Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(1): 725 – 746 (2025)
www.ejabf.journals.ekb.eg

Contamination of Pathogenic Bacteria in the Vannamei Shrimp *Litopenaeus vannamei* Pond Area along the Coast of Pangandaran, West Java Indonesia

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ARTICLE INFO

Article History:
Received: Sept. 3 2024
Accepted: Dec. 25 2024
Online: Jan. 21, 2025

Keywords:
Disease,
Habitat,
Indication,
Pathogenesis,
Sanitation

ABSTRACT

In Pangandaran Regency, shrimp farming ponds are established along a 91km coastline, but premature harvests often occur due to shrimp diseases. The environmental conditions of shrimp ponds play a critical role in shrimp health and productivity. However, research on bacterial pollution in shrimp farming environments is lacking. This study aimed to identify bacterial species and their abundance in shrimp pond water, seawater, and sediment compared to coastal areas without shrimp farming. Bacterial identification was conducted through a survey across five stations, using NA and TCBS media, staining, and PCR techniques. Results showed that the highest bacterial count on NA in sediment was 2.854×10^7 CFU/g, and the lowest in pond water was 0.658×10^7 CFU/ml. On TCBS media, sediment had the highest concentration (0.1692×10^7 CFU/g), while pond water had the lowest (0.0017×10^7 CFU/ml). The bacterial abundance on NA media was significantly higher than on TCBS. Isolated bacteria included both Gram-negative and positive types, with various shapes (rod, comma, coccus). Specific bacteria identified were *Bacillus flexus* in semi-intensive pond water, *Bacillus albus* in intensive pond water, *Vibrio fluvialis* in semi-intensive pond sediment, *Vibrio alginolyticus* in intensive pond sediment, and *Bacillus* sp. in probiotics used by farmers. In addition, pathogenic bacteria were detected in the shrimp farming ecosystem of Pangandaran.

INTRODUCTION

Aquaculture is one of the most crucial sectors for global food security, with global fish production reaching approximately 179 million tonnes, valued at around USD 250 billion. Notably, around 156 million tonnes are destined for human consumption, with aquaculture providing a supply of 20.5kg per capita. In aquaculture production, crustaceans contribute around 9.4 million tonnes, valued at USD 69.3 billion. This market

has experienced continuous growth since 2010. The main species cultivated in aquaculture are shrimp, freshwater crayfish, crabs, and lobsters **(FAO, 2020)**.

Shrimp, which are Decapoda crustaceans belonging to the superfamily Penaeidae, are a key resource for both commercial fisheries and aquaculture in many countries, accounting for over 30% of global crustacean consumption **(Pérez-Farfante & Kensley, 1997)**. During the 1980s, aquaculture advanced through the intensification of pond numbers and stocking densities of units production supported by advancements in biotechnology that enhanced cultivation practices, improving both efficiency and production quality across all stages **(Flegel, 2019)**.

However, environmental changes have become a major factor in the increased frequency of disease outbreaks, leading to a significant decline in shrimp production **(Defoirdt *et al.*, 2011; Ferreira *et al.*, 2011)**. Unlike terrestrial animals, aquatic organisms are directly exposed to their surrounding water, where bacterial populations in aquaculture systems have a crucial role in nutrient absorption, water quality control, pathogen defense, antibiotic resistance, and maintaining host health **(Blancheton *et al.*, 2013; Carbone & Faggio, 2016)**. Therefore, to achieve a fundamental mechanistic understanding of microbial ecology **(Nemergut *et al.*, 2013)**, it is essential to identify and consider environmental factors within ponds as key to ensure successful production. One of the primary components in shrimp pond management is the quality of water and sediment, which are most influenced by the presence of microorganisms, especially bacteria.

Pangandaran Regency is a newly established autonomous region that has become an important and strategic area in West Java. In several coastal locations along the Pangandaran, which has a coastline of 91km, shrimp farming ponds have been developed. However, research on bacterial contamination in coastal areas of shrimp farming locations has not yet been conducted. Recently, there have been frequent instances of early shrimp harvesting, which may be linked to the tendency of shrimp being affected by diseases. Based on the issues described above, this research was conducted with the aims of identifying the types and abundance of bacteria in pond water, sea water, and sediment in shrimp farming at five research stations, four of which (Stations 1, 2, 3, and 4) are located in areas where vannamei shrimp farming activities take place, while one station (Station 5) is located in an area without any shrimp farming activities.

MATERIALS AND METHODS

1. Research location

Sampling of seawater (input and output), pond water, and sediment was carried out in the conservation area which includes both core and limited utilization zones with five stations, as exhibited in Table (1) and Fig. (1).

Table 1. Detail of sampling locations

Station	Type of Shrimp Farming	Location	Coordinate	Conservation Status
1	Semi-intensive	Kertamukti	7°48'46.23"S, 108°23'1.60"E	Limited use zone
2	Semi-intensive	Legok Jawa	7°49'08.00"S, 108°25'14.20"E	Core conservation zone
3	Intensive	Legok Jawa	7°49'8.50"S, 108°26'05.90"E	Core Conservation Zone
4	Intensive	Madasari	7°48'19.15"S, 108°28'22.98"E	Limited use zone
5	Not a shrimp farm	Bagolo	7°41'1.851"S, 108°44'14.00"E	Limited use zone

In addition, we also took a sample from the probiotics used by the shrimp farmers at station 4.

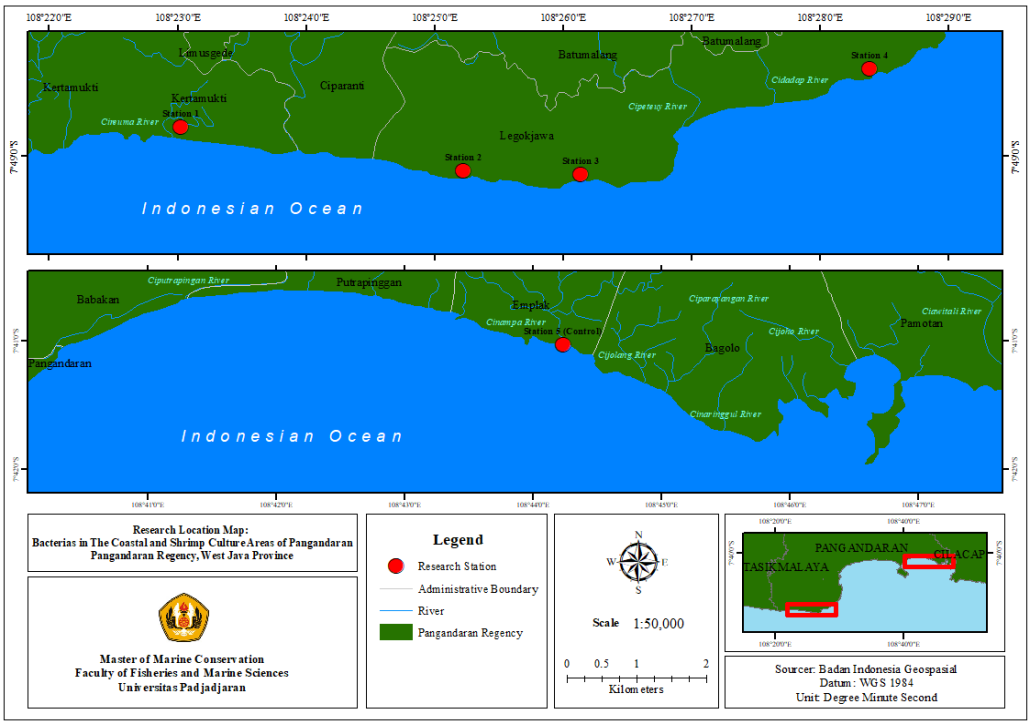


Fig. 1. Research map station

The selection of research locations refers to the presence and absence of shrimp farming activities along the coastal area. Stations 1, 2, 3, and 4 represent coastal areas where vannamei shrimp farming ponds are located, while Station 5 is a control station with no shrimp farming activities (Fig. 1). The procedures for seawater and pond water sampling followed the methods outlined by **APHA *et al.* (1998)**, and sediment sampling was conducted following **Sulaeman *et al.* (2005)**.

2. Tools and materials

The tools and materials used in the dilution of bacterial suspensions are mortar and pestles, test tubes, a test tube rack, volumetric pipettes, vortex, bunsen burner and spiritus, autoclaves, microbial isolates and environmental samples, physiological NaCl, gauze, cotton, and tissues.

The tools and materials used in bacterial isolation sterile petri dishes, sterile Erlenmeyer flasks, inoculating needles, volumetric pipettes, L-Glass, Vortex, incubator, incubator shaker, Nutrient agar (NA) with peptone composition, meat extract, sodium chloride (NaCl) and distilled water to support the growth of various bacteria. Additionally, Thiosulfate Citrate Bile Salt Sucrose (TCBS) media, containing yeast extract, peptone, sucrose, sodium thiosulfate ($Na_2S_2O_3$), sodium citrate ($C_6H_5NaO_7$), sodium cholate ($C_{24}H_{39}NaO_5$), oxgall, NaCl, ferric citrate ($C_6H_5FeO_7$), bromthymol blue ($C_27H_{28}Br_2O_5S$), thymol blue ($C_{27}H_{30}O_5S$), water and Nutrient Broth (NB) and hand counter were also utilized.

3. Procedure

3.1 Bacterial suspension dilution procedure

The first step was sterilizing the required tools and materials. The Physiological NaCl or seawater as then placed into each test tube, with the first tube containing 10mL of Physiological NaCl and the subsequent six tubes each containing 9mL of Physiological NaCl. A 1-gram sample was weighed and mixed using a vortex until a homogeneous suspension was achieved. The microbial isolate was then ground with Physiological NaCl using a mortar before being transferred to test tube I. A 1mL aliquot of the suspension from test tube I was taken using a sterile volumetric pipette and was transferred to test tube II, with a brief vortex applied to ensure homogeneity. This process was repeated sequentially through test tube VII.

3.2 Total plate count (TPC) and bacterial isolation procedures

A total of 20mL of melted Nutrient Agar (NA) medium, preheated on a hot plate, was poured into a sterile Petri dish and allowed to solidify. Once the medium solidified, 500µL of the suspension from Dilution Tube VII was placed onto the solidified agar and evenly spread using an L-glass. The Petri dish was then sealed with plastic wrap and was incubated in an inverted position at 30°C for 24 hours. After incubation, the colonies that grew were observed and counted using a colony counter and hand counter. The number of countable colonies ranged between 30 and 300. The final count was calculated by multiplying the number of colonies counted by the dilution factor.

The formula used to measure bacterial density **(Gleeson *et al.*, 2013; Boor *et al.*, 2017; FSAI, 2019)** was as follows:

$$TPC = \frac{CFU}{sample\ volume\ x\ dilution} \quad \text{.....(1)}$$

Where:
CFU = bacterial colony
Volume = volume of inoculation sample spread on agar medium

The formula (2) used to measure the proportion of Vibrio (%) that can survive on TCBS and NA media:

$$Proportion\ of\ vibrio\ (\%) = \frac{Total\ Bacteria\ in\ TCBS\ Media}{Total\ Bacteria\ in\ NA\ Media}\ x\ 100 \quad \text{.....(2)}$$

Then, isolation and sub-culture of bacterial colonies were performed using the streak plate method in a consistent medium derived from the TPC plates. Morphological characteristics and cell forms of colonies were observed. Gram staining was also performed to observe and distinguish particular colonies during purification of bacterial isolates, based on differences in the composition and structure of cell walls **(Agung *et al.*, 2021)**.

3.3 Gram staining procedure

The bacterial smear was prepared, and 1 drop of Stain I (Gentian Violet) was applied to the smear area, left for 20 seconds, then gently rinsed with distilled water and allowed to sit for 2 seconds. Next, 1 drop of Iodine Solution was applied to the smear and left for 30 seconds to 1 minute. The smear was then rinsed with alcohol until the flowing solution became colorless (approximately 10-20 seconds), gently rinsed with distilled water, and allowed to sit for 2 seconds. 1 drop of Stain II (Safranin) was applied and left for 20 seconds, followed by a gentle rinse with distilled water and a 2-second waiting period. The smear was then dried at room temperature and was observed under a microscope using a 100x objective lens with immersion oil **(Coico, 2006)**.

3.4. Bacterial genomic DNA isolation

The kit used in the isolation procedure was the Wizard® Genomic DNA Purification Kit A1120. DNA isolation involved pelleting 1ml of overnight bacterial culture by centrifugation at 13,000–16,000 × g for 2 minutes, followed by the removal of the supernatant. For Gram-positive bacteria, the pellet was resuspended in 480µl of 50mM EDTA, 120µl of lytic enzyme(s) (e.g., lysozyme or a mix of lysozyme and lysostaphin for specific strains) was added, and it was incubated at 37°C for 30–60 minutes to weaken the cell wall. After centrifugation and removal of the supernatant, 600µl of Nuclei Lysis Solution was added, incubated at 80°C for 5 minutes, and cooled to

room temperature. The lysate was treated with 3µl of RNase Solution, mixed, and incubated at 37°C for 15–60 minutes.

200µl of Protein Precipitation Solution was added to the lysate, vortexed, and incubated on ice for 5 minutes before centrifugation to separate the proteins. The supernatant was transferred to a new tube containing 600µl of isopropanol, mixed gently to form a visible DNA pellet, and centrifuged. The pellet was washed with 70% ethanol, air-dried, and rehydrated in 100µl of DNA Rehydration Solution by incubating at 65°C for 1 hour or overnight at 4°C. The DNA is stored at 2–8°C.

3.5 Amplification of 16S rRNA and gryB genes using polymerase chain reaction (PCR)

Amplification of 16S rRNA and gyrB was performed using PCR following the procedure of **Delidow *et al*. (1993)**. PCR was performed using the primers listed in Table (1).

Table 2. Primers used

Primers		Nucleotide (5′→3′)	Product Target	Ref
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	1460 bp	**Lane (1991)**
	1492R	TACGGYTACCTTGTTACGACTT		
gyrB	VF1	AARCARGGNCGTAACCGTAA	510 bp	**Wang et al. (2024)**
	VR1	HGGGTADCGRCGRCTCAT		

The total volume of reagents used was 35µL, consisting of 17.5µL of Green Taq Master Mix 2x, 11.9µL of nuclease-free water, 1.4µL of forward primer, 1.4µL of reverse primer, and 2.8µL of DNA template. For the P16s primers, the PCR settings included 30 cycles with a pre-denaturation step at 94°C for 90 seconds, followed by denaturation at 95°C for 30 seconds, annealing at 48.5°C for 30 seconds, and extension at 72°C. After the 30 cycles, a final extension was carried out at 72°C for 5 minutes. For the gyrB PCR conditions, a pre-denaturation was performed at 94°C for 4 minutes, followed by denaturation at 94°C for 1 minute, annealing for 1 minute, and extension at 72°C for 1 minute, repeated for 35 cycles. A final extension was conducted at 72°C for 10 minutes, and the reaction was stored at 4°C **(Wang *et al.*, 2024)**.

3.5 Sequencing analysis

The PCR results were subjected to Single Pass DNA Sequencing at 1st Base DNA Sequencing. The obtained sequences were then analyzed using BioEdit and MEGA11: Molecular Evolutionary Genetics Analysis version 11 **(Kumar *et al.*, 2018)**. DNA sequences, with similarity below the threshold of 98.65%, were considered as potential new species. MEGA was used to align the DNA sequences and construct a phylogenetic

tree using the neighbor-joining method for the isolates and related sequences. A total of 1,000 bootstrap replicates were used in the construction of the phylogenetic tree.

RESULTS

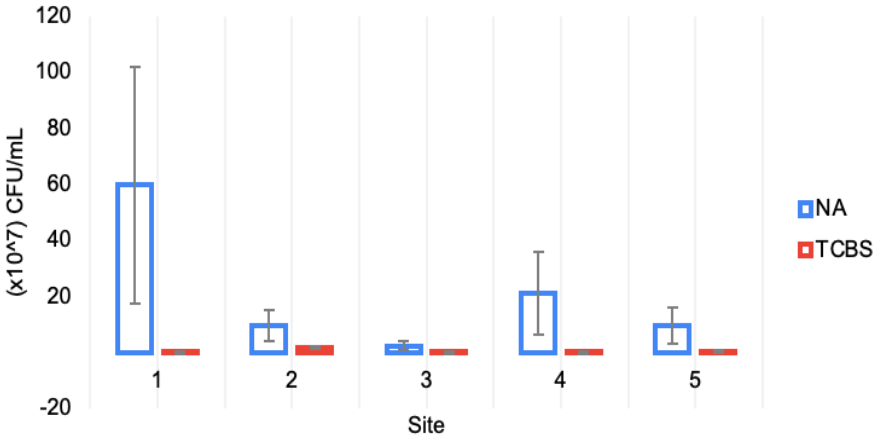
1. Bacterial abundance in seawater, pond water, and pond sediment

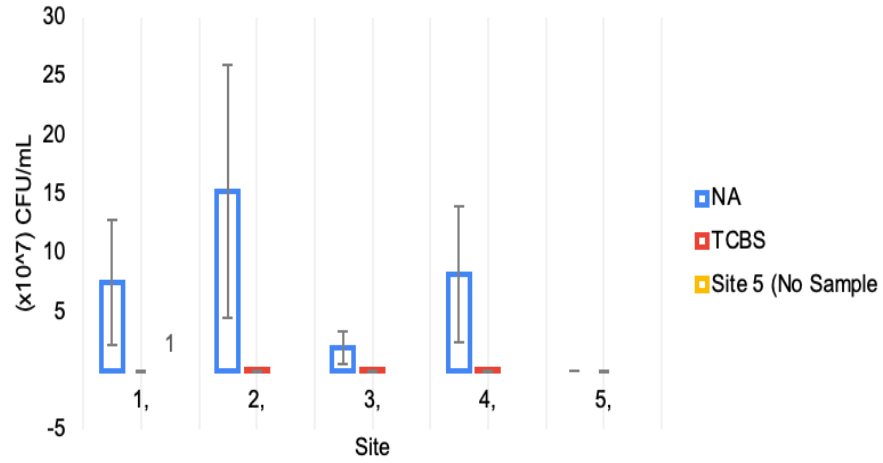
Marine ecosystems cover more than half of the Earth's surface, providing a range of habitats for marine life. Their extensive areas and depths have fostered an extraordinary ecological wealth, including extreme environments with high pressure and no light, as well as stable systems like coral reefs **(Li & Qin, 2005)**. These features create unique, diverse, and large microbial communities, providing ideal breeding grounds for antagonistic interactions among microorganisms **(Peterson *et al.*, 2020)**. Bacteria, which are single-celled prokaryotes, are morphologically "primitive" compared to fungi (eukaryotes). Despite this, they have a vital role in the cycling of organic and inorganic materials in nature, and it has been found that bacteria are the dominant lignocellulose decomposers in aquatic ecosystems **(Benner *et al.*, 1986)**.

Marine bacteria represent a large and diverse group of organisms in terms of taxonomy and metabolism, and are included in the microplankton of oceans and estuaries, where they constitute one of the largest biomasses in the sea **(Sherr, 2000)**. The main species of bacteria found in seawater belong to the genera *Pseudomonas* sp., *Vibrio* sp., *Achromobacter* sp., *Flavobacterium* sp., and *Micrococcus* sp. **(Baharum *et al.*, 2010)**. However, the genus Streptomyces has been a significant source of new molecules to date **(Blunt *et al.*, 2018)**. Marine bacteria are believed to have different physiological, biochemical, and molecular properties distinct from terrestrial bacteria, which may lead to the production of unique compounds **(Siddharth & Vittal, 2018)**. Consequently, marine bacteria are considered promising candidates for discovering new molecules with antibacterial properties, especially since majority of antibacterial drugs are derived from terrestrial actinomycetes **(Butler *et al*., 2013)**.

Vibrio sp., which are Gram-negative rod-shaped bacteria, are found in a various aquatic environments, from rivers and estuaries to the open sea and deep-sea habitats **(Valente & Wan, 2021)**. More than 140 *Vibrio* species have been reported, with 12 species identified as human pathogens and 16 species with the potential to infect aquatic animals including fish, crustaceans, and molluscs **(Beaz-Hidalgo *et al.*, 2010; Cuéllar-Anjel *et al*., 2014; Mohamad *et al.*, 2019)**. These bacteria are also present in the water column and are part of biofilms on submerged surfaces. They can be found in healthy animals, aquaculture systems, and animal tissues, often non-pathogenic as part of the natural microbiota **(Kennedy *et al.*, 2006; Castex *et al*., 2014; Gao *et al*.m 2019)**, and their presence does not necessarily indicate an impending epizootic **(Zheng *et al*., 2017)**.

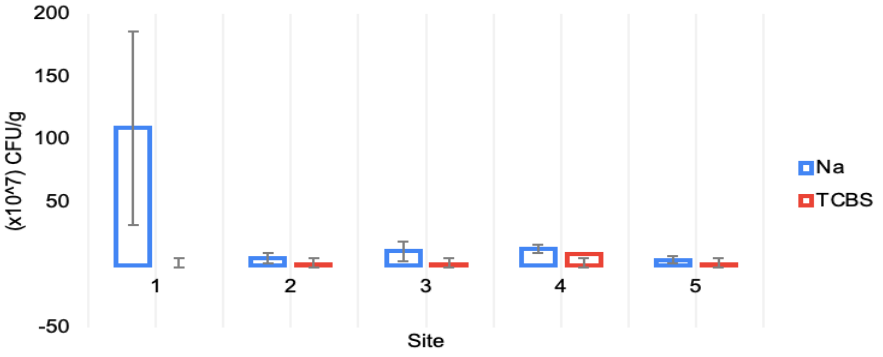
Measurements of bacterial abundance in seawater, pond water, and sediment from five research stations, cultured on nutrient agar for 24 hours with 3, 4, and 5 dilutions, showed variation in bacterial abundance across different stations and sample types, as illustrated in Fig. (2).


120
100
80
60
40
20
-20
(x10^7) CFU/mL
1
2
3
4
5
Site
NA
TCBS


30
25
20
15
10
5
-5
(x10^7) CFU/mL
1
1,
2,
3,
4,
5,
Site
NA
TCBS
Site 5 (No Sample

(a)

(b)


200
150
100
50
-50
(x10^7) CFU/g
1
2
3
4
5
Site
Na
TCBS

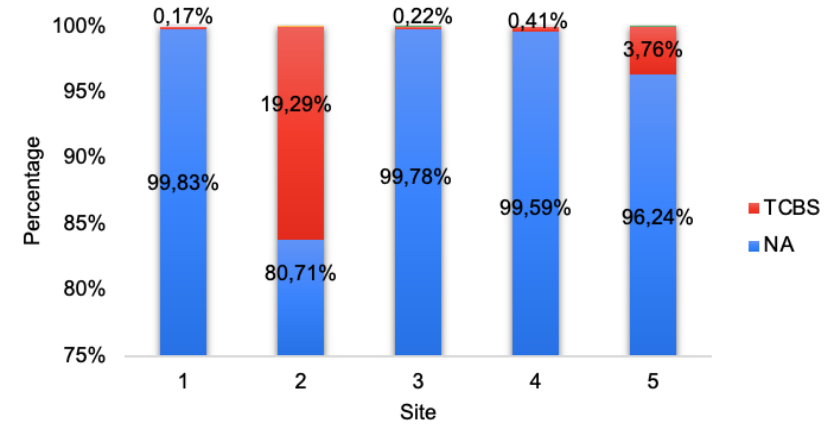
(c)

Fig. 2. Total plate count of bacteria on the Pangandaran coast using NA and TCBS media (a : sea water, b : pond water, c : pond sediment)

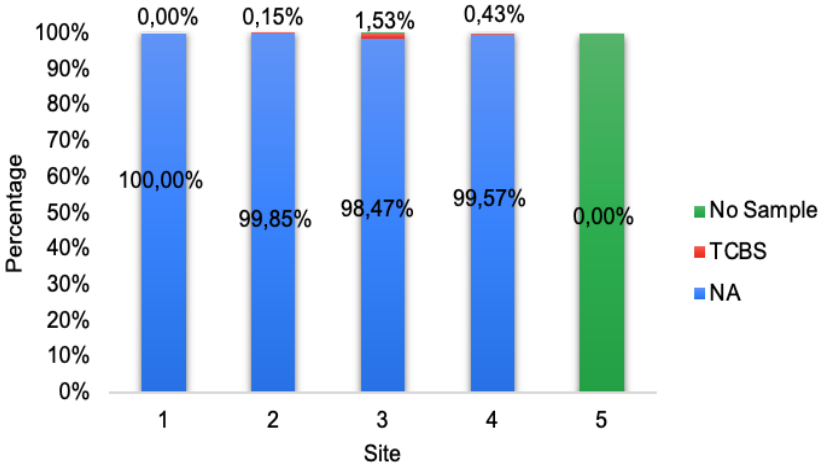
Fig. (2) presents a comparison of the number of bacterial colonies (CFU/mL or CFU/g) on Nutrient Agar (NA) and Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS) from seawater, pond water, and sediment across the five research stations under study. Based on the average NA data obtained from sea water, pond water, and sediment samples, the highest value was found in sediment, with an average of 2.854×10^7 CFU/g. Meanwhile, the lowest value was found in pond water, with an average of 0.658×10^7 CFU/ml Furthermore, based on the analysis of the average data on TCBS media, the highest bacterial concentration was found in sediment, with an average of 0.1692×10^7 CFU/g. In contrast, the lowest bacterial concentration was found in pond water, with an average of 0.0017×10^7 CFU/ml.

2. Proportion of bacterial abundance in NA and TCBS media

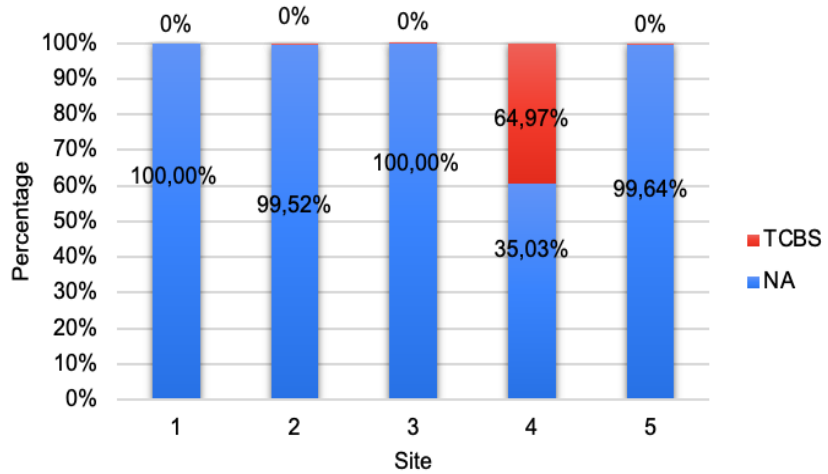
Fig. (3) shows the proportion of bacterial abundance on Nutrient Agar (NA) and Thiosulfate-Citrate-BileSalts-Sucrose Agar (TCBS) media across five stations for samples from seawater, pond water, and sediment.



(a)



(b)



(c)

Fig. 3. Proportion of the number of bacterial growth on NA and TCBS media (a : Sea water; b: pond water; c: sediment)

In seawater samples, the proportion of bacteria growing on NA media was significantly dominant, with the highest proportion at station 1 (99.83%) and the lowest at station 2 (80.71%). On TCBS media, the highest proportion was found at station 2 (19.29%) and the lowest at station 1 (0.17%). In pond water samples, most bacteria grew on NA media, with the highest proportion recorded at station 1 (100%) and the lowest at station 3 (98.47%). On TCBS media, the highest proportion was found at station 4 (0.43%), while no bacterial growth was observed at station 1 (0%). Analysis was not conducted at station 5 due to the absence of shrimp farming activities. In sediment samples, the highest bacterial abundance on NA media was found at Sites 1, 3, and 5, with 100% growth, while at Site 4, the abundance on NA media was 64.97%, the lowest value among all sites. On TCBS media, the highest bacterial abundance was found at Site 4 (35.03%), while no growth was detected at Sites 1, 2, 3, and 5 (0%).

3. Morphological characteristics of bacterial colonies and cells

Molecular identification using selective media and Gram staining of bacteria from five research stations and probiotics revealed both Gram-positive and Gram-negative bacteria. These included bacilli-shaped, rod-shaped, comma-shaped, coccus, and spiral forms, with colony colors varying from white, light green, dark green, yellow, light yellow, orange, pink, to black, as shown in Table (3).

Table 3. Colony morphology and Gram groups of bacteria growing on NA and TCBS media

No.	Sample Code	Morphology		Gram
		Color of Colony	Cell Shape	
1	A.1.N	White	Bacilli, Coccus, Spiral	Positive
2	A.1.T	Green	Comma, Coccus	Negative
3	A.2.N	White	Coccus, Bacilli	Positive
4	A.2.T	Yellow	Comma, Coccus, Spiral	Negative
5	A.3.N	White, Pink	Coccus, Bacilli	Positive
6	A.3.T	Yellow	Spiral, Comma, Coccus	Negative
7	A.4.N	White	Bacilli, Coccus, spiral	Positive
8	A.4.T	Yellow, Light Green, Dark Green	Comma, coccus	Negative
9	A.5.N	White, Yellow, Orange	Coccus, Bacilli	Positive

No.	Sample Code	Morphology		Gram
		Color of Colony	Cell Shape	
10	A.5.T	Yellow	Comma, Spiral, Coccus	Negative
11	B.1.N	White	Coccus, Bacilli	Positive
12	B.1.T	Yellow	Comma, Coccus	Negative
13	B.2.N	White	Coccus, Bacilli	Negative
14	B.2.T	-	-	-
15	B.3.N	White, Pink, Yellow	Bacilli, Coccus	Positive
16	B.3.T	Yellow, Light Green, Orange, Dark Green, Black	Comma, Coccus	Negative
17	B.4.N	White	Bacilli	Positive
18	B.4.T	Yellow	Comma, Coccus	Negative
19	B.5.N	-	-	-
20	B.5.T	-	-	-
21	C.1.N	White	Bacilli, Coccus	Positive
22	C.1.T	Green	Comma, Coccus	Negative
23	C.2.N	White, Yellow, Pink	Bacilli, Coccus, Spiral	Positive
24	C.2.T	Dark Green, Light Green, Yellow, Brown	Spiral, Comma, Coccus	Negative
25	C.3.N	White, Orange, Pink	Bacilli, Coccus, Spiral	Positive
26	C.3.T	Yellow	Coccus, Comma	Negative
27	C.4.N	White	Bacilli, Coccus, Spiral	Positive
28	C.4.T	Yellow	Coccus, Comma	Negative
29	C.5.N	White, Light Yellow	Coccus, Bacilli	Positive
30	C.5.T	Yellow	Spiral, Comma	Negative
31	P.N	White, Pink, Yellow	Bacilli	Positive
32	P.T	Yellow	Bacilli	Negative

Descriptions: A = Sea water, B = Pond water, C = Sediment, P = Probiotics; 1-5 = Stasion; N = NA, T = TCBS

Based on observation results listed in Table (3), molecular analysis was performed on five samples: seawater from station 2 on NA media (A.2.N), pond water from station 3 on NA media (B.3.N), sediment from station 2 on TCBS media (C.2.T), sediment from station 4 on TCBS media (C.4.T), and probiotics on NA media (P.N). These five samples were selected to represent different morphometric characteristics and to assess differences between samples from seawater, pond water, sediment, and probiotics. By focusing on these five similar samples, researchers could explore specific genetic aspects without dealing with excessive diversity from different samples.

4. Electrophoresis and molecular characterization of bacterial isolates

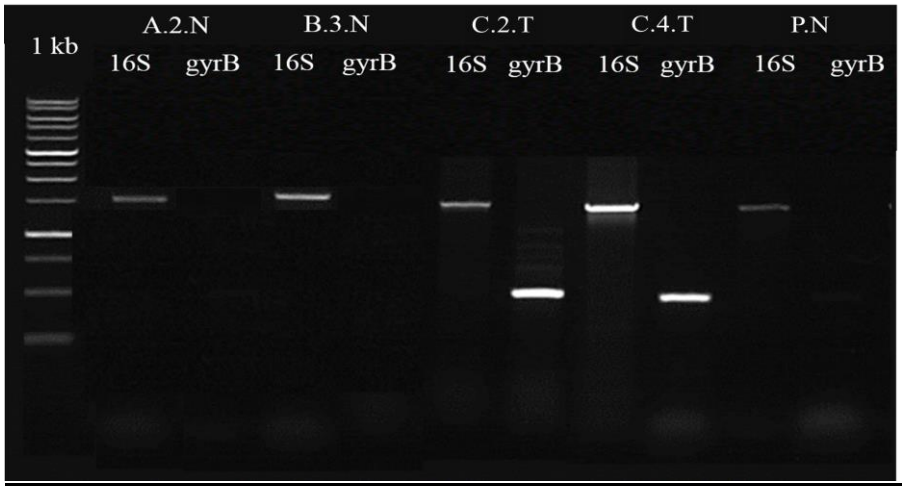


Fig. 4. Electropherogram of 16s and GyrB Gene amplification results from selected isolates. Description: A.2.N = Sea water Station 2 NA media; B.3.N= Pond water station 3 NA media, C.2.T= Sediment station 2 TCBS media, C.4.T= Sediment station 4 TCBS media, P.N= Probiotic from NA media.

Electropherogram in Fig. (4) shows patterns of bacteria on NA media with primer P16s, producing DNA bands of 1440bp for seawater samples at station 2 (A.2.N), pond water at station 3 (B.3.N), and probiotics (P.N). Bacteria appearing with primer gyrB, with DNA bands of 560bp were found in sediment samples from station 1 (C.2.T) and station 4 (C.4.T). The absence of samples A.2.N, B.3.N, and P.N with primer gyrB indicates that these samples did not contain Vibrio genus bacteria. Therefore, to ensure these samples were further analyzed through sequencing stage, samples that appeared in the amplification results and are indicated by the bands visible in electrophoresis were sequenced at 1st Base to determine their genetic relationships with samples in GenBank.

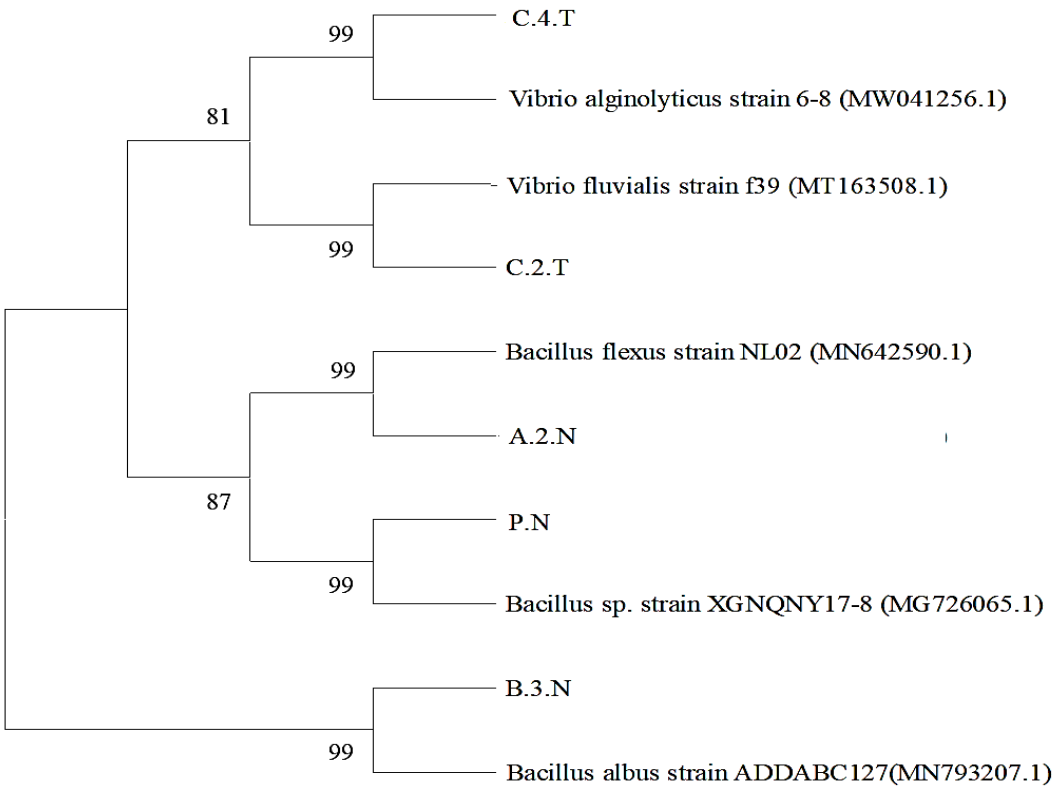


Fig. 5. Phylogeny tree of selected bacterial isolates. Description: A.2.N= Sea water station 1 NA media; B.3.N= Pond water statin 3 media NA media, C.2.T= Sediment station 2 media TCBS, C.4.T= Sediment station 4 TCBS media, P.N= Probiotic from NA media

Based on the sequencing results of the selected samples shown in Fig. (5), the data were analyzed using BioEdit and MEGA 11 software to generate a phylogenetic tree that illustrates the evolutionary relationships among species or samples based on their genetic sequences. The strains displayed in the phylogenetic tree originate from various sources and culture media, each demonstrating close evolutionary relationships supported by bootstrap values.

Samples C.4.T and C.2.T, isolated from sediment at stations 4 and 2 using TCBS media, fall within the Vibrio clade alongside *V. alginolyticus* and *V. fluvialis*. This close relationship, with a bootstrap value of 99, suggests that these samples likely contain Vibrio species from marine environments and organic-rich sediments.

On the other hand, samples A.2.N, B.3.N, and P.N, cultured on NA media, form a group within the Bacillus clade. Sample A.2.N, derived from seawater at station 1, and B.3.N, isolated from pond water at station 3, exhibit a close relationship with *Bacillus flexus*. Meanwhile, sample P.N, obtained from a probiotic culture, shows a strong connection with *Bacillus* sp. XGNQNY17-8. The high bootstrap value (99) within this clade confirms strong confidence in these phylogenetic relationships.

Overall, the grouping reflects consistency between sample origins, culture media, and evolutionary relationships, with sediment samples linked to the genus *Vibrio* and water and probiotic samples associated with the genus *Bacillus*.

Table 4. BLAST results 16s and GyrB Gene sequence matching with NCBI database

Sample Codes	Closest Species Relative	Genbank Accession	Similarity	Genes	
				GyrB	16SrRNA
A.2.N	*Bacillus flexus* strain NL02	MN642590.1	97.29%		✓
B.3.N	*Bacillus albus* strain ADDABC127	MN793207.1	98.52%		✓
C.2.T	*Vibrio fluvialis* strain f39	MT163508.1	92.94%	✓	✓
C.4.T	*Vibrio alginolyticus* strain 6-8	MW041256.1	98.64%	✓	✓
P.N	*Bacillus sp.* strain XGNQNY17-8	MG726065.1	97.52%		✓

Description: A.2.N= Sea water station 2 NA media; B.3.N= Pond water station 3 NA media, C.2.T= Sediment station 2 TCBS media, C.4.T= Sediment station 4 TCBS media, P.N= Probiotic from NA media

DISCUSSION

The research on bacterial identification and abundance in shrimp farming activities provides a valuable foundation for assessing the level of contamination in shrimp farming areas of Pangandaran Coastal Waters. Global aquaculture has grown rapidly over recent decades. With the increasing demand for high-quality seafood products, aquaculture must strive to meet these demands **(Bostock *et al.*, 2010)**. However, producing more aquatic products sustainably, without damaging the environment and depleting natural resources, remains a challenge. Sustainable aquaculture development requires careful attention to the interactions between the environment and aquatic biology **(Burgos-Aceves *et al.*, 2018; Guzzetti *et al.*, 2018; Vajargah *et al.*, 2018)**. Therefore, it is crucial to establish effective microbial ecological strategies to support successful shrimp farming processes and fully understand the characteristics of microflora in aquatic ecosystems **(De Schryver & Vadstein, 2014; Xiong *et al.*, 2016)**.

The results indicate that the highest average bacterial count on NA was found in sediment 2.854×10^7 CFU/ml, while the lowest was in pond water of 0.658×10^7 CFU/ml. On TCBS media, sediment also had the highest average bacterial concentration 0.1692×10^7 CFU/g, with the lowest in pond water 0.0017×10^7 CFU/ml. This indicates that sediment generally has higher contamination levels compared to pond water and seawater in most locations. This is due to several factors, such as organic waste from uneaten shrimp feed, shrimp feces, and plant residues, which serve as nutrient sources for

bacteria **(Mirzoyan *et al.*, 2010)**. The accumulation of this waste in the sediment promotes bacterial growth including pathogenic bacteria. The high accumulation of organic matter in the sediment leads to a decrease in dissolved oxygen due to increased microbial decomposition and aquatic respiration **(Junior *et al.*, 2021)**, which affects habitat availability for cultured animals.

The use of fertilizers and medications (such as antibiotics) in shrimp farming can lead to bacterial resistance and an increase in pathogenic bacterial populations. When these medications accumulate in the sediment, they can disrupt microbial balance. Antibiotics are also known to persist in pond sediments because they degrade more slowly once adsorbed **(Thuy *et al.*, 2011)**. Therefore, shrimp pond sediments may have higher levels of antibiotic-resistant bacteria compared to pond water, and indeed, sediments have been shown to contain higher levels of antibiotics and antibiotic-resistant bacteria than pond water **(Le & Munekage, 2004; Le *et al.*, 2005)**.

The abundance of bacteria in the sediment on TCBS media in the intensive shrimp ponds of Madasari Village (Station 4) was recorded to be high, reaching 64.97% (Fig. 3c). In addition, the pathogenic bacteria *V. alginolyticus* was identified (Table 3), a species known to cause vibriosis in shrimp. This indicates a potential risk to shrimp health in the area, but this does not negatively affect the farming activities. This indicates that the intensive farming system in Pangandaran is able to manage this condition effectively. Based on communication with the farmers at Station 4, one key factor in reducing the negative impact of pathogenic bacteria such as *Vibrio* is the use of probiotics. In the shrimp ponds at this station, farmers have utilized probiotics to prevent diseases in the shrimp, which helps reduce the risk of pathogenic bacterial infections. These probiotics help maintain the balance of microflora in the ponds and support shrimp health, so despite the high bacterial abundance in TCBS media, the use of probiotics keeps the shrimp healthy and does not affect farming outcomes. The identified probiotic species is *Bacillus*.

Bacillus species have been widely utilized as probiotics, both for humans and in the aquaculture sector. These bacteria are naturally found in soil and water, with the ability to adhere and survive in the shrimp digestive tract, which contains acid and bile salts **(Kesarcodi *et al.*, 2008)**. As Gram-positive spore-forming bacteria, *Bacillus* can form spore layers within vegetative cells, enabling them to remain dormant for extended periods. Additionally, *Bacillus* produces various extracellular compounds, such as trypsin, lipase, amylase, and antimicrobial peptides, which are effective against pathogens **(Yilmaz *et al.*, 2006; Wang, 2007)**.

Several studies have demonstrated the benefits of *Bacillus* as probiotics in improving growth, enhancing immune systems, and aiding disease management in shrimp farming **(Arisa, 2015)**. **Balcázar and Rojas-Luna (2007)** revealed that *Bacillus* produces antimicrobial compounds effective against several pathogenic *Vibrio* species, including *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, and *Vibrio harveyi*. Moreover,

Irianto (2007) explained that probiotics can secrete digestive enzymes, such as amylase and protease, which enhance feed digestion efficiency. This finding is supported by **Macey and Coyne (2005)**, who reported that probiotics increase protease enzyme activity in the gut, thereby facilitating digestion and nutrient absorption. Therefore, the application of *Bacillus* in shrimp farming in Pangandaran can be an effective strategy in controlling the population of pathogenic *Vibrio* bacteria, strengthening the immune system, and improving shrimp health, ultimately supporting the success of shrimp production even in environments with high bacterial loads.

The abundance of *Vibrio* bacteria in the seawater at Karapyak Beach (Station 5) is higher compared to other stations, reaching 3.76% (Fig. 3a), but it does not negatively affect the shrimp. This is due to the fact that the marine ecosystem is a natural habitat for *Vibrio*, which is commonly found in such environments. Additionally, the presence of *Vibrio* in the seawater is balanced with the natural microbial community, which helps maintain ecosystem stability and prevent adverse effects on shrimp production. The study by **Sha *et al.* (2016)** also indicates that wild-caught tiger prawns from the ocean exhibit better immune responses to infections. This strong immune response is linked to the influence of their natural environment, where exposure to natural microorganisms in their habitat leads to the development of more effective immunity against pathogens like *Vibrio*.

Bacteria are primarily categorized based on their morphological characteristics (shape, presence or absence of flagella, and flagellar arrangement), substrate utilization, and Gram staining. Another important characteristic is their growth patterns on solid media, as different species can produce highly varied colony structures **(Christopher & Bruno, 2003)**. In this research, bacteria identified using Gram staining in seawater, pond water, and sediment samples were dominated by Gram-negative bacteria. Microorganisms that live in shrimp farming areas on the Pangandaran coast are influenced by nutrient availability. This is evident from the high growth of bacteria cultured on NA media and the relatively lower growth on TCBS media, as presented in Figs. (2, 3). According to **Pelczar and Chan (2008)**, all organisms, including bacteria, require nutrients for growth, whereas TCBS is more selective for certain bacteria, such as *Vibrio* sp.

Based on the alignment results of the samples with data from GenBank, as shown in Table 3, the bacterial species identified in seawater at station 2 (A.2.N) was *Bacillus flexus* strain NL02. *Bacillus flexus* is found in various habitats, including soil, water, and organic-rich environments. It is known for its ability to degrade organic material and is often found in contaminated or waste-accumulating environments. *B. flexus* has been shown to have significant antibacterial activity and the ability to detoxify saxitoxin, a toxin harmful to humans and animals **(Ikhwani *et al.*, 2003)**. The presence of these bacteria can provide important information that represents seawater samples at other stations. This information can be used for ecological conditions and environmental health

in seawater as an antibacterial against potential risks to human health if exposed to pathogenic microorganisms.

In the pond water at station 3 (B.3.N), a microorganism related to *Bacillus albus* strain ADDABC127 was identified (Table 3). *Bacillus albus* is a Gram-positive, rod-shaped bacterium commonly found in soil and water; it produces robust spores and can efficiently grow using inexpensive carbon and nitrogen sources **(Kewcharoen & Srisapoome, 2019)**. This bacterium is known to have probiotic properties that can benefit the health of the pond ecosystem by playing a role in the decomposition of organic matter and the nutrient cycle. As explained in the study by **Kim *et al.* (2021)**, probiotics in shrimp farming are generally used to improve water quality by decomposing organic matter and nitrogen compounds, such as ammonia and nitrite, which can be toxic to shrimp. *Bacillus albus* was found to be effective in removing ammonia and nitrite from the water, improving overall water quality, and potentially reducing harmful algae blooms caused by excess phosphorus. Additionally, the study of **Kim *et al.* (2021)** highlights that microorganisms like *B. albus* can help reduce stress in shrimp, improve their health, and enhance their growth performance by creating a better farming environment.

In the sediment samples, *Vibrio* species were identified. At station 2 (C.2.T), bacteria from the genus *Vibrio* were identified, specifically *Vibrio fluvialis* strain F39 (Table 3). *Vibrio fluvialis* is a pathogen commonly associated with coastal environments. In large numbers, these bacteria can cause disease outbreaks, such as diarrhea and sporadic extraintestinal infections, making *V. fluvialis* a pathogenic bacterium. According to the analysis by **Ramamurthy *et al.* (2014)**, this bacterium shows close phenotypic similarities to both *Vibrio cholerae* and *Aeromonas* spp. However, molecular tools make it easy to differentiate *V. fluvialis* from other clinical and environmental samples. *V. fluvialis* has also been identified as a pathogen in lobsters **(Tall *et al.*, 2003)**.

At station 4 (C.4.T), sediment samples revealed the presence of *Vibrio* species, specifically *Vibrio alginolyticus* strain 6-8 (Table 3). This bacterium is considered part of the normal marine flora **(Carli *et al.*, 1993)**. *Vibrio alginolyticus* is a major cause of vibriosis in fish and shrimp. This infection can lead to clinical symptoms such as skin lesions, swelling, and sudden death in infected animals. It can also infect humans, causing ear infections, skin infections, and food poisoning from contaminated seafood, particularly in individuals with weakened immune systems. In a study by **Wang *et al.* (2016)**, *V. alginolyticus* infection in shrimp larvae resulted in a significant decrease in survival rates compared to controls.

On the other hand, the probiotic samples were associated with *Bacillus* sp. strain XGNQNY17-8 (Table 3), a Gram-positive, chemoheterotrophic, rod-shaped bacterium that is endospore-forming and typically moves with peritrichous flagella. It is aerobic or facultative anaerobic and catalase-positive **(Waites *et al.*, 2008)**. Members of the *Bacillus* genus are commonly found in soil and exhibit a wide range of physiological capabilities,

allowing them to thrive in diverse environments due to their ability to form highly resistant spores and to produce metabolites with antagonistic effects on other microorganisms **(Kuta *et al.*, 2008)**. According to the study of **Amin *et al.* (2015)** on the isolation and identification of bacteria from soil, *Bacillus* species have been used as antibiotics. These bacteria can synthesize various metabolites with antimicrobial activity, which have been widely utilized in medicine and the pharmaceutical industry. One of their notable capabilities is controlling diseases in animals, humans, and plants when applied as biological control agents.

CONCLUSION

The results of the research on bacterial identification and abundance in Pangandaran Coastal Waters related to shrimp farming are summarized as follows:

1. Based on the study, the bacteria identified in seawater used for irrigating semi-intensive shrimp ponds in Legok Jawa Village were *Bacillus flexus* strain NL02. Water from intensive shrimp ponds in Legok Jawa Village contained *Bacillus albus* strain ADDABC127. Sediment from semi-intensive shrimp ponds in Legok Jawa was found to contain *Vibrio fluvialis* strain F39, while sediment from intensive shrimp ponds in Madasari Village contained *Vibrio alginolyticus* strain 6-8. The probiotic sample commonly used by farmers to maintain water quality was identified as *Bacillus* sp. strain XGNQNY17-8.
2. Based on the identification of bacterial species and their abundance, shrimp farmers in Pangandaran need to be more vigilant about bacterial contamination levels in the pond environment, particularly in the sediment, which showed a higher bacterial count compared to pond water or seawater. High concentrations of pathogenic bacteria, such as *Vibrio alginolyticus*, identified in the sediment and known to cause vibriosis in shrimp, pose a threat to shrimp health and the sustainability of shrimp farming. To mitigate this risk, farmers are advised to routinely perform proper pond maintenance, including the use of probiotics that have been proven effective in controlling pathogenic bacterial populations.

ACKNOWLEDGMENT

This research is supported by Universitas Padjadjaran (Universitas Padjadjaran Lecturer Competency Research) Funded Research Project 2024 with contract number : 1821/UN6.3.1/PT.00/2024, under the Chairperson of Dr. Titin Herawati., and also providing financial assistance for Article Publishing Charge (APC). Thanks to shrimp farmers on the coast of Pangandaran who allowed for the sampling of water, sediment and shrimp, as well as to the researchers for their togetherness in the field and laboratory.

Thanks to all the committee members of the International Seminar of Indonesian Seas: Catalyst for Ocean Sustainability (ISCO) 2024, who facilitated the publication

process of this manuscript until being published in the Egyptian Journal of Aquatic Biology and Fisheries.

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