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Isolation and characterization of lactic acid bacteria from *Apis mellifera* and their potential as antibacterial using in vitro test against growth of *Listeria monocytogenes* and *Escherichia coli*

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A B S T R A C T

Food spoilage can caused by pathogenic bacteria and can cause disease (food-borne pathogens). Foods that are vulnerable to pathogenic bacteria are foods that contain lots of protein, such as meat, milk and eggs. *Listeria monocytogenes* and *Escherichia coli* are commonly found in fresh meat. The solution for the problem is microbiological preservation by using Lactic Acid Bacteria (LAB). The purpose of this study is to isolate LAB from *Apis mellifera* honey, find out its characteristics, and test for antibacterial growth of *L. monocytogenes* and *E. coli*. LAB have been isolated with pour plate. Morphological characterization have been done by observing the colonies macroscopically and microscopically. LAB have been enumerated by Total Plate Count (TPC). Antibacterial Test have been done by well diffusion method. Experimental design was using Completely Randomized Factorial Design (CRFD). Statistical analysis have been done by univariate test with SPSS 16.0. 1st factor is LAB, 2nd factor is pH of supernatant and 3rd factor is pathogenic bacteria. Total 9 isolates of LAB were obtained from *A. mellifera* honey. LAB population is 9.6 x 10^5 CFU/mL. The result of screening LAB antibacterial assay is 2 potential isolates, they are MA 3 and MA 4. The best supernatant of LAB that could inhibit the growth of *L. monocytogenes* and *E. coli* is MA 3 with average diameters 12,05 mm and 12,07 mm. Pathogenic bacteria that is more sensitive is *E. coli,* because of the antibacterial compound from LAB is composed by organic acid that can break down the outer membrane and lower the pH, so pathogenic bacterial cannot be adapted to acidic environment.

Keywords: lactic acid bacteria, Apis mellifera honey,antibacterial, Listeria monocytogenes, Escherichia coli

I. INTRODUCTION

Food spoilage or food decay is caused by pathogenic bacteria. These pathogenic bacteria make food as a substrate where they live. Food-borne disease is the entry of bacteria into the body through contaminated food and as a result of the body's reaction to bacteria or its metabolic products. Foods that are vulnerable to pathogenic bacteria are foods that contain lots of protein. There are 2 names of pathogenic bacteria, they are *Listeria monocytogenes* and *Escherichia coli* (Behravesh *et al.* 2012).

L. monocytogenes is found in ready-to-eat foods such as sausages, meat and vegetables that are stored in refrigerators. *L. monocytogenes* can survive in cold temperatures where the majority of other groups of bacteria cannot survive under these conditions. (Woraprayote *et al.* 2016). *E. coli* strain *K12* found in chicken meat which is stored in the refrigerator. Some types of *E. coli* have harmful effects on the human body. The *E. coli* O15: H7 strain produces Shiga-Toxin which can occur when the body consumes undercooked processed meat (Lew, 2011). Food preservation method in order to avoid contamination of *L. monocytogenes*, *E. coli* and *Salmonella* is done by thermal processing and vacuum packaging. This method can reduce the number of bacteria, but *L. monocytogenes* does not show significant results (McMinn *et al.* 2018). So it is necessary to apply food preservation in another way, with food preservation microbiologically, namely with Lactic Acid Bacteria.

LAB in this study will be isolated from honey produced by *Apis mellifera* bees. LAB has ability to adapt to environments with high levels of sugar or carbohydrates, such as pure honey produced by *Apis mellifera* (Olofsson & Vasquez, 2014). Total of 42 LAB isolates were found in the stomach of honey bees, royal jelly, bee bread and pure honey produced by *Apis ceranaindica, Apis mellifera and Apis dorsata*. In general, LAB isolates found belong to the genera Lactobacillus, *Streptococcus, Microccocus* and *Enterococcus* (Mathialagan *et al.* 2018). LAB is used in the food industry to produce fermented foods with an antibacterial effect that is produced as a biopreservative agent that can extend the storage period and nutrients in food are maintained. LAB also produces antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocin (Ozogul *et al.* 2017)

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II. MATERIALS AND METHODS

Materials

Materials that used in this research are *Apis mellifera* honey, *de Man Rogosa* (MRS) medium, agar, $CaCO_3$, culture of *L. monocytogenes* and *E. coli,* chloramphenicol, NaCl, aquadest, H_2O_2, Gram stain, Pepsin enzyme, NaOH.

Methods

LAB Enumeration and Isolation

LAB was isolated from *Apis mellifera* honey by pour plate method. LAB populations were enumerated using multilevel dilution and cup count methods with 100 µL samples taken from 10^{-1} to 10^{-5} dilutions. The results of the dilution were transferred to the MRS agar. $CaCO_3$ is added to the media to increase bacterial growth and as an indicator that the bacteria produce acid. Then the bacteria were incubated at 37°C for 48 hours to be enumerated in the form of Colony Forming Units (CFU / ml) (Mathialagan *et al.* 2018).

The morphology of the LAB colony has the potential to grow separately and form a clear zone around it. The medium was MRS agar which had been added with $CaCO_3$ with a concentration of 0.75-1%. The isolates were subcultured into MRS agar by quadrant streak plate method to obtain pure LAB isolates. Pure isolates then inoculated in slant agar (Mathialagan *et al.* 2018).

Morphological Characterization of LAB from *Apis mellifera* honey

Morphological characterization was done macroscopically and microscopically. Macroscopic characterization was done by observing LAB colonies on MRS agar 1% $CaCO_3$. Colonies were observed in the shape, color, edges, texture, elevation and size of the colony. Microscopic characterization was done by the Gram staining method and the cells were observed under a microscope at 1000x magnification.

Physiological Characterization of LAB from *Apis mellifera* honey

Catalase Test

Isolate from slant agar were taken and then applied to a glass object that was cleaned with alcohol. Glass object was dripped with 3% H_2O_2 solution. It was observed by formation of gas bubbles on the object glass. If there is a gas bubble it means that the catalase test is positive, if it is not present then the catalase test is negative (Mathialagan *et al.* 2018).

Low Temperature Tolerance Test

LAB isolates from slant agar were taken and then it was cultured at 10 mL MRSB and incubated at 8°C for 5 days to see its potential as a food preservation application at low temperatures. LAB growth was calculated by spectrophotometer. A total of 3 mL of LAB suspension in the MRSB was put in the cuvette. MRSB is used as a standard solution. The suspension is then measured by the value of optical density with a wavelength of 620 nm. LAB growth is expressed as ΔOD over a range of days from 0 to 5 (120 hours) (Barbosa *et al.* 2018).

Sodium Chloride Tolerance Test

LAB isolates from slant agar were taken and then it was cultured on 10 mL MRSB which has been added by 2%, 4% and 6% NaCl. After that, it was incubated at 37°C for 48 hours. LAB growth was calculated by spectrophotometer. A total of 3 mL of LAB suspension in the MRSB was put in the cuvette. MRSB is used as a standard solution. The suspension is then measured by the value of optical density with a wavelength of 620 nm. LAB growth is expressed as ΔOD in the time range from day 0 to day 2 (48 hours) (Barbosa *et al.* 2018).

Fermentation Type Test

LAB isolates from slant agar were taken and the it was cultured on 10 mL MRSB with a durham tube inside it. Durham tube was placed upside down. Furthermore, the culture was incubated for 24 hours at 37oC. Bacteria that contain gas in durham tubes are stated as heterofermentative, whereas those that do not contain gas are homofermentative (Ismail *et al.* 2017).

Motility Test

LAB isolates from slant agar were taken and the inserted into the MRS to semi-solid agar. MRS semi-solid agar was made using 0.4-0.5% agar. LAB was incubated at 37°C for 24 hours. Observation of motility is indicated by bacterial growth on the media. Bacteria that only grow in a straight line prick showed negative results. While the bacteria that spread to reach the surface showed positive results (Ismail *et al.* 2018).

Screening of LAB Producing Antibacterial with Antibacterial Tests

Pure LAB isolates were taken by inoculating loop. Then the isolate was inoculated into MRSB. The turbidity was measured by Optical Density (OD) using a spectrophotometer. LAB turbidity was measured by Optical Density (OD) because LAB tends to be clear and not too visible in turbidity on MRSB media. LAB turbidity was measured using a spectrophotometer with a wavelength of 620 nm (Ramadass *et al.* 2014). *L. monocytogenes* and *E. coli* from stock cultures were taken and mixed into 0.9% NaCl. The turbidity is equal to Mc Farland's standard 0.5 (Cockerill, 2012). The screening process of LAB producing antibacterial compounds was done by streak plates with a cotton swab so that *L. monocytogenes* and *E. coli* were distributed evenly to all parts of the petri dish. Screening has done by the well diffusion method. The well was made with a stainless steel borer with a diameter of 9 mm. Each well was filled with 80 µL LAB. Then they were incubated at 37°C for 48 hours. Inhibition zones were measured using caliper (Goyal *et al.* 2018). The control used for this test was 4 mg chloramphenicol as positive control of which was dissolved into 10 mL of sterile distilled water to obtain 0.4 µg / mL chloramphenicol stock solution and negative control of MRSB solution which was not given any additional substances (Goyal *et al.* 2018). Antibacterial test with CFS LAB used 2 best isolates selected from screening results using LAB cells. Two of the best isolates that met the criteria were consistent in producing inhibition zones against *L. monocytogenes* and *E. coli*.

Growth Curve of LAB from *Apis mellifera* Honey

Starters were made by culturing the best isolates that incubated for 24 hours taken with one inoculating loop and inoculated into 10 mL MRSB. The starter was incubated with a rotary shaker at 37°C for 48 hours. Then 10% of the starter was inoculated into an erlenmeyer flask containing 50 mL MRSB then incubated in a rotary shaker at 37°C with speed of 120 rpm for 24 hours (Thomas, 2011).

Growth measurements were done by taking 3 mL samples every 4 hours to measure the absorbance using a spectrophotometer with a wavelength of 620 nm. Growth measurements were carried out for 32 hours. The results of the LAB growth measurements are made in the form of LAB growth curve up to the stationary phase. The stationary phase is characterized by absorbance values which show a steady growth of LAB over time (Riazi *et al.* 2008).

Antibacterial Test with CFS (Cell Free Supernatant) of LAB

Bacterial inhibition test was carried out by the well diffusion method. Bacteria liquid culture was taken 15 mL and then put into centrifuge. Then it was centrifuged at 4,000 rpm for 2 x 20 minutes to get CFS (Culture Free Cell Supernatant). *L. monocytogenes* and *E. coli* were inoculated with the streak plate method with a cotton swab. Then 4 holes were made on the media using a stainless steel borer. The diameter of the well is 9 mm. Each well is filled with 80 µL supernatant. Furthermore, it was incubated at 37°C for 24 hours. The inhibition zone has measured with a caliper (Thomas, 2011).

Inhibition Substance Analysis

Inhibition substance analysis was done by 1 mL CFS given 20 mg / ml pepsin enzyme. The test was carried out by the well diffusion method with 3 repetitions for each isolate. Then they were incubated at 37°C for 30 minutes. The enzymatic reaction was stopped by boiling the mixture for 5 minutes. (Goyal *et al.* 2018).

Statistical Analysis

Experimental design was using Completely Randomized Factorial Design (CRFD). Statistical analysis have been done by univariate test with SPSS 16.0. 1st factor is LAB, 2nd factor is pH of supernatant and 3rd factor is pathogenic bacteria.

III. Results and Discussion

Results of Enumeration and Isolation of LAB on *A. mellifera* Honey

The result of LAB enumeration is 9.6 x 10^5 CFU/mL. The things that can distinguish the number of LAB colonies in honey are the geographical area of the honey-producing bee habitat and the plants associated with honey bees, especially in the nectar and pollen parts. According to Pajor *et al.* (2018), microorganisms identified in honey produced in different geographical areas have different numbers and types of bacteria. These microorganisms come

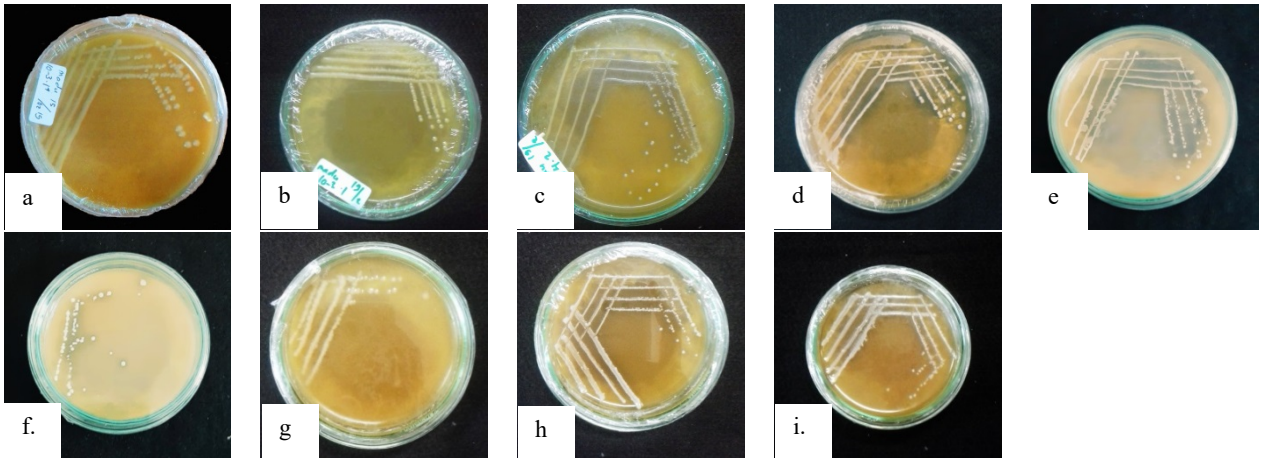
from plants associated with honey bees. During the process of making honey, honey bees introduce bacteria from their digestion into nectar.

LAB isolation from honey *A. mellifera* produced total nine isolates of LAB. The characteristic of LAB isolates is forming clear zone around the colony. This happens because organic acids produced by LAB when reacting with $CaCO_3$ will form a clear zone.

LAB produces organic acids such as lactic acid and acetic acid as the main products. Lactic acid produced by LAB reacts with $CaCO_3$ (Calcium carbonate) to produce calcium lactate, which is the clear zone around the LAB colony. According to Yusmarini *et al.* (2010), the function of adding $CaCO_3$ is to distinguish LAB from bacteria that are not acid-producing, which is indicated by the presence of a clear zone in the media. $CaCO_3$ in the media will react with acids produced by lactic acid bacteria and form calcium lactate which is soluble in the media which is indicated by the formation of clear zones around the colony.

Morphological Characterization of LAB from *A. mellifera* Honey

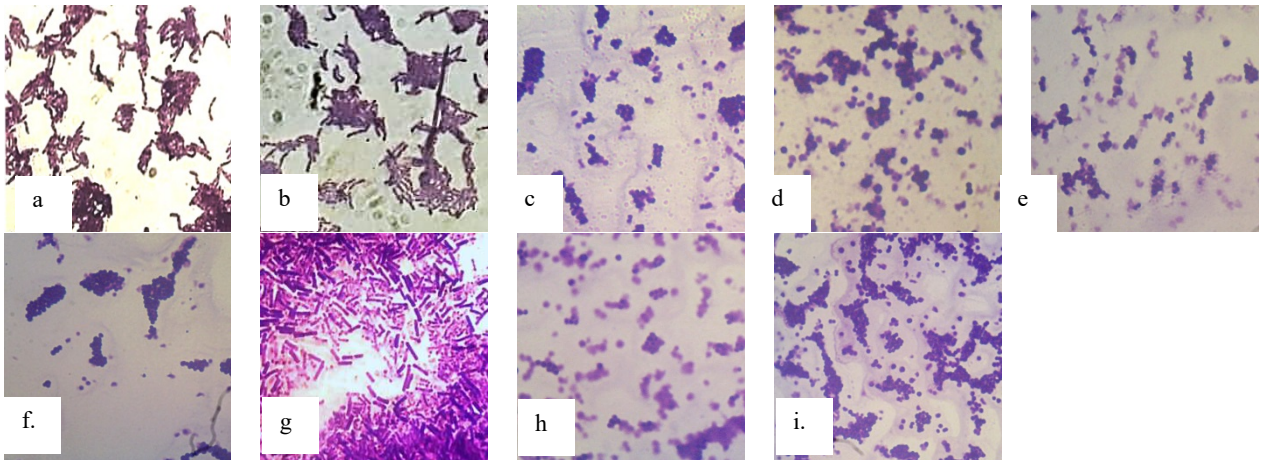
LAB isolates were then further characterized through macroscopic and microscopic morphology. LAB isolates have round shape, white or creamy colors, smooth margin and clear zone around its colony.



Information :
a. MA 1 c. MA 3 e. MA 5 g. MA 7 i. MA 9
b. MA 2 d. MA 4 f. MA 6 h. MA 8

Figure 1 LAB colony on MRS Agar $CaCO_3$ 1%

LAB morphology microscopically isolated from honey has the form of cocci cells and bacilli some are single or form chains. All 9 isolates are Gram positive bacteria. This can be used to determine the genus of these bacteria.



Information :
a. MA 1 c. MA 3 e. MA 5 g. MA 7 i. MA 9
b. MA 2 d. MA 4 f. MA 6 h. MA 8

Figure 2 Gram Positive LAB isolates observed microscopically under microscope with 1000x magnification

According to Mathialagan *et al.* (2018), LAB isolated from honey has the form of cocci or bacillus, either singly, tetrad or chain. The genus of LAB is *Lactobacillus, Micrococcus, Leuconostoc, Enterococcus, Pediococcus,* and *Streptococcus.*

Physiological Characterization of LAB from *A. mellifera* Honey

Catalase Test

The catalase test results showed that 9 LAB isolates from honey produced *A. mellifera* were negative catalase. LAB is an anaerobic bacteria. So that the O_2 gas produced from the breakdown of H_2O_2 is toxic to LAB.

Low Temperature Tolerance Test

LAB growth with the highest OD was isolate 1, which was Δ log = 0.611 and the lowest was isolate 3, which was ΔOD = 0.141. According to Barbosa *et al.* (2018), LAB growth is highly dependent on the availability of nutrients in growth media, pH and temperature. That is because LAB is classified as a selective bacterial (it is not easy to grow on normal media) and requires a nutrient-rich media and suitable environmental conditions to be able to carry out a multiplication process (doubling).

Sodium Chloride Tolerance Test

Isolates 1 to 9 are able to grow at NaCl 2%, 4% and 6%. The nine isolates had different growth rates after incubation for 48 hours. Isolate 4 has the highest ΔOD, which is 0.749 and isolate 2 has the lowest ΔOD, which is 0.059. LAB which was grown in 4% NaCl concentration had the highest ΔOD growth rate was isolate 9 with ΔOD = 0.876 and the lowest ΔOD was isolate 2 with ΔOD = 0.199. LAB grown in the concentration of 6% NaCl has the highest growth rate of ΔOD is isolate 3 with Δ OD = 0.373 and the lowest ΔOD is isolate 2 which is ΔOD = 0.074. Growth measurements with Optical Density from MA 1 to 9 cultured on MRSB with an additional 6% NaCl ranged from 0.074 to 0.373. According to Menconi *et al.* (2014), some genera of LAB can grow when tested on media that are given 6.5% NaCl concentrations with a minimum OD of 0.4 to a maximum of 1.0. Each isolate has a different growth rate. With the presence of excess NaCl can affect physiology, enzyme activity and cell metabolism.

Fermentation Type Test

MA 1, MA 2, MA 7 and MA 8 produce gas after 24-hour incubation. The gas produced is CO_2. According to Ganzle (2015), the type of LAB fermentation is divided into 2, namely homofermentative and heterofermentative based on carbohydrate metabolism and fermentation products.

Motility Test

All LAB isolates showed negative results on the motility test. LAB grows in 1 straight line made by a sharp inoculating loop on the semi-solid MRS medium. According to Ismail *et al.* (2018), the motility test aims to find out which bacteria are motile or non-motile. A bacterium is motile if it has a flagellum for movement.

In accordance with the table above, some isolates have similar characteristics, ranging from morphology and physiological characterization including catalase test, tolerance at low temperatures, tolerance to sodium chloride, fermentation type and motility, so that the possibility of these isolates are in the same genus. MA 1, MA 2, and MA 7 have the probability of the genus *Lactobacillus*. MA 3, MA 5, MA 6 has the probability of the genus *Streptococcus*. MA 4 and MA 9 have the probability of the genus *Lactococcus*. MA 8 has the probability of the genus *Leuconostoc*.

Screening of LAB Producing Antibacterial with Antibacterial Tests

LAB isolates that showed the best potential as inhibitors of growth of *L. monocytogenes* and *E. coli* were MA 3 and MA 4. MA 3 produces inhibition zones of 9.5 mm and MA 4 of 12.5 mm on the growth of *L. monocytogenes*. As for the growth of *E. coli*, MA 3 produces inhibition zones of 2.5 mm and MA 4 of 5.5 mm.

The positive control used was 4 µg / ml chloramphenicol which was poured 40 µl into the well. While negative controls use MRSB media which is not overgrown with anything. The inhibition zone produced by positive control of chloramphenicol was 21.33 against *L. monocytogenes* and 23.67 against *E. coli*. Whereas MRSB did not produces any inhibitory zone to the growth of these two pathogenic bacteria. According to Kumayas *et al.* (2015), the negative control used had no effect on the antibacterial test, so that the inhibition formed was not influenced by the solvent, but because of the activity of the compounds present on LAB. Positive control showed a significant,

Table 1. Physiological Characteristics of LAB from *Apis mellifera* Honey

LAB	Catalase	Temperature of 8°C	NaCl 6%	Fermentation Type	Motility
MA 1	-	+	-	+	-
MA 2	-	+	-	+	-
MA 3	-	-	-	-	-
MA 4	-	+	-	-	-
MA 5	-	-	-	-	-
MA 6	-	-	-	-	-
MA 7	-	-	-	+	-
MA 8	-	+	-	+	-
MA 9	-	+	-	-	-

Note:
Low temperature + = OD> 0.370 - = <0.370 NaCl + = OD> 0.4 - = OD <0.4
Fermentation type: + = heterofermentative - = homofermentative
Motility: + = motile - = nonmotile

because it produced the greatest antibacterial activity against test bacteria compared to negative control, as well as antibacterial from LAB.

Growth Curve of LAB from *Apis mellifera* Honey

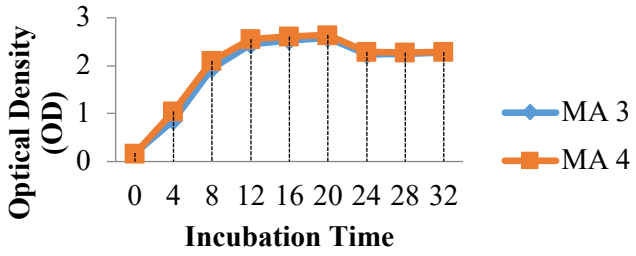


Figure 3. Growth Curve of LAB from *Apis mellifera* Honey

Sampling for growth measurements carried out for 32 hours. LAB undergoes an exponential phase after 4 hours. Then LAB begins a stationary phase after 12 to 20 hours of incubation. The stationary phase ends after 20 hours. Optical density MA 3 at incubation for 20 hours was 2.580 while MA 4 was 2.641. The influencing factors include growth media and pH. According to Goyal *et al.* (2018), LAB strains begin to end the logarithmic phase after 9-12 hours of incubation. The highest activity of secondary metabolite production takes place on average after 22 hours. *Lactococcus* has the highest secondary metabolite production activity at the beginning of the stationary phase, which is after incubation for 20 hours. The time of production of secondary metabolites for *Lactobacillus* takes place at an incubation time of 19-29 hours.

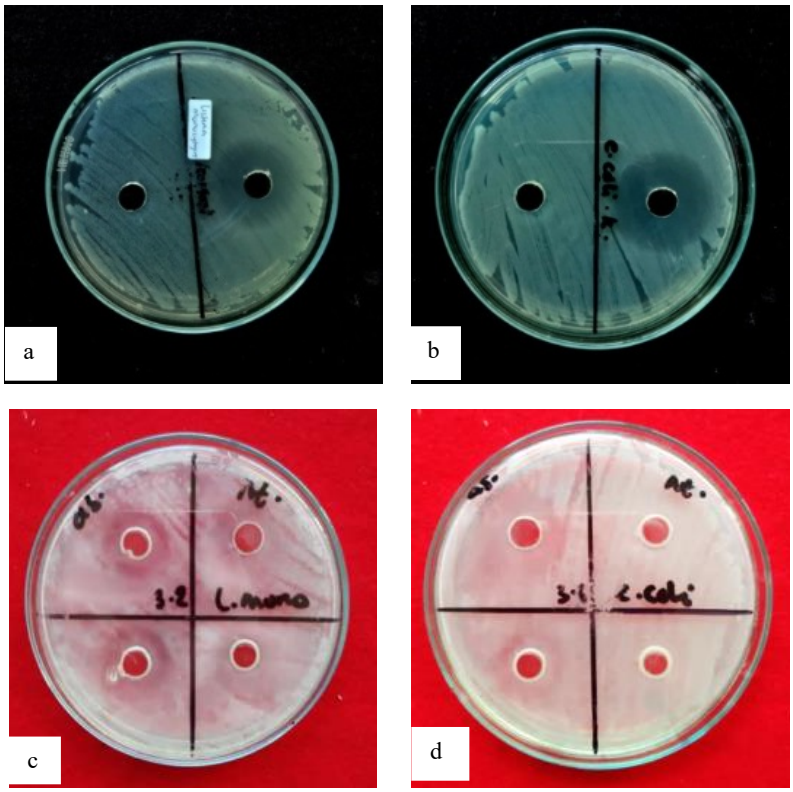
Antibacterial Test with CFS (Cell Free Supernatant) LAB

The inhibitory zone produced can be categorized as strong, but cannot yet match the positive control of chloramphenicol. This can be influenced by the type of bacteria and its resistance to antibacterial. Resistance of a pathogenic bacteria can be a problem. According to Prestinaci *et al.* (2015), antibacterial resistance can be caused by excessive and inappropriate use of antibacterial, so that it can cause genetic changes in pathogenic bacteria.

The results of data analysis with SPSS 16.0 with univariate test showed that the two isolates of LAB, MA 3 and MA 4 had a significance value of 0.001. So the two LAB isolates were stated to have significant differences from each other. Univariate test pH of supernatant results showed a significance value of 0,000. So the pH of supernatant, which is acidic and neutralized, is stated to have significantly different effect on one another. The univariate test results of the two pathogenic bacteria, *L. monocytogenes* and *E. coli* showed a significance of 0.017, so that both pathogenic bacteria had significant differences from each other. Univariate test results regarding the effect of the treatment given, with independent variables in the form of LAB isolates (MA 3 and MA 4), pH of

Table 1. Diameters of Inhibitory Zones by CFS of LAB Against Growth of *Listeria monocytogenes* and *Escherichia coli*

CFS	pH	*L. monocytogenes* ± SD	*E. coli* ±SD
MA 3	**Acid**	**12,05±1,0**	**12,07±1,0**
MA 3	Neutralized	1,11±0,6	1,80±0,4
MA 4	**Acid**	**8,80±0,8**	**11,32±0,1**
MA 4	Neutralized	2,07±0,3	1,21±0,1



Note:
a. Negative and positive controls on *L. monocytogenes*
b. Negative and positive controls on *E. coli*
c. CFS MA 3 against *L. monocytogenes*
d. CFS MA 3 against *E. coli*

Figure 4. Negative Control, Positive Control and CFS of MA 3 against the growth of *Listeria monocytogenes* and *Escherichia coli*

supernatant, and pathogenic bacteria (*L. monocytogenes* and E.coli) and the dependent variable in the form of inhibitory zones produce a significance value of 0,000 . This means that the combination of LAB isolates, pH of supernatant, and pathogenic bacteria has a significantly different effect on the inhibition zone formed. *L. monocytogenes* is a Gram positive bacteria and *E. coli* is a Gram negative bacteria. The two pathogenic bacteria have different sensitivity to the antibacterials produced by MA 3 and MA 4. The biggest inhibitory zone is produced by MA 3 in acidic conditions towards the growth of *E. coli*. *E. coli* which is a Gram-negative bacteria was damaged by its outer membrane permeability. According to Reis *et al.* (2012) the main component produced by LAB, organic acids, can reduce the pH value and change the environment into a growth medium that is not suitable for the development of some pathogenic bacteria and food contamination.

Inhibition Substance Analysis

There are 2 types of antibacterials produced by LAB, namely antibacterial which is composed of peptides and non-peptides. Antibacterial which is composed of peptides is bacteriocin, while non-peptides are acetic acid and lactic acid. Antibacterial can inhibit affected by pH conditions. LAB MA 3 and MA 4 inhibit both pathogenic bacteria under acidic pH. Low pH causes low bacteriocin activity. So it is possible that the substances inhibiting the growth of pathogenic bacteria are organic acids produced by LAB. According to Mataragas *et al.* (2002), the optimum level of bacteriocin production is at pH 5.5-6.5. In addition there are also hydrogen peroxide and diacetyl (2,3-butandion) which function as antibacterial substances produced by LAB.

LAB isolation from *A. mellifera* honey produced a total of nine pure isolates. Their characteristics are bacilli and cocci, Gram positive, catalase negative, non-motile, homofermentative and heterofermentative, tolerance resistant to low temperatures and not tolerance to high salt concentration.

The isolates that have the best ability to inhibit the growth of *L. monocytogenes* and *E. coli* are isolates MA 3 and MA 4. Antibacterial test with CFS produces the best inhibition zone produced by MA isolates 3. Inhibition of the growth of *L. monocytogenes* and *E. coli* occurs in an acidic state, so that the possibility of antibacterial substances produced comes from organic acids produced by LAB.

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