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IDENTIFICATION OF LACTIC ACID BACTERIA (BAL) FROM THE INTESTINES OF *Pangasianodon hypophthalmus* THAT WAS FEEDED BY FERMENTED HERBAL AND THE ABILITY TO INHIBIT *Aeromonas hydrophila* BACTERIA

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ABSTRACT

Lactic acid bacteria can improve digestibility because they can be absorbed directly by the body. The purpose of this research was to obtain lactic acid bacteria types from the intestines of striped catfish (*Pangasionodon hypophthalmus*) that had been fed fermented herbal feed. This research was conducted from June 2019 to April 2020. The treatment used in this research was with different doses and stocking densities. The test was carried out using the disc diffusion method or the Kirby-Bauer method, which was done by measuring the inhibition zone around the paper disc. Identification of LAB was carried out by phenotypic and genotypic. The results showed that as many as four of the selected isolates were able to inhibit the growth activity of the pathogenic bacteria *Aeromonas hydrophila*. Isolates with code K had an inhibition zone of 6.7 mm against *A.hydrophila*, an inhibition zone of 7.5 mm for P_1D_2 isolates, P_2D_1 isolates had an inhibition zone of 7.0 mm, P_2D_2 isolates had an inhibition zone of 7.8 mm. Types of LAB that were identified phenotypically and genotypically include *Lactobacillus casei*, *L.plantarum*, and *L. acidhopilus*

Keywords: Striped catfish, antimicrobial, Fermented herbal, BAL.

I. INTRODUCTION

High stocking densities in intensive aquaculture activities will cause fish to be susceptible to stress and disease, resulting in slow fish growth. In addition, catfish are omnivorous fish that require high protein feed for their survival. Feed is an important factor in aquaculture activities, most of the costs incurred in aquaculture activities are for feed costs. Around 60-70% of aquaculture, production costs are concentrated in feed [1].

According to [2] so that the feed given can work optimally and be able to increase fish weight, a supplement is needed that is mixed in the feed. One of the efforts to improve fish health, farmers often adds supplements. One of the supplements that can be given is fermented herbs that can be added to fish pellets as a supplement to increase fish appetite, feed digestibility and fish health. Fermented herbal medicine is processed by fermentation of the BAL starter type Lactobacillus sp. derived from fermented beverages. These bacteria are used for the fermentation process because the antibacterial (bactericidal) compounds produced by BAL can inhibit other microorganisms. Several groups of LAB include safe microorganisms and are called food grade microorganisms [3].

An alternative that can be done to improve feed quality and fish growth is to add fermented herbs to commercial pellets. Fermented herbal medicine is processed by fermentation of the BAL starter type

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Lactobacillus sp. derived from fermented beverages. BAL can naturally be found in the digestive tract of fish, namely the intestines. Thus, by adding BAL in feed, it is expected to obtain BAL types that can improve feed quality, growth, and fish meat quality. Based on this explanation, the authors are interested in conducting further research on the identification of BAL from the intestines of striped catfish fed fermented herbal feed and conducting antimicrobial activity tests against the pathogenic bacteria *Aeromonas hydrophila.*

2. RESEARCH METHOD

Time and Place

The research was conducted from June 2019 to April 2020.

Method

The treatments used in the research were stocking density and doses of fermented herbs.

Research Procedure

Maintenance of Fish

In accordance with the stocking density treatment used, namely 50 fish/m^3 and 75 fish/m^3. Furthermore, the fish were reared for two months (60 days), by providing commercial pellet feed added with fermented herbs at doses of 100 mL/Kg, 200 mL/Kg, and 300 mL/Kg. Feed is given as much as 10% of body weight and done three times a day (08.00, 13.00 and 17.00 WIB) ad satiation.

Fermented Herbal Feed

Materials used in the manufacture of fermented herbs, such as; turmeric, aromatic ginger, and curcuma, each peeled and washed, then weighed as much as 100 g of each ingredient. The clean material is sliced thin and then mashed using a blender, and then the fine material is filtered to obtain a solution. Then, clean water is added to 3 L of the herbal ingredients solution. The solution that has been mixed with water is boiled until boiling, and then cooled to room temperature. After the cold solution was added as much as 175 mL of molasses, 65 mL of fermented drink, and 50 mg of yeast then stirred until evenly distributed. Next, the solution is put into jerry cans and tightly closed to ferment for 7-10 days. During fermentation, the container is opened 1-2 times a day to allow the gaseous vapors to escape.

Bacterial Isolation from Fish Intestine

BAL isolation was carried out on the fish intestines from each treatment were put in a petri dish, each containing 0.9% NaCl as a physiological solution for further dilution and isolation of bacteria, then 0.1 g of intestinal contents were suspended in 10 mL of NaCl solution in a tube. Reaction, followed by dilution. Dilution of the sample in stages was carried out by transferring 1 mL of the sample solution aseptically into the diluent (9 mL of distilled water). From the first dilution a 10^{-2} dilution was obtained, then 1 mL of sample from 10^{-2} was transferred into 9 mL of diluent so that a 10^{-3} dilution was obtained and 1 mL of sample was taken again up to a 10^{-4} dilution. Dilution was carried out with the aim of obtaining separate bacterial colonies when grown into the medium.

The growing colonies were then taken using a loop and put into an Eppendorf tube containing 1 mL of NaCl solution that had been conditioned at pH 2, 4 and 6 respectively using HCl or NaOH. The pH measurement was carried out using a pH indicator. Next, it was homogenized using a vortex, then 100 µL was taken to grow on MRS agar media using the pour plate technique.

Antimicrobial Activity

Antimicrobial activity test was carried out with the aim of knowing the ability of BAL to inhibit growth and kill

pathogenic bacteria. The method used is the disc method, which refers to the method according to Kirby Bauer. Pure pathogenic bacteria (*Edwardsiella tarda, A.hydrophila*, and *Staphylococcus aureus*) were taken as much as 25 µL and then planted in the test medium namely NA, then spread evenly using a glass spreader. Next, disc paper that had been dripped with 5 µL of BAL was planted in the NA medium. Oxytetracycline was used as a control. After that, it was incubated at 37 ºC anaerobically. Then the diameter of the inhibition zone was measured. BAL inhibition of pathogenic bacteria is indicated by the clear zone formed on the test medium.

Identification of Lactic Acid Bacteria (BAL)

Biochemical test is a test performed on the biochemical properties of bacteria. This test is performed using pure bacterial cultures. Tests include oxidase test, catalase test, SIM test, O/F test, and H test$_2$S.

Oxidase Test

The oxidase test was carried out by taking the Konvack Reagent solution using a pipette and then dripping it on filter paper, then the bacterial colonies were taken aseptically using an ose needle and streaked onto the filter paper. If the paper turns dark blue/purple, this means that the bacteria is positive (+) oxidase, and if there is no color change, it is negative (-) oxidase.

Catalase Test

The catalase test was carried out using a 3% H_2O_2 solution that was dripped onto an object glass, and then the bacterial culture was taken using an ose and then mixed with a 3% H_2O_2 solution. If the bacteria emit air bubbles (foam) then the bacteria are catalase positive (+), and if they do not produce bubbles (foam) it means negative catalase (-).

Motility Test

Motility test was carried out using SIM media to determine bacterial motility. The bacterial culture was taken using an ose needle and then planted perpendicularly on the SIM medium in a test tube. Motility was observed in young cultures at 18-24 hours of incubation. If the bacteria grows spreading from the puncture line, it means the bacteria are motile, conversely, if the bacteria only grows in the puncture line, it means the bacteria are non-motile.

O/F test

The O/F test is carried out on two tubes containing solid O/F media. Each O/F media was inoculated with bacteria, and one of the tubes that had been inoculated with bacteria was given paraffin liquid to cover the surface of the media 1 cm thick. Then incubated for 18-24 hours at 37^0C, and then observed the color changes that occur. If both mediums change color to yellow and bubbles appear on the medium, which is given liquid paraffin, it means that the bacteria are fermentative. If the color changes to yellow only on media without paraffin, then the bacteria are oxidative.

H_2S test

The H_2S test was carried out on TSIA media in a test tube. Bacteria are inoculated by zig-zag and puncture. Furthermore, incubated for 18-24 hours at 37^oC. An indication of the presence of H2S is when there is a black discoloration on the puncture marks and if gas is formed it is indicated by the presence of bubbles in the media or the media in the tube is lifted upwards. Isolates that were tested for identification using the PCR method were isolates that had an inhibition zone value against pathogenic bacteria *A.hydrophila*, namely Control (without treatment), P1D2, P2D1, and P2D2 isolates. DNA extraction (DNA isolation), amplification, and electrophoresis.

3. RESULT AND DISCUSSION

Water Quality Measurement

The results of isolation of LAB from the intestines of catfish that have been given feed with the addition of fermented herbs. After purification 5 times, uniform and separate colonies were obtained. The purified bacteria can be seen in Figure 1, and the results of morphological observations can be seen in Table 1.

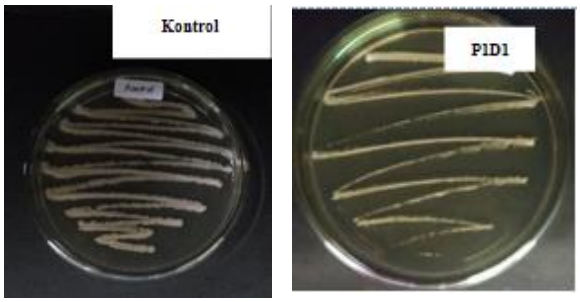

Figure 1. LAB isolates from the intestines of striped catfish

Table 1. Observation of LAB Colony Morphology

Kode Isolat	pH Media	Warna	Bentuk Koloni	Elevasi	Tepian	Ukuran
K		Putih Susu	Bulat	Timbul	Licin	Sedang
P_1D_1		Putih Susu	Bulat	Timbul	Licin	Kecil
P_1D_2		Putih Susu	Bulat	Timbul	Licin	Kecil
P_1D_3	2	Putih	Bulat	Timbul	Licin	Kecil
P_2D_1		Putih Susu	Bulat	Timbul	Licin	Kecil
P_2D_2		Putih Susu	Bulat	Timbul	Licin	Kecil
P_2D_3		Putih	Bulat	Timbul	Licin	Kecil
K		Putih Susu	Bulat	Timbul	Licin	Sedang
P_1D_1		Putih Susu	Bulat	Timbul	Licin	Kecil
P_1D_2		Putih Susu	Bulat	Timbul	Licin	Kecil
P_1D_3	4			Tidak Tumbuh		
P_2D_1		Putih Susu	Bulat	Timbul	Licin	Kecil
P_2D_2		Putih susu	Bulat	Timbul	Licin	Kecil
P_2D_3		Putih	Bulat	Timbul	Licin	Kecil
K		Putih Susu	Bulat	Timbul	Licin	Sedang
P_1D_1				Tidak Tumbuh		
P_1D_2		Putih Susu	Bulat	Timbul	Licin	Kecil
P_1D_3	6			Tidak Tumbuh		
P_2D_1		Putih Susu	Bulat	Timbul	Licin	Kecil
P_2D_2				Tidak Tumbuh		
P_2D_3		Putih Susu	Bulat	Timbul	Licin	Kecil

Keterangan:
K = Kontrol
P_1D_1 = Padat tebar 50 ekor/m², Dosis 100 mL/Kg pakan
P_1D_2 = Padat tebar 50 ekor/m², Dosis 200 mL/Kg pakan
P_1D_3 = Padat tebar 50 ekor/m², Dosis 300 mL/Kg pakan
P_2D_1 = Padat tebar 100 ekor/m², Dosis 100 mL/Kg pakan
P_2D_2 = Padat tebar 100 ekor/m², Dosis 200 mL/Kg pakan
P_1D_3 = Padat tebar 100 ekor/m², Dosis 300 mL/Kg pakan

Based on Table 1, it is known that the colonies produced from bacterial isolates have a milky white color, rounded colony shapes with convex elevations and small smooth edges that can be seen from the growth of the colonies after purification in MRS Agar bacterial growth media. In accordance with the results of [4], that found several isolates of lactic acid bacteria isolated from soursop fruit, namely with round and round colony shapes, smooth edges, convex elevations and milky white and cream colors. [5], stated that the morphology of the colonies which were round (circular), flat edges (entire), convex surfaces (convex) and yellowish white were the morphological characteristics of lactic acid bacteria.

Furthermore, the results of the biochemical test showed that the seven bacterial isolates had the same characteristics. The results of the biochemical tests of bacterial isolates can be seen in Table 2.

Table 2. Results of LAB Biochemical Tests from the Intestines of Striped Catfish

Kode Isolat	Uji Biokimia						
	Gram	Katalase	Oksidase	Motilitas	H_2S	Glukosa	O/F
K	+	-	-	-	-	+	F
P_1D_1	+	-	-	-	-	+	F
P_1D_2	+	-	-	-	-	+	F
P_1D_3	+	-	-	-	-	+	F
P_2D_1	+	-	-	-	-	+	F
P_2D_2	+	-	-	-	-	+	F
P_2D_3	+	-	-	-	-	+	F

Keterangan:
+ = Terjadi reaksi menghasilkan hasil positif
- = Tidak terjadi reaksi menghasilkan hasil negatif
F = Fermentatif

The results of microscopic observations can be seen in Figure 2.

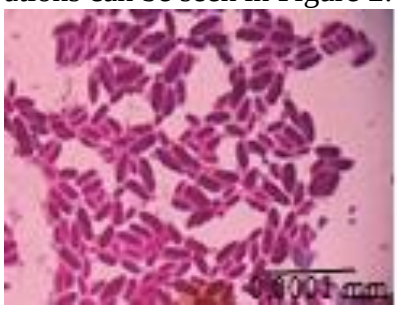

Figure 2. Observation of bacteria under a microscope with a magnification of 1000x

The results obtained from Gram staining are in accordance with the characteristics of lactic acid bacteria,

namely Gram-positive bacteria that do not produce spores and are usually in the form of bacilli or cocci [6].

BAL that has been isolated from the intestines of striped catfish with feed added with fermented herbs is able to inhibit the growth of pathogenic bacteria. This shows that the antimicrobial activity produced from lactic acid bacteria digestion of striped catfish that is given feed with the addition of fermented herbs is quite large. Antimicrobial activity in research can be seen in Table 3.

Table 3. Antimicrobial activity test results against *A.hydrophila*

Isolate	***A. hydrophila*(mm)**
Antibiotics (Tetracycline)	12,6
K	6,7
P1D1	-
P1D2	7,5
P1D3	-
P2D1	7.0
P2D2	7,8
P2D3	-

The mean value of the inhibition zone of bacterial isolates originating from striped catfish gut with fermented herbal feed against *A.hydrophila* pathogenic bacteria ranged from 6.7 to 7.8 mm. P2D2 isolate had the highest inhibition zone value against *A.hydrophila* bacteria of 7.8 mm. As can be seen in Figure 3.

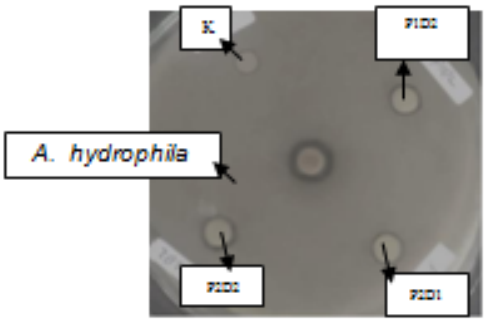

Figure 3. Antimicrobial activity test results against *A.hydrophila*

The ability of BAL to inhibit the growth of pathogenic bacteria, not only produces lactic acid and acetic acid (organic acids), but also other compounds such as bacteriocins which have antibacterial activity. The antimicrobial activity of BAL is indicated by the size of the inhibition zone produced. The greater the inhibition zone produced, it can be concluded that the bacterial isolates are more effective in inhibiting the pathogenic bacteria *A.hydrophila.*

The control isolates had an inhibitory effect only on the pathogenic bacteria *A.hydrophila*, which was 6.7 mm. This could happen due to the nature of *A.hydrophila* as a secondary attacker on weak hosts. Its opportunistic nature is susceptible to attacking fish that come in contact with it, this contact can cause infection depending on the species and its virulence level. *A.hydrophila* often attacks catfish, it can even be said that this bacterium is the main pathogen of freshwater fish. The bacteria are able to multiply in the intestine, causing a mucuous-desquamative haemorrhagic inflammation (excessive mucus production).

The toxic metabolites of *A.hydrophila* are absorbed from the gut and induce poisoning. Capillary bleeding occurs on the surface of the fins and in the abdominal submucosa. Hepatic and epithelial cells of the renal tubules show degeneration. The glomeruli are destroyed and the tissue bleeds, with an exudate of serum and fibrin. Lactic acid bacteria are known to have the ability to produce bioactive compounds in the form of hydrolytic enzymes that can degrade and damage the structural components of the cell walls of pathogenic bacteria [7].

Isolates that were tested for identification using the PCR method were isolates that had an inhibition zone value against the pathogen *A.hydrophila*. Identification was carried out using the

PCR-sequence analysis method, which is a technique that is considered the best for viewing the biodiversity of a group of organisms.

In principle, polymorphism is seen from the DNA sequence or sequence of a particular fragment of an organism's genome. The first stage in the PCR method is to perform DNA extraction, then amplification and electrophoresis processes are carried out to produce PCR products from the selected BAL isolate DNA. BLAST analysis was carried out with the aim of comparing the results obtained with the results of DNA sequences from around the world from the results deposited in the public sequence genbank database.

The identification results of each bacterial isolate based on the BLAST results can be seen in Table 4.

Table 4. BLAST analysis results

Kode Isolat	Spesies	Kode Akses	Homologi	Strain
K	*Lactobacillus casei*	AY196976.1	100 %	ATCC4646
P_1D_2	*Lactobacillu acidophilus*	NR_043182.1	99 %	BCRC10695
P_2D_1	*Lactobacilllus plantarum*	NR_113338.1	99%	NBRC15891
P_2D_2	*Lactobacillus acidophilus*	HG518161.1	99%	LM6

BAL obtained from the results of identification with the PCR method showed that the four bacteria could grow in the intestines of striped catfish in both control (without feed treatment with the addition of fermented herbs) and those fed fermented herbs. Three types of BAL were obtained by tracing the BLAST system, namely *L.casei* with a 100% homology level, 2 *L.acidophilus* bacteria with a 99% homology level, and *L.plantarum* bacteria with a 99% homology level.

Furthermore, after performing BLAST analysis, the sequences obtained were reconstructed for phylogenetic trees using the Molecular Evolutionary Genetics Analysis (MEGA) 5 computer program [8]. The phylogenetic tree of each bacterial isolate can be seen in Figure 4

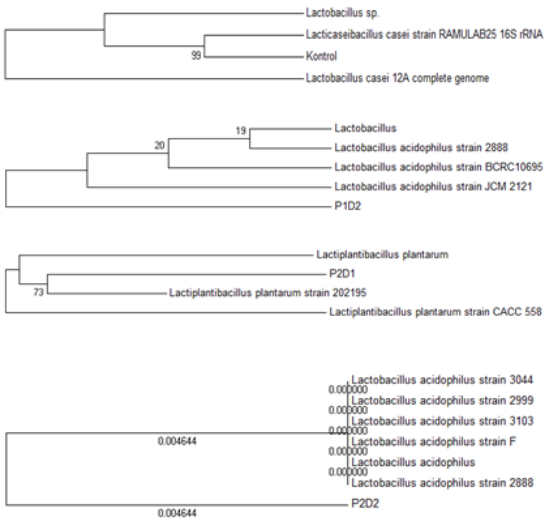

Figure 4. The phylogenetic Trees

The less the values of the genetic distance between two organisms, the closer the kinship between the two organisms [9]. According to [10], the neighbor joining tree method selects sequences which when combined will provide the best estimate of the length of the branch that closest reflects the real distance between the sequences. The phylogenetic tree was tested statistically using the bootstrap method with 1000 replications.

The research that has been done shows that the identified control isolate is LAB *L.casei* that is able to inhibit the growth of the pathogenic bacteria *A.hydrophila. L.casei* helps limit the growth of pathogenic bacteria in the gut. *L.plantarum* produces lactic acid in the digestive tract. This BAL species also helps the absorption of vitamins and antioxidants and removes toxic components from food. *L.plantarum* is one of the Lactobacillus type LAB that is dominant in the digestive tract. LAB is a probiotic bacterium because it has an important role in improving gut health in organisms and a barrier to the growth of pathogenic bacteria [11].

An *L.acidophilus* bacterium thrives in an acidic environment (pH 4-5 or lower) and grows optimally at 45^0C. *L.acidophilus* naturally exists in the intestines of humans and animals as well as the vagina.

L.acidophilus lives throughout the digestive tract and is present in very large quantities in the small intestine. *L.acidophilus* is a natural micro flora in the digestive tract and can produce lactic acid as the main product of sugar fermentation. *L.acidophilus* bacteria were identified on P2D2, which had the largest inhibition zone on the tested pathogenic bacteria.

4. CONCLUSION

The types of LAB that were identified phenotypically and genotypicly included *L.casei, L.plantarum,* and *L.acidhopilus*. Further research is needed to determine the digestibility of fish on fermented herbal feed.

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