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Detection of multidrug resistant *Vibrio parahaemolyticus* and anti-*Vibrio Streptomyces* sp. MUM 178J

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Abstract: The growing global population has increased the demand for seafood, making the aquaculture industry a vital food source. However, climate change has negatively impacted the industry as natural inhabitants of aquatic environments such as *Vibrio parahaemolyticus* are thriving in the warming waters. These foodborne pathogens can cause disease in marine animals, resulting in lowered production rates and substantial economic losses. *V. parahaemolyticus* is transmitted from these marine animals in aquaculture to humans via raw or undercooked seafood, resulting in gastroenteritis outbreaks. In addition, the extensive use

of antibiotics in aquaculture to treat infections within marine animals has led to the development of multidrug-resistant (MDR) *V. parahaemolyticus*, which threatens the effectiveness of antimicrobial treatments. Thus, it is vital to explore the alternatives of antibiotics in aquaculture to manage the spread of antibiotic resistance. This study aimed to investigate the prevalence of MDR *V. parahaemolyticus* to provide insight into the current antibiotic resistance patterns of this pathogen and to determine potential anti-*Vibrio* properties of *Streptomyces* MUM 178J which was previously isolated from the soils of a mangrove forest in Sarawak, Malaysia. Colony morphology and *toxR*-assay indicated that all samples tested positive for *V. parahaemolyticus*. Further identification with genomic analyses confirmed that 64% (64/100) of the isolates were *V. parahaemolyticus*. The multiple antibiotic resistance index (MAR) of the isolates ranged between 0.07 and 0.57, with 81% (52/64) of the isolates resistant to more than one antibiotic. The isolates were most resistant to ampicillin (98.4%), followed by ceftazidime (53.1%). In contrast, the isolates were highly susceptible to nalidixic acid (98.4%), tetracycline (93.8%), and sulfamethoxazole/trimethoprim (90.6%). They also demonstrated susceptibilities of 89.1% to levofloxacin, oxytetracycline, and gentamicin. *Streptomyces* sp. MUM 178J exhibited antagonistic effects on select *V. parahaemolyticus* strain, RP0132, with a minimum inhibitory concentration of 12.5mg/mL and a minimum bactericidal concentration of 50mg/mL.

Keywords: *Vibrio parahaemolyticus*; antibiotic resistance; prevalence; aquaculture; *Streptomyces*; SDG 3 Good health and well-being

1. Introduction

The aquaculture industry has been steadily expanding following improvements in knowledge and technology, whereby increased yield and seafood production can be seen throughout the years to fulfill the demand for human consumption [1]. In the 1990s, total production from world fisheries and aquaculture was only 110.7 million tons; in 2020, there was a 60.6% increase to 177.8 million tons [2]. The increase in the global population has also contributed to the rise in seafood consumption, further prompting the expansion of the aquaculture industry [3]. The global aquaculture market was valued at USD 204 billion in 2020 and has a projected compound annual growth rate of 3.6%, estimated at USD 262 billion by the end of 2026 [4]. The aquaculture market provides the producers in the industry a means of generating income and continuously brings opportunities for employment for many individuals worldwide [5]. It was reported that approximately 59.6 million people were engaged in the primary sector of capture fisheries and aquaculture in 2016 [6]. Furthermore, seafood is an important of the human diet as it supplies a plethora of macro- and micronutrients such as protein, omega-3 fatty acids, selenium, taurine, and vitamins B12 and D [7, 8]. These nutrients are essential for promoting growth and maintaining a healthy immune system.

However, climate change negatively affected the aquaculture industry, affecting its production to satisfy global seafood demand [9]. For instance, the rise in global temperatures has brought about an increase in infectious diseases, including zoonotic diseases worldwide [10-12], such as the most recent COVID-19 pandemic [13-15], which was attributed to the sale of wild animals in a seafood market in China [16-18]. The COVID-19 pandemic not only caused high fatalities worldwide but also affected the healthcare system badly [14, 19-22]. As such, COVID-19 patients were prioritized [23, 24], while other non-severe cases, such as foodborne infection cases, were treated as outpatient care. This led to underreported cases and a lack of surveillance on foodborne-related infections.

The rise in water temperatures [25], makes the aquatic environments more favorable for bacteria like *Vibrio parahaemolyticus* to thrive in [26, 27]. Consequently, there is an increased spread of diseases in the marine animals that inhabit these waters [28]. *V. parahaemolyticus* is a Gram-negative, rod-shaped, halophilic bacteria that is autochthonous to aquatic environments such as ponds, rivers, estuaries, and oceans [29]. First discovered in 1950, this pathogen was the causative agent for an outbreak of gastroenteritis in Japan, resulting in 20 deaths associated with the consumption of *shirasu* [30]. Since then, multiple outbreaks of gastroenteritis associated with *V. parahaemolyticus* with common symptoms such as nausea, vomiting, fever, abdominal pain, and diarrhea have been reported in various countries [31-35]. The increased presence of *V. parahaemolyticus* in water environments raises concern regarding food safety, as these foodborne bacteria can accumulate in animals from aquaculture and be transmitted to humans upon consuming contaminated seafood [29, 36-38]. Polymerase chain reaction (PCR), multiplex PCR, nucleic acid sequence-based amplification (NASBA), or loop-mediated isothermal amplification (LAMP) are among the rapid diagnostic methods which can be deployed to detect this foodborne pathogen to monitor its prevalence in the environment to prevent outbreaks of gastroenteritis [39, 40].

Pathogenic strains of *V. parahaemolyticus* possess virulence factors known as thermostable direct hemolysin (*tdh*) and TDH hemolysin (*trh*) to elicit their pathogenicity [29, 41]. These virulence factors are associated with *V. parahaemolyticus* hemolysis and cytotoxicity of cells in the gastrointestinal tract of the host. In addition, it is documented that *V. parahaemolyticus* with *trh* typically produces urease, which is important for colonizing intestinal epithelial cells and triggers inflammatory cytokine production [42]. The correlation between the presence of virulence genes in *V. parahaemolyticus* and the pathogenicity of the bacteria is well known, but clinical strains without the virulence genes have also been documented [43-45]; thus, the mechanisms of pathogenesis for these *V. parahaemolyticus* isolates remain unknown. Infections caused by *V. parahaemolyticus* are generally self-limiting, whereas in severe cases, antimicrobial treatment is administered to reduce the microbial load in the host [46, 47]. However, multidrug-resistant (MDR) *V. parahaemolyticus* is emerging. Traditionally, they have been susceptible to various antimicrobials, such as penicillins, cephalosporins, tetracyclines, and fluoroquinolones, to name a few. Still, recent reports have shown that *V. parahaemolyticus* isolates are developing resistance to these

antibiotics [48-54]. The evolution of their antibiotic resistance patterns is worrying as this indicates that current treatments may no longer be effective in managing *V. parahaemolyticus* infections in aquaculture and humans. The rise in MDR *V. parahaemolyticus* can be attributed to the misuse of antimicrobials in the aquaculture industry as prophylaxis and disease management [55-57]. The dissemination of antimicrobial residues into the waters creates environmental pressure for MDR *V. parahaemolyticus* to survive and propagate, thus increasing their prevalence in aquatic environments [58]. These factors further drive the intra- and interspecies transmission of antibiotic-resistance genes via horizontal gene transfer. Hence, there is a need for continuous surveillance and monitoring of the prevalence and antibiotic resistance patterns of *V. parahaemolyticus* to ensure food safety and preserve the efficacy of the antimicrobials that are currently available to maintain public health.

Moreover, exploring the alternatives to antibiotics to manage *V. parahaemolyticus* populations in aquaculture is needed to effectively control the spread of antibiotic resistance while maintaining the health of the farmed animals and consumers. Alternatives to antibiotics that have been well studied include bacteriophages, prebiotics, probiotics, and vaccines, which are deployed in managing diseases in aquaculture [55, 59-61]. Vaccines provide immunity to the farmed aquatic animals to fight against potential infections, while bacteriophages can cause lysis of bacterial cells during infection [55]. Meanwhile, prebiotics and probiotics can help strengthen the immune system of aquatic animals via the modulation of their gut microbiota while promoting their growth and disease prevention [62-64]. Microbial-derived natural products have also been shown to be promising sources of antagonistic compounds against bacterial pathogens [65]. For instance, *Streptomyces* sp. has been deemed a promising candidate for inhibiting the growth of *Vibrios* in aquatic environments [66, 67]. *Streptomyces* sp. are a group of Gram-positive, filamentous bacteria which can produce secondary metabolites with various bioactivities [68-72]. These include antioxidant, antibacterial, antibiofilm, anticancer, cytotoxic, and even anti-*Vibrio* properties, making them potential candidates as anti-*Vibrio* agents. For instance, *Streptomyces rubrolavendulae* M56 isolated in India caused a decline in viable *Vibrio* count. Researchers speculated the production of enzymes by M56 was the reason for growth inhibition within the *Vibrios* in the co-culture experiments [73]. Yang et al. isolated a marine *Streptomyces* sp., S073, with antagonistic properties against *V. parahaemolyticus*. Carboxylate-type siderophores are produced by S073, which creates an iron-limiting condition lethal to *V. parahaemolyticus* [74], thereby effectively inhibiting their growth. Available evidence leads researchers to believe that *Streptomyces* sp. will continue to be a valuable source of prolific compounds to manage the proliferation of *V. parahaemolyticus* in aquaculture. The current study aims to explore the prevalence of MDR *V. parahaemolyticus* from seafood and anti-*Vibrio* properties of *Streptomyces* sp. MUM 178J. Through this research, we hope to harness the potential of *Streptomyces* sp. MUM 178J which could be used as a probiotic in managing *Vibrio* infections in aquaculture settings, subsequently reducing antibiotic dependency.

2. Methods

2.1. Sampling

In this study, white prawns (*Fenneropenaeus indicus*), speckled shrimps (*Metapenaeus monoceros*), venus clams (*Paratapes textilis*), blood clams (*Tegillarca granosa*), and flower crabs (*Portunus pelagicus*) we sampled. The seafood samples were purchased from a local wet market (3.3512° N, 101.2520° E) in April 2022. Upon purchase, the samples were stored in an ice box and immediately transported to the laboratory for processing. All the seafood samples were processed at the laboratory within 3 hours of collection.

2.2. Isolation of presumptive Vibrio parahaemolyticus isolates in seafood samples

The methodology of isolating *V. parahaemolyticus* was adapted from Reshma et al. [75] with some minor modifications. Ten grams of each sample were weighed and homogenized with 90 mL of alkaline peptone water (APW) supplemented with 2% w/v sodium chloride (NaCl) (Vivantis, United States) in a sterile homogenizer bag. The samples were then homogenized for 1 minute using a stomacher (Bagmixer 400W, Interscience, St Nom, France) and incubated under aerobic conditions at 37°C for 18 hours. After incubation, a loopful of enriched broth was streaked onto chromogenic selective agar (HiCrome™ Vibrio agar, Himedia) plates and incubated under aerobic conditions at 37°C for 18 hours. On HiCrome™ Vibrio Agar, the growth of *V. parahaemolyticus* in bluish-green colonies is well differentiated from other *Vibrio* species. A total of 100 presumptive *V. parahaemolyticus* were isolated from the seafood samples.

2.3. Purification of presumptive V. parahaemolyticus isolates

After incubation, the bluish-green colonies were picked and purified by re-streaking streaked onto Tryptic Soya Agar (TSA) plates supplemented with 2% NaCl and incubated under aerobic conditions at 37°C for 18 hours. The purified colonies were streaked onto sterile TSA slant agar supplemented with 2% w/v sodium chloride (NaCl) (Vivantis, United States) and stored until further molecular identification.

2.4. DNA extraction

The extraction of genomic DNA was done according to the instructions of GF-1 bacterial DNA Extraction Kit (Catalog No. BA-100, Vivantis, Malaysia). One loopful of isolates was picked from the TSA slants and inoculated into Tryptone Soy Broth (TSB) supplemented with 2% w/v sodium chloride (NaCl) (Vivantis, United States) and incubated at 37°C at 220 rpm for 18 hours. 1-3 mL of the overnight culture was centrifuged at 6,000g for 2 minutes and the supernatant was discarded. The cells were resuspended with 100µl of Buffer, centrifuged at 10,000g for 3 minutes and the supernatant was discarded. The pellet

was resuspended in 180µl of Buffer R2 and 20µl Proteinase K, then incubated at 65°C for 20 minutes in a water bath with mixing every 5 minutes. After incubation, 400µl of Buffer BG was added and the tube was inverted several times to achieve a homogenized solution. The tubes were subsequently incubated at 65°C for 10 minutes. 200µl of absolute ethanol was added with immediate mixing to prevent precipitation of DNA and transferred (maximum volume 650µl) into a clean column and centrifuged at 10,000g for 1 minute. The flow through was discarded and 650µl of wash buffer was added to the column and centrifuged at 10,000g for 1 minute in which the flow through was discarded. The column was centrifuged again at 10,000g for 1 minute to remove residual ethanol. The column was then placed into a clean microcentrifuge tube and 100µl of sterile water was added, and the tube was left to stand for 2 minutes. The DNA was then eluted via centrifugation at 10,000g for 1 minute. The DNA was then stored for further analysis at 4°C.

2.5. Molecular identification of Vibrio parahaemolyticus using toxR-based polymerase chain reaction

The *toxR*-based polymerase chain reaction (PCR) assay was performed to identify *V. parahaemolyticus* from all the presumptive isolates by using the primers: *toxR*-F (5′-GTC TTC TGA CGC AAT CGT TG-3′) and *toxR*-R (5'-ATA CGA GTG GTT GCT GTC ATG-3′). The expected amplicon size is 368 bp [76]. A final volume of 20µl for the reaction mixture was prepared with 1µl of DNA template, 10µl of 2x *Taq PLUS* PCR Smart mix 1 (SolGent™, Korea), 7µl of sterile distilled water and 1µl of each primer. The *toxR*-based PCR amplification was performed using a PCR thermocycler (Kyratec, SuperCycler Thermal Cycler, Australia) with the cycling conditions: initial denaturation at 95°C for 4 minutes, 35 cycles of 94°C for 1 minute, 68°C for 1 minute and 72°C for 30 seconds, and a final elongation step at 72°C for 5 minutes. The PCR products were then separated in 1.5% agarose gel and visualized under a gel documentation system (ChemiDoc™ XRS, Bio-Rad, USA).

2.6. Genomic and Phylogenetic Analyses

Polymerase chain reaction amplification of the 16S rRNA gene of all the *toxR*-positive *V. parahaemolyticus* was performed following the protocol described by Thomas et a. with slight modifications [77]. The 16S rRNA gene sequence of each isolate was aligned with representative sequences of related type strains of *V. parahaemolyticus* retrieved from the GenBank database using ClustalW software [78]. Manual verification and adjustment of the alignment were made, followed by the utilization of MEGA11 [79] to construct phylogenetic trees with neighbor-joining [80] and maximum-likelihood [81] algorithms. In the neighbor-joining algorithm, the evolutionary distances were computed using Kimura's two-parameter model [82]. The GenBank server was used to calculation the level of sequence similarity and bootstrap based on 1,000 re-sampling method by Felsentein [83] was used to analyze the stability of the resultant tree topologies.

2.7. Antibiotic susceptibility testing

Fourteen antibiotic discs (Oxoid, UK) infused with amikacin (30µg), ampicillin (10g), ampicillin/sulbactam (30µg), cefotaxime (30µg), ceftazidime (30µg), chloramphenicol (30µg), gentamicin (30µg), imipenem (10µg), kanamycin (30µg), levofloxacin (5µg), nalidixic acid (30µg), oxytetracycline (30µg), sulfamethoxazole/trimethoprim (25µg), and tetracycline (30µg) were used to determine the antibiotic susceptibility of the identified *V. parahaemolyticus* isolates via the Kirby-Bauer disk diffusion method [84].

The antibiotic discs were dispensed on Mueller Hinton agar (HiMedia, India) (MHA) plates supplemented with 2% w/v NaCl (Vivantis, United States) with bacterial lawn and incubated at 37°C for 18 hours. After incubation, the inhibition zones were measured and interpreted according to the guidelines from Clinical and Laboratory Standard Institute (CLSI) (2010) M45-A2 [85]. The multiple antibiotic resistance (MAR) index was calculated using the formula first developed by Krumperman in 1983 [86]. The ratio between the number of antibiotics that an isolate is resistant to and the total number of antibiotics the organism was exposed to is represented by the MAR index.

$$\textbf{Multiple antibiotic resistance } (\textbf{MAR}) = \frac{\textbf{Number of antibiotics that an isolate is resistant to}}{\textbf{Total number of antibiotics the isolate is exposed to}}$$

2.8. Evaluation of anti-Vibrio activity of Streptomyces sp. extract

The *V. parahaemolyticus* strain chosen for the cross-streak assay was based on its antibiotic resistance profile and the neighbor-joining phylogenetic tree. RP0132 was chosen for the evaluation of anti-*Vibrio* effects of *Streptomyces* sp. as the strain was multidrug resistant with MAR of 0.36, and it is from a distinct clade in the neighbor-joining phylogenetic tree with a bootstrap value of 75%. Ten presumptive *Streptomyces* sp. isolates used in the cross-streak assay were previously isolated from the soils of the mangrove forest of Sarawak.

2.8.1. Maintenance and growth condition of V. parahaemolyticus (RP0132)

The RP0132 isolate was grown in tryptic soy broth (TSB) (HiMedia, India) 2% w/v sodium chloride (NaCl) (Vivantis, United States) at 37◦C for 18 h under constant agitation.

2.8.2. Maintenance and growth condition of Streptomyces sp.

The *Streptomyces* sp. isolates were maintained in the International *Streptomyces* Project-2 Medium (ISP2) slants. They were inoculated into TSB and incubated at 28°C, with constant shaking at 200 rpm for 7 days [87].

2.8.3. Cross-streak assay

The cross-streak assay was adapted from Yoshida et al. [88] with slight modifications. Each *Streptomyces* sp. was streaked onto MHA plates supplemented with 2% NaCl and incubated at 28°C for 7 days. After incubation, a loopful of RP0132 grown in TSB supplemented with 2% w/v sodium chloride NaCl was streaked perpendicularly to the line of *Streptomyces* isolate growth. Antagonism was observed based on the inhibitory interaction between the *Streptomyces* isolate and RP0132 after an overnight incubation.

2.8.4. Molecular identification of Streptomyces sp. MUM 178J based on 16S rRNA gene

DNA extraction and PCR amplification of the 16S rRNA gene for *Streptomyces* sp. MUM 178J was performed [89, 90]. The sequence similarities between the acquired sequence and its related type strains were determined via BLAST search on the EzBioCloud database (http://www.ezbiocloud.net/).

2.8.5. Preparation of Streptomyces MUM 178J extract

MUM 178J was grown in TSB at 28°C at 200rpm for 14 days to be used as a seed medium for fermentation. Fermentation was done in a 500mL Erlenmeyer flask with 200mL of sterilized Han's Fermentation Media (HFM1) (BioMerge, Malaysia) inoculated with 200µl of seed medium. The fermentation medium was cultured at 28°C, 200 rpm, for 10 days. Upon completion, the medium was centrifuged at 12000g for 15 minutes, and the supernatant was filtered and collected. The clear filtrate was subjected to freeze-drying, then the freeze-dried samples were extracted using methanol for 72 hours [91]. Extraction with methanol was repeated twice under the same conditions at 24-hour intervals. The methanol-containing extract was collected and concentrated by using a rotary vacuum evaporator at 40°C to remove the extracting solvent [92]. The final extract was suspended in sterile ultrapure water and stored at -4°C.

2.8.6. Measurement of minimum inhibitory concentration (MIC) for MUM 178J extract

The MIC of MUM 178J extract was determined using a standard serial dilution method in TSB supplemented with 2% NaCl. 50µL of the MUM 178J extract was added to each well in a microtiter plate, followed by 50µL of RP0132 to give a final inoculum of 10^6 colony-forming units/mL. The final testing concentrations of MUM 178J extract were 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 mg/mL. The microtiter plate was then incubated at 37°C for 18 hours, and the turbidity of the individual wells was observed. The absorbance value of each well was also measured at 600 nm to confirm the results. The MIC was determined at the lowest concentration of MUM 178J where growth of RP0132 was inhibited [93, 94]. Positive controls of 50µL florfenicol at concentrations of 8, 4, and 2µg/mL followed by 50µL of RP0132 were also prepared to validate the results further, while wells acting as negative controls contained RP0132 only.

2.8.7. Measurement of minimum bactericidal concentration (MBC) for MUM 178J extract

After measuring the MIC, aliquots of 100µL from the wells without bacterial growth were streaked onto TSA supplemented with 2% w/v sodium chloride NaCl and incubated at 37°C for 18 hours. The MBC was determined at the lowest concentration when no bacterial growth was observed on the agar plates after incubation.

2.9. Statistical analysis

Data analysis was performed with SPSS statistical analysis software version 29. The statistical analysis was performed to determine whether there was any significant difference between the two types of seafood samples (crustaceans and bivalves) and the MAR index of resistant isolates using the independent t-test. The significance level was set at $p \leq 0.05$. One-way analysis of variance (ANOVA) followed by *post* hoc test (Tukey) was performed to determine the significant difference between the four types of seafood samples which were positive for MDR *V. parahaemolyticus* (white prawn, speckled shrimp, blood clam, flower crab) and the MAR index of resistant isolates. A difference was considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Prevalence of Vibrio parahaemolyticus in seafood

In the present study, *V. parahaemolyticus* was isolated from 10 seafood samples comprised of white prawns (*Fenneropenaeus indicus*), speckled shrimps (*Metapenaeus monoceros*), venus clams (*Paratapes textilis*), blood clams (*Tegillarca granosa*), and flower crabs (*Portunus pelagicus*). One hundred presumptive isolates were isolated from HiChrome Vibrio agar based on colony morphology. Out of the 100 presumptive isolates, 25% (25/100) were isolated from white prawns, 18% (18/100) were from speckled shrimps, 36% (36/100) were isolated from blood clams, 7% (7/100) were from venus clams, and 14% (14/100) of the presumptive *V. parahaemolyticus* were isolated from flower crabs. The *toxR*-PCR assay demonstrated positive amplification of the *toxR* gene of 368 bp amplicon bands in 75% (75/100) of the presumptive *V. parahaemolyticus* isolates (Figure 1). 22.7% (17/75) of the isolates from white prawns, 18.7% (14/75) of isolates from speckled shrimps, 42.7% (32/75) of isolates from blood clams, 4% (3/75) isolates from venus clams, and 12% (9/75) of isolates from flower crabs were *toxR* positive.

3.2. Genomic and phylogenetic analyses

All 75 *toxR*-positive *V. parahaemolyticus* strains were positive for the 16S rRNA gene, but upon alignment with representative sequences of related type strains of *V. parahaemolyticus* from GenBank, 64 isolates were confirmed to be *V. parahaemolyticus*. The phylogenetic tree was constructed based on these 64 strains and the analysis showed that

closely related strains include RP0132 (OQ540693), P0151 (OQ540591), K0231 (OQ533122), K01311 (OQ533125), and K0139 (OQ533047). The isolates are separated into five major clades (A to E) and isolates within the same clade of the phylogenetic tree are closely related (Figure 2).

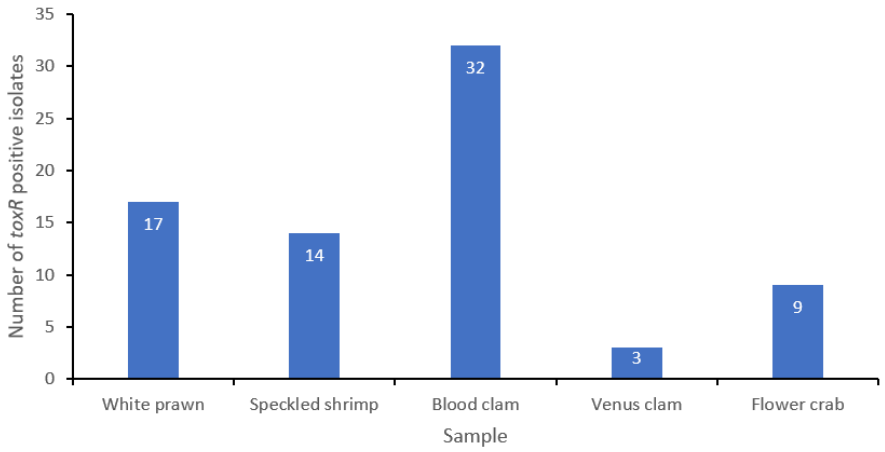

Figure 1. The total number of *toxR* positive *V. parahaemolyticus* isolates from each seafood sample.

3.3. Antimicrobial susceptibility of V. parahaemolyticus isolates

The commonly used antibiotics in aquaculture and the treatment of *V. parahaemolyticus* infections were included in this study [55, 95]. The antimicrobials are aminoglycosides (amikacin, gentamicin, kanamycin), amphenicols (chloramphenicol), carbapenems (imipenem), cephalosporins (ceftazidime and cefotaxime), fluoroquinolones (levofloxacin), folate pathway inhibitors (sulfamethoxazole/trimethoprim), penicillins (ampicillin and ampicillin/sulbactam), quinolone (nalidixic acid), and tetracyclines (oxytetracycline and tetracycline). The antibiotic resistance of the 64 confirmed strains of *V. parahaemolyticus* is summarized in Table 1.

The antibiotic susceptibility test found that 98.4% (63/64) of the *V. parahaemolyticus* isolates were resistant to ampicillin, the highest resistance recorded in the current study. This is followed by resistance towards cephalosporins, with 53.1% (34/64) for ceftazidime and 39.1% (25/64) for cefotaxime. Interestingly, 23 out of 64 (35.9%) isolates were resistant to the antimicrobial combination of ampicillin and sulbactam. Among the *V. parahaemolyticus* isolates, only 10.9% (7/64) were resistant towards the carbapenem, imipenem, and aminoglycoside, kanamycin, respectively. The study found that the *V. parahaemolyticus* isolates were highly susceptible to nalidixic acid (98.4%), tetracycline (93.8%), sulfamethoxazole/trimethoprim (90.6%), and shared similar susceptibilities (89.1%) to

gentamicin, levofloxacin, and oxytetracycline. This is followed by their susceptibility to chloramphenicol with 87.5% (56/64).

The antibiograms and MAR indices for each of the *V. parahaemolyticus* isolates are summarized in Table 2. The MAR index for the isolates ranged from 0.07 to 0.57, with more than half (53.1%) of the isolates with a MAR index greater than 0.2, indicating these isolates were from a high-risk source of contamination where multiple antibiotics were used (Figure 3) [96]. 81% of the isolates had a MAR index over 0.07, meaning that 52 out of 64 isolates were resistant to more than one antibiotic, making the majority of the isolates MDR *V. parahaemolyticus*.

Table 1. Antibiotic resistance of *V. parahaemolyticus* isolates.

Antibiotics	Resistant (%)	Intermediate (%)	Sensitive (%)
Penicillin			
Ampicillin (10μg)	63 (98.4)	0 (0)	1 (1.6)
Ampicillin/sulbactam (30μg)	23 (35.9)	11 (17.2)	30 (46.9)
Cephalosporin			
Ceftazidime (30μg)	34 (53.1)	22 (34.4)	8 (12.5)
Cefotaxime (30μg)	25 (39.1)	27 (42.2)	12 (18.8)
Carbapenem			
Imipenem (10μg)	7 (10.9)	2 (3.1)	55 (85.9)
Aminoglycoside			
Amikacin (30μg)	5 (7.8)	16 (25)	43 (67.2)
Gentamicin (30μg)	4 (6.3)	3 (4.7)	57 (89.1)
Kanamycin (30μg)	7 (10.9)	43 (67.2)	14 (21.9)
Amphenicol			
Chloramphenicol (30μg)	6 (9.4)	2 (3.1)	56 (87.5)
Folate pathway inhibitors			
Sulfamethoxazole/trimethoprim (25μg)	4 (6.3)	2 (3.1)	58 (90.6)
Tetracyclines			
Oxytetracycline (30μg)	2 (3.1)	5 (7.8)	57 (89.1)
Tetracycline (30μg)	4 (6.3)	0 (0)	60 (93.8)
Fluoroquinolone			
Levofloxacin (5μg)	2 (3.1)	5 (7.8)	57 (89.1)
Quinolone			
Nalidixic acid (30μg)	0	1 (1.6)	63 (98.4)

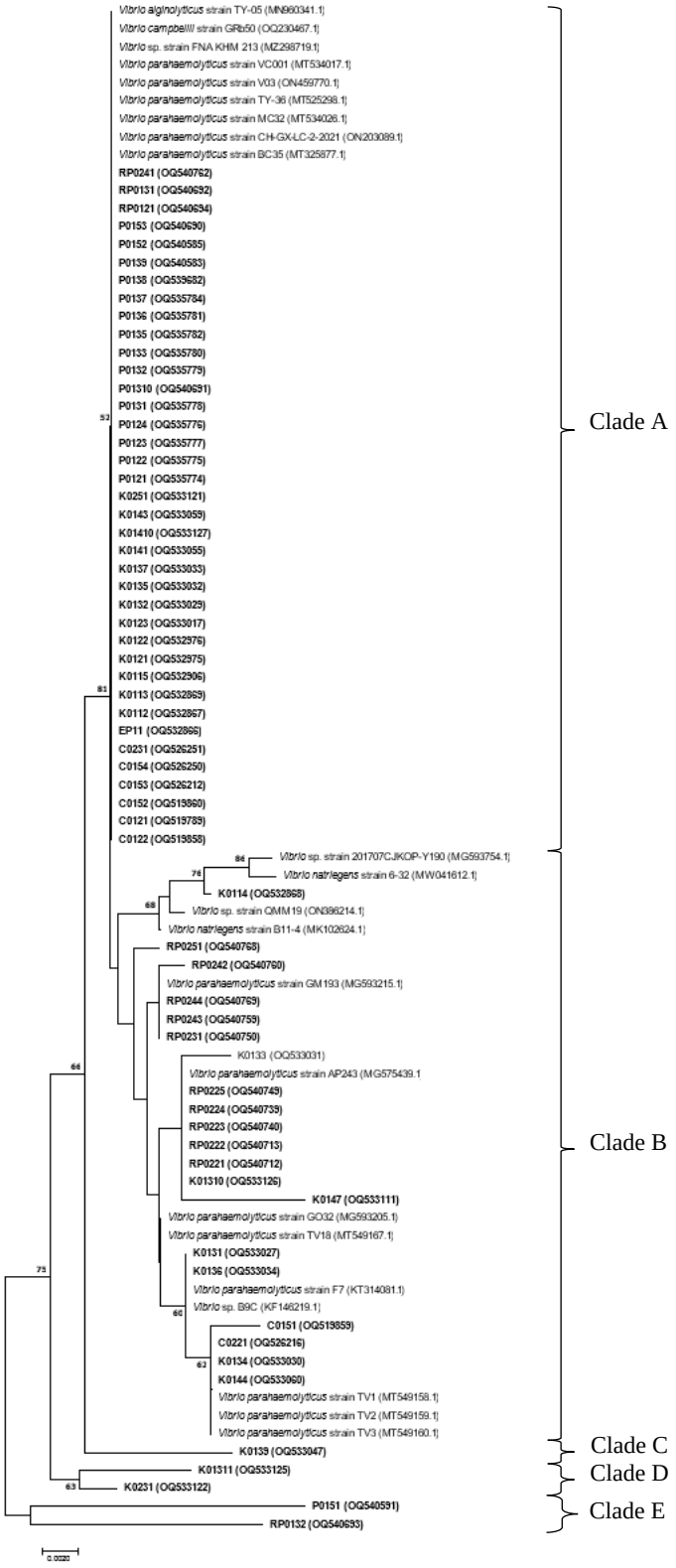

Figure 2. Neighbor-joining phylogenetic tree based on 823 nucleotides of 16S rRNA gene sequence showing the relationship between a total of 85 *Vibrio* strains and representatives of related taxa. Number and nodes indicate percentages (>50%) of 1000 bootstrap re-sampling. Bar: 0.002 substitutions per site.

The statistical analysis done using an independent t-test revealed no significant difference between the type of seafood samples (crustaceans and bivalves) and the MAR index of the resistant isolates. It was found that there was a greater diversity of MAR indices in the resistant isolates from crustaceans compared to bivalves. In addition, the one-way ANOVA analysis indicated no significant difference between the four types of seafood samples which were positive for AMR *V. parahaemolyticus* and the MAR index of the resistant isolates. However, *post-hoc* test (Tukey) showed that resistant isolates from white prawns and their MAR index were significantly different from other seafood samples. The higher MAR indices from resistant isolates in white prawns could have been due to a higher antibiotic exposure compared to other seafood sources.

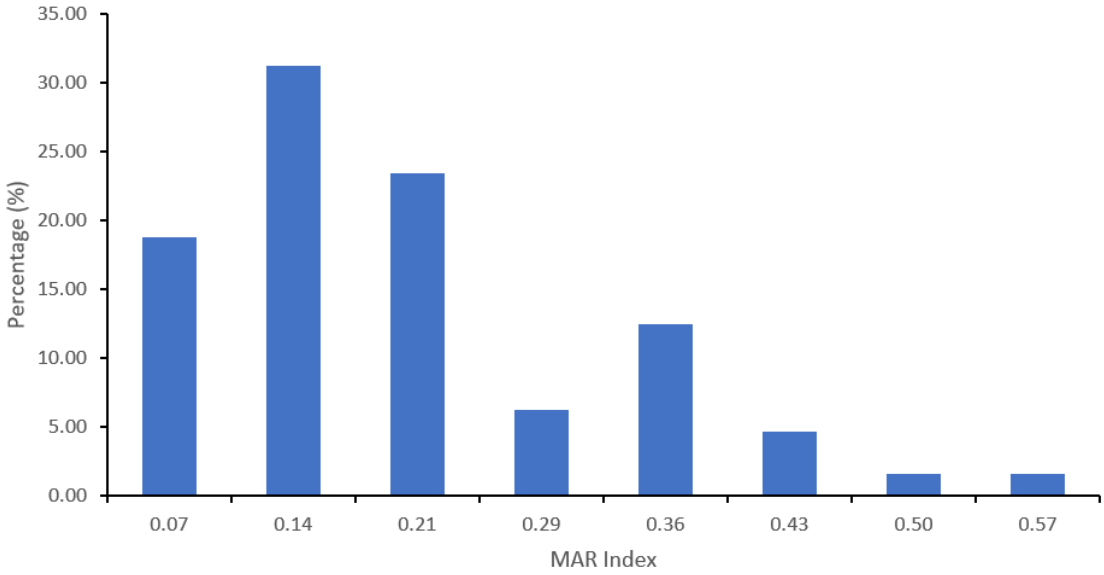

Figure 3. Percentage occurrence of MAR index (0.07-0.57) of *V. parahaemolyticus* isolates from all seafood samples.

Table 2. Antibiogram of the 64 confirmed *V. parahaemolyticus*.

Antibiogram*	**Strain**	**Total antibiotic resistance**	**MAR index**
AMP/CAZ/CTX/CN/K/LEX/SAM/TE	P0139	8	0.57
AMP/AK/C/CAZ/CN/CTX/ SAM	P0137	7	0.50
AMP/AK/CAZ/CN/CTX/ SAM	P0121	6	0.43
AMP/AK/CAZ/CTX/K/SAM	P01310	6	0.43
AMP/C/CAZ/CTX/IMI/SAM	P0135	6	0.43
AMP/AK/CTX/K/SAM	RP0251	5	0.36
AMP/CAZ/CTX/K/IMI	K0136	5	0.36
AMP/CAZ/C/CTX/SAM	K0151	5	0.36
AMP/CAZ/CN/CTX/SAM	RP0132	5	0.36
AMP/CAZ/CTX/K/SAM	P0122, P0138	5	0.36
AMP/CAZ/CTX/SAM/TE	P0124	5	0.36
AMP/CAZ/CTX/SAM/SXT	P0132	5	0.36
AMP/AK/CTX/SAM	K0139	4	0.29
AMP/CAZ/CTX/OT	K0123	4	0.29
AMP/CAZ/CTX/SAM	RP0241	4	0.29
AMP/CTX/K/SAM	P0133	4	0.29
AMP/CAZ/IMI	K0133	3	0.21
AMP/CAZ/LEV	K0121	3	0.21
AMP/CAZ/OT	K0114	3	0.21
AMP/CAZ/TE	K0144	3	0.21
AMP/CAZ/CTX	P0131, P0211, K01310, K0143, K01410	3	0.21
AMP/CAZ/SAM	RP0131, K0112, C0154	3	0.21
AMP/IMI/SAM	K0131	3	0.21
AMP/CTX/SAM	P0151	3	0.21
AMP/SAM/SXT	C0122	3	0.21
AMP/C	RP0243, RP0221, C0152	2	0.14

Antibiogram*	Strain	Total antibiotic resistance	MAR index
AMP/CAZ	P0152, K0113, K0134, K0137, K01311, K0251, C0121	2	0.14
AMP/IMI	RP0242, RP0244, K0115	2	0.14
AMP/TE	C0231	2	0.14
AMP/CTX	K0135	2	0.14
CAZ/CTX	K0141	2	0.14
AMP/SAM	P0153, K0132	2	0.14
AMP/SXT	RP0224, C0151	2	0.14
AMP	P0123, P0136, RP0121, RP0222, RP0223, RP0225, RP0231, K0122, K0147, K0231, C0153, C0221	1	0.07

*Amikacin: AK, Ampicillin: AMP, Ampicillin/sulbactam: SAM, Cefotaxime: CTX, Ceftazidime: CAZ, Chloramphenicol: C, Gentamicin: CN, Imipenem: IMI, Kanamycin: K, Levofloxacin: LEV, Nalidixic acid: NA, Oxytetracycline: OT, Sulfamethoxazole/trimethoprim: SXT, Tetracycline: TE.

3.4. Evaluation of anti-Vibrio activity of Streptomyces sp. extract

Based on the antibiotic susceptibility test results and the neighbor-joining (NJ) phylogenetic tree generated based on the 16S rRNA sequence of the *V. parahaemolyticus* strains, RP0132 was chosen for the cross-streak assay. RP0132 was found to be resistant to five types of antibiotics, namely ampicillin, ampicillin/sulbactam, cefotaxime, ceftazidime, and gentamicin, and it had a MAR index of 0.36 (Table 2). In addition, RP0132 appeared to be in a distinct phylogenetic clade with a bootstrap value of 75% (Figure 2). Out of the ten presumptive *Streptomyces* isolates chosen for the cross-streak assay in the current study, MUM 178J elicited the strongest antibacterial effects on RP0132, where the growth of the latter was completely inhibited. The BLAST results based on 16S rRNA gene from EzBioCloud show that MUM 178J indeed belonged to the *Streptomyces* genus as it showed 98.69% sequence similarity to *Streptomyces fragilis* NBRC 12862^{T}. Following that was the evaluation of the anti-*Vibrio* effects of the crude extract of MUM 178J by measuring its MIC and MBC against RP0132. Based on visual observation of the microplate, the wells supplemented with 12.5, 25, and 50mg/mL of MUM 178J extract and all the positive control wells containing florfenicol were clear to the naked eye, indicating no bacterial growth. Further confirmation was done using a microplate reader at 600nm, which validated the results. For the MBC determination, no bacterial growth was observed on the agar plate spread with RP0132, which was treated with 50mg/mL of MUM 178J extract. In summary, the MIC and MBC of MUM 178J extract are measured at 12.5mg/mL and 50mg/mL, respectively.

4. Discussion

There is an abundance of *V. parahaemolyticus* in aquatic animals, evident from the frequent reports of their detection in various types of seafood worldwide [97-102]. The findings from this study align with those from the literature whereby all the samples were positive for *V. parahaemolyticus*. The accumulation of *V. parahaemolyticus* in the crustaceans in this study, such as white prawns, red shrimps, and flower crabs, is inevitable as the bacterium is ubiquitous in aquatic environments. This impacts human health if the pathogen is transmitted to humans during consumption and heavily impacts the shrimp farming industry. In 2020, 11.2 million tonnes of crustaceans valued at USD 81.5 billion were produced, and marine shrimp currently dominate the production of crustaceans. They are an important source of foreign exchange earnings for developing countries in Asia and Latin America [2]. *V. parahaemolyticus* can cause acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS), in shrimp. AHPND causes severe atrophy of the shrimp hepatopancreas, and the disease progresses to sloughing of the digestive tract epithelial cells [103, 104]. AHPND affects the early stages of the shrimp life cycle, whereby it affects shrimp postlarvae 20-30 days after stocking and can cause up to 100% mortality [105]. The very first AHPND outbreak was recorded in China in 2009, and it quickly spread to multiple countries such as Thailand [106], Malaysia [107], Philippines [108], Mexico[109], the United States of America [110, 111], and South America [112]. AHPND outbreaks have been reported to cause a collective loss of approximately USD 43 billion in shrimp aquaculture across China, Malaysia, Thailand, Vietnam, and Mexico [113]. The molluscan bivalves included in this study (blood clams and venus clams) are filter feeders that filter large volumes of water in their surroundings for sustenance [114]. During the feeding process, there will be a natural bioaccumulation of pathogenic organisms such as *V. parahaemolyticus* in these bivalves which can harbour unsafe levels of bacterial load to cause disease upon consumption. In 2020, the total harvest of 17.7 million tonnes of molluscs that was mostly bivalves amassed a total of USD 29.8 billion [2]. The huge supply of bivalves to the global market also potentially increases the spread of pathogenic *V. parahaemolyticus* via international trade of seafood products.

As seafood consumption is steadily increasing worldwide, with a projected increase of 15% from 20.2kg per capita in 2020 to an average of 21.4kg per capita in 2030 [2], the increasing prevalence of *V. parahaemolyticus* is worrying. Moreover, the ever-increasing global demand for food and climate change are the driving forces in the expansion of fisheries [26]. Compared to terrestrial food production, seafood has the advantage of being nutritionally diverse and having a lower carbon footprint, making them the more sustainable option as a

food source [115]. Therefore, the constant detection of *V. parahaemolyticus* in seafood threatens global food safety and public health as the population relies on seafood as a food source. In addition, consuming raw seafood is common in numerous cultures, such as *sashimi* from Japan, *hoe* from Korea, *ceviche* from Peru, *poke* from Hawaii, and *stroganina* from the indigenous people in northern Russia. The consumption of raw or not thoroughly cooked seafood increases the risk of exposure to *V. parahaemolyticus*, thereby increasing the chances of developing gastroenteritis. Hence, consuming seafood that has been cooked thoroughly is strongly advised to reduce the risk of *V. parahaemolyticus* infections to maintain gastrointestinal health [116]. The contamination of the foodborne pathogen in seafood meant to be eaten raw or cooked can be minimised with the enforcement of sanitation and food safety regulations by authorities in seafood processing plants [117]. The continuous and extensive surveillance of these seafood products to ensure their conformance to regulations can instil confidence in the consumers regarding their food safety. As consumers, it is important to purchase high-quality seafood from reputable sources and to implement safe handling practices when handling seafood. For example, separating seafood from other meats or fresh produce is important to prevent cross-contamination when purchasing and storing seafood. By processing and handling seafood with these considerations in mind, the risk of *V. parahaemolyticus* contamination can be greatly reduced.

The presence of *V. parahaemolyticus* in aquaculture is a major concern as it can seriously impact harvest and production, leading to huge economic losses. From a public health perspective, *V. parahaemolyticus* is a major foodborne bacterium that causes gastroenteritis outbreaks associated with consuming these crustaceans and molluscan bivalves [118-120]. Hence, it is crucial to constantly monitor the prevalence of *V. parahaemolyticus* in seafood to preserve food safety and minimise the risk of gastroenteritis outbreaks. Furthermore, there is an emergence of MDR *V. parahaemolyticus* mainly attributable to the over- and misuse of antibiotics in the aquaculture industry [121]. Antimicrobial residues can also be introduced into the aquatic environments via the wastewater from aquaculture or livestock, hospital effluents, or sewage discharge [122-124]. The environmental pressure produces MDR *V. parahaemolyticus* that can survive and propagate to populate the water bodies. Another vital issue is that MDR *V. parahaemolyticus* can transfer their resistance genes either inter- or intraspecies, which further increases antibiotic resistance in the ecosystem. This threatens global public health as infections become more difficult to treat, and managing outbreaks will be increasingly challenging. The findings from the current study showed that the *V. parahaemolyticus* isolates were resistant

to at least one out of 14 antimicrobial agents from the following classes of antibiotics: penicillin, cephalosporin, carbapenem, aminoglycosides, amphenicol, folate pathway inhibitors, tetracyclines, and fluoroquinolones.

Penicillin is a class of antibacterial agents that was a serendipitous discovery by Alexander Fleming in 1928 and is widely used as a first-line agent in empirical therapy of bacterial infections [125, 126]. They are β-lactams that disrupt the formation of peptidoglycan, a major component of bacterial cell walls. Without the structural integrity of the cell wall, the bacterial cell lyse and the infection is effectively managed with minimal side effects [127]. Among the tested antimicrobials, ampicillin and ampicillin/sulbactam fall under this category. Antibiotic resistance towards penicillin has been amply documented over the years [49, 52, 128, 129] and the current study also demonstrated that 98.4% of the isolates were resistant to ampicillin. The high levels of antibiotic resistance towards ampicillin jeopardise the role of ampicillin as a first-line therapy for bacterial infections, but ampicillin is still being used in aquaculture [130]. 35.9% of the isolates in this study were resistant to ampicillin/sulbactam, which is a combination of a β-lactam and sulbactam as a β-lactamase inhibitor. Sulbactam prevents ampicillin degradation by the β-lactamase enzyme in bacterial cells, allowing ampicillin to elicit its therapeutic effect [131]. The development of resistance towards this combination medication will negatively impact its efficacy in treating infections. To add, the *V. parahaemolyticus* isolates from this study have also shown resistance to ceftazidime (53.1%) and cefotaxime (39.1%), which are β-lactams known as third-generation cephalosporins. Compared to their predecessors, they are known to have better stability than common β-lactamases produced by Gram-negative bacilli. They have a broad spectrum of activity and are well-tolerated; thence, they are commonly prescribed as first-line therapy in bacterial infections [132]. Antibiotic resistance towards third-generation cephalosporins [133, 134] is concerning as they are heavily used as a first-line agent to treat an array of bacterial infections in hospitalized patients before susceptibility profiles of the pathogenic organisms are confirmed [132].

Besides, 10.9% of the *V. parahaemolyticus* isolates from this study were resistant to imipenem, belonging to the class of carbapenems, a newer class of β-lactam antibiotics. Imipenem has a broader antibacterial spectrum and potency than the traditional β-lactams, making them suitable for treating many bacterial infections [135]. It also has been indicated in severe infections, including mixed infections involving MDR pathogens [136]. The resistance of *V. parahaemolyticus* towards this carbapenem could be due to their expression of carbapenemase, which reduces the effectiveness of the drug. In 1993, carbapenemase was

first detected in Enterobacteriaceae isolated from the United States [137], and this form of antibiotic resistance has spread to different species of bacteria, including *V. parahaemolyticus* worldwide [138, 139]. If the resistance continues to spread rampantly, the therapeutic effects of imipenem and other carbapenems could be greatly diminished, causing a rise in untreated severe infections that lead to a surge in mortality rates. On another note, the antimicrobial susceptibility testing showed that the MDR *V. parahaemolyticus* had intermediate results towards amikacin (25%) and kanamycin (67.2%), which are aminoglycosides. This suggests that the dose of these medications will require adjustments during administration as the efficacy of the drug is compromised [140]. Aminoglycosides are highly effective against a broad range of Gram-negative bacteria as they have both bacteriostatic and bactericidal activity by inhibiting polypeptide synthesis in the target. However, resistance towards aminoglycosides may increase the dosage required and prolong the course of treatment for full recovery. This puts the patient at a higher risk of experiencing side effects such as nephrotoxicity and ototoxicity, which have been notoriously linked to the use of aminoglycosides [141]. Given these negative effects, it is vital to control the spread of antibiotic resistance to alleviate the burden on public health systems.

Findings from the current study show that the *V. parahaemolyticus* isolates are highly susceptible to chloramphenicol, gentamicin, levofloxacin, nalidixic acid, oxytetracycline, sulfamethoxazole/trimethoprim, and tetracycline. Although this is the case, if the spread of antibiotic resistance is not adequately managed, the current trajectory of the rise in antibiotic resistance in *V. parahaemolyticus* will lead to total ineffectiveness of the antibiotics. As of 2021, the World Health Organization (WHO) recorded a total of 258 antibiotics that are available for use, but the emergence of MDR bacteria will reduce the effectiveness of these drugs in treating diseases [142-145]. Lujiwa et al. revealed that from 2008-2018, the top 15 major producers in aquaculture were using 67 different antibiotics, including previously banned antibiotics such as chloramphenicol, rifamycin, and penicillin [130]. This accounts for more than 25% of the available antibiotics, an alarming statistic given the increasing prevalence of MDR pathogens, including *V. parahaemolyticus* [146-149]. The outcomes of this study also showed that MDR *V. parahaemolyticus* is plentiful in seafood, where 81% of the isolates were resistant to more than one antibiotic, giving them an MAR index greater than 0.02. This further indicates that the environment which the foodborne bacteria reside has been highly contaminated by multiple antibiotics. The rise in MDR *V. parahaemolyticus* will eventually lead to the spread of antibiotic resistance to other species of pathogens, thereby causing a butterfly effect where bacterial infections become more difficult to treat,

endangering the lives of those infected. The future of treating infectious diseases and outbreaks is at stake, given the dangerously high levels of antibiotic resistance which continue to rise [150]. Therefore, policymakers and the authorities need to implement strict rules and regulations on the antimicrobial agents used in aquaculture. The choice of drugs should be decided based on the specific region's most recent data on MDR *V. parahaemolyticus* and other MDR pathogens that are relevant in aquaculture. This is to manage infections of marine animals effectively and to minimise their impact in the development of antibiotic resistance. Following the implementation of stricter policies, the use of antimicrobial agents in aquaculture should be monitored under continued vigilance to ensure compliance.

Moreover, utilising alternatives to antibiotics to prevent and manage diseases is imperative in controlling the spread of antibiotic resistance. With that, researchers have been actively searching for new antimicrobial compounds from natural products derived from various bioresources, including plants [151-153], animals [154] and microorganisms [155, 156]. Within the microbial world, *Streptomyces* sp. is a group of filamentous Gram-positive mycelial *Actinobacteria* that are well-established candidates with antimicrobial, antioxidant, anticancer and immunosuppressant effects [157-164]. Their ability to produce a myriad of compounds piques the interest of researchers to identify valuable compounds that could hinder the growth of *Vibrios* [143, 165-167]. In the cross-streak assay, *Streptomyces* MUM 178J demonstrated antagonistic effects against MDR *V. parahaemolyticus*, RP0132. Subsequent examination of the MIC and MBC of MUM 178J crude extract also validated the results from the cross-streak assay. The MIC was determined to be 12.5mg/mL, while the MBC was 50mg/mL. This shows that MUM 178J may possess the ability to produce bioactive compounds with anti-*Vibrio* activity which inhibits the growth of Gram-negative pathogens like *V. parahaemolyticus*. Hence, MUM 178J could potentially be utilised as an anti-*Vibrio* agent. In addition, there have been studies which report on the anti-*Vibrio* activity of crude extract from *Streptomyces* sp. For instance, Thirumurugan and Vijayakumar found that the crude extract from *Streptomyces* sp. ECR77 was able to inhibit the growth of *V. parahaemolyticus* [168]. Kumaran *et al.* also determined that the extract from *Streptomyces enissocaesilis* SSASC10 was able to inhibit the growth of *V. parahaemolyticus*. Furthermore, Rateb *et al.* [169] was able to determine the presence of chaxalactins in *Streptomyces* sp. C34 extract which conferred antibacterial activity against *V. parahaemolyticus*. Therefore, the mechanisms of the antagonistic activity of MUM 178J will require more in-depth analysis and further *in-vivo* studies involving animal models need to be executed to ensure its safety,

efficacy, and sustainability to be used in aquaculture. Nevertheless, studies into multiple strains of *Streptomyces* sp. have displayed antibacterial effects against *V. parahaemolyticus*. An anti-*V. parahaemolyticus* compound, Actinomycin D was discovered in *Streptomyces parvus* during molecular docking studies done by Liu *et al.* [165]. Actinomycin D was proposed to bind strongly to the flagellar motor switch protein FliN of *V. parahaemolyticus*, thereby impairing its motility and preventing biofilm formation [165]. You *et al.* [166] isolated several *Streptomyces* sp. from nearshore marine sediments, demonstrating antagonistic effects against *V. parahaemolyticus*. In marine environments, iron supply is limited; hence *Streptomyces* sp. needs to compete with other marine bacteria to acquire iron for growth. As a means to adapt, *Streptomyces* sp. produces siderophores, compounds with low molecular weight that have a high affinity for iron [170]. The production of siderophores depletes the iron supply for other bacteria, such as *V. parahaemolyticus*, ultimately resulting in cell death.

5. Conclusion

As shown in the current study, *V. parahaemolyticus* remains prevalent in seafood and they were most likely exposed to the pathogen before harvest. The prevalent nature of *V. parahaemolyticus* in our surrounding environment is a concern, as this can result in gastroenteritis outbreaks, thereby compromising the integrity of our public health systems. This situation is further worsened by the surge in detection of MDR *V. parahaemolyticus*, where the antibiotics that were effective in treating *V. parahaemolyticus* infections are no longer useful. Most notably, *V. parahaemolyticus* has been found to be resistant towards carbapenems, the last line of antibiotics used to treat bacterial infections. This is corroborated by the findings of the current study, whereby carbapenem-resistant *V. parahaemolyticus* isolates were detected from seafood samples. In an effort to explore potential agents that have anti-*Vibrio* properties, this study tested the antagonistic effects of *Streptomyces* sp. MUM 178J against an MDR *V. parahaemolyticus*, RP0132 from a distinct clade. It was found that the MUM 178J bacterial isolates were able to inhibit the growth of RP0132 in the cross-streak assay. Additionally, continued probing on the antagonistic effects of MUM 178J was done by using its crude extract to test against RP0132, and it was discovered that MUM 178J extract had an MIC of 12.5mg/mL and MBC of 50mg/mL. This shows that *Streptomyces* sp. MUM 178J could be potentially utilized as an anti-*Vibrio* agent which could be employed in disease control in aquaculture. In summary, this study found 64 confirmed *V. parahaemolyticus* strains out of 100 presumptive isolates. All 64 isolates exhibited antibiotic resistance to at least one antibiotic, with the highest resistance against ampicillin, followed by ceftazidime, cefotaxime, ampicillin/sulbactam, imipenem, and kanamycin. The isolates were most susceptible to nalidixic acid, followed by tetracycline, sulfamethoxazole/trimethoprim, gentamicin, levofloxacin, oxytetracycline, and chloramphenicol. The present study showed that MDR *V. parahaemolyticus* remains prevalent, and they are a concern as they can contribute to an increased spread of antibiotic

resistance both inter- and intraspecies. Nonetheless, the discovery of the antagonistic effects of MUM 178J against MDR *V. parahaemolyticus* provides a positive outlook in mitigating the propagation of antibiotic resistance.

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