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Diversity and abundance of culturable *vibrionaceae* in the rearing tanks of three different consumer types of aquaculture commodities

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ABSTRACT

Vibrio spp. bacteria species are heterotrophic and halophilic bacteria, which are commonly found in various habitats such as brackish, marine waters and animals. They are widely used as an indicator of water quality and cultured animal health. This study investigates the diversity and abundance of *Vibrionaceae* populations in the rearing water of three distinct consumer types of aquaculture commodities, namely abalone, lobster and sea cucumber, during six months sampling. We isolated *Vibrionaceae* bacteria from the rearing tanks of three different consumer types of aquaculture commodities. We then inoculated 100 μ L of undiluted to 10^{-5} diluted water samples onto petri dish containing selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar. We characterised bacteria colonies based on the biochemical test for identification. *Vibrio metschnikovii* was the predominant bacteria species in abundance value from the rearing water of cultured lobster and sea cucumber. Meanwhile, *Vibrio anguillarum* was the most noticeable bacteria species in abundance from the rearing water of cultured abalone. These data provide information and facilitate the risk assessment of these pathogenic *Vibrio* species in aquaculture commodities.

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1. Introduction

The human population is expanding rapidly, which is directly related to the growing demand for food sources, including food derived from the sea. Therefore, aquaculture plays a vital role in providing sustainable food sources (Bentzon-Tilia, Sonnenschein, & Gram, 2016). In aquaculture, microorganisms play an essential role, particularly bacteria. Bacteria of the genus *Vibrio* are highly abundant in the marine environment that has two distinct roles, namely a positive role (nutrients recycling and organic matter degrading) and a negative role (parasite, infectious diseases) as well as the harmful role (Wendling et al., 2014). The application of artificial feed, high stocking density, excess food and low water quality leads to a high bacterial population, particularly pathogenic *Vibrio*. They are opportunistic pathogens, which can survive and multiply in the absence of their host, thus causing diseases in the aquaculture environment (Brown et al., 2012; Defoirdt, 2016). *Vibrio* is responsible for disease and water- and food-borne pathogen (Ganesh et al., 2010). Vibriosis is the most common bacterial disease causing severe economic tolls in the aquaculture industry worldwide. Besides, the presence of specific human pathogenic species of *Vibrio* in the aquaculture environment can indicate public health safety of water and

food that destined for human consumption (Lekshmy et al., 2014; Bentzon-Tilia et al., 2016; Cáceres-Martínez et al., 2019).

The *Vibrionaceae* family belongs to gram-negative *Gammaproteobacteria* which is ubiquitous in aquatic environments, including brackish, marine and sediments. They are usually motile rods and have a facultative fermentative metabolism. They generally grow on marine agar and the selective medium thiosulfate-citrate-bile salts-sucrose (TCBS) agar. They are primarily oxidase-positive (Mechri et al., 2012). The *Vibrionaceae* family has 142 classes and consists of 7 genera, including *Aliivibrio*, *Echinimonas*, *Enterovibrio*, *Grimontia*, *Photobacterium*, *Salinivibrio* and *Vibrio* (Sawabe et al., 2013). *Vibrio* is a nutrient cycling agent that utilises dissolved organic compounds and the decomposition of an organic compound to provide simple biomolecules (Sherr & Sherr, 2002). *Vibrio* species, a pathogen for marine biota, make its monitoring *Vibrio* in aquaculture processes crucial (Froelich et al., 2016; Nicolas et al., 2002). Several aspects of aquaculture, such as artificial feed, excessive stocking density, food surplus and poor water quality, contribute to a large bacterial population, especially the virulent *Vibrio*. They are opportunistic pathogens that may live and reproduce in the absence of their host, causing illnesses to aquaculture animals (Defoirdt, 2016). Several *Vibrio* species such as *V. parahaemolyticus* and *V. variabilis* was

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discovered to be in a significant number when skin ulceration diseases (SKUDs) occurred in sea cucumber *Holothuria scabra*. Other *Vibrio* species such as *V. splendidus*, *V. cyclitrophicus*, *V. splendidus*, *V. harveyi* and *V. tasmaniensis* are similarly affected by this condition in *A. japonicus* (Delroisse et al., 2020). *Vibrio* has also been discovered in crustaceans such as shrimp and lobster, as well as bivalve molluscs (Destoumieux-Garzón et al., 2020).

Vibrio is quickly attached to the particle in the water column, including animals (Baker-Austin et al., 2018). A previous study reported that seaweed was a reservoir for diverse *Vibrio* species, and several pathogens were reported in macroalgae (Barberi, 2020). *Vibrio* is also found in fish and shellfish with diverse concentrations and species (Austin & Zhang, 2006; Beaz-Hidalgo et al., 2010). *Vibrio* with abundance concentration in the bottom waters (Ganesh et al., 2010; Plotieau et al., 2013). Seaweed was a food source for abalone farming, and fish was commonly used for lobster feeding. At the same time, sea cucumber utilises organic decomposition matter to complete its nutrition need (Mactavish et al., 2012; Robinson et al., 2019). Information about the difference of diverse *Vibrio* bacteria among these three consumer types at the trophic level is vital to support the aquaculture process. This study aims to provide the data and information about diversity, abundance and correlation *Vibrio* spp. in the rearing water of these three consumer types of animals.

2. Materials and Methods

2.1 Seawater sampling

Sampling was investigated from July 2017 to January 2018 at Aquaculture Laboratory of the Research and Development Division for Marine Bio Industry – Indonesian Institute of Sciences (LIPI), North Lombok, West Nusa Tenggara, Indonesia. Natural seawater was changed regularly with 50% of the total water volume twice a week and 100% of the total water volume once a week. The pumped seawater was filtered using sand and coral rubbles, seawater which source coming from Teluk Kodek seawater into these ponds. It allows water quality in each concrete pond to be similar to a natural condition. We regularly measured water quality parameters in the rearing tanks, such as pH, temperature, salinity and dissolved oxygen (DO). We used three different cultured and trophic level species because these animals have been reared in this research institution for over five years and monitor pathogens mainly, the *Vibrionaceae* in the rearing tanks. Lobsters were fed with fresh fish meat once daily, abalone were fed with fresh seaweed (*Gracilaria sp.*) ad libitum once daily and then sea cucumbers were fed with fermented

seagrass pulp once daily. All of these animals were reared in ± 300 L concrete ponds for each species. Water samples were collected aseptically in sterile containers 100 mL, twice a month for seven months (July 2017 – January 2018) from rearing water of the adult stage or broodstock of lobster (*Panulirus homarus*), abalone (*Haliotis squamata*) and sea cucumber (*Holothuria atra*) ponds. Then, these samples were brought directly to the Microbiology Laboratory and adequately prepared for further detailed analysis.

2.2 Bacterial isolation and characterisation

Enumeration of *Vibrio* spp. was conducted by using serial dilution and plating method. Serial dilutions of the samples were performed using sterile seawater. *Vibrio* was isolated by inoculating 100 μ L of undiluted to 10^{-5} diluted water sample onto petri dish containing selective thiosulfate-citrate-bile salts-sucrose (TCBS) agar medium (Hi-Media Laboratories, Mumbai, India) containing 2% NaCl, specific for the *Vibrionaceae* biota in sterile seawater, followed incubation at 35 °C for 20 – 24 h. The bacteria colonies were counted and the population density was expressed as colony-forming units (CFU) per mL. We selected all yellow, green and blue-green colonies for biochemical characterisation tests such as 0%, 3%, 6%, 8% and 10% NaCl growth, motility, indole production and lysine ornithine decarboxylase (LDB), sucrose, glucose and fructose fermentation. These colonies were characterised based on the classical morphological and biochemical test, as described in Bergey's Manual of Systematic Bacteriology (Krieg & Holt, 1984). We measured *Vibrio* bacterial diversities based on the Shannon diversity index, using the $H' = -\sum p_i \ln(p_i)$ formula (Shannon, 1984). Shannon's index of evenness was calculated according to $E = H' / \ln S$, where $\ln S$ represents H' max. Statistical differences among the bacteria diversity and evenness indexes in rearing tanks of different trophic level species commodities were performed by ANOVA analysis, followed by the least significant difference (LSD) test. Pearson's correlation measured the number of bacteria colonies in the rearing tank of three different level trophic species and sampling months. P-value < 0.05 and P-value < 0.01 being accepted as a significant level using IBM SPSS version 27 software for Windows.

3. Results

3.1 Water quality parameters

Water quality parameters data showed a lower tendency occurred in three months of the wet season. Characteristics of sampled rearing water are measured physically, biologically and chemically in Table 1.

Table 1. Physical, biological and chemical characteristics of sampled rearing water in aquaculture tanks.

Sampling month	Water quality parameters				
	Temperature (°C)	pH	Salinity (‰)	Dissolved Oxygen (DO) (mg/L)	Total <i>Vibrio</i> (CFU/100 mL)
August 2017	29 \pm 1.03	8 \pm 0.2	34 \pm 0.7	7.2 \pm 0.5	6.5 x 10 ³ -1.9 x 10 ⁴
September 2017	29 \pm 1.04	8 \pm 0.3	34 \pm 0.4	7.5 \pm 0.3	2.1x 10 ³ -2.03 x 10 ⁴
October 2017	28 \pm 1.08	8 \pm 0.2	33 \pm 0.5	6.8 \pm 0.4	1.8 x 10 ² -2.1 x 10 ³
November 2017	27 \pm 0.7	8 \pm 0.1	33 \pm 0.2	7.4 \pm 0.2	6.5x10 ² -8.2 x 10 ³
December 2017	27 \pm 1.01	7.8 \pm 0.3	32 \pm 0.6	6.5 \pm 0.3	7.1x10 ² -5.2 x 10 ³
January 2018	27 \pm 0.9	7.8 \pm 0.5	32 \pm 0.1	7 \pm 0.1	5.1x10 ² -6.1 x 10 ³

Data are presented as means \pm SD.

Table 2. Diversity bacteria of the family *Vibrionaceae* in the rearing water of lobster, abalone and sea cucumber evaluated by Shannon's diversity index (H'), species evenness (E') and species richness (S).

Aquaculture commodity species	Shannon diversity index (H')	Shannon evenness index (E')	Species richness (S)
Lobster (<i>Panulirus homarus</i>)	2.02 ^a	0.71 ^a	17 ^a
Abalone (<i>Haliotis squamata</i>)	2.03 ^a	0.77 ^a	14 ^a
Sea cucumber (<i>Holothuria atra</i>)	1.56 ^b	0.59 ^b	14 ^a

Different letters indicate significant differences among three different cultured species commodities ($P < 0.05$) using the LSD test.

The pH was highly stable throughout the six months. Meanwhile, temperature and salinity tended to decrease in November, December and January. The higher temperature was found in August, September and October. On the other hand, lower salinity values were associated with heavy rain in the wet/rainy season.

Total *Vibrionaceae* populations in rearing water of three different trophic level species demonstrated higher populations of *Vibrionaceae* occurred in August and September, while other four months presented lower *Vibrionaceae* populations than two previous months. During the six months, the value of dissolved oxygen in the rearing water of three different trophic level species showed slight fluctuations.

3.2 Shannon's diversity index of *Vibrionaceae* bacteria

The diversity and evenness of the *Vibrionaceae* populations in the rearing water of lobster and abalone were moderate for all sampling occasions. However, sea cucumber's diversity and evenness indexes were significantly lower than lobster and abalone (Table 2). The diversity index of lobster and abalone was 2.02 and 2.03, respectively. The lowest value of diversity index was shown in sea cucumber, which was 1.56. Meanwhile, the evenness index values of *Vibrionaceae* populations in rearing water of lobster and abalone were not a significant difference, and it was 0.71 and 0.77, respectively. The contrary result was shown to the evenness index value of *Vibrionaceae* populations in the rearing water of sea cucumber with a remarkable distinction compared to others, which was 0.56. The number of distinctive *Vibrionaceae* species in the rearing water of lobster was 17, followed by rearing water of abalone and sea cucumber which were 14.

3.3 Identification and comparison of culturable *Vibrionaceae*

Vibrionaceae species in the rearing water of cultured lobster (*Panulirus* sp.) demonstrated that *Vibrio metschnikovii* was the most incredible relative abundance. Then, it was followed by the lowest number of *Vibrionaceae* colonies, which was *Vibrio damsela* (Fig.1). Some bacterial species from the *Vibrionaceae* and *Aeromonadaceae* families were found only in the rearing water samples of the lobster tanks, which was *Vibrio comitans*, *Vibrio damsela*, *Aeromonas salmonicida* biovar *masoucida* and *Aeromonas molluscorum* (Fig.1). In the rearing water of abalone (*Haliotis asinina*) sample, *Vibrio anguillarum* showed the highest relative abundance value, followed by *V. metschnikovii*, *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus*. However, the smallest relative abundance value of *Vibrio* bacteria was *Vibrio pacinii* (Fig. 2). Interestingly, *Vibrio proteolyticus* was only found in the abalone (*Haliotis asinina*) tank; however, it was not obtained in the rearing water sample of other cultured animals tanks (Fig. 3). A similar result of prominent *Vibrio* species from rearing water of lobster, *Vibrio metschnikovii* was found in the rearing water of sea cucumber as well. Two different *Vibrio* species with the most negligible relative abundance were discovered in the rearing water of sea cucumber. It was *Vibrio brasillensis* and *Aeromonas salmonicida* (Fig. 3). *Vibrio agarivorans* and *Vibrio angustum* were two specific *Vibrio* species found only in the rearing water of cultured sea cucumber.

The difference of *Vibrionaceae* colony number in three different consumer types during six months has presented that *Vibrionaceae* bacteria populations in the rearing water of lobster were the highest in August and September, then decreased sharply in October. At the same time, those *Vibrionaceae* populations differ significantly compared to two other cultured animals, namely abalone and sea cucumber (Fig. 4). The number

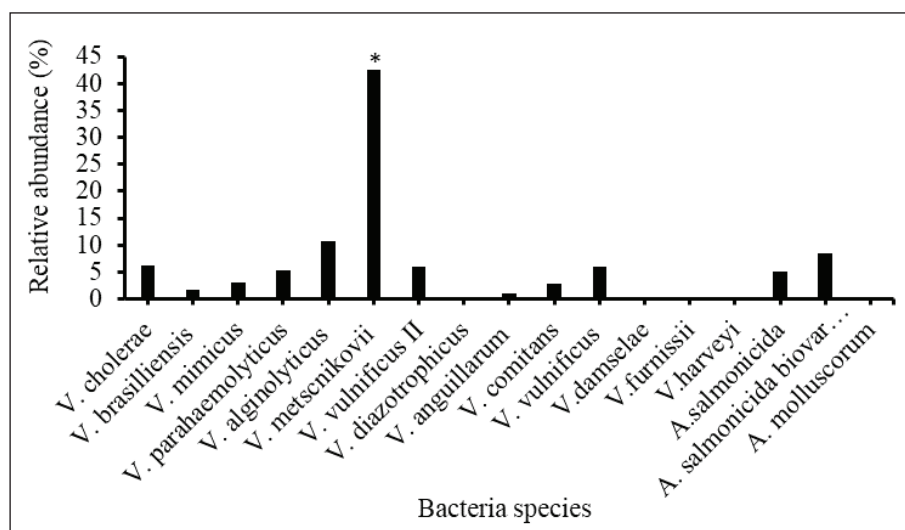


Fig. 1. Relative abundance of *Vibrionaceae* species in the rearing water of cultured lobster (*Panulirus homarus*).

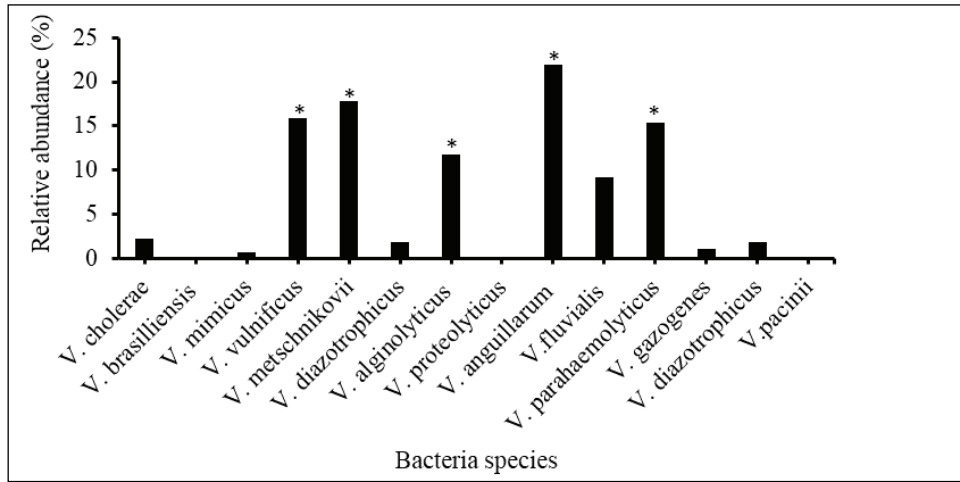


Fig. 2. Relative abundance of *Vibronaceae* species in the rearing water of cultured abalone (*Haliotis asinina*).

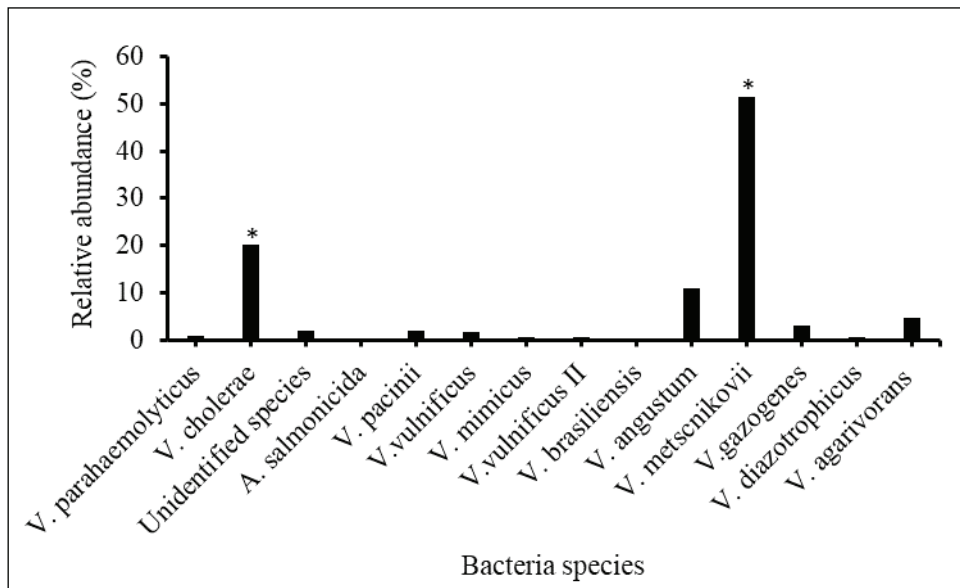


Fig.3. Relative abundance of *Vibronaceae* species in the rearing water of cultured sea cucumber (*Holothuria atra*).

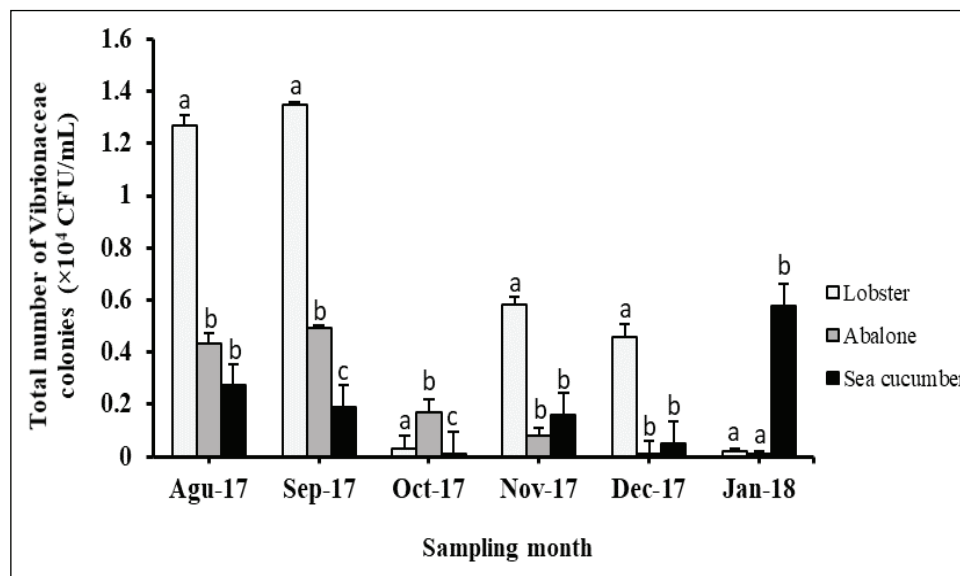


Fig. 4. Fluctuations of *Vibronaceae* populations in different sampling months and rearing seawater tanks of different cultured animal species

of *Vibrionaceae* populations in the rearing water of abalone tended to drop gradually during six months of sampling. In contrast, the highest number of *Vibrionaceae* populations in the rearing water of sea cucumber was found only in January. All results of *Vibrionaceae* populations number in distinct rearing water of three different consumer types of aquaculture commodities and sampling month showed a decreasing tendency in bacteria population number from August to January. However, only in January, did the *Vibrionaceae* populations in rearing water of cultured sea cucumber rise sharply.

3.4 Correlation between *Vibrionaceae* abundance and water quality

Pearson's correlation analysed that *Vibrionaceae* abundance and water quality parameters represented a highly positive relationship between *Vibrionaceae* abundance in the rearing water of cultured lobster and abalone and water quality parameters such as temperature and salinity during sampling month. However, Pearson's correlation index showed a significantly negative relationship between *Vibrionaceae* abundance in the rearing water of culture sea cucumber and water quality parameters during sampling month, which were -0.12 and -0.18 for temperature and salinity, respectively (Table 3).

4. Discussion

Data of water quality parameters demonstrated that temperature and salinity values slightly declined for three months during the rainy season. Even though its value was relatively low, it probably promoted some chemical, physical and biological changes in the rearing water column. Consequently, those changes may impact the diversity, abundance, richness of the microbiota community, particularly the *Vibrionaceae* family, in the rearing water of cultured animals. Some previous studies regarding the effect of water quality on aquatic animal health and microbiota community in aquaculture activities have been investigated. According to Takemura et al. (2014), there may be ecological relationships between *Vibrio* and plankton, while salinity and temperature showed different patterns between total *Vibrio* and individual species and within each other species. In natural aquatic environments, bacteria communities respond rapidly to changes and may lead to activation or inactivation of their particular metabolic pathway in composition and functionality.

Shannon's diversity and abundance index of *Vibrionaceae* populations in the rearing water of cultured lobster and abalone showed moderate species diversity and abundance. These results differed from *Vibrionaceae* populations in the rearing water

of cultured sea cucumber, which was slightly lower (1.56 and 0.59). It was interesting data as to be known that sea cucumber is a deposit feeder and a decomposer biota type, which uses high bacteria concentration for degrading organic matter from sediment, faeces and detritus. However, these index values, *Vibrionaceae* diversity and abundance in the rearing water of cultured sea cucumber presented the contrary and the lowest one. These results may occur because the populations of *Vibrionaceae* in the rearing water of cultured sea cucumber may be filtered by sea cucumber and collected in their gut or body colon. These results showed in agreement with previous studies that sea cucumber obtain food by filtration of seawater or by ingestion of marine sediment (Ward-Rainey et al., 1996). Besides, these results also revealed that water quality did not strongly influence *Vibrionaceae* diversity and abundance in the rearing water of cultured sea cucumber because of its feeding behaviour.

The distinct biological-types consumers of cultivated species in aquaculture tanks probably influence bacteria density and variation species in rearing water samples of cultured species. The highest bacterial populations in the rearing water of farmed lobster may be related to the accumulation of nitrogenous compounds or protein particles concentration from faeces and the excess feed of fresh fish meat as its food. In the water column and sediment, these organic matters are then decomposed by aerobic heterotrophic bacteria, including the *Vibrionaceae* family, to their metabolism and growth (Naeem & Li, 1998; Hargreaves, 1998). Hargreaves (1998) described that ammonia is released as the end product of protein catabolism, and it may be toxic if allowed to accumulate in aquaculture ponds. *Vibrio metschnikovii*, a bacteria species with the highest density in the rearing water of cultured lobster is described microbiologically as gram-negative, catalase-positive and oxidase-negative, slightly curved rods and opaque colonies 2 to 3 mm in diameter. It can be found in various aquatic habitats such as lakes, marine waters, sewage and marine biota, for instance, shrimp, crabs and shellfish (Linde et al., 2004). Human infection by *V. metschnikovii* based on human clinical samples, in contrast, is an infrequent event. Despite a deadly rare case, this bacteria can lead to a fatal human infection such as severe septic shock, cardiac arrest, septicaemia and wound infection (Jensen & Jellinge, 2014; Linde et al., 2004; Hansen et al., 1993). The previous study reported that *V. metschnikovii* produce protease enzyme (Jellouli et al., 2009). This enzyme is probably used to degrade protein particles of feeds and faeces waste sources in the rearing water column. Interestingly, some *Vibrio* bacteria were only found in the rearing water of cultured lobster; 1. *Vibrio comitans* produce alginase enzyme (Sawabe et al., 2007), 2. *Vibrio damsela* produce the cytolytic toxin, which causes wound infection to humans as well as pathogenic to marine animals (Hundenborn et al.,

Table 3. Pearson's correlation index between *Vibrionaceae* abundance and two vital parameters of water quality.

Cultured species	Pearson's correlation index	
	Temperature (°C)	Salinity (‰)
Lobster	0.76 ^a	0.82 ^a
Abalone	0.98 ^a	0.95 ^a
Sea cucumber	-0.12 ^b	-0.18 ^b

Different letters indicate significant differences among three different cultured species commodities ($P < 0.05$).

2013; Rivas et al., 2013; Akram et al., 2015), 3. *Aeromonas salmonicida* biovar *masoucida* and *Aeromonas molluscorum*, the two *Aeromonas* genus members are ubiquitous, water-borne bacteria, predominantly pathogenic to poikilothermic animals and humans. In human infection, these bacteria from mesophilic strains can cause gastroenterics (Tomás, 2012; Janda & Abbott, 2010; Igbinsosa et al., 2012; Miñana-Galbis et al., 2004).

Vibrio bacteria density in rearing water of cultured sea cucumber showed a lower bacterial density. It may be affected by the biological consumer type of sea cucumbers (*Holothuria atra*), which is detritivores (deposit-feeding or consuming particulate matter, including bacteria). Hence, a high concentration of *Vibrio* populations in the rearing water was probably fed by sea cucumbers. Those results demonstrated in an agreement that sea cucumbers obtain food by filtration of seawater as well as the ingestion of large quantities of marine sediment (Gao et al., 2014; Plotieau et al., 2013; Lin et al., 2016). Sea cucumbers play a crucial role in the marine ecosystem in the recycling and remineralisation of organic matters by bioturbation and feeding in reef sands (Purcell et al., 2016; Lee et al., 2017). However, according to Plotieau et al. (2013), sea cucumbers significantly differ in microhabitat and behaviour among species. Furthermore, *Vibrio metschnikovii* were found predominant density in the rearing water of farmed sea cucumber (*Holothuria atra*), a similar result with notable *Vibrio* species density in the rearing water of cultured lobster. *Vibrio metschnikovii* was found in all of the rearing water samples of different farmed animals, including reservoir as control. These obtained results revealed that this bacteria species is a natural heterotrophic bacteria inhabitant, which is ubiquitous in marine waters and infected sea animals and has been well-known as a causative agent with virulence gene expresses (Jensen & Jellinge, 2014; Hansen et al., 1993). Unlike previous results of the outstanding *Vibrio* species densities in rearing water of cultured lobster and sea cucumber, *Vibrio anguillarum* is the highest *Vibrio* species density found in the rearing water of farmed abalone. *V. anguillarum* is described as a Gram-negative and comma-shaped rod bacterium belonging to the *Vibrionaceae* family. It is polarly flagellated halophilic and facultatively anaerobic halophilic bacteria. Furthermore, *V. anguillarum* is the causative agent of vibriosis, a deadly haemorrhagic septicemic disease affecting various marine, fresh or brackish water fish, molluscs and crustaceans. This disease, vibriosis, is responsible for severe economic losses worldwide (Frans et al., 2011; Gao et al., 2018; Larsen et al., 2004). Some previous studies reported that there are 12 *Vibrio* species have been implicated in human infections, however, particularly *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* are certainly the most frequently isolated species in human diseases (Hansen et al., 1993; Dalsgaard, 1998; Baker-Austin et al., 2018; Wang et al., 2015; Latinoamericana de et al., 2007). There was a correlation relationship between *Vibrionaceae* diversity and water quality. These data were in accordance with a previous study that stated temperature and salinity correlate strongly to the *Vibrionaceae* population in an aquaculture environment (Takemura et al., 2014). However, the previous study reported that some major factors substantially influence the distribution of *Vibrio* species, such as geographic location, water temperature and salinity (Tey et al., 2015). In the aquaculture system, the pathogenic *Vibrio* bacteria density in aquaculture tanks should be controlled under 1000 CFU mL⁻¹ (Ganesh et al., 2010).

5. Conclusion

The current study reveals the diversity and abundance of *Vibrionaceae* species in the rearing water of three different consumer types of cultured commodities, as well as its correlation to the aquaculture environment. Some pathogenic *Vibrio* species were found in the rearing water of three different consumer types of cultured commodities. It can be vital information to prevent mass mortality and diseases outbreak in aquaculture activities. These bacteria are common in the natural water environment, however, they could be pathogenic bacteria to animals and humans as well. While, *Vibriolginolyticus*, *Vibrioparahaemolyticus*, and *Vibriovulnificus* are the most causative *Vibrio* species.

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