# Screening of Probiotic Candidate from Mangrove Leaf Litter and Sediment for a Sustainable Shrimp Farming

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**Abstract.** Infectious diseases pose a significant challenge in shrimp farming. However, the use of antibiotics to control these diseases has been prohibited due to their negative effects. Probiotics offer an environmentally friendly and sustainable alternative to minimize the use of antibiotics for disease prevention. This study aims to identify potential probiotic candidates from litter leaf and sediment in the Mangrove Center Bengkak (MCB), Banyuwangi, East Java. The methods used included sampling litter and sediment from the mangrove, identifying probiotic candidate isolates, testing their inhibition zones against pathogens (specifically *Vibrio harveyi*), and assessing their resistance to different temperatures, salinity and pH. The results identified several bacteria genera, including *Bacillus*, *Pseudomonas*, *Aerococcus*, and *Enterococcus*, from mangrove litter while *Bacillus*, *Achromobacter*, *Actinobacillus*, *Flavobacterium*, *Micrococcus*, and *Neisseria* were identified from mangrove sediments, which showed potential as probiotics. The genus of *Bacillus* displayed the highest inhibitory effect againsts *Vibrio harveyi* compared to other genera. The resistance tests demonstrated that all bacterial genera exhibited broad tolerance to various environmental conditions. This result was firstly reports the potential of probiotic candidate from MCB. Further studies were needed to apply the probiotic bacteria in feed supplementation or direct into rearing media to assess the potential effect as probiotic agent.

## Introduction

Bacterial diseases, such as luminous vibriosis, are common infections in shrimp. Luminous vibriosis is a highly lethal disease in aquaculture organisms caused by *Vibrio harveyi*. It has been reported to infect various species, including vaname shrimp (*Litopenaeus vannamei*) [1] and blood clams (*Tegillarca granosa* [2], *Vibrio* spp. bacteria generally infect shrimp during the zoea and early post-larval stages, thereby disrupting the production of healthy shrimp fry. Initial symptoms typically appear within 1-3 days and require immediate termination.

Disease control in aquaculture organisms generally uses antibiotics and other chemical compounds but the use of these chemicals has a harmful impact on the quality of the aquatic environment. Furthermore, the continuous use of antibiotics can also increase resistance to target organisms [3]. To minimize the use of antibiotics and similar chemicals, alternative materials can be used to control diseases caused by *V. harveyi*, including probiotics.

Probiotics are live organisms added to aquaculture systems to improve water quality, feed consumption, immune response, and inhibit the growth of pathogenic bacteria. Probiotics commonly used in aquaculture are isolated from lactic acid bacteria, photosynthetic bacteria, fungi (yeasts), bacteriophages, and microalgae. Probiotic bacteria are biological control agents because they can play a role in combating the pathogenic microorganisms by producing natural antimicrobial compounds, including bacteriocins, siderophores, lysozyme, proteases, cellulases, hydrogen peroxide or organic acids Probiotic bacteria that have been used to control *V. harveyi* are *Bacillus*, *Lactobacillus* and *Photobacterium* [4].

Mangrove ecosystems are located on coral beaches, coral reef land, and on alluvial soil beaches, therefore they are strongly influenced by tides and their sediments are always inundated with water. The abundance of microorganisms in sediments in mangrove ecosystems is influenced by the interaction between biological and physical factors in mangrove ecosystems. Physical factors include the formation of sediment deposits that are carried along with river and sea flows and trapped in mangrove. Meanwhile, biological factors that affect the

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abundance of microorganisms in mangrove sediments are the decomposition of organic matter into nutrients, which are used as a food source for live microorganisms in sediments [5].

Mangrove Center Bengkak (MCB) is located on the coast of Bengkak Beach, Wongsorejo District, Banyuwangi Regency, East Java. There are several types of mangrove species, including *Rhizhopora*, *Avicennia*, *Nypa*, *Pemphis acidula,* and others. The environmental characteristics in MCB include sandy and muddy soil textures, temperatures ranging from 23-33 °C, pH levels between7-8, and salinity levels of 23-36‰. The total area of MCB is approximately 5 hectares, which is divided into 4 blocks and is close to an organic waste disposal site, causing sedimentation. This condition affects the availability of nutrients for bacteria growth.

Isolation and identification of probiotic candidate bacteria from MCB, Banyuwangi, East Java has never been reported before. Therefore, this study aims to obtain isolates of potential probiotic candidates to prevent Luminous vibrio infection in shrimp. The results of this study will be used as an antibiotic alternative that is safety and more environmentally friendly.

# METHODS

## 2.1 Time and Location

This study was conducted from January to March 2023 in the Instrument Laboratory, Faculty of Health, Medicine and Life Sciences at Airlangga University in Banyuwangi, East Java, Indonesia. The sampling location was at the Mangrove Center Bengkak (MCB) in Wongsorejo Subdistrict, District of Banyuwangi, East Java, Indonesia (Figure 1). Station 1/S1 (Coordinates 8º02’01’’S and 114º26’00’’E) is located close to a residential area. Station II/S2 (Coordinates 8º02’01’’S and 114º26’59’’E), is close to human household waste. Station III/S3 (Coordinates 8º01’55’’S and 114º25’58’’E), is near shrimp ponds. Station IV/S4 (Coordinates 8º01’57’’S and 114º26’01’’E) is the nearest to the coastline. Station V/S5 (Coordinates 8º01’52’’S and 114º25’57’’E) is close to a river that serves as a waste disposal from ponds and industrial activity. The sampling locations selected for this study are presented in Figure 1.

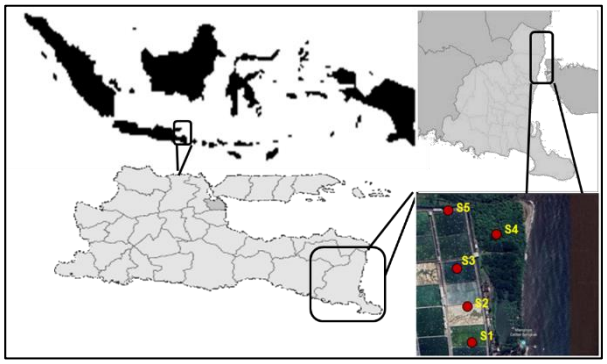


**Fig. 1.** Sampling site.

## 2.2 Mangrove leaf litter sampling

Litter was collected directly from each station by using a litter-trap (2x2 meters in size, mesh size 1 mm, nylon, weighted in the bottom center to maintain the stability). The litter-traps were set at a height of 1 meter above the highest tide line. One litter trap was installed at each observation station on a tree branch. Litter collection was carried out over a period of 5-10 days, depending on the density of mangroves. After the mangrove litter was collected, it was air-dried and placed into a litter bag.

## 2.3 Mangrove sediment sampling

Sediment samples were taken directly from the sampling site, approximately 10 g using a scoop, to a depth of 10 cm below the sediment surface. The samples were stored in ziplock plastic bags sterilized using 70% alcohol, placed in a cool box, and delivered to the laboratory.

## 2.4 Bacterial identification

1 g of mangrove litter leaf powder and sediment from each station was diluted with 9 mL physiological saline solution. 1 mL from the first dilution was taken and mixed with 9 mL of physiological saline solution to obtain the fifth dilutions ($10^5$). Then, 0.1 ml from each dilution was spread onto TSA/Tripticase Soy Agar (Merck, Germany), and incubated at 37°C for 24 hours. For sediment, 1 g of sediment from each station was serially diluted with 9 mL of physiological saline solution as stated previously ($10^5$). After 24 h, the colonies were purified using the quadrant method until a single colony was obtained for characterization. The identification method was based on biochemical characteristics, including Gram staining, oxidase, and catalase reactions, motility activities, indole production (using Sulphite Indol Motility/SIM, Merck, Germany), glucose fermentation (using Oxidative-Fermentative/OF Medium (Merck, Germany) and Triple Sugar Iron Agar/TSIA (Merck, Germany), citrate acid production (using Simmone Citrate Agar/SCA (Himedia, India), and acid production from glucose fermentation (using MRVP Medium (Himedia, India).

## 2.5 Antibacterial activity against Vibrio harveyi

A pure culture of *V. harveyi* was obtained from the collection of the Microbiology Laboratory, Center of Brackiswater Aquaculture, Jepara, Center of Java. The pathogen was re-cultured in selective medium (TCBS/Thiosulphate Citrate Bile Salt Agar, HiMedia, India) and diluted using physiological solution until reaching $10^6$ $CFUmL^{-1}$. The antibacterial activity test was conducted using the Kirby-Bauer method for each probiotic candidate. The positive control used a ciprofloxacin disc containing 0.5 $mgmL^{-1}$ (Oxoid, USA), and the negative control used physiological solution. After 24 hours, the clear zone was measured using a digital pulse (Taffware, China) with alevel of accuracy of 0.05 mm. The clear zones were categorized into four groups based on diameter: very strong (≥ 20

mm), strong (10 – 20 mm), moderate (5 – 10 mm), and weak (≤ 5 mm) [29].

### 2.6 Resistance test against environmental factors

#### *2.6.1 Temperature resistance*

The probiotic isolate was inoculated into three test tubes containing Nutrient Broth/NB medium (Merck, Germany), and incubated at three different temperatures (i.e. 20°C, 30°C and 40°C) for 24 hours. After incubation, the bacterial density was measured using a spectrophotometer (Shimadzu, Japan) at 600 nm.

#### *2.6.2 pH resistance*

The probiotic isolate was inoculated into a test tube containing NB medium with three different pH covering theacidic, neutral, and basic levels. The acidic condition was treated with the addition of HCl (Merck, Germany) to obtain pH 5, while the basic condition was adjusted with the addition of KOH (Merck, Germany) to obtain pH 10. Meanwhile, for neutral condition, the medium was maked using standard procedures. All tubes were incubated at 37°C for 24 hours, and the bacterial density was determined using a spectrophotometer (Shimadzu, Japan) at 600 nm.

#### *2.6.3 Salinity resistance*

The probiotic isolate was cultured in different test tubes containing NB medium with three salinity levels (i.e. 15 ppt, 25 ppt, and 35 ppt). The salinity level was adjusted using NaCl (Merck, Germany) based on each treatment. All tubes were incubated at 37°C for 24 hours, and the bacterial density was measured using a spectrophotometer (Shimadzu, Japan) at 600 nm.

### 2.6 Data Analysis

The characterization of probiotic bacteria was described in table. Meanwhile, the bacterial density under different environmental conditions (temperature, pH and salinity) was analyzed statistically using t-Test - Paired Two Sample for Means ($\alpha$ = 95%) in Microsoft Excel 2013.

## RESULT

### 3.1 Bacterial identification from mangrove litter and sediment in MCB

The results of bacterial identification from the litter demonstrated four genus bacteria, including *Bacillus*, *Aerococcus*, *Enterococcus*, dan *Pseudomonas* (Table 1). On the other hand, bacteria isolated from mangrove sediment were belonged to *Bacillus, Achromobacter, Actinobacillus, Flavobacterium, Micrococcus,* and *Neisseria*. The bacterial isolates obtained from mangrove litter and sediment samples were characterized based on their morphological and phenotypic characteristics. The results of the characterization are presented in Table 1.

### 3.2 Antibacterial activity of probiotic candidates from leaf litter and sediment of mangrove against *Vibrio harveyi*

The Antibacterial activity tests indicated that all bacterial isolates could inhibit the growth of *V. harveyi* as shown by the clear zone with different abilities (Table 2). The greatest inhibitory activity was shown by *Bacillus* bacteria (S1A).

**Table 1.** Characterization of bacteria isolates collected from litter and sediment of mangrove in MCB

| No. | Sample | Code | Colony Colour | Shape | Margin | Elevation | Cell Shape | Gram | Motility | Indole | MR | VP | O/F | TSIA | SCA | Catalase | Oxidase | Identified Genus |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Litter | L1A | Cream | Punctiform | Entire | Convex | Rod | Negative | - | + | - | - | Facultative | K/K/+ (Air) | + | + | - | *Pseudomonas* |
| 2 | Litter | L2A | Cream | Circular | Entire | Convex | Round | Positive | - | - | - | - | Facultative | K/K/- (Air) | - | + | - | *Aerococcus* |
| 3 | Litter | L2B | Cream | Circular | Entire | Flat | Rod | Positive | + | + | - | - | Facultative | K/K/- (Air) | + | + | - | *Bacillus* |
| 4 | Litter | L3B | White | Circular | Entire | Convex | Rod | Positive | + | - | - | - | Facultative | M/K/- (Air) | + | + | - | *Bacillus* |
| 5 | Litter | L4B | White | Punctiform | Entire | Convex | Rod | Positive | + | - | - | - | Facultative | K/K/- (Air) | + | + | + | *Bacillus* |
| 6 | Litter | L5A | Yellow | Circular | Entire | Convex | Round | Positive | - | - | - | + | Facultative | K/K/- (Air) | + | - | - | *Enterococcus* |
| 7 | Litter | L5B | Cream | Circular | Entire | Pulvinate | Round | Positive | - | - | - | - | Facultative | K/K/- (Air) | + | + | - | *Aerococcus* |
| 8 | Litter | L5C | White | Punctiform | Entire | Raised | Rod | Negative | + | - | - | - | Facultative | K/K/+ (Air) | + | - | + | *Pseudomonas* |
| 9 | Sedimen | S1A | White | Punctiform | Entire | Flat | Rod | Positive | + | - | + | - | Facultative | K/K/+ (Air) | + | + | + | *Bacillus* |
| 10 | Sedimen | S1B | White | Circular | Entire | Flat | Rod | Negative | + | - | - | - | Oxidative | K/K/+ (Air) | + | + | + | *Achromobacter* |
| 11 | Sedimen | S1C | White | Circular | Entire | Raised | Rod | Negative | - | - | - | - | Fermentative | K/K/- (Air) | + | + | + | *Actinobacillus* |
| 12 | Sedimen | S2A | Cream | Circular | Entire | Convex | Rod | Negative | - | - | - | - | Fermentative | K/K/- (Air) | + | + | + | *Actinobacillus* |
| 13 | Sedimen | S2B | Cream | Circular | Entire | Convex | Rod | Negative | - | - | - | - | Oxidative | K/K/+ (Air) | + | + | + | *Flavobacterium* |
| 14 | Sedimen | S3B | Cream | Circular | Entire | Convex | Rod | Negative | - | - | - | - | Oxidative | K/K/+ (Air) | + | + | + | *Flavobacterium* |
| 15 | Sedimen | S3C | White | Punctiform | Entire | Convex | Round | Positive | - | - | - | - | Oxidative | K/K/+ (Air) | + | + | + | *Micrococcus* |
| 16 | Sedimen | S3D | White | Circular | Entire | Flat | Rod | Positive | + | - | + | - | Oxidative | K/K/+ (Air) | + | + | + | *Bacillus* |
| 17 | Sedimen | S4A | White | Circular | Entire | Flat | Rod | Negative | + | - | + | - | Oxidative | K/K/+ (Air) | + | + | + | *Flavobacterium* |
| 18 | Sedimen | S4B | Cream | Circular | Entire | Convex | Rod | Negative | + | - | - | - | Oxidative | K/K/+ (Air) | + | + | + | *Flavobacterium* |
| 19 | Sedimen | S5A | White | Punctiform | Entire | Raised | Round | Negative | - | - | - | - | Oxidative | K/K/+ (Air) | + | + | + | *Neisseria* |

**Note:** MR: methyl red; VP: Voges–Proskauer; O/F: Oxidative/Fermentative; TSIA: Triple Sugar Iron Agar; K: Alkali; M: Acid; SCA: Simmons Citrate Agar.

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**Table 2.** Anti-vibrio activity from bacterial isolate against *V. harveyi*

| Sample Code | Genus | Average diameter of clear zone (mm) | Category |
|---|---|---|---|
| L1A | *Pseudomonas* sp. | 1.7 | Weak |
| L1C | *Enterococcus* sp. | 3.35 | Weak |
| L2A | *Aerococcus* sp. | 11.9 | Strong |
| L2B | *Bacillus* sp. | 3.1 | Weak |
| L2B | *Bacillus* sp. | 3.1 | Weak |
| L3B | *Bacillus* sp. | 6.85 | Moderate |
| L4B | *Bacillus* sp. | 15.3 | Strong |
| L5A | *Enterococcus* sp. | 1.5 | Weak |
| L5B | *Aerococcus* sp. | 1.43 | Weak |
| L5C | *Pseudomonas* sp. | 7.4 | Moderate |
| S1A | *Bacillus* sp. | 31.1 | Strong |
| S1B | *Achromobacter* sp. | 18.65 | Strong |
| S1C | *Actinobacillus* sp. | 12.25 | Strong |
| S2A | *Actinobacillus* sp. | 10 | Strong |
| S2B | *Flavobacterium* sp. | 2.6 | Weak |
| S3B | *Flavobacterium* sp. | 9.15 | Moderate |
| S3C | *Micrococcus* sp. | 9.85 | Moderate |
| S3D | *Bacillus* sp. | 3.1 | Weak |
| S4A | *Flavobacterium* sp. | 10.4 | Strong |
| S4B | *Flavobacterium* sp. | 13.15 | Strong |
| S5A | *Neisseria* sp. | 15.95 | Strong |
| control – | Aquades | 0 | - |
| control + | Ciprofolaxin | 35.65 | Very Strong |

### 3.3 Resistance tests of Probiotic Candidates at different conditions

Based on the resistance test results, all bacterial isolates can grow in different temperature, pH, and salinity conditions ($p<0.01$). The bacterial isolates were able to grow in a wide temperature range of 20-40ºC; wide pH range of 5-10 and wide salinity range from 15 - 35 ppt.

## DISCUSSION

Probiotic is defined as live microorganisms that had positive impact to host, including increasing the growth, enhancing the immune response and modifying the normal flora in intestine. Probiotic candidate in aquaculture can be isolated from fish intestine and environment. Several bacterial genus including *Bacillus*, *Lactobacillus* and *Bifidobacterium* have been known as a popular probiotic bacteria and have beneficial effect to host [6].

*Bacillus* was found in leaf litter and sediment of mangroves from three different sampling locations, indicating that this genus can grow in varied conditions, including extreme conditions. *Bacillus* can produce endospores and exhibit resistance to various environmental changes, including low nutrient content, water, and temperature fluctuations. *Bacillus* is one of the endophytic bacteria that symbiotically colonizes plant tissue; therefore, it can be found in all mangrove vegetation.

The diversity of bacteria species depends on the characteristics of each sampling site. The river area (S5), which was near a shrimp pond and industrial waste, contains a more diverse range of bacteria types. Bacterial abundance can be influenced by several factors, including organic matter and waste. Organic matter in the BMC comes from domestic waste, pond waste, and the decomposition of mangrove leaf litter. The presence of factories and agricultural waste affects to the preferred environment for the growth and development of bacteria. Furthermore, domestic waste from household activities is used as a habitat for microorganisms including bacteria. Organic matter and essential nutrients are needed for metabolism and also affect the growth of microorganism. The high concentration of organic matter in the area affects the abundance of bacteria in the waters [7].

Nutrients in the sediment not only come from litter decomposition but also depend on tidal activity. During high tide, the mass of seawater has a high ionic strength, therefore the process of dissolving nutrients back from the solid phase into dissolved phase occurs. The mixing process between freshwater and sea water causes high turbidity, and bottom sediments become resuspended. This process contributes to the input of nutrients such as phosphate from sediments to the water column and has an impact on the growth of microorganisms [8].

The diversity and abundance of bacteria in leaf litter and sediment samples in mangroves depend on the decomposition process that occurs in the mangrove forest. The decomposition process can produce nutrients that are useful for bacteria. Furthermore, bacteria can mineralize organic matter and convert it into ammonia, nitrite and nitrate. The rate of decomposition contributes to the amount of organic matter that plays a role in the growth and development of plants, macroorganisms (for example fish, shrimp, crabs) and other microorganisms in mangrove forests [9].

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Antibacterial activity tests indicated that all bacterial isolates could inhibit the growth of *V. harveyi* as shown by the clear zone with different abilities (Table 2). The greatest inhibitory activity was shown by *Bacillus* bacteria. *Bacillus* can produce antibacterial substances, including bacteriocins, that have the potential to inhibit the growth of pathogens [10].

Bacteriocin compounds produced by *Bacillus* consist of bacitracin, surfactin, polymyxin, colistin, circulin, and subtilin. These compounds can cause damage to the cell membrane. Damaged cell membranes can cause disruption of metabolic processes and result in the cell death. In addition, damaged cell membranes will interfere with the biosynthesis of essential enzymes involved in cell metabolic processes, so metabolic processes will decrease and affect the death of cells [11]. Bacitracin can cause the inhibition of cell wall synthesis. Moreover, surfactin compounds cause a decrease in membrane surface tension. Colistin compounds cause the disruption of cell wall permeability and increase the risk of plasma membrane leakage, therefore the metabolic process does not proceed normally. Furthermore, circulin has the ability to damage the peptidoglycan component of the cell wall and cause incomplete cell wall synthesis. Polymyxin plays a role in disrupting the ion balance, which causes cell swelling due to the disturbance of ion transport balancing [12].

Based on the resistance test results all bacterial isolates can grow in different temperature, pH, and salinity conditions ($p<0.01$). The bacterial isolates were able to grow in a wide temperature range of 20-40ºC. This indicates that all isolates include mesophile bacteria, which can grow at temperatures between 20-40ºC. *Bacillus* is naturally found in the mangrove environment because of their ability to produce endospores. Endospores are a strategy of defence against environmental stresses such as nutrient deficiencies, high mineral composition, pH, temperature, and high cell density. Endospores are resistant to extreme temperatures due to adaptive resistance mechanisms, including the protection of endospore DNA by small-acid-soluble proteins, accumulation of divalent cations in the endospore core, dehydration of the endospore core, and the presence of dipicolinic acid (DPA) [13]. *Bacillus* will undergo the sporulation process when exposed to extreme conditions such as nutrient deficiencies, excessive mineral composition, and high temperatures. Endospore formation is crucial for several bacteria as the thick endospore structure can function as a heat shield.

The results of the resistance tests against pH indicated that all the isolates were able to grow in a wide pH range of 5-10. This phenomenon showed that the isolates can survive in various acidic and alkaline environmental conditions. Bacteria can survive in low and high pH levels due to pH homeostasis or the ability of microbes to control their internal pH in order to survive. This mechanism is carried out by activating the ATP-ase enzyme, which affects energy production and pumps the protons outside the cell, resulting in an increase in pH in the cell cytoplasm [14].

The salinity experiment revealed that all bacterial isolates were able to grow in a wide salinity range from 15 - 35 ppt, indicating that all bacterial isolates were tolerant of salinity changes. Salinity is closely related to osmotic pressure, which affects bacterial growth. Bacteria can grow in a wide range of environmental conditions by maintaining the osmotic balance of their cells. Bacteria can adapt and survive changes in the environment by accumulating solutes, also known as osmoprotectants [15]. Osmoprotectants can stabilize enzymes, protecting cells from various kinds of stressors such as osmotic stress, high temperature, frezing, and drying. Furthermore, cells will produce small molecules, called osmolytes (consisting of glycine, betaine, choline or proline) to balance the difference in osmolarity between the internal and external cell environments [14].

## CONCLUSION

Bacterial isolate that successfully identified from litter leaf and mangrove of sediment MCB Banyuwangi consist of *Bacillus*, *Aerococcus*, *Enterococcus*, dan *Pseudomonas* from litter leaf. Meanwhile, bacteria genera including *Bacillus, Achromobacter, Actinobacillus, Flavobacterium, Micrococcus,* and *Neisseria* was isolated from sediment with the highest anti-vibriocidal activity was showed in *Bacillus*. All of candidate probiotics were highly tolerance in wide temperature, pH and salinity. Further studies were needed to apply the probiotic bacteria in feed suplement or direct into rearing media to assess the potential effect as probiotic agent.

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