positive and Gram negative result in differences in the properties of the coloring.

Cell and Shape Morphology

Morphology of LAB isolates used were either single or rod chains. Cell shape isolates (Isolate A1) with Gram stain can be seen in Fig. 2.

Lactic acid bacteria that are commonly isolated from breast milk, according to some researchers that has potential as probiotics are: Bifidobacterium longum, B. animalis, B. bifidum, and B. catenulatum. Existence of lactobacilli in breast milk are: L. gasseri, L. fermentum, and L. salivarius [9].

Determination of Lactic Acid Bacteria Species of Breast Milk Isolates with API 50 CHL Kit

LAB species identification of breast milk isolates using API 50 CH kit and API 50 CHL medium version 5.1 (Biomerioux), followed by processing and analysis using software APIWEB with the data results of the characterization of lactic acid bacteria phenotypically. The test results in the ability to ferment simple sugars with API 50 CHL kit can be seen in Table 2.

TABLE 2.
THE TEST RESULTS IN THE ABILITY TO FERMENT SIMPLE SUGARS WITH API 50 CHL KIT

No	Isolate Code	Species	Isolate Name
1	A1	*Lactobacillus plantarum 1*	*Lactobacillus plantarum 1*A1
2	A6	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus* A6
3	A8	*Lactobacillus plantarum 1*	*Lactobacillus plantarum 1*A8
4	B8	*Lactobacillus plantarum 1*	*Lactobacillus plantarum 1* B8
5	B10b	*Lactobacillus rhamnosus*	*Lactobacillus rhamnosus* B10b

The five isolates of LAB isolated from breast milk, were successfully identified into two species of indigenous lactic acid bacterial species: 3 species of Lactobacillus plantarum-1 and 2 species of Lactobacillus rhamnosus.

Lactobacillus plantarum-1 species that were identified: L. plantarum-1 A1 very good category of identification (99.7%), L. plantarum-1 A8 good category of identification (97.1%), and L.plantarum-1 B8 acceptable category of identification (of 88.7%). Two species of Lactobacillus rhamnosus that were identified: L. rhamnosus A6 good category of identification (98.7%), and L. rhamnosus B10b very good category identification (99.9%). The test results

of LAB breast milk isolates with API 50 CHL kit can be seen in Fig. 3

A B C

Fig 1. LAB Isolate (A1) in API 50 CHL kit; a. Before incubation, b. after 24 hours incubation, c. after 48 hours incubation

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