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Isolation and characterization of lactic acid bacteria from *Apis mellifera* stomach and their potential as antibacterial using in vitro test against growth of *Staphylococcus aureus* and *Salmonella typhimurium*

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A B S T R A C T

Food contaminated by pathogenic bacteria is very dangerous to consume especially if the bacteria can produce toxins. *Staphylococcus aureus* and *Salmonella typhimurium* are two examples of pathogenic bacteria that cause demage to food because they produce enterotoxins that can cause food poisoning to consumers. The use biological agents as antibacterial agents can be used as a solution to overcome this. Lactic acid bacteria (LAB) can produce compounds thet can inhibit the growth of pathogenic bacteria in food. This study aims to obtain local LAB isolates from the stomach of *Apis mellifera* in Indonesia and determine their characteristics as potential biopreservatif foods, to test the inhibition of LAB bacteriocin against Staphylococcus *aureus* and *Salmonella typhimurium* in vitro. The isolation method was carried out using multilevel dilution and streak plates on MRSA media. Morphological identification was carried out macroscopically on BAL and microscopic colonies with gram staining. Antibacterial test using the well method. LAB isolation result obtained eight isolatates. Antibacterial activitytest results for cells, all isolates showed inhibition of *Staphylococcus aureus* and *Salmonella typhimurium, for* further antibacterial testing using 2 potential isolates namely PAM 3 and PAM 4. The two isolates were further tested for antibacterial with cell free supernatant using two treatments namely pH acidic and neutral, both isolates showed antibacterial activity at acidic ph better than neutral ph, wheares for the bacteriosin analysis test all isolates had no inhibition.

Keywords: lactic acid bacteria, Apis mellifera, antibacterial, Staphylococcus aureus, Salmonella typhimurium.

I. INTRODUCTION

Food consisting of meat and milk has perishable properties, so it has a shorter lifespan. Food can be said to be damaged or rotten when changes occur that cause the food can no longer be consumed by consumers. One of the damages that occurs in food is microbiological damage that can be caused by the presence of pathogenic bacteria. Examples of pathogenic bacteria that contaminate food ingredients are *Staphylococcus aureus* and *Salmonella typhimurium*. According to Heredia et al., (2009), Staphylococcus aureus and Salmonella typhimurium are known as food poisoning bacteria, because they produce heat-resistant enterotoxins (100^0 C for 30 minutes).

Salmonella typhimurium is a Gram-negative bacterium found in dairy products. According to Monica et al., (2013), *Salmonella typhimurium* bacteria are pathogenic and can infect humans and animals, these bacteria usually damage food such as milk, chicken, beef, and eggs. Staphylococcus aureus belongs to a group of Gram-positive bacteria and can damage food such as meat and milk. *S. aureus* is also one of the bacteria that causes poisoning after drinking milk. Jorgensen et al., (2005). In some countries in Europe, such as Norway, S. aureus is one of the bacteria that causes poisoning after drinking milk, this bacterium can also damage processed foods derived from meat.

This food damage can be prevented by preservation that will extend the shelf life of food. The preservation method itself can be done in three ways, namely physical preservation, chemical preservation, and microbiological preservation. This research emphasizes the preservation of food in microbiological terms, because preservation in microbiological terms is safe and can increase the nutritional value of food. Microbiological preservation can be done using Lactic Acid Bacteria and the metabolites it produces.

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Lactic Acid Bacteria (LAB) is a gram-positive bacterium. This bacterium is one example of beneficial microorganisms and has an important role in the food industry. Lactic Acid Bacteria are also good bacteria that can inhibit pathogenic bacteria. According to Audisio et al. (2011), lactic acid bacteria produce antibacterial in the form of organic acids, bacteriocin, hydrogen peroxide, diacyl, carbon dioxide, acetaldehyde and reduce the pH of the environment by expressing compounds that can inhibit pathogenic bacteria. In addition, Leverentz et al. (2006) states that LAB is a microorganism that can be used in controlling the growth of pathogenic bacteria in food because it can reduce pH and produce bacteriocin which can inhibit the growth of pathogenic bacteria such as *Staphylococcus aureus* and *Salmonella typhimurium.*

LAB in this study will be isolated from the stomach of honeybees (*Apis mellifera*). According to Segueni et al. (2011), *Apis mellifera* honeybees have an advantage in the high amount of honey production and are not aggressive or benign. Lyapunov et al. (2008), LAB can be found in the digestive tracts of mammals and vertebrate animals and from existing research found Lactobacillus in the honeybee channel. Olofsson & Vasquez (2014), there are 7 species of LAB found in the stomach of honeybees which are the central organs for nectar collection.

Therefore, this study will examine the isolation of BAL from the stomach of *Apis mellifera* which is expected to improve the quality of food. LAB isolation from the stomach of Apis mellifera itself in Indonesia has not been done much, especially research that specifically examines the isolation of BAL from the stomach Apis mellifera which has the potential as an antibacterial against the growth of pathogenic bacteria in food for example *Staphylococcus aureus* and *Salmonella typhimurium.*

II. MATERIALS AND METHODS

Materials

The tools used are petri dishes, test tubes, autoclaves, ovens, microwaves, measuring cups, test tube racks, digital scales, erlenmeyers, bunsen, vortex, round ose, microtube and its container, micropipette, blue tip, yellow tip, sprayer, stove , heater, pan, laminar air flow, needles, tweezers, mortar, centrifuge, magnetic stirer, rotary shaker, microscope, UV spectrophotometer, stainless steel borer, ruler, beaker and cover glass.

The materials used are honey bee Apis mellifera, culture stock of Staphylococcus aureus ATCC 25923 and Salmonella typhimurium ATCC 202117,, MRS, agar, CaCO3, distilled water, NA, sodium chloride, barium chloride, hydrochloric acid, sulfuric acid, Salmonella typhimurium ATCC 202117,, MRS, agar, CaCO3, distilled water, NA, sodium chloride, barium chloride, hydrochloric acid, sulfuric acid, chloramphenicol, pepsin enzyme, reagent Gram stain, H2O2, alcohol, cotton, gauze, plastic wrap, aluminum foil, heat-resistant plastic, matches, labels, disinfectants.

Methods

LAB Isolation and Enumeration

LAB isolation in the stomach of Apis mellifera was carried out using serial delution by pour plate method. The surface sterilization of honey bees is done to remove the bee surface from contamination. The next step after surface sterilation is to separate the bees' stomach from other parts, after the bee's stomach has been crushed using a mortar until it is smooth. LAB populations are enumerated using multilevel dilution and cup count method from 10-1 dilution to 10-5 dilution. The result of dilution is grown into a petri dish and incubated at 37° C for 48 hours, after growing the bacterial colony is enumerated in the form of Colony Forming Units (CFU/ gram) (Musnelina et al., 2004).

The isolates that have been grown are then sub-cultured on new media using the streak plate method to obtain a single colony. Bacteria that have been grown are taken with ose and then rubbed into new media. Bacteria were incubated at 37 ° C for 48 hours. LAB colonies were cultured until they were single isolates. This means that the bacterial colonies from the streak plate are uniform and there are no different colonies (Musnelina et al., 2004).

Gram Staining

Isolates taken from sloping agar are updated using Gram stain / stain painting. Glass objects are sterilized using alcohol. Then sterile distilled water is dripped on glass. Ose sterilized by heating using a Bunsen flame until it blazed. Isolates from sloped agar were taken using ose. The isolate was flattened over sterile distilled water on a glass slide. Then fixed above Bunsen. 1 drop of Gram A (violet crystal solution) is dripped on top of the isolate. Wait for 2 minutes. Then washed using distilled water. 1 drop of Gram B (mordan solution) is dropped onto the isolate. Wait for 1 minute. Then washed using distilled water. 1 drop of Gram C (alcohol) is dropped on the isolate. Wait for 30 seconds. Then washed using distilled water. 1 drop of Gram D (safranin solution) is dripped

on top of the isolate. Wait for 1 minute. Then washed using distilled water. The isolate was then identified under a microscope If the isolate is purple, the isolate is gram + (positive) bacteria, if it is red, the isolate is gram- (negative) bacteria (Malik and Mustafa, 2017).

Catalase Test

Isolate from sloping agar is taken one ose, then applied to glass objects that have been sterilized with alcohol. Glass objects dropped with a solution of H2O23%. It is observed the formation of gas bubbles in the preparation. If there are gas bubbles, the catalase test is positive (Buntin, 2008).

Resistance Test at Low Temperature

LAB isolates were taken then cultured on MRSB 5 ml media and incubated at 8oC for 48 hours. LAB growth was measured using a spectrophotometer that is by taking 1 mL of LAB suspension on the MRSB which was inserted into the cuvette. Aquades are used as blanks. The suspension is then measured by the absorbance value with a wavelength of 625 nm. LAB growth is expressed as Δ log CFU / mL over a range of days from 0 to 48 hours (Todorov, 2017).

Resistance Test for Sodium Chloride (NaCl)

LAB isolates were cultured on 10 mL MRSB media that had been added with NaCl with concentrations of 2%, 4%, and 6% (m / l). The suspension was taken 3 ml to measure the absorbance value using a spectrophotometer as the initial OD with a wavelength of 625 nm. Then incubated at 37o C for 48 hours. LAB growth was measured after 48 hours of incubation by calculating the absorbance value as the final OD, ie by taking 3 mL of BAL suspension on the MRSB which was put into the cuvette. MRSB media is used as a blank. The suspension is then measured by the absorbance value with a wavelength of 625 nm. LAB growth is expressed as Δ OD CFU/mL (Todorov, 2017).

Fermentation Test

Fermentation type tests are used to classify lactic acid bacteria into homofermentative or heterofermentative groups. The test was carried out by growing bacterial culture in liquid MRS as much as 10 ml in a test tube containing a durham tube. Incubation for 24 hours at 37^0 C. Observations were made by looking at the formation of air bubbles in the durham tube (Romadhon et al., 2012).

Motility Test

Bacterial isolates were inserted into the MRSB containing 0.5% agar (agar soft) using acute ose. Incubation is carried out at room temperature for 24 hours. If bacterial growth spreads, the bacteria are motile and if bacterial growth does not spread, only in the form of a line along the puncture then the bacteria are non-motile (Aisyah et al., 2014).

Antibacterial testing of *Staphylococcus aureus* and *Salmonella typhimurium*

Bacteria Preparation Test

Rejuvenation of Salmonella typhimurium and Staphylococcus aureus isolates which will be tested for antibacterial is done by inoculating the stock culture of Salmonella typhimurium and Staphylococcus aureus in NA media. Making Nutrient media Agar by weighing as much as 2 grams of NA is inserted into the erlenmeyer, dissolved with 100 ml of distilled water. Then homogenized with a magnetic stirrer on the hot plate. Furthermore, it is sterilized by autoclaving (Arulanatham et al. 2012).

LAB Screening Test for Antibacterial Producing Against *S. aureus* and *Salmonella typhi.* Growth.

BAL isolates that have been purely inoculated into MRSB media and calculated turbidity with OD. Staphylococcus aureus and Salmonella typhimurium from stock cultures were taken 1 ounce each and mixed into 0.9% NaCl. The turbidity is equal to Mc Farland's standard 0.5 (Cockerill, 2012).

The screening process of BAL producing antibacterial compounds is carried out by Staphylococcus aureus and Salmonella typhimurium dispread on NA media until it is evenly distributed to all parts of the media on the petri dish. Screening is done by the well diffusion method. The well is made with a media hole (stainless steel borer) with a diameter of 9 mm. Each well hole is filled with 80 µL BAL. Then incubated at 37oC for 48 hours. Inhibited zones formed were measured using a ruler (Goyal et al. 2018).

This antibacterial activity test uses two dick namely positive control and negative control as a comparison. Positive control of chloramphenicol which has been dissolved into sterile distilled water, and negative control of MRSB media that is not overgrown with bacteria.

Antibacterial Test with Cell Free Supernatant (CFS) LAB and Analysis of Subtance Inhibitors of Growth of *S. aureus* and *Salmonella typhi.*

15 ml of bacterial liquid culture was centrifuged at 4000 rpm for 2 x 20 minutes, so that a cell-free supernatant was obtained. The filtrate was then put into a sterile tube for further testing (Fortuna et al., 2014).

The supernatant that has been obtained is further divided into three tubes of 3 ml each, ie the initial supernatant has an acidic pH, supernatant with neutralized pH, with supernatant for analysis of antibacterial substances. whereas supernatants with neutralized pH are given NaOH up to a pH value of 7 (neutral). Antibacterial test using cell-free supernatant using the well method with each isolate was repeated. The well is made with a media hole (stainless steel borer) with a diameter of 9 mm. Each well hole is filled with 80 µL suspension. After the inhibition zone is formed around the wellbore, the zone is measured using a ruler.

Analysis of antibacterial substance on LAB was carried out by adding the pepsin enzyme into the supernatant with neutralized pH, then heated for 5 minutes to stop the enzymatic reaction. Antibacterial test for antibacterial substance analysis using the well method. The well is made with a media hole (stainless steel borer) with a diameter of 9 mm. Each wellbore is filled with 80 µL suspension. After the inhibition zone is formed around the wellbore, the zone is measured using a ruler.

Data Analysis

Research using a Completely Randomized Design (CRD), with three factors. The first factor is LAB isolates, as many as two potential isolates that have antibacterial activity against test bacteria. The second factor is the different pH conditions at the supernatant, namely acidic and neutral pH. The third factor is test bacteria, namely two food-spoiling pathogenic bacteria Staphylococcus aureus and Salmonella typhimurium. Each treatment was repeated six times. Quantitative data in the form of inhibition zone diameters were analyzed using SPSS 16.0 software.

III. RESULTS AND DISCUSSION

Isolation and Enumeration of LAB from the Stomach of *Apis mellifera*

Isolation of lactic acid bacteria (LAB) from the stomach of Apis mellifera was carried out using MRS (de Man Rogosa Sharpe) media aimed at optimizing growth and obtaining the expected colony of lactic acid bacteria. MRS media used were added agar and CaCO3. The addition of CaCO3 is used as an indicator of a bacterial colony capable of producing acids, by showing the growth of colonies that form a clear zone (clear zone). According to Subagiyo et al., (2016), MRS agar medium added with CaCO3 obtained colonies showing clear zones.

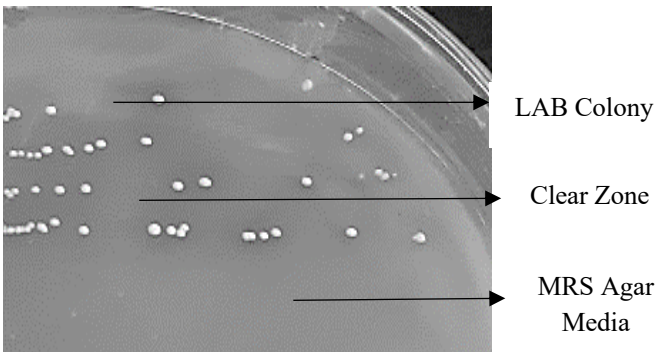


Figure 1. LAB colony on MRS Agar media

LAB isolation from the stomach of Apis mellifera was carried out by the pouring method or pout plate with multilevel dilution. Calculation data on the number of BAL colonies is presented in Table 1.

Table 1. LAB counts from the stomach *Apis mellifera*

Dilution Factor					CFU/mg	Information
10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}		
TBUD	TBUD	TBUD	47	32	$4{,}7 \cdot 10^5$	$\frac{32 \cdot 10^5}{47 \cdot 10^4} = 6{,}8 \geq 2$

Based on the calculation table the number of BAL colonies from the stomach of A. mellifera shows that the dilutions of 10-1, 10-2, and 10-3 show the growth of TBAL BAL (cannot be counted), because the number of colonies is more than 300. Whereas in dilutions with smaller concentrations of 10-4 and 10-5, each has a colony of 47 and 32, and is included in the calculation because it is in the range of 30-300. The results of the colony calculation from dilution 10-5 are divided by dilution 10-4. The results obtained show a number greater than 2, so based on these results the number of colonies used came from a 10-4 dilution. The total LAB isolated from Apis mellifera's stomach was 4.7. 105 CFU / mg. According to Wasteson and Hornes (2009), the goal of multilevel dilution is to reduce or reduce the number of microbes that are suspended in a liquid. Determination of the amount or amount of dilution depends on the estimated number of microbes in the sample. 1: 9 ratio is used for the sample and first and subsequent dilutions. According to Fardiaz (2004), SPC is a method to get the results of the number of microbes with a range of 30 - 300 CFU (Colony Forming Unit) / ml from dilution.

LAB isolates that have been obtained by the pour plate method are then purified on MRS media so that the petri dish uses the streak plate method. The isolate was continuously transferred into new media in a petri dish by the streak plate method until a single LAB isolate was obtained. LAB isolates obtained from the stomach of Apis mellifera totaled 8 isolates. The pure isolates were subsequently cultivated into slanted agar as a culture stock. The culture stock obtained was used for further tests.

Characterization of LAB from the stomach of *Apis mellifera*

Morphological Characterization of LAB

LAB single isolate was further characterized microscopically and macroscopically. Macroscopic characterization is done by observing the shape, color, texture, edges, surface, and elevation of the colonies that are formed directly. ased on macroscopic characterization of LAB colonies, they have a round colony shape with a milky white color with a colony diameter of 1-2 mm. Microscopic characteristics are performed by looking at the shape of LAB cells by the Gram staining method.

The eight LAB isolates from the stomach of Apis mellifera were found to have microscopic characteristics as follows;

Observation on a microscope with Gram staining shows the results of testing that bacterial cells are purple, this shows that the isolated bacteria belong to the class of Gram positive bacteria. According to Ibrahim (2015), Gram-positive bacteria have cell wall characteristics with thicker peptidoglycan so that the absorption of color from violet crystalline paint that is absorbed in the cell will survive even though washing is done using color paint (a solution of alcohol-lugol) which is expected to wear off the first color paint.

Physiological Characterization of LAB

Physiological characterization of BAL includes catalase test, NaCl resistance test, resistance test at low temperature, motility test, and fermentative test.

Catalase test showed that the eight isolates of BAL showed negative catalase results in which bubbles did not form on glass objects containing H_2O_2 liquid. According to Ibrahim et al., (2015), lactic acid bacteria in the catalase test are negative catalase, this is because lactic acid bacteria do not produce the enzyme catalase which can convert hydrogen peroxide into water and oxygen and is related to the ability of lactic acid bacteria that only need a little oxygen to be able to live.

Further characterization was carried out to see the potential of LAB as a food preservation application material by testing the LAB resistance at low temperatures. LAB incubated at 8 ° C and the results of LAB growth measurements

were carried out in the range of days 0 to day 5. The measurement results on isolate 1 to isolate 8 are different. The BAL growth with the highest OD was isolate 1 which was Δ log = 0.807 and the lowest was isolate 8 which was Δ log = 0.1220.

NaCl resistance test was carried out with concentrations of NaCl 2%, 4%, and 6%. Based on the LAB growth data for the NaCl variations tested, isolat BAL 1 hingga 8 memiliki tingkat pertumbuhan yang berbeda-beda. NaCl 2% concentration, isolate 3 had the highest growth rate with absorbance value of 0.860 CFU / ml, and isolate 5 had the lowest growth rate with an absorbance value of 0.391 CFU / ml. LAB isolates were grown at 4% NaCl, the highest growth was in isolate 3 with absorbance value of 0.697 CFU / ml and the lowest growth was in isolate 7 with absorbance value of 0.484 CFU / ml. NaCl with a concentration of 6%, isolate 1 had the highest growth with an absorbance value of 0.249 and the lowest growth was isolate 5 with an absorbance value of 0.038 CFU / ml. Based on these data LAB grows well at 2% and 4% NaCl concentrations, and begins to be intolerant at 6% NaCl concentrations. According to Adnan et al., (2006), lactic acid bacteria are tolerant of 2% NaCl concentration and begin to be intolerant at NaCl concentrations of more than 5%. NaCl concentrations that are too high will affect the growth of LAB, where the production of bacteriocin or substances to inhibit the growth of pathogens will also be disrupted.

The fermentation test is carried out to determine the LAB that is isolated is homofermentative or heterofementative. According to Purwohadisantoso et al. (2009) and Suryani et al. (2010), states that no gas formation indicates that the isolate is a homofermentative LAB, while those that produce acids, CO2 gas, alcohol and other evaporating compounds are included in heterofermentative fermentation type bacteria. Based on data of PAM1, PAM2, PAM6, PAM7, and PAM8 classified as LAB with heterofermentative fermentation type, whereas PAM3, PAM4, and PAM5 are classified as LAB with homofermentation type fermentation.

Motility test is done to see the movement of bacteria. Based on tests that have been carried out after 24 hours incubation, on 8 isolates of LAB showed that LAB was non motile or not moving characterized by the growth of strains that did not spread on the media, LAB only grows on the path formed by ose prick. According to Desniar et al., (2012), LAB is non-motile or does not move, this is because LAB does not have flagella or locomotor. The motility test shows that the growth of LAB strains is only a straight line on semi-solid media.

PAM 1 and PAM 6 in the form of cocoa or round colony, positive at incubation temperature of 8 0C, negative in the 6% NaCl test, and heterofermentative in the fermentation test possibly included in the genus Lactococcus. PAM 2, PAM 7, and PAM 8 in the form of rounded colonies, negative at incubation temperature of 8 0C and 6% NaCl test, bersifat heterofermentatif pada uji fermentasi kemungkinan termasuk dalam genus *Streptococcus.* PAM 3 in the form of coccus or globular colonies, positive at incubation temperature of 8 0C, negative in the 6% NaCl test, and homofermentative in the fermentation test may be included in the genus Lactococcus. PAM 4 and PAM 5 in the form of rounded colonies, negative at incubation temperature of 8 0C and 6% NaCl test, are homofermentative in the fermentation test possibly included in the genus Streptococcus. According to Adiguna and Oedijani (2017), the genera Streptococcus and Lctococcus have a round or coccus cell shape.

Table 2. Characterization and Probability of the LAB Genus of Stomach *A. mellifera*

Isolate	Characterization					Probabilitas Genus
	Cell Shape	Temp. 8 ^{0}C	NaCl 6%	Fermentation Test	Motilitas Test	
PAM 1	Coccus	+	-	Heterofermentatif	-	*Lactococcus*
PAM 2	Coccus	-	-	Heterofermentatif	-	*Streptococcus*
PAM 3	Coccus	+	-	Homofermentatif	-	*Lactococcus*
PAM 4	Coccus	-	-	Homofermentatif	-	*Streptococcus*
PAM 5	Coccus	-	-	Homofermentatif	-	*Streptococcus*
PAM 6	Kokus	+	-	Heterofermentatif	-	*Lactococcus*
PAM 7	Coccus	-	-	Heterofermentatif	-	*Streptococcus*
PAM 8	Coccus	-	-	Heterofermentatif	-	*Streptococcus*

Note: Low Temperature + = OD ≥0,370 -=≤0,370 NaCl + = OD ≥0,4 -=OD ≤0,4

Antibacterial Test

LAB Screening Produces Antibacterial

Screening is done to get potential isolates, this method for antimicrobial testing using LAB cells. Anti-bacterial-producing LAB screening for *S. aureus* and *Salmonella typhi.* performed by the well diffusion method.

Based on data that has been obtained from screening results, PAM 3 and PAM 4 have the best inhibition zone when compared with other isolates. Both isolates were also stable in inhibiting the growth of pathogens both on the first and second repetitions in 24-hour incubation of the two test bacteria, so PAM 3 and PAM 4 are chosen as potential isolates which will be used for further antimicrobial tests. PAM 3 has an average inhibition zone of 13.29 mm in inhibiting the growth of *S. aureus*, and 10.51 mm to inhibit *Salmonella typhi.* PAM 4 has a zone of inhibition of 16.11 mm in inhibiting the growth of *S. aureus*, and 12.42 mm to inhibit *Salmonella typhi*.

The control used is chloramphenicol as a positive control and MRSB as a negative control. According to Nuria (2009), positive control functions as a control of test substances, by comparing the diameter of the inhibitory zone (inhibitory zone) formed, while the negative control serves to determine whether there is an effect of solvents on bacterial growth so that it can be seen that which has antibacterial activity is a substance from LAB not from a substance contained in the media. The inhibition zone diameter of the positive control of the *S. aureus* test bacteria was 20.82 mm while the negative control showed no inhibitory zone. Inhibitory zone diameters of positive control of *Salmonella typhi.* test bacteria. was 22.42 mm, and negative controls did not show any inhibitory zones.

Antibacterial Test using Cell Free Supernatant (CFS) against *S. aureus* and *Salmonella typhi.*

PAM 3 and PAM 4 are two potential isolates obtained from screening results, which has the largest inhibition zone when compared to other isolates. The next stage is making the BAL growth curve to determine at what time the BAL is at the stationary stage. BAL stationary stage is very important to note because in that phase BAL will produce secondary metabolites in the form of antibacterial substances, which later will play a role to inhibit the growth of pathogenic bacteria. According to Yuliana (2008), after the logarithmic phase which is the stationary phase of organic acids produced by BAL such as lactic acid, acetic acid, or pyruvic acid results in the accumulation of acid end products and a decrease in pH which causes growth retardation. Products that might inhibit growth other than lactic acid, can also be carbon dioxide, and other neutral components.

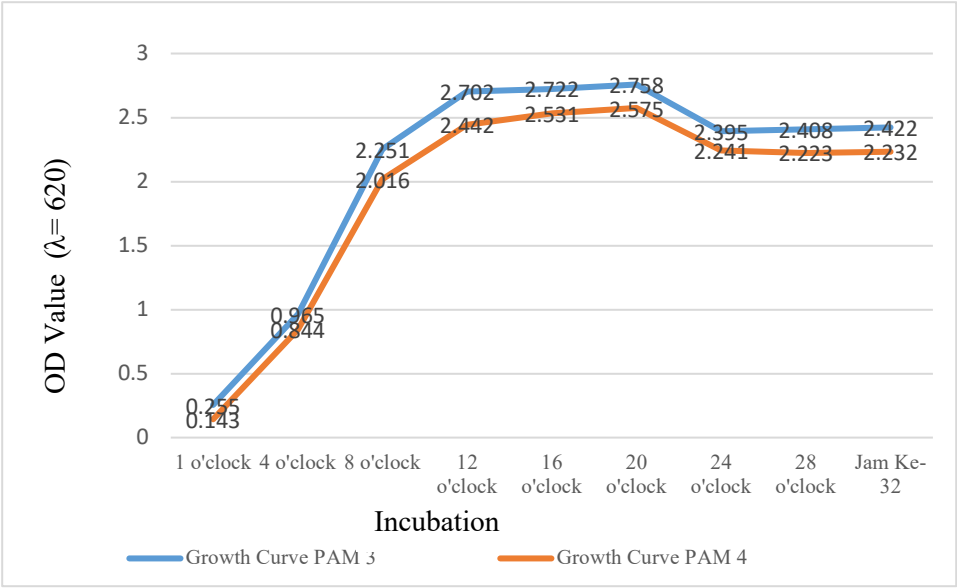
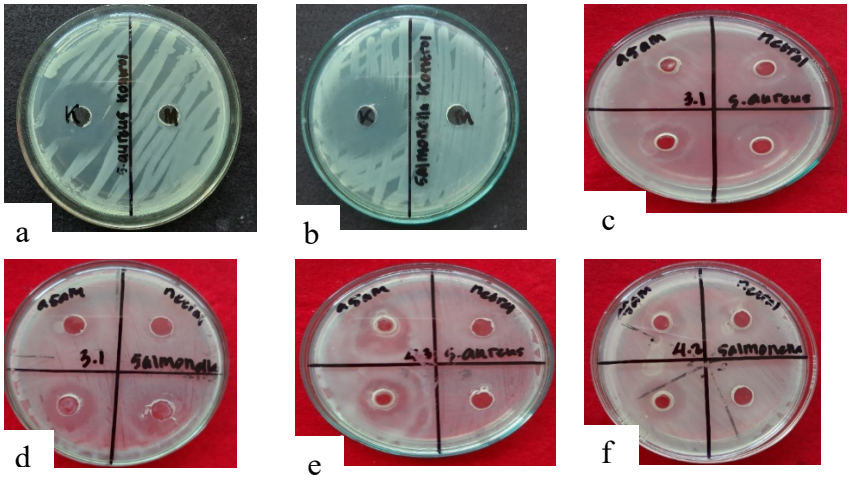


Figure 2. LAB Growth Curve

Based on the LAB growth chart, the two isolates namely PAM 3 and PAM 4 have a final stationary phase time at the 20th hour. The stationary phase is the phase where BAL produces its secondary metabolites, one of the secondary metabolites produced by LAB is bacteriocin or antibacterial substances. According to Yuliana (2008), the stationary

phase of organic acids produced by BAL such as lactic acid, acetic acid, or pyruvic acid results in the accumulation of acid end products and a decrease in pH which causes growth inhibition. Possible products inhibit growth in addition to lactic acid, can also be in the form of carbon dioxide, and other neutral components.

The next antibacterial test uses CFS, using a supernatant with a different pH, namely acid as the initial pH of the supernatant and neutralized supernatant. BAL suspension made after measured pH has a sufficiently acidic pH which is around 4-5, it is caused by the production of lactic acid produced by BAL. For the neutralized treatment, add NaOH until the pH reaches 7. According to Audisio et al. (2011), LAB can produce several metabolites such as organic acids (lactic acid and acetic acid), hydrogen peroxide, diacetyl and bacteriocin.



a. Positive and negative controls on *S. aureus*
b. Positive and negative controls on *Salmonella typhi.*
c. CFS PAM 3 bacterial test against *S. aureus*
d. CFS PAM bacterial test against *Salmonella typhi.*
e. CFS PAM 4 bacterial test against *S. aureus*
f. CFS PAM 4 bacterial test against *Salmonella typhi.*

Table 3. Antibacterial Activity with Cell Free Supernatants

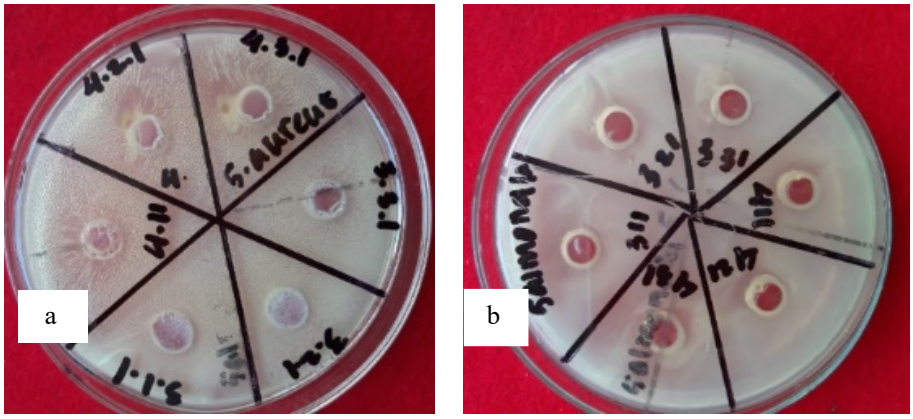
		Obstacles Zone ± Standart Deviation	
Isolate	CFS	*St. aureus*	*Salmonella typhi.*
PAM 3	Supernatant	11,50 ± 2,6	12,57 ± 1,3
	Netralis Supernatant	8,44 ± 1,5	3,17 ± 1,8
PAM 4	Supernatant	13,82 ± 2,6	11,50 ± 1,3
	Netralis Supernatant	4,80 ± 2,9	4,88 ± 1,9

Based on the results of data analysis using SPSS 16.0 with univariate test shows that the two isolates of BAL, namely PAM 3 and PAM 4 have no significant effect in inhibiting the growth of pathogens, meaning that both PAM 3 and PAM 4 have the same ability to inhibit the growth of pathogens. Univariate test results for supernatant conditions with

different pH showed significantly different values, yaitu pH awal yakni asam dan pH yang dinetralkan dinyatakan memiliki perbedaan pengaruh yang nyata secara signifikan satu sama lain terhadap penghambatan pertumbuhan patogen. The results of the univariate test of both pathogenic bacteria, S. aureus and Salmonella typhi. show significant differences from each other. Univariate test results regarding the effect of the treatment given, with independent variables in the form of BAL isolates (PAM 3 and PAM 4), pH, and pathogenic bacteria (S. aureus and Salmonella typhi.) and the dependent variable in the form of inhibitory zones of bacteria have significantly different effect. This means that the interaction between LAB isolates, different pH conditions, and pathogenic bacteria has a significantly different effect on the inhibitory zone formed. According to Mawan (2018), testing for antibacterial activity, if the inhibitory zone that is formed is more widespread then it can be caused by the higher antibacterial compounds produced.

Analysis of Substance Inhibitors produced by LAB

The last antibacterial test is the CFS test to determine the inhibiting substances produced by LAB, both organic acids and bacteriocin. Organic acids and bacteriocin are antibacterial substances produced by LAB. According to Adnan and Tan (2006), LAB is able to produce organic acids, reduce the pH of its environment and excrete compounds that can inhibit pathogenic microorganisms such as H2O2, diacetyl, CO2, acetaldehyde, d-isomers of amino acids and bacteriocin.



a. LAB inhibition zone against *S. aureus*

b. LAB inhibition zone against *Salmonella typhi.*

Based on the analysis of the inhibitory substances produced by LAB that have been carried out by the well's method, no inhibitory zone is formed from the supernatant suspension and from previous antibacterial tests in supernatant conditions with a neutralized pH the resulting inhibition zone is also not sensitive enough. This shows that the inhibition zone formed from the antibacterial test is the role of organic acids produced by LAB, but it does not rule out the possibility that bacteriocin plays a role but in a small frequency. LAB produces a lot of organic acids which causes an acidic pH, a decrease in pH can also be an inhibiting factor for the growth of pathogenic bacteria tested. According to Audisio et al. (2011), states that the main role of LAB is to break down carbohydrates so as to cause a decrease in pH, it is important to inhibit spoilage bacteria and pathogens so as to maintain food quality.

LAB isolated from the stomach Apis mellifera isolates PAM 3 and PAM 4 had antibacterial activity against *S. aureus* and *Salmonella typhi.* test bacteria. Antibacterial activity with the best effect was carried out by supernatant isolate PAM 4 against *S. aureus.*

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