Journal of Advanced Zoology

ISSN: 0253-7214

*Volume **44** Issue **02 Year 2023** Page **120:131***

The Effectiveness of Lactic Acid Bacteria on the Immune System of Vannamei Shrimp Infected with Bacteria Vibrio Parahaemolyticus

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Article History

Received: 06 May 2023
Revised: 11 August 2023
Accepted:13 August 2023

Abstract

Diseases are a major obstacle in shrimp farming because they can cause relatively high mortality and decrease the quality of the aquaculture environment. One dangerous shrimp disease is caused by Vibrio bacteria. Today, people are turning to biological control methods by utilizing lactic acid bacteria that live as microflora in the digestive tract of aquaculture animals. This study aimed to analyze the potential of lactic acid bacteria isolates from the gut of vannamei shrimp as probiotic candidates and to analyze selected lactic acid bacteria isolates that were effective in increasing the immune response and survival of vannamei shrimp infected with Vibrio parahaemolyticus bacteria. Four BAL isolates were isolated, designated as 8A, 11B, F1, and G2. BAL isolates showed antibacterial activity against Vibrio parahaemolyticus bacteria with an inhibition zone of up to 11.5 mm, capable of living at acidic to alkaline pH (1.5-7.2), bile salts (3000 ppm) and negative catalase. The characterization of several tests such as gram-positive, inhibition test and sugar fermentation test and comparison of LAB characterization showed that LAB isolates (G2) from the intestine of vannamei shrimp were classified as Pediococcus acidilactici. The application of probiotics, namely LAB (G2) to vannamei shrimp through feed can stimulate the vaname shrimp immune system after being challenged with V. parahaemolyticus, as indicated by an increase in the number of hemocytes (THC), phagocytosis activity, and suppressing the population of V. parahaemolyticus in vannamei shrimp.

Keywords: *Lactic Acid Bacteria, Probiotics, Immune System, Vannamei Shrimp*

1. Introduction

Shrimp cultivation development business cannot be separated from the presence of disease. Disease is a major obstacle in shrimp farming because it can cause relatively high mortality and decrease the quality of the aquaculture environment. One of the most dangerous shrimp diseases is shrimp disease caused by Vibrio bacteria (Apriliani et al., 2016).

One type of Vibrio that infects vannamei shrimp is the bacterium Vibrio parahaemolyticus. Vibrio parahaemolyticus is an infectious agent that can infect farm animals and humans. In addition, this bacterium can also cause Acute Hepatopancreatic Necrosis Disease (AHPND) or Early Mortality Syndrome (EMS) caused by the pathogenic bacterium Vibrio parahaemolyticus which can cause huge losses in shrimp farming.

One alternative prevention against vibriosis is the administration of probiotic bacteria, especially lactic acid bacteria. Lactic acid bacteria are popular probiotic strains used to combat bacterial, fungal and viral pathogens. Lactic acid bacteria are beneficial for enhancing immunity, digestion, protection against pathogens, and promoting the growth and reproduction of fish and shellfish. Several studies have shown that probiotics can serve as an alternative to antibiotics (Chizhayeva et al., 2022).

The use of probiotics derived from animals themselves is generally more effective than probiotics derived from other sources because they are more adaptable to the environment and the host. These probiotics are called indigenous probiotics which are bacteria originating from the digestive tract and environment that are the same/similar to the host animal (Yulvizar et al., 2014). Today, people are turning to biological control methods, namely by utilizing lactic acid bacteria that live as microflora in the digestive tract of aquaculture animals. So, in this study, isolation and identification of lactic acid bacteria isolate taken from the intestines of vannamei shrimp will be carried out to be used as probiotic candidates, where the probiotic candidates will be infected with Vibrio parahaemolyticus bacteria to see the immune response of vannamei shrimp.

The purpose of this study was to analyze the potential of lactic acid bacteria isolates from the intestines of vannamei shrimp as probiotic candidates and to analyze selected lactic acid bacteria isolates that were effective in increasing the immune response and survival of vannamei shrimp infected with Vibrio parahaemolyticus bacteria.

2. Materials And Methods

Isolation of lactic acid bacteria (LAB) from vannamei shrimp intestines. This research was carried out in June 2022 - March 2023, namely the process of isolating bacteria from the intestines of Vaname shrimp, preparation for challenge tests, and immune responses were carried out at the Laboratory of Parasites and Fish Diseases, Department of Fisheries, Faculty of Marine Sciences and Fisheries, Hasanuddin University. The LAB Probiotic Candidate Application and Challenge Test in this study were carried out in April 2023 at the Hatchery of the Faculty of Marine Sciences and Fisheries, Hasanuddin University, Makassar. The sample used is vannamei shrimp obtained from farmers. The isolated shrimp samples were 25. Shrimps were sliced from dorsal to anus with a sterile knife, then to take the intestine was done with sterile tweezers as much as approximately 1 g of the intestinal sample was crushed and put into a test tube containing 10 mL of sterile 0.9% NaCl solution as stock and then homogenized using vortex. Then proceed with making multilevel dilutions, to make a 10-1 dilution, 1 mL of the stock solution is taken and then put in a test tube containing 9 mL of 0.9% NaCl physiological solution. The 10-2 dilution was made by taking 1 mL of the 10-1 solution and then homogenizing using a vortex for 5 minutes. And so on until the 10-3 dilution. Then 15 mL of warm MRSA medium is poured into a petri dish and allowed to solidify. Then the results of the 10-1 to 10-3 dilutions of 100 µL each were poured into different Petri dishes and spread evenly using a hockey stick and then labeled. Then incubated for 2x24 hours at 37^{o}C.

The LAB isolates found were then identified morphologically, biochemically and molecularly. Morphologically, growing bacterial colonies were identified based on differences in color, shape, edges, and elevation. Then it was re-inoculated on GYPA+CaCO3 media by streaking method. After obtaining colonies that grew separately, were white and there was a clear zone around them, they were isolated again and stored on slanted MRSA media. Biochemically, identification was carried out using Viantek 2 at the Makassar Health Laboratory.

Identification of lactic acid bacteria (LAB) from vannamei shrimp intestines. Identification of LAB isolates as probiotic candidates were based on gram staining, catalase test, pH tolerance test, bile salt tolerance test, sugar fermentation test, and inhibition test. An inhibition test was carried out. The test was carried out by inoculating 100 µL of Vibrio parahaemolyticus bacteria. evenly into the solid TSA media in a petri dish using a cotton bud. then isolates 8A, 11B, F1, and G2 as a result of incubation

were then centrifuged for 15 minutes at 6000 rpm to separate bacterial cells and supernatant (solution containing antibacterial compounds) and then filtered using 0.2 µm Syringe Filters Sterile. 50 µL of the supernatant was dropped on a paper disc and allowed to dry. After drying, it is then placed on the agar media which has solidified after adding Vibrio parahaemolyticus. Then, incubated at 37oC for 24 hours. As a positive control, 50 µL of Ciprofloxacin solution was used. Bacteria that are capable of producing antibacterial compounds will inhibit pathogenic bacteria as evidenced by the presence of a clear zone around the paper disc. The diameter of the inhibition zone was then measured using a ruler.

The pH tolerance test was measured as follows: BAL isolates were grown on MRSB media for 24 hours at 37°C in an incubator shaker. Then 1 mL of the growing bacteria was distributed to each test tube containing 9 mL of PBS with different pH: 1.50; 3.0; and 7.2, then incubated for 3 hours. To measure the concentration of LAB, a serial dilution technique was carried out and 100 µL of the dilution was poured and spread on MRSA media. The number of colonies growing on MRSA media was counted after 24 hours of incubation at 37°C

LAB resistance test against bile salts was carried out by LAB isolates grown on MRSA solid media with a Bile Salt concentration of 3000 ppm. Then incubated for 24 hours at 37°C. In this test, the number of colonies was also counted after 24 hours of incubation.

The catalase test was carried out by dripping approximately 2 drops of 3% H2O2 on a culture that was 24 hours old. Lactic acid bacteria are catalase-negative bacteria. The results of the catalase test reaction do not form air bubbles which means no gas is formed. Tests for sugar fermentation are carried out by putting the test solution into a test tube of as much as 6 ml and then wet sterilization in the autoclave, then taking 1 ose of bacteria is put into a test tube that has been sterilized. Then incubated for 24 hours. A yellow color change indicates a positive result and no color change is a negative result.

Preparation of Probiotic Bacteria

The probiotic bacteria used in this study was a type of lactic acid bacteria (LAB) from the results of pure isolates from the intestines of vannamei shrimp, namely pure isolates from the intestines of vannamei shrimp, namely isolate G2 which, after investigation, included lactic acid bacteria. Then the bacteria were grown back on Trypticase Soy Agar (TSA) media and incubated for 24 hours at 37°C to become stuck. All research processes were carried out aseptically and carried out in a laminar chamber to prevent contamination from other microorganisms.

Addition of Probiotic Bacteria to Feed

The test feed used in this study was commercial feed. Before being mixed into the feed, 1 mL of bacteria were grown on Trypticase Soy Broth (TSB) media in a 500 mL Erlenmeyer and incubated in a shaker incubator for 24 hours at 29ºC at 140 rpm, then centrifuged for 15 minutes at 6000 rpm. The probiotic bacteria obtained are then weighed and mixed into the feed.

After that, the method of mixing probiotics in the feed refers to the method of Aslamsyah (2006), namely the bacteria are first diluted with Buffer Peptone Water and fish oil (with a ratio of 1 mL probiotic 3 mL Buffer Peptone Water 1 mL fish oil) into 100 grams of feed. This mixture is then sprayed on the feed evenly using a sprayer and then air-dried.

Vibrio Parahaemolyticus Challenge Test

The treatment of probiotics through feed was carried out for 14 days of maintenance, then a challenge was tested on day 15 by injection of Vibrio parahaemolyticus with a concentration of 107 CFU/ml in the ventral sinus in the second abdominal segment (Huang et al. 2013). Bacterial isolates of V. parahaemolyticus were obtained from the collection of the Fish Health Laboratory, BRPBAP3 Maros, South Sulawesi. The V. parahaemolyticus bacterial isolate to be used was first cultured in Trypticase Soy Broth (TSB) and incubated for 24 hours on an incubator shaker. To obtain the density of bacteria to be injected into the test shrimp, the bacterial stock was diluted in stages using a physiological solution (0.85% NaCl).

Treatment and Research Design

The containers used in the maintenance of vannamei shrimp are fiber tubs measuring 50x120 totaling 9 pieces which are placed outdoors and each is equipped with aeration to maintain the stability of dissolved oxygen and temperature. The container is filled with 70 liters of sterile seawater with 15 ppt salinity.

The study was designed in a completely randomized design (CRD) with 3 treatments with 3 replications. The treatments tested in this study were: A: Control (Feeding Without Probiotics Without Challenge Test), B: Feeding Without Probiotics + Challenge Test and C: Feeding + LAB Isolates + Challenge Test.

The feed used is a commercial feed which is applied at a dose of 5% of the weight of the biomass with a frequency of feeding 4 times a day, given at 08.00, 10.00, 14.00 and 16.00 WITA

Calculation of Intestinal Microflora Density of Vaname Shrimp. The calculation of the number of bacteria that was carried out was the calculation of the number of V. parahaemolyticus bacteria and the total bacteria in the shrimp digestive tract. Samples of the shrimp digestive tract that had been taken from each treatment were put into a sample tube that had previously been added with 0.9% sodium chloride. The sample tube was then tightly closed and placed in a cool box. Calculation of the number of Vibrio parahaemolyticus bacteria and total intestinal bacteria according to the method carried out by Yunita et al., (2015). The sample will be tested after administration of probiotics and after the challenge test.

Immune Response

After the challenge test, observations of the immune response were carried out, namely Observation of Total Hemocyte Count (THC), Observation of Differential Hemocyte Count (DHC), Phagocytosis Activity, Lysozyme Activity and Survival

Statistic Analysis

Data on several LAB parameters such as inhibition test, pH tolerance test, bile salt resistance test, sugar fermentation test and catalase test were analyzed descriptively. Data on microflora density of vannamei shrimp intestine, immune response such as THC, DHC, phagocytosis activity, lysozyme activity and vannamei shrimp salinity were analyzed using ANOVA.

3. Results and Discussion

The results of bacterial inoculation from vannamei shrimp intestine, on MRSA media, produced 25 isolates and then on GYPA+CaCO3 media produced 4 isolates of lactic acid bacteria with different colony sizes and showed clear zones around the bacterial colonies. The four isolates are 8A, 11B, F1 and G2.

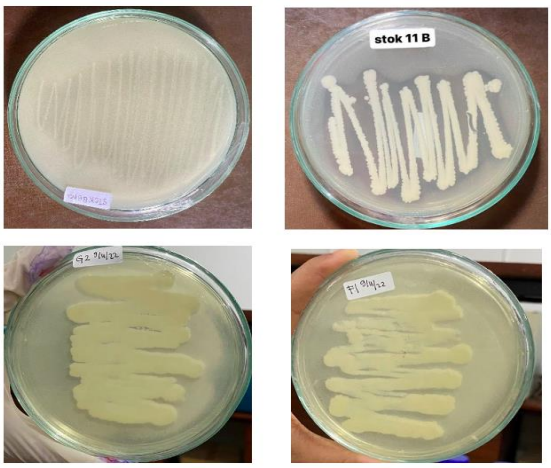

Figure 1. BAL colonies of vaname intestine (8A, 11B, F1, G2) on GYPA+CaCO3 media show a clear zone around the colony.

Selected bacterial isolates from the intestines of vannamei shrimp as many as 4 isolates namely 8A, 11B, F1 and G2 have morphological characteristics that can be seen in Table 1.

Table 1. Morphology of BAL bacteria from the intestines of vannamei shrimp

Isolates	Colony Form	Banks	Elevation	Colony color
8A	Small Round	Flat	Convex	Milky White
11B	Large Round	Flat	Convex	Milky White
F1	Large Round	Flat	Convex	Milky White
G2	Small Round	Flat	Convex	Milky White

Identification of lactic acid bacteria (LAB) from vannamei shrimp intestines. The results of observing the characterization of Gram staining on isolates C1, 8K, 23P and D3 with the cell shape of all isolates in the form of bacilli (Figure 2), it was suspected that all isolates belonged to lactic acid bacteria because the results of Gram staining were Gram-positive according to Lutfiah's statement (2015) that bacteria Lactic acid is Gram-positive, round or rod-shaped, does not form spores, is able to ferment carbohydrates, is catalase negative and is a microaerophilic group.

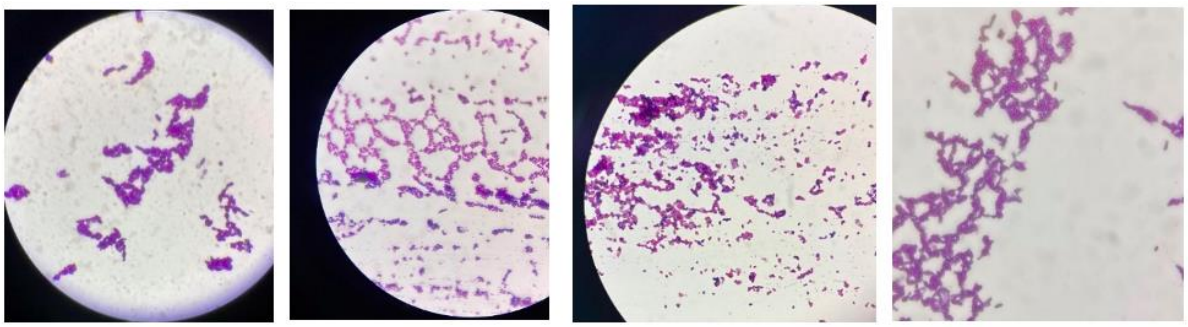

Figure 2. Gram staining test results of bacterial isolates from the intestines of vannamei shrimp

Table 2. Gram staining results of a bacterial isolate from the intestines of vannamei shrimp

Isolate	Gram Staining	Cell Shape
8A	+	Basil
11B	+	Basil
F1	+	Basil
G2	+	Basil

pH tolerance test

This test was carried out by setting the initial pH of 1.5, 3 and 7.2 in the media against 4 potential isolates namely 8A, 11B, F1 and G2 (Table 3). The results of the pH tolerance test showed that the four isolates were able to survive at pH 3 and at pH 1.5 experienced poor growth, these four isolates grew optimally at pH 7.2. One of the conditions for a microbe to be used as a probiotic bacteria is to be able to grow in acidic conditions.

Table 3. BAL isolate density in pH tolerance test

Isolate Code	Number of Bacteria (103 CFU)		
	pH 1,5 ± SD	pH 3 ± SD	pH 7,2 ± SD
8A	28 ± 15,6	56 ± 4,2	270 ± 8,5
11B	1,5 ± 0,1	276,5 ± 33,2	300 ± 0
F1	2,5 ± 0,1	85,5 ± 19,1	292 ± 11,3
G2	19,5 ± 9,2	109,5 ± 17,7	300 ± 0

Bile salt resistance test

This test was carried out to determine its resistance to bile salt or bile salts. LAB isolates were grown on MRSA media with a bile salt concentration of 3000 ppm. In this test, the number of colonies was also counted after 24 hours of incubation with the number of colonies for two repetitions (Duplo).

Table 4. The density of BAL isolates on the bile salt resistance test

Isolate Code	Number of Bacteria (103 CFU)	
	24 hours	48 hours
8A	4 ± 2,83	22,5 ± 3,5
11B	3 ± 1,41	17 ± 4,2
F1	1,5 ± 0,71	4,5 ± 1,0
G2	50,5 ± 6,36	267,5 ± 3,5

Catalase test. The catalase test in Table 5 can find out that all isolates have negative catalase results. Negative catalase is indicated by the absence of oxygen-filled gas bubbles when BAL isolates are dripped with H2O2 solution.

Table 5. Catalase Test Results of bacterial isolates from the intestines of vannamei shrimp

Isolate Code	8A	11B	F1	G2
Catalase Test	(Negative)	(Negative)	(Negative)	(Negative)

Sugar fermentation test. The results of the sugar fermentation test of LAB isolates from the intestines of vannamei shrimp, namely the sugar fermentation test using a carbon source for glucose produced positive results for all isolates, lactose produced positive results for isolate F1 and negative results for isolates G2, 8A and 11B, sucrose produced negative results on isolate G2 and positive on isolates F1, 8A and 11B, Mannitol produces negative results on all isolates, Maltose produces positive results on isolates 8A and negative results on isolates F1, G2 and 11B, then Sorbitol produces negative results on all isolates, then Xylose produced negative results on isolate 11B and positive on isolates F1, G2 and 8A, finally Rhamnose produced negative results for all isolates and on the sugar fermentation test which can be seen in Table 10 Isolates 8A, 11B, F1 and G2 compared to some fermented sugars several lactic acid bacteria namely Pediococcus acidilactici, Pediococcus dextrinicus, Pediococcus pentosaceus, and Tetragenococcus halophilus referred to from (Cai et al., 1999) that isolate G2 is the isolate closest to the fermented sugar of Pediococcus acidilactici.

Table 6. Sugar Fermentation Test Results

Biochemical Test	Isolate			
	F1	G2	8A	11B
Glucose	+	+	+	+
Lactose	+	-	-	-
Sucrose	+	-	+	+
Mannitol	-	-	-	-
Maltose	+	-	+	-
Sorbitol	-	-	-	-
Xylose	-	+	+	-
Rhamnose	-	-	-	-

Information:

\+ : Positive

1. : Negative

Table 7. Comparison of 4 Isolates of Lactic Acid Bacteria and Some Lactic Acid Bacteria

Source Sour	Results For :							
	8A	11B	F1	G2	Pediococcus acidilactici	Pediococcus dextrinicus	Pediococcus pentosaceus	Tetragenococcus halophilus
Glucose	+	+	+	+	+	+	+	+
Lactose	-	-	+	-	-	-	+	+
Sucrose	+	+	+	-	-	-	+	-
Mannitol	-	-	-	-	-	-	-	-
Maltose	+	-	+	-	-	+	-	-

Sorbitol	-	-	-	-	-	-	-	-
Xylose	+	-	-	+	+	-	+	+
Rhamnose	-	-	-	-	+	-	-	-

Resistance Test

The inhibition test of LAB isolates showed different values. In Petri Dishes I obtained the inhibition zone for control (Ciprofloxacin) which was 11.5 mm and for 8A 6.37 mm, 11B 7.2 mm, F1 6.0 mm and G2 10 .7mm. Where at the level of antibacterial activity the smallest inhibition zone was found in Petri dishes I with G2 and antibiotics (Ciprofloxacin) (Medium), 8A, 11B and F1 (weak). At the highest level of antibacterial activity, the inhibition zone was found in Petri dishes II with G2 and antibiotics (Ciprofloxacin) (Medium), 8A, 11B and F1 (weak). Then at the level of antibacterial activity, the inhibition zone was moderate in Petri dishes III with G2 and antibiotics (Ciprofloxacin) (Medium), 8A, 11B and F1 (weak). According to Zainuddin (2006), the level of antibacterial activity based on the inhibition zone with a diameter of ≥ 20 is very good, 15-20 is good, 10-15 is moderate and ≤ 10 is weak.

Table 8. Results of the BAL Isolate Inhibitory Power Zone from the Intestines of Vaname Shrimp

BAL Inhibitory Zone Yield(mm)			
BAL isolate	**I**	**II**	**III**
8A (1)	6,37	7,0	8,0
11B (2)	7,2	8,0	7,7
F1 (3)	6,0	6,2	6,5
G2 (4)	10,7	11,2	11,5
+ (Ciprofloxacin)	11,5	13,2	11,0

Vaname Shrimp Intestine Microflora Density

The results of the analysis of variance showed that the addition of lactic acid bacteria isolates had a significant effect ($p < 0.05$) on the total bacteria in the intestines of vannamei shrimp. Tuckey's further test results showed that the lowest total bacterial value was found in treatment A, namely control and the highest total bacterial value, namely treatment C, namely the treatment of adding lactic acid bacteria isolates. The results of the Tuckey test showed that treatment C was different from treatments A and B.

Table 9. Total bacteria in the intestines of vannamei shrimp

Treatment	Total Bakteri (x 10^3 CFU/mL) ± SD
A	3.67 ± 1.53^a
B	5.67 ± 1.53^a
C	$18,33 \pm 3,06^b$

Description: significant effect between treatments ($p < 0.05$)

Total Vibrio after the challenge test

The results of variety analysis showed that the addition of lactic acid bacteria isolates had a significant effect ($p < 0.05$) on the total vibrio bacteria in the intestines of vannamei shrimp. The results of Tuckey's follow-up test showed the highest result value in treatment B and the lowest value in treatment A. Tuckey's follow-up test results showed that treatments B and C were significantly different from treatment A which was not infected with Vibrio parahaemolyticus bacteria at all.

Table 10. Total *Vibrio* bacteria in vannamei shrimp

Treatment	Total Bakteri (x 10^3 CFU/mL) ± SD
A	2.00 ± 1.00^a
B	$13,33 \pm 3,51^b$
C	$11,67 \pm 2,52^b$

Description: significant effect between treatments ($p < 0.05$)

Immune Response of Vaname Shrimp

Total Hemocyte Count. The results of observations of total hemocytes after the addition of lactic acid bacteria isolates had a high value compared to other treatments. After the challenge test with Vibrio parahaemolyticus, based on the results of the analysis of variance, it was shown that the addition of lactic acid bacteria isolates had a significant effect ($p < 0.05$) on the total hemocyte count before and after the challenge test. Tuckey's further test results showed that treatments A and B were significantly different from treatment C.

Table 11. *Total Hemocyte Count* (THC) of Vaname Shrimp

Treatment	After Treatment	After the Challenge Test
A	7.25 ± 0.90^{a}	9.15 ± 1.10^{a}
B	5.14 ± 0.92^{a}	6.25 ± 0.75^{a}
C	$11,9 \pm 2,70^{b}$	$13,68 \pm 1,86^{b}$

Description: significant effect between treatments ($p < 0.05$)

Differential Hemocyte Count

The results of variance analysis after treatment of vannamei shrimp had no significant effect ($p > 0.05$) on hemocyte count differential.

Table 12. Differential Hemocyte Count (DHC) after treatment

Treatment	Average (%) SD		
	Granular	Semigranular	Hialin
A	33.47 ± 3.36th	31.53 ± 5.56^{a}	35.00 ± 2.23^{a}
B	32.33 ± 0.85th	29.23 ± 3.32^{a}	38.43 ± 2.80^{a}
C	34.43 ± 1.60th	31.60 ± 1.10^{a}	33.97 ± 1.38^{a}

Description: no noticeable effect between treatments ($p>0.05$)

Table 13. Hemocyte Count (DHC) differentiation after the challenge test

Treatment	Average (%) SD		
	Granular	Semigranular	Hialin
A	35.33 ± 1.44^{a}	28.30 ± 4.15^{a}	36.37 ± 3.01^{a}
B	33.37 ± 1.80^{a}	29.63 ± 3.02^{a}	37.00 ± 2.29^{a}
C	35.80 ± 1.76^{a}	29.17 ± 1.04^{a}	35.03 ± 1.46^{a}

Description: no noticeable effect between treatments ($p>0.05$)

Phagocytosis activity

The results of observations of phagocytosis activity in vannamei shrimp after treatment, namely the addition of lactic acid bacteria isolate and after the challenge test showed results that had a real effect ($p < 0.05$) on phagocytosis activity. The results of the Tuckey test showed that after the treatment of adding lactic acid bacteria the value between treatments tended to increase, as well as phagocytosis activity after the challenge test the value between treatments tended to increase but the results of the tuckey test showed that treatment A was different from treatment B and C.

Table 14. Phagocytosis activity in vannamei shrimp

Treatment	Phagocytosis Activity	
	After Treatment	After the Challenge Test
A	34 ± 1.00^{a}	31 ± 2.08^{a}
B	39 ± 2.08^{b}	41 ± 2.08^{b}
C	$44 \pm 1,00^{c}$	$49 \pm 3,61^{b}$

Description: significant effect between treatments ($p < 0.05$)

Lysozyme activity

The results of variance analysis showed that each treatment had no real effect ($p > 0.05$) on the lysozyme activity of vannamei shrimp.

Table 15. Lysozyme activity in vannamei shrimp after a challenge test treatment

Treatment	Track (mm) ± SD
A	0.84 ± 0.03^a
B	0.91 ± 0.03^a
C	0.97 ± 0.02^a

Description: no noticeable effect between treatments ($p>0.05$)

Survival

The results of the variance analysis showed that each of the treatments had no real effect ($p > 0.05$) on the survival of vannamei shrimp.

Table 16. Vaname shrimp survival after challenge test with Vibrio parahaemolyticus bacteria

Treatment	Average (%) ± SD
A	73.33 ± 0.58^a
B	53.33 ± 0.58^a
C	60.00 ± 1.00^a

Description: no noticeable effect between treatments ($p>0.05$)

The results of isolating bacteria from the intestines of vannamei shrimp produced 4 isolates of lactic acid bacteria with different colony sizes and showing clear zones around the bacterial colonies. This is because during their growth period, lactic acid bacteria produce lactic acid and this lactic acid will react with CaCO3 which is not soluble in the media to form Ca-Lactate which is soluble so that a clear area is visible around the growing bacterial colonies. Lactic acid bacteria will use glucose in the media as an energy source and produce energy secondary metabolites in the form of acids that are visible in the zone around the colony (Nuryady et al., 2014).

Identification of superior isolates aims to find out which species produce bacteriocins. In this identification, several tests were carried out, namely morphological identification, inhibition test, gram staining, catalase test, sugar fermentation test, bile salt resistance test and pH tolerance test. These tests were carried out on 4 superior isolates namely 8A, 11B, F1 and G2. In morphological identification, the four isolates had the same shape and color. The gram staining showed a positive result, which means that the four isolates are types of lactic acid bacteria. In the catalase test, the four isolates had the same results, namely producing negative results. The nature of the reaction to the catalase test was determined by the formation of gas bubbles which indicated the formation of water and oxygen from hydrogen peroxide (H2O2) (Tjahjaningsih et al., 2016).

In the pH tolerance test, it can also be seen that the four isolates can grow in acidic conditions, namely at pH 1.5, pH 3 and pH 7 which is characterized by the growth of bacterial colonies on the four isolates tested. According to Ansumar and Fibriarti (2019), probiotic bacteria generally live in the digestive tract and quality with the host's body and live in the pH range of 2-4. Another opinion according to Natali and Zubaidah (2013) LAB isolates can survive pH conditions 2.5 and 3.0. This can be due to the ability of these isolates to maintain a higher pH in their cells neutral than the environment, it can also be because the bacterial cell membrane is more resistant to exposure to acids in the environment. Meanwhile, in the bile salt resistance test, the results of the four isolates were resistant to salt, especially bile salts which is an important characteristic of lactic acid bacteria. However, it can be seen from the results in the table above that isolate G2 has good growth, which can be seen from the number of colonies. This result is in line with the opinion of Adawiyah et al. (2015) the degree of resistance to salt, especially bile salts, is an important characteristic of probiotic lactic acid bacteria, because it affects their activity in the digestive tract, especially the upper intestinal tract where bile is secreted and LAB will be tolerant of high salt levels to start metabolic processes. which will produce acids that can inhibit the growth of pathogenic bacteria. Furthermore, in the sugar

fermentation test which can be seen in Table 7 Isolates 8A, 11B, F1 and G2 were compared with some of the fermented sugars of several lactic acid bacteria, namely Pediococcus acidilactici, Pediococcus dextrinicus, Pediococcus pentosaceus, and Tetragenococcus halophilus referred to from (Cai et al., 1999) that isolate G2 is the isolate closest to the fermented sugar of Pediococcus acidilactici. In the inhibition test, it can be seen that isolate G2 has the highest clear zone results among the other three isolates, namely 8A, 11B and F1. So it can be concluded from several test results that have been carried out and compared with the characteristics of lactic acid bacteria in the reference isolate, it shows that isolate G2 is Pediococcus acidilactici.

The total yield of bacteria is always higher than the abundance of Vibrio in the culture water and shrimp bodies. This is presumably because the probiotic bacteria (Bacillus megaterium) given to the culture water and feed have had a positive effect on suppressing the Vibrio population (Anjasmara et al, 2018). This opinion is in line with the results of research in this study where the results showed that the control treatment and the treatment with the addition of probiotics had a higher value than the abundance of Vibrio in the shrimp intestine. Susana (2017) put forward the bacteria Bacillus sp. tested with pathogenic bacteria (Vibrio algynolyticus, Aeromonas hydrophila and Pseudomonas sp) forming barriers, these bacteria produce antibiotic products. In the shrimp body, probiotic bacteria are thought to compete for space in the exoskeleton and digestive tract of shrimp. Probiotic bacteria produce pathogenic antimicrobial compounds through competition for nutrients and attachment sites on the intestinal wall, changing bacterial metabolism by increasing or decreasing enzyme activity, and stimulating immunity through increasing antibody levels or macrophage activity (Anjasmara et al., 2018). The intestine contained in the digestive tract is very closely related to the health of the shrimp body. Lactobacillus bacteria are bacteria belonging to the lactic acid bacteria (LAB) which produce several antimicrobial components, in this case, bacteriocins. Bacteriocins are protein-like toxins secreted by the bacteria concerned to inhibit the growth of other harmful bacteria (Jannah et al, 2018).

The isolation of lactic acid bacteria in vannamei shrimp as a probiotic against the immune system of vannamei shrimp showed that this treatment could improve immune parameters in vannamei shrimp as seen from the increase in the total number of hemocytes after the V. parahemolyticus challenge test. Hemocytes are one of the defense systems in vannamei shrimp which are responsible for phagocytosis, nodulation and encapsulation. A high number of hemocytes indicates a good level of shrimp health. The results of observations of total hemocytes after the addition of lactic acid bacteria isolates had a high value compared to other treatments. Thus, it can be presumed that the addition of lactic acid bacteria isolates isolated from vannamei shrimp intestines can increase the THC value. Total hemocytes in the body of crustaceans are very important in maintaining resistance to pathogens. If the total hemocytes are high, it can increase the blood's ability to phagocytize (Jannah et al, 2018). In this study, it was seen that treatment C, namely the administration of probiotics, namely bacterial isolates isolated from the intestines of vannamei shrimp, was able to produce the highest total hemocytes after infection with V. parahaemolyticus bacteria compared to Control so that it could increase the shrimp's immune system against infection with V. parahaemolyticus bacteria. This is in accordance with the results of a study conducted by Vieira et al., (2010) which showed that the administration of probiotic bacteria in the form of Lactobacillus plantarum at a dose of 0.7 x 108 CFU/ml gave shrimp body resistance to Vibrio harveyi bacterial disease.

Hemocytes consist of three types of cells that are distinguished based on the granules in the cytoplasm of each cell; namely hyaline, granular, and semi-granular. The three types of cells function to destroy foreign particles that enter the shrimp body through phagocytosis, encapsulation, nodule formation, and production of humoral components stored in haemocytic granules, namely anticoagulant proteins, agglutinins, PO enzymes, antimicrobial peptides, and protease inhibitors (Jayasree et al., 2006) in Jannah et al (2018). Van de Braak et al. (2002) stated that the immune response of shrimp can be known from the activity of hemocyte cells, namely hyaline, semi-granular and granular. The results of the study showed an increase in granular cells and hyaline cells both after the addition of lactic acid bacteria and after a challenge test on vannamei shrimp, these results were in line with the study of Anton et al, (2020), namely the results of observations on differential hemocytes (DHC) showed an increase in the number of cells hyaline on day 5 post-challenge test in all treatments and controls. The high presentation of hyaline cells in the reared shrimp was due to the efforts of the shrimp to defend

themselves from the high number of V. parahaemolyticus that had previously been infected to the shrimp's body due to the lack of support from probiotic bacteria to fight the pathogen. Hyaline cells are a type of immune cell that plays a role in phagocytosis of pathogens and are involved in the process of coagulation and cuticle formation (Anton et al, 2020).

Phagocytosis activity is a function of a non-specific immune response which is the initial defense mechanism against attacks by microorganisms and high phagocytic activity indicates that an organism can produce more phagocytic cells so that when exposure to pathogenic microorganisms occurs, blood cells are ready to carry out phagocytic processes (Widanarni et al. al, 2020). The increase in total hemocytes in this study was also followed by an increase in phagocytosis activity. Phagocytic activity increased after administration of lactic acid bacteria as probiotics through feed and after being challenged. The increase in phagocytosis after the challenge test led to an increase in phagocytosis, so that when challenged with V. parahaemolyticus it could survive pathogens because the function of hyaline cells for phagocytosis increased (Kalsum, 2021). The results of phagocytosis activity in this study had the highest percentage in treatment C compared to other treatments because the increase in phagocytosis activity was related to the peptidoglycan content in Bacillus bacteria which is one of the components that can stimulate the immune system in shrimp and activate phagocytic cells (Sukenda et al, 2007) is also in line with the opinion that increased phagocytic activity of hemocytes is an indicator of increased immune defense in vannamei shrimp, the entry of foreign bodies into the host's body will be responded to by the occurrence of a phagocytosis process. Phagocytosis is a non-specific defense mechanism that is generally able to protect against pathogen attack (Kurniawan et al, 2018).

The activity of lysozyme is a major defense factor of humoral immunity in cellular defense mechanisms and its ability to break down pathogenic cell walls makes lysozyme fight against harmful microorganisms such as parasites, bacteria and viruses naturally (Rahim et al, 2020). Lysozyme plays an important role in the defense mechanism against infectious diseases. The results of this study indicate that the administration of lactic acid bacteria as probiotics produces the highest value compared to other treatments. The increase in lysozyme activity in this study indicated an increase in the humoral non-specific immune system in vannamei shrimp. These results can be attributed to the structure of the β-1,3 GF complex and its ability to activate vannamei shrimp immunity (Eissa et al, 2023). Circulating shrimp haemocytes are the main source of lysozyme. Therefore, the hemocyte spike in this study coincided with an increase in lysozyme activity. The activity of lysozyme is also related to phagocytosis activity, where phagocytosis is an active process that begins with the engulfing of pathogens by macrophage cells, then the pathogen will be inserted into the phagosome which will undergo an oxidase-reduction reaction so that the degree of acidity increases.

The identification from the several tests above shows that isolate G2 is included in Pediococcus acidilactici. Pediococcus acidilactici F-11 is a bacteriocin-producing homofermentative lactic acid bacterium isolated from fermentation products.

4. Conclusion

Four isolates of lactic acid bacteria from vannamei shrimp intestine (8A, 11B, F1, and G2) were successfully isolated. The isolates were Gram-positive cocci, catalase, tolerant to acidic to alkaline pH, tolerant to bile salts and showed antibacterial activity against Vibrio parahaemolyticus. The G2 isolate tested stimulated the vannamei shrimp immune system by increasing THC and phagocytic activity and suppressing the development of Vibrio parahaemolyticus. Analysis of the inhibition test, gram staining and sugar fermentation test of LAB isolate (G2) showed that isolate G2 belongs to Pediococcus acidilactici.

Conflict of interest:

The authors declare no conflict of interest.

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