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Identification and antibiotic-resistant properties of *Vibrio owensii* and *V. alginolyticus* isolated from the Spermonde Islands, Indonesia

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Abstract. *Isnansetyo A, Istiqomah I, Anshary H, Sriwulan S, Yudiati E, Subagiyo S, Arif A, Kartikasari DW. 2022. Identification and antibiotic-resistant properties of* Vibrio owensii *and* V. alginolyticus *isolated from the Spermonde Islands, Indonesia. Biodiversitas 23: 5995-6005.* The Spermonde Islands are located in Makassar Strait, southern Sulawesi, Indonesia, and have attracted the attention of researchers for decades because of their mega biodiversity and abundance of bioresources. However, no study has evaluated the potential mariculture diseases in this area. The present study assessed the potential bacterial fish diseases based on the current status of mariculture in the Spermonde Islands. The samples were collected from three marine aquaculture sites at Barrang Caddi and the Samaloa Islands. *Vibrio* was isolated on Thiosulfate-citrate-bile-sucrose (TCBS) agar medium. The most common bacterial diseases were observed by bacterial isolation, morphological and biochemical observations, and molecular identification based on 16S rDNA sequence. The microbial, physical, and chemical water qualities at Barrang Caddi and Samaloa Island were suitable for marine aquaculture. Although cultured species in this area did not exhibit gross disease signs, potential opportunistic pathogens of *Vibrio* were found. The 16S rDNA sequences analysis indicated that the bacterial isolates closed to *Vibrio owensii*, *V. alginolyticus*, and *V. neocaledonicus*. Multi-drug resistant *V. owensii* was identified as a potential pathogen in marine aquaculture. This is the first report on *V. owensii* isolated from Indonesian marine waters, particularly from the Spermonde Islands. *V. alginolyticus* was another potential opportunistic pathogen in this area. These results gave the alarm to develop countermeasure methods for potential diseases to minimize the possible outbreak of vibriosis.

Keywords: 16S rDNA, anti-microbial, marine aquaculture, molecular identification, opportunistic pathogen

INTRODUCTION

Mariculture is an aquaculture sector that significantly contributes to world seafood production. The Food and Agriculture Organization has stated that the contribution of marine aquaculture in 2000 reached 14% of total aquaculture production, and increased steadily to 37% in 2016 (FAO 2018). In 2018 marine aquaculture production reached 63,071,939 tons, while inland aquaculture has reached 51,409,304 tons (FAO 2020), indicating that marine aquaculture has contributed 55.1% of total aquaculture production in 2 years. This contribution must increase considering that the world's seafood demand is increasing, whereas production from wild-catch fisheries is decreasing.

The Spermonde Islands in Indonesia are being developed into an aquaculture center and a tourist spot, and are expected to provide a balanced contribution of aquaculture and tourism. The potential areas on the Spermonde Islands are 29.39 ha for coastal tourism, 742.47 ha for marine tourism, 2,438.27 ha for floating net cages, and 136.98 ha for seaweed culture (Kasnir 2018). The Spermonde Islands has been widely studied by Indonesian and world researchers because of their abundant natural resource and mega biodiversity. Several researchers have stated that this archipelagic area is vulnerable to climate change (Yusuf et al. 2012) and anthropogenic impacts (Kench et al. 2017), which greatly affect the susceptibility of cultured marine species to diseases and the sustainability of marine aquaculture.

Vibriosis is the main disease in marine aquaculture worldwide. Istiqomah et al. (2020) have reviewed the pathogenic vibrio that infects marine fish and shrimp in Indonesia. There are 14 reported pathogenic Vibrio species in Indonesia namely *Vibrio anguillarum*, *V. alginoluticus*, *V. azureus*, *V. cincinnatiensis*, *V. carchariae*, *V. damsela*, *V. fluvialis*, *V. furnisii*, *V. harveyi*, *V. methcnikovii*, *V. mimicus*, *V. ordalii*, *V. parahaemolyticus*, and *V. vulnificus*. However, reports on *Vibrio* species isolated from the Spermonde Islands are limited available. Moreover, *V. owensii* isolated from Indonesia has not been reported so far.

Genus *Vibrio* is Gram-negative bacteria, short rod or curve, fermentative, and motile by polar flagella. *Vibrio harveyi*, *V. parahaemolyticus*, *V. anguillarum*, and *V. alginolyticus* are frequently identified as the causative agent of vibriosis in marine aquaculture. In addition, *Vibrio*

owensii was proposed as a new species in 2010. This bacterium is an important pathogenic *Vibrio* that causes significant diseases in mariculture species, such as amberjack *Seriola dumerili* (Nishiki et al. 2018), spiny lobster *Panulirus ornatus*, giant tiger shrimp *Penaeus monodon* (Cano-Gómez et al. 2010), and *Litopenaeus vannamei* (Liu et al. 2018). This bacterium has also been isolated from the coral *Montipora capitate*, which causes *Montipora* white syndrome (Ushijima et al. 2012), and from green algae (Lin et al. 2015). The previous reports indicate that *V. owensii* is a potential pathogenic *Vibrio* in marine aquaculture and the marine ecosystem. *V. alginolyticus* is well known pathogenic bacterium that infects almost all marine aquaculture species worldwide (Ina-Salwany et al. 2019). Istiqomah et al. (2020) have reviewed *V. alginolyticus* as the causative agents of vibriosis in Indonesian marine aquaculture, including in the species of tiger shrimp (*Penaeus monodon*), humpback grouper (*Cromileptes altivelis*), tiger grouper (*Ephinepelus fuscoguttatus*), hybrid grouper cantang (*E. lanceolatus* × *E. fuscoguttatus*), and seabass (*Lates calcarifer*). Recently, an infection of *V. alginolyticus* concomitant with *Amyloodinium ocellatum* caused mass mortality of European seabass (*Dicentrarchus labrax*) fry in the hatchery. The gross signs of infected fry are asphyxia, ascites, darkening, lethargy, and velvety skin appearance (Ragab et al. 2022). *V. alginolyticus* and *V. owensii* are also reported to be resistant to antibiotics.

Vibrio alginolyticus is the second most common bacterial species in marine aquaculture with a high resistance rate. *V. alginolyticus* isolated from the aquatic environment of marine aquaculture area in China indicates the resistance rates of the bacterium are 1.0-100 %. This bacterial species is highly resistant to ampicillin, cephalexin and neomycin (Yu et al. 2022). Yang et al. (2022) have reported that *V. alginolyticus* isolated from Pacific Oyster (*Crassostrea gigas*) is resistant to 17 antibiotics from a total of 30 antibiotics tested. The above reports indicate that *V. alginolyticus* is a pathogenic bacterium with a high rate of resistant, and should be paid attention to minimize the negative impact on marine aquaculture, public health and the environment. *V. owensii* is also a pathogenic in marine aquaculture with a high rate of resistance to antibiotics. This Vibrio species is resistant to ampicillin, erythromycin, kanamycin, ofloxacin, rifampicin, methicillin, tetracycline and vancomycin (Thillaichidambaram et al. 2022). Liu (2021) reported that *V. owensii* isolated from *Fenneropenaeus chinensis* is resistant to 18 antibiotics among the 30 antibiotics tested. Antibiotic resistance of *V. alginolyticus* isolated from Indonesian waters is rarely reported, and there are not even reports on antibiotic resistance of *V. owensii* isolated from Indonesian waters.

The report on potential causative agents of diseases in marine aquaculture species from the Spermonde Islands is limited availability. An early assessment will be valuable to propose a fish health management strategy to minimize the risk of disease outbreaks in the future. Therefore, the purposes of the present study were to determine the possibility of bacterial diseases in marine aquaculture species in the Spermonde Islands by isolating and identifying the bacterial isolates based on morphological, biochemical, molecular characters, and antibiotic resistance property.

MATERIALS AND METHODS

Sampling sites

Sampling was carried out at Barrang Caddi Island (S 05°04.868'; E 119°19.306'; and S 05°04.670', E 119°19.200') and Samalona Island (S 05°07.332 '; E 119°20.484') (Figure 1).

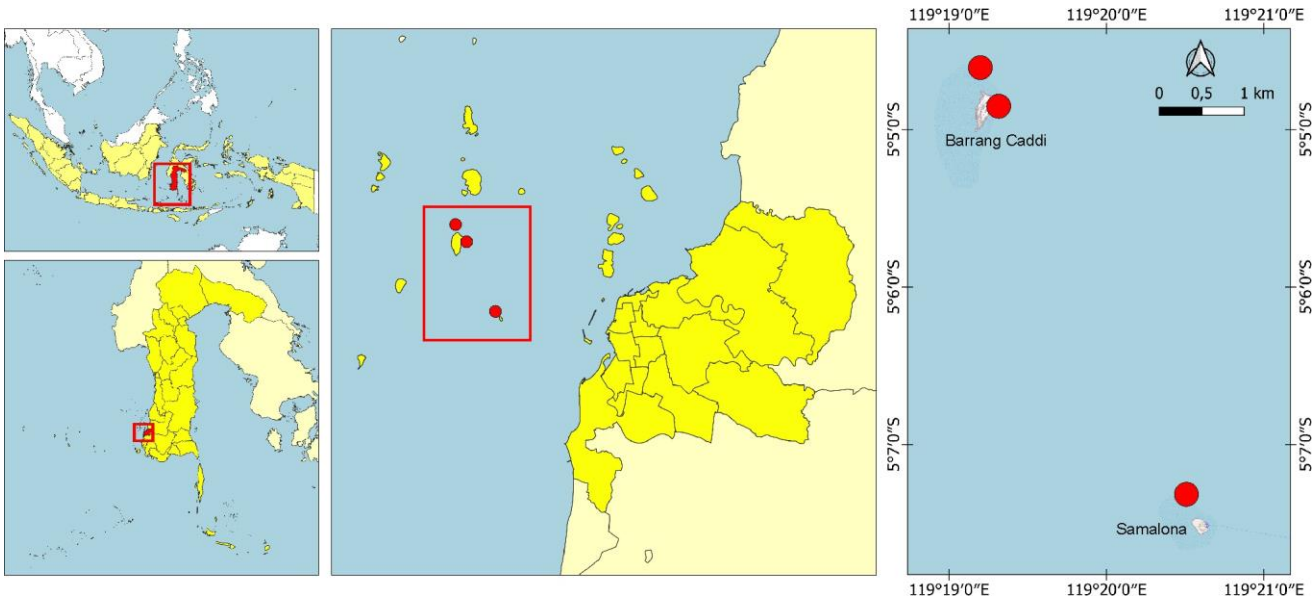

Figure 1. Map of study site in the Spermonde Islands, Makassar Strait, southern Sulawesi, Indonesia. The latitude and longitude were drawn using GPS Coordinate Web (https://www.gps-coordinates.net/)

Bacterial isolation

The isolation of potentially pathogenic bacteria was carried out on-site using fish reared at the sampling site, such as red snapper (*Lutjanus bitaeniatus*) (n = 3), grouper (*Epinephelus fuscoguttatus*) (n = 8), *Loligo duvauceli* (n = 2), *Loligo octopus* (n = 3), cuttlefish (*Sepia officialis*) (n = 5), and milkfish (*Chanos chanos*) (n = 5) that were reared in floating net cages. They were reared in floating net cages. Bacteria were isolated on site from the internal organs and wounds when found on the body surface of the sample. Bacteria were isolated by selective media, including glutamate starch phenol red agar (GSP, Merck, Germany) for *Pseudomonas*, thiosulfate-citrate-bile salts-sucrose agar (TCBSA, Merck, Germany) for *Vibrio*, and KF streptococcal agar (KF, HiMedia Laboratories, India) for *Streptococcus*. *Vibrio* and *Pseudomonas* were isolated from the kidneys, and *Streptococcus* was isolated from the eyes and brain. Incubation was carried out at 30°C for 24 h for the GSP and TCBS agar plates and 48 h for the KF *Streptococcus* plate.

Phenotypic characterization of the bacteria

Bacteria were identified based on phenotypic observations and molecular analysis. Cell morphology, as well as fermentative-oxidative and oxidase tests, were carried out based on the standard procedures of Smibert and Krieg (1994). The other biochemical properties were tested with the HiVibrio Identification KIT (HiMedia Laboratories, India).

Antibiotic susceptibility testing

An antibiotic susceptibility test was conducted based on the 96-well microplate microdilution method (NCCLS, 1994) in Mueller-Hinton broth medium at 30°C for 24 h. Antibiotic susceptibility was tested in triplicate to six antibiotics, namely oxytetracycline, enrofloxacin erythromycin, chloramphenicol (Sigma, USA), ampicillin, and kanamycin (Wako Pure Chemical Industries, Japan). The antibiotics concentrations were in the ranges of 0.45 to 40 µg mL^{-1}.

Molecular identification of the bacteria based on the 16S rDNA sequence

Molecular identification was conducted based on the 16S rDNA sequence. Total bacterial DNA was isolated from bacterial cultures in Zobell broth medium at 30°C for 24 h. One mL of bacterial cell culture was transferred to a sterile microtube and centrifuged at 13,000 × *g* for 2 min. Genomic DNA was isolated using a Genomic DNA Mini Kit (Promega, USA) according to the manufacturer's protocol.

The bacterial genomic DNA was amplified by targeting the 16S rDNA using a Thermal cycler (BioRad Laboratories, USA). The reaction mixture consisted of 24 µL of Kappa Mix (Merck), 20 µL of ddH_2O, 2 µL of the 27F primer, 2 µL of the 1492R primer, and 2 µL of DNA template. The forward and reverse primers used in this study were from Isnansetyo et al. (2009). Amplification was carried out by pre-denaturation (95°C for 3 min), denaturation (95°C for 30 sec), annealing (55°C for 30 sec), and elongation (72°C for 1 min 30 sec) for 30 cycles. The final extension was carried out at 72°C for 5 min. The amplified 16S rDNA was detected by 1% agarose gel electrophoresis (Invitrogen, Carlsbad, CA, USA), and sequenced using First Base services (PT Genetika Sains, Indonesia).

Analysis of the 16S rDNA sequences

The 16S rDNA sequences were analyzed using the BLAST (Basic Local Alignment Search Tools, http://www.ncbi.nlm.nih.gov/BLAST) algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the identity of the sequence to the sequences in the database. The representative rDNA sequences were aligned with the Clustal W program (http://clustalw.ddbj.nig.ac.jp/index.php?lang=en) to construct a phylogenetic tree using the neighbor-joining method with 1,000 resamplings. TreeView software was used to construct the phylogenetic tree.

Water quality testing

Water quality tests were carried out directly in the floating net cages for dissolved oxygen and temperature using a portable dissolved oxygen meter (Az-8403, AZ Instrument Corp, Taiwan). pH was determined with a pH meter (Hanna Hi98107, Singapore), and water salinity was determined with a refractometer (Atago, Singapore). NH_4-N was measured by the alkali phenol-hypochlorite reaction and detected spectrophotometrically at 630 nm (Isnansetyo et al. 2014). The bacterial count in the water was enumerated on marine agar medium (HiMedia Laboratories) for the total bacterial count and on TCBS agar medium for total *Vibrio* spp.

RESULTS AND DISCUSSION

We isolated the BC SIPUT A, S5 USUS A, S5 USUS C, SMI 1 A, BCSP1C, and S4 USUS A bacterial strains as normal microflora of marine organisms. The results of morphological characterization showed that the isolated bacteria were Gram-negative rods and oxidase-positive. The strains were biochemically tested except S5 USUS C because this strain was very closely associated with S5 USUS A based on the 16S rDNA sequence. The β-galactosidase activities and the utilization of citrate and mannitol varied among the strains (Table 1). Only strain BC SIPUT A was β-galactosidase positive. The antibiotic susceptibility test indicated that the bacterial strains were resistant to oxytetracycline, ampicillin, erythromycin, kanamycin, and enrofloxacin with minimum inhibitory concentrations (MICs) > 40 µg mL^{-1}. Bacterial strains BC SIPUT A, BCSP1C, and S4 USUS A were sensitive to chloramphenicol with MICs ranging from 0.95 to 3.75 µg mL^{-1} (Table 2). Strains SIM 1A and S5 USUS A were intermediately resistant and resistant to chloramphenicol with MICs of 10 and 20 µg mL^{-1}, respectively. Furthermore, these bacterial isolates were molecularly identified based on the 16S rDNA sequence.

Table 1. Morphological, biochemical, and antibiotic susceptibility properties of the bacterial strains isolated from the Spermonde Islands, southern Sulawesi, Indonesia

Properties	Bacterial strains				
	***V. owensii* (BC SIPUT A)**	***V. alginolyticus* (S5 USUS A)**	***V. neocaledonicus* SMI 1A**	***V. neocaledonicus* BCSP1C**	***V. neocaledonicus* S4 USUS A**
Cell morphology	Gram-Negative, curved-rod	Gram-Negative, curved-rod	Gram-Negative, curved-rod	Gram-Negative, curved-rod	Gram-Negative, curved-rod
Oxidase	+	+	+	+	+
Fermentative/oxidative	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Voges Proskauer	-	-	-	-	-
β-galactosidase	+	-	-	-	-
Utilization of					
Arginine	+	+	+	+	+
Citrate	+	-	+	+	-
Ornithine	+	+	+	+	+
Mannitol	-	-	+	-	-
Arabinose	-	-	-	-	-
Sucrose	+	+	+	+	+
Glucose	+	+	+	+	+
Salicin	-	-	-	-	-
Cellobiose	-	-	-	-	-
Antibiotic susceptibility (Minimum Inhibitory Concentration; μg mL^{-1})					
Oxytetracycline	>40	>40	>40	>40	>40
Chloramphenicol	1.25	20	10	3.75	0.95
Ampicillin	>40	>40	>40	>40	>40
Erythromycin	>40	>40	>40	>40	>40
Kanamycin	>40	>40	>40	>40	>40
Enrofloxacin	>40	>40	>40	>40	>40

Note: The antibiotics concentrations ranged from 0.45 to 40 μg mL^{-1}. The MIC test was conducted in triplicate. >40, bacteria were resistant to the tested antibiotics at 40 μg mL^{-1}. +, the bacteria gave the positive reaction (The bacteria produced enzyme for enzyme production test and were able to utilize carbon sources for carbon sources utilization test. –, the bacteria gave the negative reaction (The bacteria did not produce enzyme for enzyme production test and were not able to utilize the carbon sources for carbon sources utilization test

Analysis of the 16S rDNA sequences and construction of the phylogenetic tree

The results of the BLAST analysis showed that the bacterial strains were closely related to *Vibrio* and were generally in the *V. harveyi* clade (Table 2-4). BC SIPUT A was closely related to *V. owensii* CAIM 1854 (Accession number NR_117424.1) with a sequence identity of 99.72%. Strains S5 USUS A and S5 USUS C were closely related to *V. alginolyticus* NBRC 15630 (Accession number NR_122050.1) with sequence identities of 99.58 and 99.45%, respectively. Similarly, *V. alginolyticus* NBRC 15630 was closely related to strains SMI 1 A, BCSP1C, and S4 USUS A with sequence identities of 99.51, 99.51, and 99.86% respectively.

The results of the phylogenetic tree based on the neighbor-joining method placed strain BC SIPUT A on the same branch with *V. owensii*, *V. jasicida*, and *V. hyugaensis* with high bootstrap values (Figure 2). However, this strain was not on the same branch as *V. alginolyticus*. In contrast, strains SMI 1 A, BCSP 1 C, and S4 USUS A were placed on the same branch with *V. neocaledonicus* and *V. alginolyticus* (Figure 3). This branch was somewhat different from the branch of strains S5 USUS A and S5 USUS B as only *V. alginolyticus* was included in the corresponding branch (Figure 4). The branch was supported with high bootstrap values.

Table 2. 16S rDNA sequence identity of strain BC SIPUT A and the most closely related *Vibrio* species

***Vibrio* species**	**Accession number**	***V. owensii* (BC SIPUT A)**
V. owensii CAIM 1854	NR_117424.1	99.72
V. jasicida CAIM 1864	NR_113182.1	99.43
V. hyugaensis strain 090810a	NR_145569.1	99.28
V. rotiferianus CAIM 577	NR_118091.1	99.23
V. alginolyticus strain NBRC 15630	NR_113781.1	99.16
V. campbellii CAIM 519	NR_119050.1	99.09
V. natriegens NBRC 15636	NR_117890.1	98.95
V. parahaemolyticus strain ATCC 17802	NR_118569.1	98.83
V. natriegens NBRC 15636	NR_113786.1	98.81
V. azureus LC2-005 16S	NR_117997.1	98.74

Table 3. 16S rDNA sequence identity of strains S5 USUS A and S5 USUS C, and the most closely related *Vibrio* species

***Vibrio* species**	**Accession number**	**Sequence identity**	
		***V. alginolyticus* (S5 USUS A)**	***V. alginolyticus* (S5 USUS C)**
V. alginolyticus NBRC 15630	NR_122050.1	99.58	99.45
V. natriegens NBRC 15636	NR_117890.1	98.96	98.82
V. parahaemolyticus ATCC 17802	NR_041838.1	98.96	98.82
V. azureus LC2-005	NR_117997.1	98.89	98.75
V. campbellii NBRC 15631	NR_113782.1	98.55	98.41
V. rotiferianus CAIM 577	NR_118091.1	98.62	98.48
V. owensii CAIM 1854	NR_117424.1	98.80	98.59
V. pelagius ATCC 25916	NR_119059.1	98.46	98.32
V. neocaledonicus NC470	NR_118432.1	98.80	98.59
V. azureus NBRC 104587	NR_114268.1	98.27	98.75
V. mytili CECT 632	NR_044911.1	98.40	98.26
V. harveyi NBRC 15634	NR_113784.1	97.99	97.79

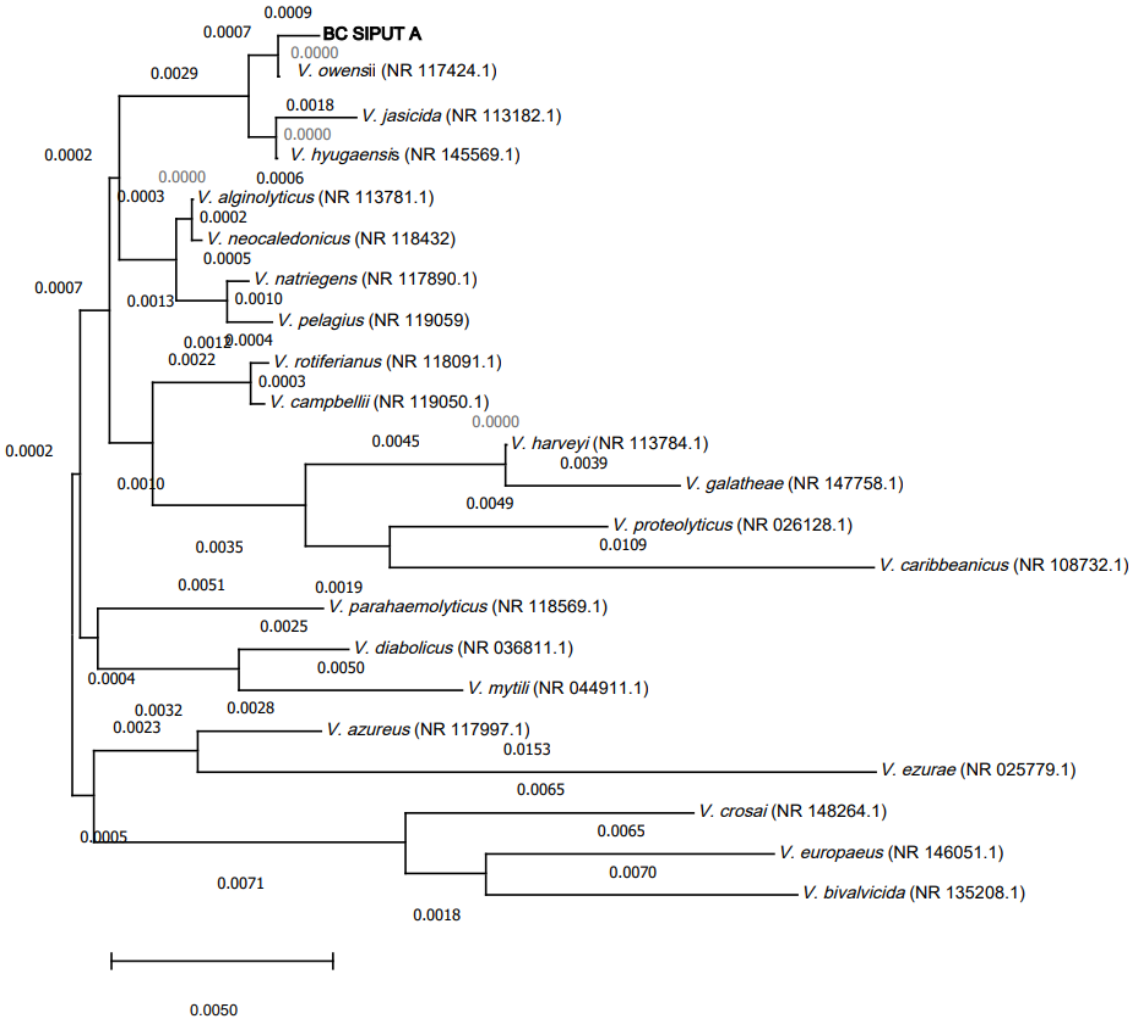

Figure 2. Phylogenetic relationships between strain SIPUT A and the most closely related *Vibrio* species based on the 16S rDNA sequences. The tree was constructed using the neighbor-joining method. Bootstrap values were generated from 1,000 replicates, and values of more than 500 are indicated at the nodes. Numbers in parentheses after the species name indicate the sequence Accession numbers. The 0.005 *Knuc* unit is indicated by the bar below the phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree is shown. (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. This analysis involved 22 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1,350 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018)

Table 4. 16S rDNA sequence identity of strains SMI 1 A, BCSP1C, and S4 USUS A, and the most closely related *Vibrio* species

***Vibrio* species**	**Accession number**	**Sequence identity**		
		***V. alginolyticus* (SMI 1 A)**	***V. alginolyticus* (BCSP1C)**	***V. alginolyticus* (S4 USUS A)**
V. alginolyticus NBRC 15630	NR_113781.1	99.51	99.51	99.86
V. natriegens NBRC 15636	NR_117890.1	99.37	99.38	99.72
V. campbellii NBRC 15631	NR_113782.1	99.03	98.96	99.30
V. rotiferianus CAIM 577	NR_118091.1	99.10	99.03	99.37
V. parahaemolyticus NBRC 12711	NR_113604.1	98.89	98.89	99.24
V. azureus LC2-005	NR_117997.1	98.82	98.75	99.10
V. owensii CAIM 1854	NR_117424.1	99.15	99.09	-
V. neocaledonicus NC470	NR_118432.1	99.15	99.02	99.30
V. pelagius ATCC 25916	NR_119059.1	98.87	98.73	99.09
V. harveyi ATCC 14126	NR_119054.1	98.47	98.34	98.82

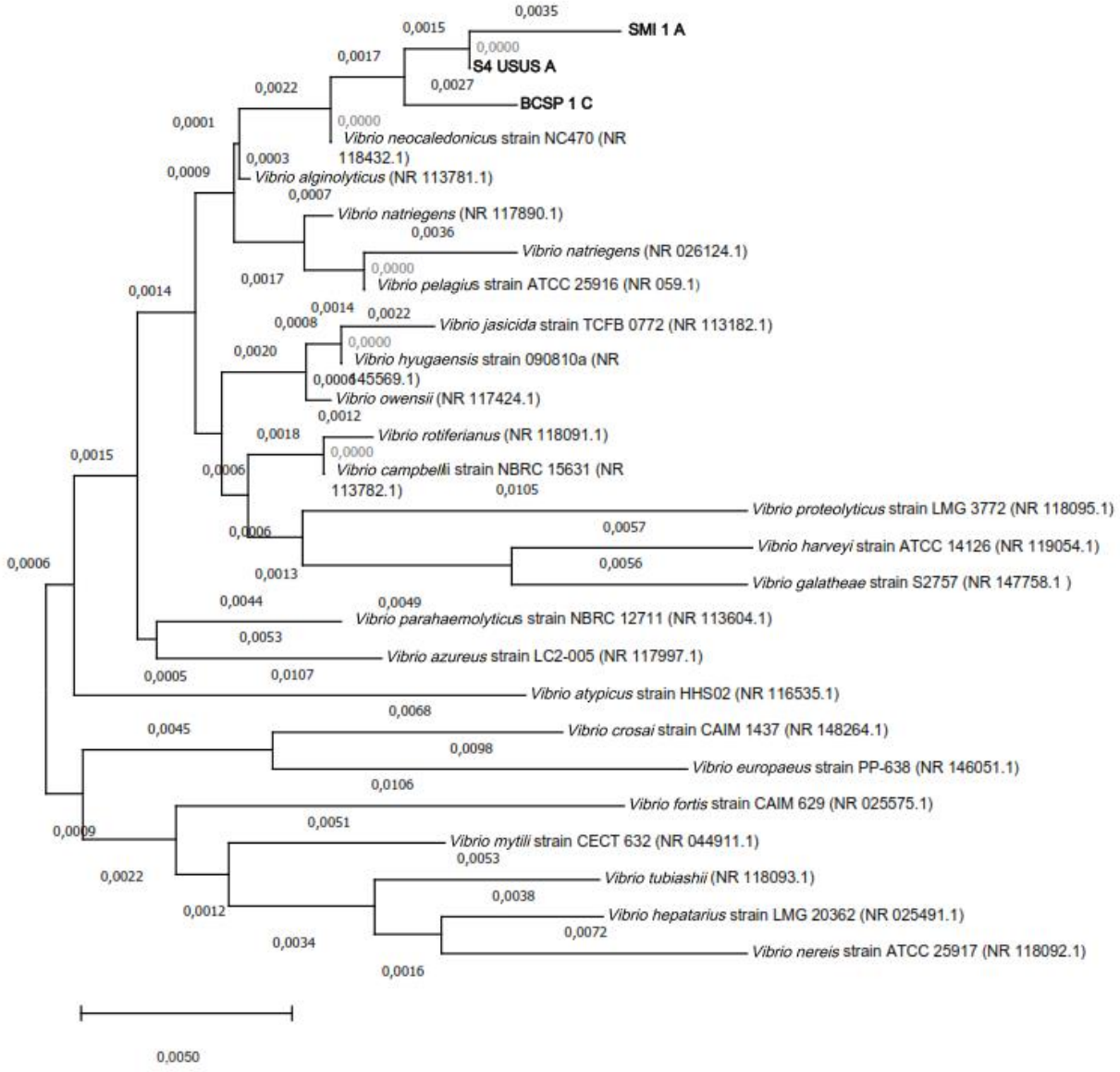

Figure 3. Phylogenetic relationships between strains SMI 1 A, BCSP 1 C, and S4 USUS A, and the most closely related *Vibrio* species based on the 16S rDNA sequences. The tree was constructed by the neighbor-joining method. Bootstrap values were generated from 1,000 replicates, and values of more than 500 are indicated at the nodes. Numbers in parentheses after the name of the species indicate the sequence Accession numbers. The 0.005 Knut unit is indicated by the bar below the phylogenetic tree

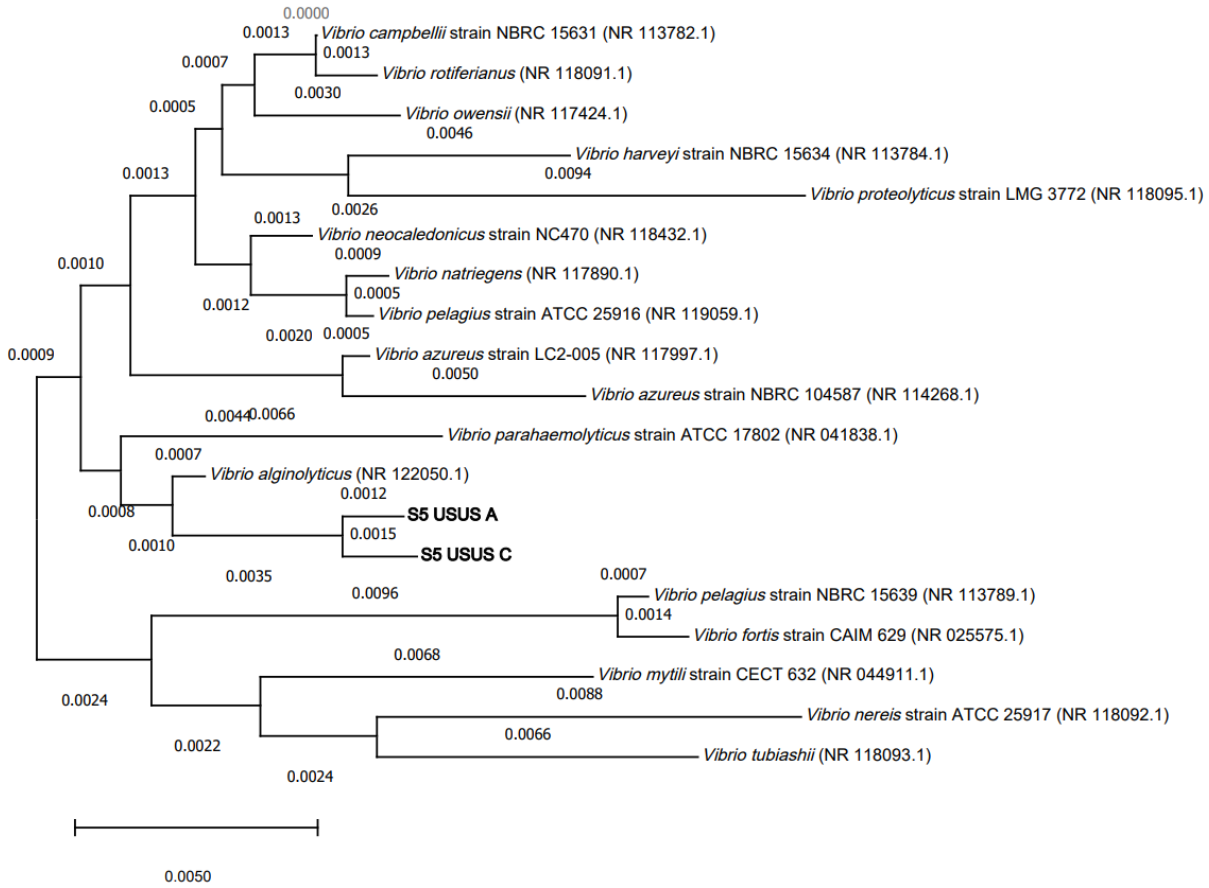

Figure 4. Phylogenetic relationships between strains S5 USUS A and S5 USUS C and the most closely related *Vibrio* species based on the 16S rDNA sequences. The tree was constructed by the neighbor-joining method. Bootstrap values were generated from 1,000 replicates, and values of more than 500 are indicated at the nodes. Numbers in parentheses after the name of the species indicate the sequence Accession numbers. The 0.005 *Knuc* unit is indicated by the bar below the phylogenetic tree

Table 5. Water quality at the marine aquaculture site on the Barrang Caddi and Samalona Islands, Spermonde Islands, southern Sulawesi, Indonesia

Water quality parameters	Barrang Caddi Island				Samalona Island				Average
	Cage 1		Cage 2		Cage 1		Cage 2		
	M*	A*	M	A	M	A	M	A	
Temperature (°C)	29	29.2	29	29.1	29.1	29.1	29.1	29.1	29.08
Salinity (ppt)	33	33	33	33	33	33	33	33	33
Disolved Oxygen (mg L^{-1})	7.7	7.5	7.6	7.7	7.7	7.8	7.7	7.7	7.67
pH	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2	8.2
Ammonia (mg L^{-1})	0.003	0.001	0.002	0.002	0.002	0.003	0.002	0.002	0.002

Note: *M, morning; A, afternoon

Physicochemical and microbiological water quality

Water quality is essential for mariculture and is a crucial factor in fish health management. Water temperature, dissolved oxygen, and ammonia ranged from 29.0-29.2°C, 7.5-7.8 mg L^{-1}, and 0.001-0.003 mg L^{-1}, respectively. Salinity and pH were stable at 33 ppt and 8.2, respectively (Table 5). These are proper ranges for mariculture. The total bacterial density and total *Vibrio* counts were low, ranging from 1×10^2-5×10^2 and 7×10^1-1×10^2 CFU mL^{-1} (Figure 5) in the Barrang Caddi and Samalona Islands, respectively.

Discussion

Mariculture has yet to take root in the Spermonde Islands. The vast majority of floating net cages are used to temporarily rear and maintain fish caught by fishermen. Only grouper is grown from seed to marketable size in the sampling site by floating net cage. During sampling, no outbreaks of fish disease were discovered at either site. The fish's good health was supported by good water quality conditions and a low density of bacteria in the water. A good fish immune system is aided by good water quality and appropriate environmental conditions for fish culture. Furthermore, good water quality reduces the growth and density of opportunistic bacterial pathogens in the water. Mariculture in this area has not been extensively developed, and floating net cages have not been stocked intensively. Therefore, the infection and transmission rates of pathogenic organisms in this area are relatively low.

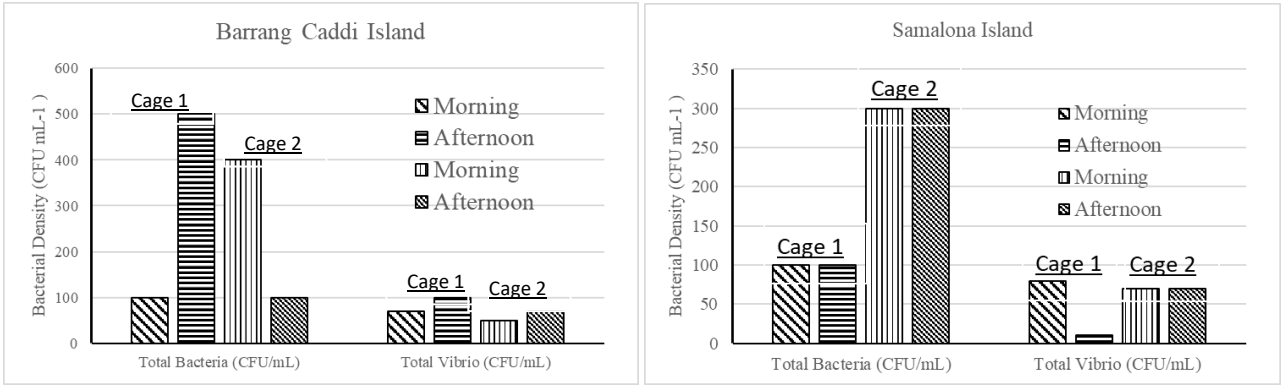

Figure 5. Bacterial density in the surface water of the floating net cage sites at the Barrang Caddi and Samalona Islands, southern Sulawesi, Indonesia

Despite the fact that no fish or marine organisms were infected with *Vibrio*, *Streptococcus*, or *Pseudomonas* at the time of sampling, bacteria from the digestive tract were successfully recovered on TCBSA. The bacterial isolates were assigned to the genus *Vibrio* based on their morphological and biochemical characteristics (Table 1). The most common opportunistic pathogen in seawater is *Vibrio*, and the bacteria surrounding the floating net cages could be a significant reservoir of opportunistic pathogens. The 16S rDNA sequences were analyzed further for identification.

According to the 16S rDNA sequence analysis, all of the isolates belonged to the *V. harveyi* clade (Go'mez et al. 2010; Yoshizawa et al. 2012). *V. harveyi* clade members are opportunistic pathogenic *Vibrio* in marine aquaculture. The 16S rDNA sequence analysis suggested that the BC SIPUT A strain was *V. owensii* and that the other five strains were *V. alginolyticus* (Tables 2-4, Figures 2-4). *V. owensii* is resistant to ampicillin, tetracycline, and erythromycin as confirmed in the present study (Table 1), and intermediately resistant to kanamycin (Liu et al. 2021). This study also discovered *V. owensii* resistance to enrofloxacin, which had not previously been reported. The other four *V. alginolyticus* strains were resistant to oxytetracycline, ampicillin, erythromycin, kanamycin, and enrofloxacin (Table 1). These findings are consistent with the responses of *V. alginolyticus* to erythromycin reported by El-Sayed et al. (2019). *V. alginolyticus* is reported to be resistant to ampicillin and cephalothin, but sensitive to ampicillin, amikacin, streptomycin, neomycin, oxytetracycline, tetracycline, chloramphenicol, erythromycin, norfloxacin, and ciprofloxacin (Hannan et al. 2019; Emam et al. 2019). The current findings suggest that *V. owensii* and *V. alginolyticus* are potential pathogenic bacteria in the marine environment.

The 16S rDNA sequence analysis suggested that the BC SIPUT A strain could be placed on the same branch with *V. owensii*, *V. jasicida*, and *V. hyugaensis* with a percent identity of 99.72, 99.43, and 99.28%, respectively (Table 2, Figure 1). Go'mez et al. (2010) isolated *V. owensii* from cultured spiny lobster *Panulirus ornatus* (Fabricius 1798) and giant tiger shrimp *Penaeus monodon* in Queensland, Australia. This Vibrio species belongs to the *V. harveyi* clade, which causes disease in fish and shrimp. *V. owensii* has been reported to be pathogenic in crustaceans and a causative agent of acute hepatopancreatic necrosis disease (AHPND) (Liu et al. 2021), the most important disease in *Litopenaeus vannamei* (Boone 2000). *V. owensii* isolated from shrimp in Shanghai carries the *pirA* and *pirB* genes, which cause a 100% mortality rate after 4 days of artificial infection (Liu et al. 2021). According to the findings, *V. owensii* is a possible causative agent of AHPND in crustaceans, particularly *L. vannamei*. Since the presence of *V. owensii* in Indonesian waters has never been reported, this is the first report of *V. owensii* isolated from Indonesian waters, specifically the Spermonde Islands waters.

BC SIPUT A, which is closely related to *V. jasicida*, was first reported in packhorse lobster (*Jasus verreauxi*), abalone (*Haliotis* sp.), and Atlantic salmon (*Salmo salar*) by Yoshizawa et al. (2012). The BC SIPUT A isolate was closely related to *V. hyugaensis* with a percent identity of 99.28% based on the 16S rDNA sequences (Table 2). *V. hyugaensis* isolated from Miyazaki coastal waters was first proposed as a new species in the genus *Vibrio* by Urbanczyk et al. (2015). No study has reported on the pathogenicity of either *V. jasicida* or *V. hyugaensis* in cultured marine species.

S5 USUS A and S5 USUS B strains were closely related to *V. alginolyticus* (Table 3). *V. alginolyticus* is a well-known opportunistic marine pathogen found in a variety of organisms, including fish (Rameshkumar et al. 2017; Liu et al. 2019; Sadok et al. 2019), crustaceans (Hannan et al. 2019), mollusks (Mechri et al. 2018), and coral (Li et al. 2019). The SMI 1 A, BCSP 1 C, and S4 USUS A strains were all closely related to *V. alginolyticus*, with 99.51, 99.51, and 99.86 percent identities, respectively (Table 4). These isolates were also closely related to *V. neocaledonicus*, which was first described as a producer of exopolysaccharides with high N-acetyl-hexosamine and uronic acid contents (Chalkiadaki et al. 2015).

The present study found that *V owensii* was resistant to oxytetracycline, ampicillin, erythromycin and enrofloxacin. Previous publications also reported similar-antibiotic resistance properties of this bacterium. *V. owensii* FcYS03 is resistant to 15 antibiotics and intermediate resistant to 7

antibiotics from 30 tested antibiotics (Liu et al. 2021). The higher resistant rate was described by the same authors for *V. owensii* Vp_{AHPND}-AG01. This strain was resistant to 19 antibiotics and intermediate resistant to 3 antibiotics from a total of 30 antibiotics tested. Recently, *V owensii* has been reported to be resistant to aztreonam, streptomycin, oxacillin, tetracycline, and minocycline; and intermediate resistant to ampicillin, piperacillin, and cefotaxime (Dai et al. 2022). Thillaichidambaram et al. (2022) also reported that *V. owensii* isolated from the Palk Bay of India is highly resistant to ampicillin, methicillin, tetracycline and vancomycin. This study and the previous publications suggest that *V. owensii* possesses a wide range of antibiotic resistance properties.

Vibrio alginolyticus is a multidrug-resistant marine bacterium. This bacterium is not only an important pathogenic Vibrio in mariculture, but also a causative agent in public health diseases (Ye et al. 2016). An emerging multidrug-resistant *V. alginolyticus* OS1T-47 isolated from the red sea has also been reported by Yasir et al. (2020). This strain possesses 22 multidrug-resistant genes including multidrug-resistant efflux pumps, β-lactams antibiotics, fluoroquilonone, elfamycin, tetracycline and peptide antibiotics. Hernández-Robles et al. (2016) have reported that 10%, 16%, 45%, 60% and 90% of *V. alginolyticus* strains are resistant to pefloxacin, cephotaxime, amikacin, cephalotin and beta-lactams antibiotics, respectively. The present study also confirms that *V. alginolyticus* is resistant to oxytetracycline, ampicillin, erythromycin and enrofloxacin.

The existence of mariculture potential pathogenic strains of *V. owensii* and *V. alginolyticus* in the present study encourages the need for methods to anticipate the occurrence of vibriosis in marine aquaculture in the Spermonde Islands in the future. Vaccination to increase fish immunity against vibriosis is the most promising alternative considering that prevention efforts have the highest potential for success in fish farming (Colquhoun and Lillehaug 2014). Although the live attenuated vibriosis vaccine was said to be safe (Chen et al. 2020), the type of fish vaccine permitted for use in mariculture in Indonesia is the inactivated vaccine (Istiqomah et al. 2020). Several types of inactivated vaccines that can be used include formalin-attenuated cells (Mohamad et al. 2021), subunit vaccines, or recombinant proteins (Ji et al. 2020) which can be applied in combination with adjuvants (Galindo-Villegas et al. 2013). Following that, a biocontrol approach, either using natural materials (Wang et al. 2022), antagonistic bacteria against *Vibrio* (Isnansetyo et al. 2009; Chau et al. 2021), or bacteriophages (Yu et al. 2013; Letchumanan et al. 2016; Kim et al. 2019), is possibly the most appropriate treatment method that needs to be developed to overcome the vibriosis.

The water quality parameters ranges assessed in this study on the Barrang Caddi and Samaloa Islands were suitable for the culture of marine fish. In addition, total bacteria and *Vibrio* densities were low, which is ideal for mariculture. According to the water quality parameters and bacterial densities in water, the floating net cage sites on the Barrang Caddi and Samalona Islands were suitable for mariculture activities.

In conclusion, the current findings suggest the possibility of opportunistic bacterial outbreaks caused by *V. owensii* and *V. alginolyticus* in the Spermonde Islands. This is the first report of *V. owensii* isolated from marine waters in Indonesia. The bacterial strains were intermediately and completely resistant to oxytetracycline, enrofloxacin, erythromycin, chloramphenicol, ampicillin, and kanamycin. These findings highlight the importance of using good aquaculture practices and integrated fish health management in the Spermonde Islands mariculture to reduce vibriosis outbreaks and avoid the development of antibiotic resistance in pathogenic marine bacteria.

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