Screening of antivibrio-producing lactic acid bacteria originated from aquatic animals as probiotic candidates

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Abstract. Acute hepatopancreatic necrosis disease has been a major challenge faced by white shrimp farmers in many shrimp-producing countries inducing Indonesia. The etiological agent of this disease was a virulent strain of\textit{Vibrio parahaemolyticus}. However, a technology to treat or to prevent the disease infection has not been established yet, and probiont could be a potential approach for this disease. Thus, this study aimed at screening 35 lactic acid bacteria (LAB) that originated from the gastrointestinal tract of wild white shrimp for the production of antivibrio compounds using an agar well diffusion. The result showed that three LAB strains (LAB-1, LAB-2, and LAB-3) showed antivibrio activity indicated by the formation of clearance zone (diameter of >10mm) on the diffusion agar plate. When the extracellular products (ECP) were incubated with proteinase K, the antivibrio activity was disappeared which indicated that the antimicrobial compounds were bacteriocin-like inhibitory substances (BLIS). Phenotypic studies suggest that the LAB were\textit{Labctobacillus} sp. (LAB-1 and LAB-2), and \textit{Enterococcus} sp. (LAB-3). These results suggest these LAB are potential candidates for preventing \textit{V. parahaemolyticus}. Infection, although, further assays by \textit{in vivo} using live white shrimp, are still required.

1. Introduction

White shrimp (\textit{Litopenaeus vannamei}) is one of the economically important aquaculture commodities in Indonesia. Currently, Indonesia is in the third-highest shrimp exporting country after China and India. Indonesian government keeps pushing shrimp production to increase shrimp export. One way to increase production is by increasing stocking density from semi-intensive, intensive to supra intensive aquaculture systems. Currently, stocking density has reached 1,000 shrimp/m³, and total production could be increased multiple times compared to semi-intensive aquaculture systems. However, the intensification has been considered as the principal cause of immune suppression and increase shrimp susceptibility to diseases [1].

The emerge of various diseases infecting white shrimps such as viruses, bacteria, and parasites has been reported in many studies. One of the most current diseases reported in shrimp farming is Acute hepatopancreatic necrosis diseases (AHPND). This disease has been affecting the global culture of shrimp by reducing total global supply from shrimp. It was firstly reported in china in 2009, and since then, it spread worldwide from southeast Asian countries such as Thailand, Vietnam, Malaysia to South
America. A study by Hastuti [2] in 2015 indicated that Indonesia was still free from AHPND disease. Since then, no study detects the presence of the disease in Indonesia yet. However, the last 4 years, there have been many cases of mass shrimp mortalities in several regions of Indonesia, and clinical signs appear to be similar to AHPND.

Technology to prevent the disease outbreak in shrimp farming is very limited, acknowledging the immune system of white shrimp. Some of the available approaches are antibiotics, vaccination, immunostimulant, and probiotics. However, the use of antibiotic has been criticized due to having short protection period, the need for repeated treatments in extended outbreaks of disease, and increased harmful residues in carcasses of cultured animals [3], and the rise of antibiotic-resistance pathogen which is considered as the most serious problem nowadays [4]. On the other hand, the reliability of vaccination when it was applied in invertebrate organisms such shrimp has been questioned due to having less developed immune systems. Furthermore, vaccination was complained due to difficulty in application, requiring laborious works, expensive, and require a long process in developing strategies for new diseases. These problems motivate aquaculture scientists to search for other safer and more reliable methods to improve the disease resistance of white shrimps [5].

Probiotic application in aquaculture industries has gained considerable interest in the prevention of disease outbreak due to the increasing demand for environmental benefits, eco-friendly alternatives for sustainable aquaculture production. Scientists and aquaculturists adopted recent effective biological control methods, such as probiotics. Probiotics are cultured products or live microbial food supplements, which beneficially affect the host by improving its intestinal microbial balance. It helps in improving the water quality, aids in food digestion, and modulates the host immune responses, increasing production efficiency and reducing disease incidence [6].

Thus, this present study aimed at developing probiotic out of indigenous bacterial communities associated with the gastrointestinal tract of white shrimps. The indigenous probiotic is expected to increase practicability in their application.

2. Materials and methods
2.1. Isolation of LAB
Wild white shrimp (Litopenaeus vannamei) was collected using a scope net from an estuarine farm in Pijot, Keruak, East Lombok, West-Nusa Tenggara Province. The shrimp was blot dried on a tissue and weighted individually on a balance. Subsequently, the shrimp was sprayed with 70% ethanol to kill microorganisms attached to the shrimp carapace, followed by rinsing with sterile water (dH₂O) and placed on a wax plate inside a laminar flow. The gastrointestinal tract was dissected out aseptically and placed inside a glass mortar for homogenization. Then, the homogenized gut was put in a 5ml de Man Rogosa Sharp (MRS) broth and incubated aerobically at room temperature (~27°C) for 24 hours. Afterward, the broth culture was streaked onto a MRS agar and incubated aerobically at room temperature for 24 h. Representative bacterial colonies (morphology, color, and surface) were purified by streaking them onto another MRS agar. Pure colonies were stored in 15% glycerol stock and placed in a freezer (~20°C) until further use.

2.2. Screening for anti-vibrio activity
The presence of anti-vibrio activity was measure determined according to a protocol of Amin, Adams, Bolch and Burke [7] with slight modifications. In brief, crude extracellular product (ECP) of each LAB was prepared according to Amin et al. [7] with slight modifications. In brief, a fresh culture of pure LAB was subcultured in 500 mL MRS broth and incubated at 37°C anaerobically for 24 h. The broth culture was centrifuged at 5,000 x g for 15 min at 4°C using an Eppendorf Centrifuge [8]. The ECP was collected, and the pH was adjusted to 6.5 by adding 1 M sodium hydroxide (NaOH) to neutralize any antagonistic activity of organic acids. Thereafter, the ECP was sterilized by filtration through 0.22 μm filter Millex Syringe Filter (Millipore). The sterilized supernatant was stored in a fridge (~4°C) until further use.
Preparation of pathogen inoculum: *V. parahaemolyticus* was obtained from Fish Quarantine Inspection Agency, Mataram, West-Nusa Tenggara Province. The pathogen was subcultured in a 10-mL bottle of Muller-Hinton broth (CM0405, Oxoid) and incubated at room temperature (~28°C) for 12 h. Afterwards, cells of the pathogen were harvested by centrifugation at 13,000 x g for 60 sec. The cells were washed with phosphate-buffered saline (PBS, pH 7.2) and rediluted in the PBS to get ~0.15 optical density (OD) at 600 nm wavelength with a spectrophotometer, which was equal to ~1.0 x 10^6 CFU.mL^-1 cell concentration.

Screening for Anti-vibrio Activity: This assay was conducted according to a modified protocol developed by Amin [4] with some modification. In brief, one ml of *V. parahaemolyticus* inoculum (~1.0 x 10^6 CFU.mL^-1) was spread on Muller-Hinton agar (CM0337, Oxoid) and air dried at a room temperature for 5 min. Then, 6 mm-diameter wells were bored on the agar using the base of a sterile pipette, followed by the addition of 10 μl of ECP from each LAB isolate in duplicate wells. After 24 h aerobic incubation at 37°C, clearance zones were measured, and an isolate which had clearance zone >10mm was selected for further studies [9].

2.3. Susceptibility to proteinase K
The effect of proteinase K on the anti-vibrio compound was tested according to a modified protocol of Abrams et al., [10]. ECP was incubated for 1 h at 37°C in the presence of 1 mg.mL^-1 (final concentration) proteinase K (Triritchium, Sigma-Aldrich), and the enzyme activity was inactivated by heating at 100°C for 3 min [11]. Thereafter, the remaining anti-vibrio activity was evaluated using a micro titer plate assay according to a modified protocol of Amin, Adams, Bolch and Burke [7]. Briefly, 230 μL of sterilized MH broth was pipetted into wells of a 96-well tissue culture plate and inoculated with a fresh culture of *V. parahaemolyticus* to give ~ 1.0 x 10^4 CFU.mL^-1. Then, 10 μL of proteinase K- treated ECP was added to the wells. As a control 15 μL of ECP was added. The treatment and control had 6 replicate wells. The growth of *V. parahaemolyticus* at room temperature was monitored by measuring optical density (OD) at 600 nm every hour for 24 h using a microplate reader.

2.4. Identification
LAB which showed antagonistic activity against the targeted pathogen (*V. parahaemolyticus*) were identified using phenotypic assay. The assay includes: colony morphology, cell shape, gram staining, catalase, oxidase, ability to ferment glucose, capacity to produce CO2.

3. Result and discussion
*V. parahaemolyticus* was considered as the current pathogen causing acute hepatopancreatic necrosis disease (AHPND) in shrimp farmers over the world. Various efforts have been developed to cure and prevent the pathogen infection. However, the technology which can be applied in shrimp was very limited, acknowledging their less developed immune systems [12]. Screening criteria for selecting probiotic candidates proposed by Food Agriculture Organization (FAO) or World Health Organization (WHO) are the ability of certain bacteria to produce antimicrobial compounds against pathogens and safe or non-toxic to culture animals [13]. Thus, this study screened 32 strains of LAB for probiotic properties. The result showed that three LAB had antagonistic activity against the pathogen target (*V. parahaemolyticus*).

3.1. LAB strains with antagonistic activity against vibrio
Fifty-two lactic acid bacteria (LAB) were isolated from the intestinal tracts of white shrimp (*Litopenaeus vannamei*). Three of them showed antagonistic activity against *V. parahaemolyticus*, indicated by the formation of an inhibition zone with >10mm diameter (Fig. 1 and Table 1). The inhibition zones obtained from LAB this study was much wider compared to the inhibition zone of probiotic candidates previously reported by Sathyabama, Vijayabharathi, Devi, Kumar and Priyadarisini [14], which was only 2, 4, 7, 10 and maximum at 12mm. This result may indicate that the LAB reported in the present study are potential probionts for preventing outbreak of *V. parahaemolyticus* infection.
Table 1. Diameter of inhibition zones from three LAB strains isolated from the intestinal tract of white shrimp against *V. parahaemolyticus*

| No | LAB Strains | Diameter of Inhibition Zone |
|----|-------------|-----------------------------|
| 1  | LAB 1       | 13.33±0.58                  |
| 2  | LAB 2       | 14.00±1.53                  |
| 3  | LAB 3       | 10.33±0.56                  |

Note: Values are average of clearance zones and standard deviations of 6 replicates

Figure 1. Clearence zone of ECP from three LAB strains agaisnt *V. parahaemolyticus*

3.2. Characterization of anti-vibrio compounds

The anti-vibrio activity of ECP was completely inactivated when it was incubated into proteinase K at final concentration of 1 mg. mL⁻¹ for one h, Figure 2. Meanwhile, in the positive control without proteinase K treatment), ECP of all LAB maintained its anti-vibrio activity, confirmed by no growth of *V. parahaemolyticus* in the microtiter plate assay. This result may suggest that the antimicrobial compounds produced by the three LAB were bacteriocin-like inhibitory substances (BLIS). According to Abrams, Barbosa, Albano, Silva, Gibbs and Teixeira [10], the inactivation of antimicrobial activity of antimicrobial compound after being treated with proteinase K may indicate that that the substance was bacteriocin like inhibitory substances.
Figure 2. The effect of ECP from LAB after being treated with proteinase K on the growth of *V. parahaemolyticus*. Growth of *V. parahaemolyticus* was measured in terms of optical density (OD) at 600nm. Values are average of OD values over times. ECP was the growth of the pathogen when treated “ECP without Proteinase K treatment”, and “ECP + proteinase K” represented the growth of pathogen after being treated with ECP with proteinase K treatment.

3.3. Identification
Phenotypically, all isolates were Gram-positive, cocci or rod cell shapes, oxidase negative, and catalase-negative, Table 2. In addition, all tested LAB showed a capacity to ferment glucose indicated by the color changes from purple to yellow in the all broth culture tube.

| Table 2. Colonial and cell morphology of LAB producing anti-vibrio |
|---------------------------------------------------------------|
| **A. Colony morphology**                                     | **Isolate 1** | **Isolate 2** | **Isolate 3** |
| - Form                                                      | Circular      | Circular     | Circular      |
| - Surface                                                   | Smooth        | Smooth       | Smooth        |
| - Optical characteristic                                    | Opaque        | Opaque       | Opaque        |
| - Consistency                                               | Mucoid        | Mucoid       | Mucoid        |
| - Pigmentation                                              | Deep yellow   | Light yellow | White         |
| - Elevation                                                | Convex        | Convex       | Convex        |
| - Margin                                                   | Entire        | Entire       | Entire        |
| **B. Cell morphology**                                      |               |              |
| - Cell                                                      | bacilli       | bacilli      | Coccus        |
| - Gram staining                                             | +             | +            | +             |
| - Catlase                                                   | -             | -            | -             |
| - Oxidase                                                  | -             | -            | -             |
| - Glucose fermentation                                      | +             | +            | +             |
| - CO2 production                                            | -             | -            | -             |
Figure 3. Cell morphology of three lactic acid bacteria (LAB) producing antivibrio activity.

All these phenotypical characteristics indicate that these bacteria belonged to a member of lactic acid bacteria [7, 15]. Similar phenotypic results had been also described by Nursyirwani, Asmara, Wahyuni, Triyanto, Fauzi and Muchlisin [16] in which LAB members were Gram-positive, coccus of the cell shape, catalase-negative, oxidase-negative, and had a capacity to ferment glucose. One of LAB in this present study (LAB 3) had very similar phenotypes; therefore, we can identify LAB 3 as *Enterococcus* sp. Meanwhile, cell shape of the other two LAB was bacilli, and according to [17], these bacteria could be *Lactobacillus* sp. However, further identification techniques such as polymerase chain reaction (PCR) by amplification of their 16S rRNA gene should be conducted in order to get more specific and accurate results.

4. **Conclusion**

Three LAB strains (LAB 1, LAB 2, and LAB 2) showed antimicrobial activity against *Vibrio parahaemolyticus*. The antimicrobial compounds appeared to very vulnerable to proteinase K which may indicate that the antimicrobial compound was bacteriocin-like inhibitory substance (BLIS). Phenotypically the bacteria were identified as lactic acid bacteria (LAB-1: *Lactobacillus* sp., LAB-2: *Lactobacillus* sp. And LAB3: *Enterococcus* sp.). However, further studies using molecular techniques are required to identify for species level. The protective capacity of these LAB toward *V. parahaemolyticus* in live shrimp should also be further studied.

5. **References**

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