Viability of Lactobacillus from Breast Milk Isolate in Upper Digestive Tract by In Vitro

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Abstract. Transformation in lifestyle and eating habits can change the stability of flora ecosystems in the gut. This condition can be altered by increasing the number of good bacteria to reduce the number of pathogenic bacteria. This can be achieved by consuming functional foods containing probiotic bacteria. Probiotics are living bacteria found in food products and supplements. These bacteria have many benefits for the health of the body, especially for the digestive tract and can help balance the intestinal microbiota. Probiotics from lactic acid bacteria (LAB) classification, especially Lactobacillus genus is part of the normal flora of the human digestive tract. One of the conditions for bacteria to be a probiotic culture is to be able to survive in high stomach acid in the digestive tract. This study used 5 species of LAB breast milk isolates: *L. plantarum 1* A1, *L. plantarum 1* A8, *L. plantarum 1* B8, *L. rhamnosus* A6, and *L. rhamnosus* B10b which were tested it's ability to survive high acid conditions in the upper digestive tract by in vitro. This is done to determine whether the five isolates have the ability as probiotic bacterias. From the results of the study it was found that at pH 2 there was a decrease between 2 - 5 log cycles where the highest resistance of LAB breast milk isolate was *L. rhamnosus* A6. At pH 3 there was a decrease between 1 - 7 log cycles, where *L. plantarum 1* A1, *L. plantarum 1* A8, and *L. rhamnosus* B10b has the highest resistance. At pH 4 there was a decrease between 1 -3 log cycles with the five isolates of LAB that were able to survive at pH 4.

Keywords : *breast milk, digestive tract, probiotic, in vitro, pH*

I. INTRODUCTION

The development of degenerative diseases is currently growing very rapidly, causing the level of public health decline. Increased income from certain groups of people leads to irregular lifestyles, fast paced and instant eating habits that are very popular with various circles in the community (Arisman, 2007). Changes of eating habits in modern society by eating foods rich in protein and fat but low in dietary fiber is thought to be one of the triggers of the emergence of various diseases associated with the digestive tract (Saarela *et al*., 2002).

One of the alternatives to overcome the problem of diseases in the digestive tract without using chemical drugs is to modify the composition of bacteria in the digestive tract. Modification of bacterial composition of the digestive tract can be done through the consumption of live bacteria to maintain beneficial bacterial balance in the digestive tract, is called probiotics (Fuller, 1989).

Lactic acid bacteria (LAB) is one of the many beneficial bacteria belonging to the probiotic group. One of the benefits of lactic acid bacteria is that it can improve the balance of intestinal microflora in the body. Lactic acid bacteria with probiotic character are widely used as food supplements. These bacteria consisting of *Lactobacillus* and *Bifidobacteria* have many positive benefits such as antimicrobial activity, anticholesterol activity, immune system stimulation effect, increase of lactose absorption by the body, prevents diarrhea and antimutagenic activity to prevent cancer, especially colon cancer (Fuller, 1992; Surono, 2004; and Hill, 1995).

In a previous study, five species of LAB breast milk isolates have been identified using API 50 CH kit: *L. plantarum 1* A1, *L. plantarum 1* A8, *L. plantarum 1* B8, *L. rhamnosus* A6, and *L. rhamnosus* B10b (Arihantana and Puspawati, 2017). One of the conditions that lactic acid bacteria must have to be developed as a probiotic culture is resistance to acids. In order to survive and grow in the gastrointestinal tract, probiotic cultures must pass through some obstacles such as high gastric acidity in the intestine that can adversely affect microbial cultures. In addition, bacteria must also be able to compete with pathogenic enteric bacteria in the digestive tract (Gilliland *et. al.*, 1984 ; Salminen dan Wright, 2004). According to Chou dan Weimer, 1999 when probiotic bacteria enters the stomach, this bacteria must be able to withstand very low pH. The time it takes the bacteria to enter in and out of the stomach is 90 minutes. Once the probiotic bacteria succeeds through the stomach, they will enter

the upper intestinal tract which is where the salt bile is secreted.

Based on this, it is necessary to test the 5 species of LAB breast milk isolates to determine survival of LAB isolates in high acid conditions in the upper human digestive tract tested by in vitro and to find out whether the five isolates have the ability as probiotic bacteria.

II. RESEARCH METHODS

The materials used in this study are: 5 species of LAB breast milk isolates: *L. plantarum 1* A1, *L. plantarum 1* A8, *L. plantarum 1* B8, *L. rhamnosus* A6, and *L. rhamnosus* B10b, MRSA Agar, NaCl, NaCl, KCl, NaHCO3, pepsin, HCl, pankreatin, Na-kolat, NaDC, 70% alcohol, cotton, aluminium foil, plastic, spiritus, and tissue paper.

The equipments used are: autoclave, incubator, vortex, analytical scale, test tubes, petri dishes, pipetman, test tubes racks, beakers, bunsen, ose needle, crooked trunks, gloves, *air laminar flow*, hot plate, *magnetic stirer*, colony counter, effendorf, *hoky stick*, shaker, and centrifuge.

Resistance to upper digestive tract

Resistance to upper digestive tract was tested using modified gastric fluid models (Fernandez *et. al.*, 2003) containing : 125 mM NaCl; 7 mM KCl; 45 mM NaHCO3; and pepsin (3 g/l). The degree of acidity (pH) of the medium is adjusted by HCl close to pH: 2, 3, and 4. Resistance to transit time on gastric simulation was done by adding culture cells to the gastric model medium pH 2, 3 and 4 for 180 minutes. After that, it is continued with resistance to simulated intestinal fluid containing 0.1% pancreatin (SIGMA); 0.3% Na-colat (SIGMA) at pH 8. Cells were soaked for 240 minutes. The cells that are resistant to the condition of the upper digestive tract are further calculated by fertilizing on MRSA media. A total of 100 µl of cell mass and 7.5 µl of pepsin (concentration 1 mg / 75 µl) were inserted into an eppendorf containing 1 ml of artificial gastric of pH 2, 3, 4, and vortexed. Furthermore, 100 µl of culture mixture and pepsin was taken for dilution with 0.9 ml saline to obtain 10^{-1} dilution. Next, dilution is graded until obtained dilution of 10^{-6} (done for each pH). Dilution results for 10-4 until 10-6, each taken as much as 100 µl then planted on the MRSA media to be spread by using hoky steak, then incubated for 24 - 48 hours at 37 ^{o}C. Furthermore, colonies that grow are then counted.

The culture mixture with the remaining pepsin from "a" is placed in a shaker with a speed of 120 rpm for 90 minutes (1,5 hour treatment). After that 100 µl of the mixture was taken and inserted into an eppendorf containing 0.9 ml of saline to obtain 10^{-1} dilution. Next, dilution is graded until obtained dilution of 10^{-6} (done for each pH). Dilution results for 10-4 until 10-6, each taken as much as 100 µl then planted on the MRSA media to be spread by using hoky steak, then incubated for 24 - 48 hours at 37 ^{o}C. Furthermore, colonies that grow are then counted. The culture mixture with the remaining pepsin is placed in a shaker with a speed of 120 rpm for 90 minutes (3 hour treatment) then continued with the exact step as "b". The rest of the culture mixture from the previous step was centrifuged at 3000 rpm, 27^{0}C for 15 minutes, the supernatant was thrown away and then add 1 ml saline, vortex, centrifuged again (washed 2 times). After being washed 2 times, 700 µl of gastric artificial fluid pH 8 was added into the mass of the cell, then added 7,5 ml of pancreatin (1mg / 75µl) and 10 µl NaDC (100mM), vortex, then placed in a shaker with speed of 120 rpm for 4 hours (4 hour treatment). 100 µl cultures which have been treated for 4 hours were taken and inserted into an eppendorf containing 0.9 ml of saline to obtain 10^{-1} dilution. Next, dilution is graded until obtained dilution of 10^{-6} (done for each pH). Dilution results for 10-4 until 10-6, each taken as much as 100 µl then planted on the MRSA media to be spread by using hoky steak, then incubated for 24 - 48 hours at 37 ^{o}C. Furthermore, colonies that grow are then counted.

III. RESULT AND ANALYSIS

Resistance on Upper Digestive Tract

Testing of lactic acid bacteria resistance in upper digestive tract is done to know the life ability of lactic acid bacteria from the stomach to the intestine. This is one criteria that is important for bacteria that has potential as probiotics. Probiotic bacteria must be able to live and survive until reached to the intestines to continue to have functions for health. The upper human digestive tract has a low acidity (pH) condition that varies from 2 to 4. The food consumed will be in the stomach for about 90 minutes to 2 hours and will reach the intestines in the range of 7 to 9 hours. Based on this, the lactic acid bacteria that are potential for probiotics will be tested by growing the isolates on the media that has been regulated the condition of acidity according to the condition of acidity in the human digestive tract.

Resistance at pH 2

Based on the testing of lactic acid bacteria resistance in upper digestive tract with medium condition of pH 2, it is known that 5 species of LAB breast milk isolates have different resistance. This is indicated by the decrease in total amount of LAB before and after inoculation in low pH medium. The data of total LAB reduction can be seen in Figure 1.

Based on Figure 1 it can be seen that the resistance of lactic acid bacteria in medium with pH 2 varies. From 5 species of LAB breast milk isolates that were tested, it was known that there has been a decrease as well as increase in total of LAB ranging from 2 to 5 log cycles for 8,5 hours during exposure in medium with pH 2.

The initial amount of LAB isolate of *L. plantarum 1* A1 was 1,1 x 10^{8} cfu/ml, where as after inoculated in medium pH 2 was decreased by 3 log cycles, after

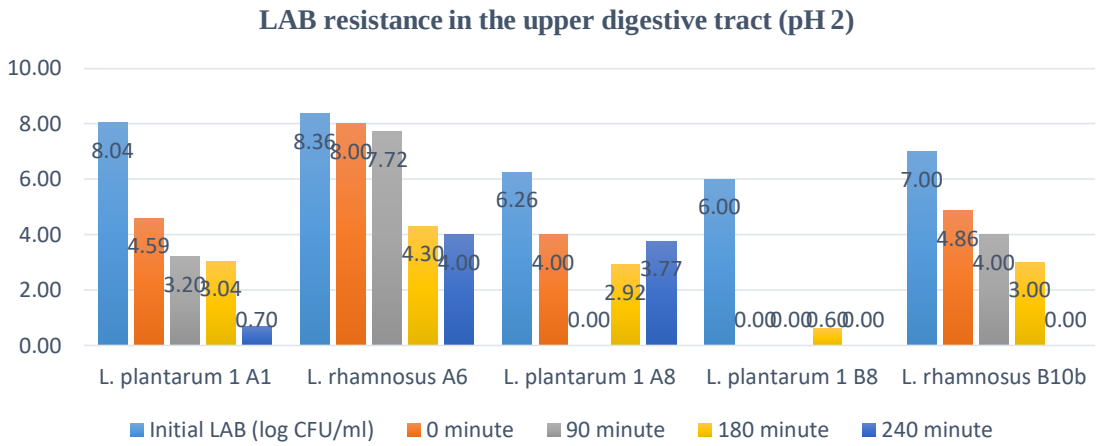

Figure 1. LAB resistance of breast milk isolates in upper digestive tract (medium with pH 2)

incubated for 90 minutes there was a decrease of 2 log cycles, after incubated for 180 minutes there were no decreases nor increases of log cycle of the total of LAB. After incubated for 240 minutes the total LAB decreased again by 2 log cycles from the total LAB before.

The decrease in total LAB due to the effect of extreme medium pH caused by the occurrence of cell death caused by bacterial cell wall lysis. According to Hong *et al.* (2005), when the bacterial cell is in a very acidic condition for a long time, then the cell membrane can be damaged and results in the loss of intracellular components from within the cell. This can cause cell death.

For isolate *L. rhamnosus* A6, the initial amount of LAB isolate was 2,3 x 10^8 cfu/ml, where as after inoculated in medium pH 2 there were no decreases nor increases of log cycle. After incubated for 90 minutes there were still no decreases nor increases of log cycle, next after incubated for 180 minutes the total LAB decreased 4 log cycles, while after incubated for 240 minutes the total LAB did not decrease nor increased.

The initial amount of LAB isolate from *L. plantarum 1 A8* was 1,8 x 10^6 cfu/ml, after inoculated in medium pH 2 was decreased by 2 log cycles . After incubated for 90 minutes there was a decrease of 3 log cycles, after incubated for 180 minutes the total LAB increased 1 log cycle. After incubated for 240 minutes the total LAB again increased 2 log cycles from the initial amount of LAB before.

For isolate *L. plantarum 1* B8, the initial amount of LAB isolate was 1,0 x 10^6 cfu/ml, after inoculated in medium pH 2 was decreased by 5 log cycles. After After incubated for 90 minutes there were no decreases nor increases of log cycle, next after incubated for 180 minutes the total LAB increased 1 log cycle, while after incubated for 240 minutes the total LAB decreased 1 log cycle.

The initial amount of LAB isolate of *L. rhamnosus* B10b was 1,0 x 10^7 cfu/ml, after inoculated in medium pH 2 was decreased by 2 log cycles. After incubated for 90 minutes there was a decrease of 1 log cycle, after incubated for 180 minutes the total LAB decreased 1 log cycle. After incubated for 240 minutes the total LAB again decreased 2 log cycles from the initial amount of LAB before.

Resistance at pH 3

Based on the testing of lactic acid bacteria resistance in upper digestive tract with medium condition of pH 3, it is known that 5 species of LAB breast milk isolates have different resistance. This is indicated by the decrease in total amount of LAB before and after inoculation in low pH medium. The data of total LAB reduction can be seen in Figure 2.

Based on Figure 2 it can be seen that the resistance of lactic acid bacteria in medium with pH 3 varies. From 5 species of LAB breast milk isolates that were tested, it was known that there has been a decrease as well as increase in total of LAB ranging from 1 to 7 log cycles for 8,5 hours during exposure in medium with pH 3.

The initial amount of LAB isolate of *L. plantarum 1* A1 was 1,1 x 10^8 cfu/ml, where as after inoculated in medium pH 3 was decreased by 2 log cycles, after incubated for 90 minutes there was a decrease of 2 log cycles, after incubated for 180 minutes decreased 1 log cycle of the total of LAB. After incubated for 240 minutes the total LAB decreased again by 2 log cycles from the total LAB before.

The decrease in total LAB due to the effect of extreme medium pH caused by the occurrence of cell death caused by bacterial cell wall lysis. According to Hong *et al.* (2005), when the bacterial cell is in a very acidic condition for a long time, then the cell membrane can be damaged and results in the loss of intracellular components from within the cell. This can cause cell death.

For isolate *L. rhamnosus* A6, the initial amount of LAB isolate was 2,3 x 10^8 cfu/ml, where as after inoculated in medium pH 3 there were no decreases nor increases of log cycle. After incubated for 90 minutes

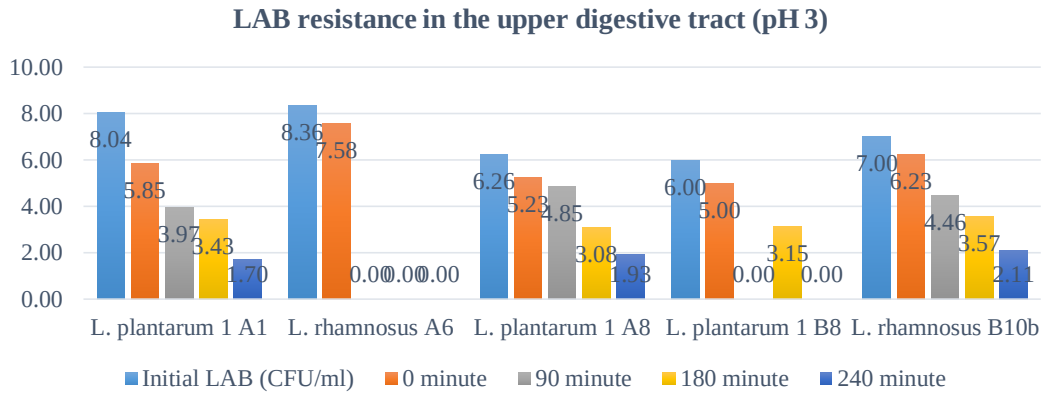

Figure 2. LAB resistance of breast milk isolates in upper digestive tract (medium with pH 3)

there was a decrease of 7 log cycles, next after incubated for 180 minutes were no decreases nor increases of log cycle, while after incubated for 240 minutes the total LAB still did not decrease nor increased.

The initial amount of LAB isolate of *L. plantarum 1 A8* was 1,8 x 10^6 cfu/ml, after inoculated in medium pH 3 was decreased by 1 log cycle. After incubated for 90 minutes there were no decreases nor increases of log cycles, after incubated for 180 minutes the total LAB decreased 2 log cycles. After incubated for 240 minutes the total LAB again decreased 1 log cycle from the initial amount of LAB before.

Resistance at pH 4

Based on the testing of lactic acid bacteria resistance in upper digestive tract with medium condition of pH 4, it is known that 5 species of LAB breast milk isolates have different resistance. This is indicated by the decrease in total amount of LAB before and after inoculation in low pH medium. The data of total LAB reduction can be seen in Figure 3.

Based on Figure 3 it can be concluded that the resistance of lactic acid bacteria in medium with pH 4 varies. From 5 species of LAB breast milk isolates that were tested, it was known that there has been a decrease as well as increase in total of LAB ranging from 1 to 3 log cycles for 8,5 hours during exposure in medium with pH 4.

The initial amount of LAB isolate of *L. plantarum 1* A1 was 1,1 x 10^8 cfu/ml, where as after inoculated in medium pH 4 was decreased by 2 log cycles, after incubated for 90 and 180 minutes there were no decreases nor increases of log cycles. After incubated for 240 minutes the total LAB decreased by 3 log cycles from the total LAB before.

The decrease in total LAB due to the effect of extreme medium pH caused by the occurrence of cell death caused by bacterial cell wall lysis. According to Hong *et al.* (2005), when the bacterial cell is in a very acidic condition for a long time, then the cell membrane can be damaged and results in the loss of intracellular components from within the cell. This can cause cell death.

For isolate *L. rhamnosus* A6, the initial amount of LAB isolate was 2,3 x 10^8 cfu/ml, where as after inoculated in medium pH 4 there was a decrease of 1 log cycle. After incubated for 90 minutes there was a decrease of 2 log cycles, next after incubated for 180 minutes there was a decrease of 1 log cycle, while after incubated for 240 minutes the total LAB decreased again for 3 log cycles.

The initial amount of LAB isolate of *L. plantarum 1 A8* was 1,8 x 10^6 cfu/ml, after inoculated in medium pH 4 there was a decrease of 2 log cycles. After incubated for 90 minutes there was an increase of 1 log cycle, after incubated for 180 minutes the total LAB decreased 1 log cycle. After incubated for 240 minutes the total LAB again decreased 1 log cycle from the initial amount of LAB before.

For isolate *L. plantarum 1* B8, the initial amount of LAB isolate was 1,0 x 10^6 cfu/ml, after inoculated in medium pH 4 was decreased by 1 log cycle. After incubated for 90 minutes there were no decreases nor increases of log cycles, next after incubated for 180 minutes the total LAB decreased 1 log cycle, while after incubated for 240 minutes the total LAB increased 1 log cycle.

The initial amount of LAB isolate of *L. rhamnosus* B10b was 1,0 x 10^7 cfu/ml, after inoculated in medium pH 4 was decreased by 1 log cycle. After incubated for 90 minutes there was an increase of 1 log cycle, after incubated for 180 minutes the total LAB decreased 2 log cycles. After incubated for 240 minutes the total LAB decreased again for 3 log cycles from the initial amount of LAB before.

IV. CONCLUSSION

From the 5 species of LAB breast milk isolates tested for survival in the upper human digestive tract, it can be concluded that:

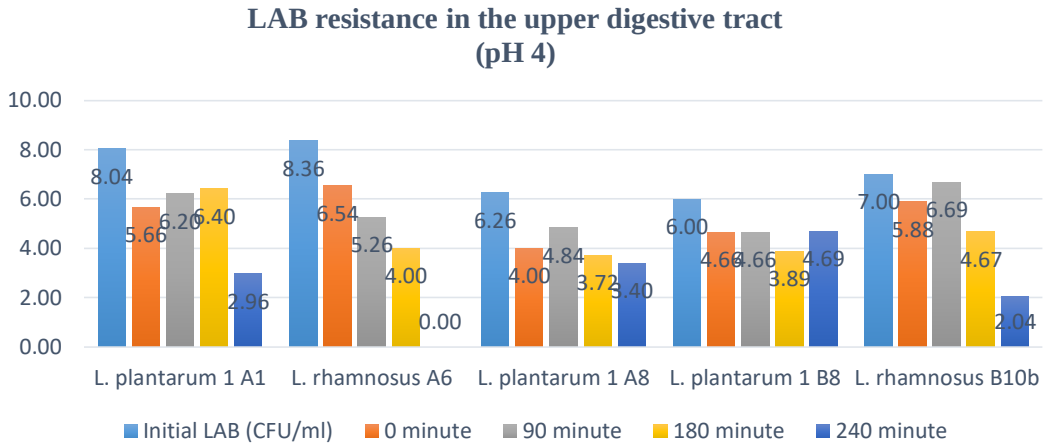

Figure 3. LAB resistance of breast milk isolates in upper digestive tract (medium with pH 4)

1. Five species of LAB breast milk isolates that were tested had different resistance. This is indicated by the decrease in total amount of LAB which varies before and after inoculation in medium with pH 2, 3, and 4.
2. There was a decrease between 2 - 5 log cycles at pH 2. The LAB isolate which had the highest resistance at pH 2 was *L. rhamnosus* A6 with exposure time of 90 - 240 minutes.
3. There was a decrease between 1 – 7 log cycles at pH 3. The LAB isolates which has the highest resistance at pH 3 was *L. plantarum 1* A1, *L. plantarum 1* A8, and *L. rhamnosus* B10b with exposure time of 90 - 240 minutes.
4. There was a decrease between 1 – 3 log cycles at pH 4. The 5 species of LAB breast milk isolates were able to survive at pH 4 with a 90 - 240 minute exposure time.

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