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Microbiological, biochemical, and molecular characterization of *Vibrio* spp. isolated from environmental samples collected from Namibe (Angola)

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Microbiological, biochemical, and molecular characterization of *Vibrio* spp. isolated from environmental samples collected from Namibe (Angola)

Erasmus Mundus Master's in Quality in Analytical Laboratories

work carried out under the supervision of Professor João CR Cardoso and Professor Deborah M Power

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Declaração de autoria de trabalho / Statement of Authorship

Microbiological, biochemical and molecular characterization of *Vibrio* spp. isolated from environmental samples collected from Namibe (Angola)

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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Abstract

Foodborne diseases have a major impact on public health, increasing rates of illness, death, and medical costs. Consumption of bivalves like mussels provide known health benefits but consuming them without knowing associated health risks can expose consumers to harmful microbes like pathogenic *Vibrio* species. The goal of this work was to identify and characterize *Vibrio* species from environmental seawater and mussel samples that might pose the risk of foodborne infections in Namibe (Angola) a province where there is a high risk of food poisoning and food insecurity. A combination of microbiological, molecular, bioinformatics, biochemical, and toxin analysis techniques were employed. The Vibrio isolates characterized in this study exhibited varied phenotypic traits, including differences in sucrose fermentation and colony characteristics on TCBS agar. The combination of 16S rRNA gene sequencing and species-specific amplification of genes, ToxR, tdh, and gyrB initially revealed all the isolates were *V. alginolyticus* but subsequent genome sequencing of three candidates species revealed that two were different *V. alginolyticus* strains and another was a different species *V. diabolicus.* Growth kinetics at 24°C and 37°C demonstrated the adaptability of the isolated species, with faster growth and shorter duplication times at 37°C, highlighting their growth optimum. Partial lysis of erythrocyte on blood agar and detection of proteolytic and lipolytic activities on agar plates indicated that these strains are likely to be virulent. Genome analysis revealed the presence of 128 virulence-related genes, involved in adherence, biofilm formation, motility, and effector delivery, and toxicity were identified and antibiotic resistant genes that differed between the genomes of the three candidates sequenced. In summary the study suggested that the *Vibrio* isolates that were characterized in this study are likely to be virulent and may represent a risk to the population when contaminated foods are consumed. This multidisciplinary approach underlines the importance of genomic and phenotypic analyses for precise identification, identification of pathogenic mechanisms, and monitoring of antibiotic resistance in Vibrio species which is crucial for the development of targeted strategies to manage vibriosis and mitigate public health risks.

Key words: pathogenic Vibrio, foodborne diseases, mussels, microbiological and molecular characterization, Virulence factors, genomes.

Resumo

As doenças transmitidas por alimentos têm um grande impacto na saúde pública, aumentando as taxas de doenças, mortes e custos médicos associados ao seu tratamento. O consumo de bivalves tal como os mexilhões proporciona benefícios conhecidos para a saúde humana, pois são uma importante fonte de proteína saudável de vitaminas e sais minerais para o humano e a sua produção em aquacultura é uma das indústrias mais sustentáveis na produção de alimento. Os bivalves são organismos marinhos filtradores de água e é desta forma que se alimentam e assim contribuem para a "limpeza" da coluna de água. Por isso para além de ser um alimento saudável para os humanos também um papel ecológico fundamental na manutenção da homeostasia do ambiente aquático. Os bivalves no seu ambiente natural estão expostos diretamente às condições ambientais e qualquer alteração no meio ambiente onde vivem devido ao efeito das alterações climáticas (temperatura, salinidade, ph) ou poluição provocada pela ação do homem (biológica – bactérias ou vírus, ou química- metais pesados poluentes) no meio marinho ou devido a causas ambientais naturais tais como afloração de biotoxinas devido ao sobre-cresciemnto de microalgas toxicas, podem ter um grande impacto na sua fisiologia ou de quem os consome quando estão contaminados por compostos químicos tóxicos ou microrganimos patogénicos. Como são organismos filtradores se estão expostos a ambientes contaminados os bivalves vão filtrar, acumular e biomagnificar dentro do seu organismo agentes patogénicos e ao consumi-los sem conhecer os riscos associados pode expor os consumidores a microrganismos patogénicos que o podem infetam e com isso desenvolvem doenças que em alguns casos podem ser fatais tal como as infeções causadas pelas espécies patogénicas de bactérias do género Vibrio, das mais comuns relacionadas com o consumo de produtos alimentares de origem marinha.

As bactérias do género Vibrio são um dos maiores e mais diverso grupi de bactérias encontradas em ambientes aquáticos que fazem parte da microbiota normal dos organismos marinhos. Existem 149 espécies descritas que possuem características bioquímicas e genéticas distintas e na sua grande maioria são não-patogénicas. No entanto existe algumas espécies que são patogénicas ou patogénicas oportunistas que são responsáveis por várias doenças em humanos e em organismos marinhos, podendo mesmo em alguns casos levar a morte em infeções muito graves. As vibrioses são as infeções causadas por Vibrios e as espécies mais conhecidas que causam infeções em humanos são o *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus* e *V. vulnificus* e algumas destas espécies podem também infectar os organismos marinhos e as epidemias causadas por infeções

por vibrio são um dos maus graves problemas de Saúde Publica e Ambiental e um dos principais problemas em Aquacultura pois são responsáveis pela morte de peixes e bivalves. Algumas destas bactérias são transmitidas ao humano através do consumo de peixe ou bivalves contaminados, especialmente quando são consumidos crus ou malcozinhados e representam um risco significativo especialmente para indivíduos com problemas de saúde, malnutridos, sistema imunológico comprometido e crianças com menos de 5 anos de idade.

O objetivo deste trabalho foi de identificar e caracterizar espécies de Vibrio provenientes de amostras ambientais de água do mar e de mexilhões que possam representar o risco de infecções de origem alimentar no Namibe, uma província costeira localizada no Sul de Angola cuja maioria da sua população vive da pesca e apanha de bivalves e onde existe uma elevada prevalência de intoxicações alimentares e insegurança alimentar cuja origem é maioritariamente desconhecida. As amostras de água do mar e de mexilhão anaizadas neste estudo foram recolhidas da Praia das Conchas (Pco) umas das zonas mais utilizadas pela população para a captura de bivalves que depois vendem no mercado. Neste estudo para caracterizar os isolados foram utilizadas uma combinação de técnicas de análise microbiológica, molecular, bioinformática, bioquímica e de analise de toxinas para identificar as espécies e caracterizar o risco associado. Foram analisados sete isolados distintos de Vibrio em culturas puras 5 isolados de mexilhão (Perna perna) e 2 de agua do mar envolvente que foram inicialmente selecionados em placas de TCBS e isolados de acordo com a sua morfologia. Os isolados caracterizados exibiram características fenotípicas e microbiológicas variadas. Analise por sequenciação do gene 16S rRNA e genes específicos de espécies gyrB (especifico para *V. alginolyticus*) e para os genes de virulência ToxR e Tdh, identificou todos os isolados como bactérias pertencentes à espécie *V. alginolyticus* uma das principais bactérias que causa infeções em humanos e organismos marinhos que é geneticamente muito idêntica ao *V. parahaemolyticus*. Estsa analise também confirmou que estávamos na presença de estirpes diferentes. No entanto a sequenciação do genoma de três isolados selecionados devido as suas características microbiológicas e moleculares revelou que existiam duas estirpes diferentes, dois dos isolados são duas estirpes diferentes de *V. alginolyticus* e um outro isolado era uma espécie diferente *V. diabolicus*, que é muito semelhante ao *V. alginolyticus* e *V. parahaemolyticus*. Caracterização microbiológicas dos sete isolados confirmou que todos têm capacidade de crescimento a 24°C e 37°C podendo ser potenciais bactérias patogénicas para os organismos marinhos e humano e o tempo de duplicação e taxa de crescimento é semelhante entre

os diferentes isolados. A lise parcial de eritrócitos em placas de ágar sangue e a deteção de atividade proteolíticas e lipolíticas em placas de ágar com diferentes substratos indicaram que estas estirpes são provavelmente bastante virulentas. A análise do genoma revelou a presença de 128 fatores genéticos de virulência, envolvidos na adesão, formação de biofilme, motilidade e entrega de efetores, e a toxicidade foi identificada e genes resistentes a antibióticos que diferem entre os três candidatos sequenciados. No entanto a analise da suscetibilidade microbiológica à presença de vários antibióticos (usados na clinica e em aquacultura) realizada utilizando o método de difusão em disco revelou que o sete isolados foram suscetíveis a todos os antibióticos testados.

No geral, o estudo realizado sugere que os isolados de Vibrio caracterizados são provavelmente virulentos e podem representar um risco para a população quando são consumidos alimentos contaminados. Esta abordagem multidisciplinar sublinha a importância das análises genómicas e fenotípicas para a identificação precisa, compreensão dos mecanismos patogénicos e monitorização da resistência aos antibióticos em espécies de Vibrio, o que é crucial para o desenvolvimento de estratégias específicas para gerir as infeções por Vibrio e mitigar os seus efeitos e riscos na Saúde Pública.

Palavras-chave: Vibrio patogénicos, doenças de origem alimentar, mexilhões, caracterização microbiana, e molecular, fatores de virulência, genomas

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List of Abbreviations

16S rRNA	16S Ribosomal Ribonucleic Acid
BAP	Blood Agar Plate
CDC	Centre for Disease Control and Prevention
CFU	Colony Forming Unit
CLSI	Clinical Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
EDTA	Ethyle Diamine Tetra-Acetic Acid
ELISA	Enzyme-Linked Immuno Sorbent Assay
EMBL	European Molecular Biology Laboratory
FAO	Food and Agriculture Organization
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA-BAM	Intergovernmental Panel on Climate Change.
gDNA	Genomic Deoxyribonucleic Acid
IPCC	Intergovernmental Panel on Climate Change
ISO	International Organization for Standardization
LAMP	loop-mediated isothermal amplification
NaCl	Sodium chloride
LC-HRMS	Liquid Chromatography-High Resolution Mass Spectrometry
LOD	Limit of detection
MDR	Multi-Drug Resistance
MH	Mueller-Hinton
OD	Optical Density
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
QS	Quorum Sensing
TAE	Tris-Acetate-EDTA

TCBS	Thiosulfate-Citrate-Bile Salts-Sucrose
TDH	Thermo-stable Direct Hemolysin.
TLH	Thermo labile hemolysin
TRH	Thermostable Related Hemolysin
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TTX	Tetrodotoxin
VFDB	Virulence Factor Database.
WGS	Whole Genome Sequencing
WHO	World Health Organization

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Introduction

1.1. The global burden of foodborne illnesses

The burden of foodborne illnesses is a significant public health concern worldwide (Long et al., 2023). According to the World Health Organization (WHO) report, each year unsafe food causes approximately 600 million cases of foodborne diseases and is associated with 420,000 deaths worldwide (WHO, 2015). This equates to nearly 1 in 10 people globally becoming ill after eating contaminated foods and children under 5 years are particularly vulnerable, accounting for 30% of foodborne deaths (WHO, 2015). Foodborne disease poses a substantial public health challenge in Africa, and this continent experiences the highest rate of foodborne illnesses globally (Grace, 2023). According to WHO estimates, 22.5 % (135 million) of illnesses related to food poisoning and 42.5 % (180.000) of the related deaths occur in Africa (WHO 2020). However, these values are likely to be underestimated (Grace, 2023) and factors such as limited resources, lack of administrative commitment, and a focus on major diseases contribute to this underestimation, hindering existing surveillance efforts in many countries. However, in countries such as America where surveillance programs exist and are implemented the Centers for Disease Control and Prevention (CDC) estimates that each year 1 in 6 Americans (or 48 million people) become sick, 128.000 are hospitalized, and 3.000 die from foodborne diseases (CDC, 2023).

The 2018 World Bank report emphasizes the lack of policy focus and investment in food safety, especially in countries with socioeconomic status (World Bank, 2018). Typically, attention is only given to foodborne diseases when there is an outbreak, and this means detection and tracking are deficient, infrastructure is lacking, there are insufficient trained personnel, no culture of safety exists or enforceable guidelines. This oversight results in a substantial economic burden, with estimated annual productivity losses of $95.2 billion and treatment costs of $15 billion in low- and middle-income nations (World Bank, 2018). These illnesses and deaths are primarily due to the contamination of foods with pathogens such as bacteria, parasites, viruses, and chemical substances either from their production site or contamination post-harvest or during food retailing. Figure 1.1 illustrates WHO global estimates of the burden of foodborne diseases in 2010.

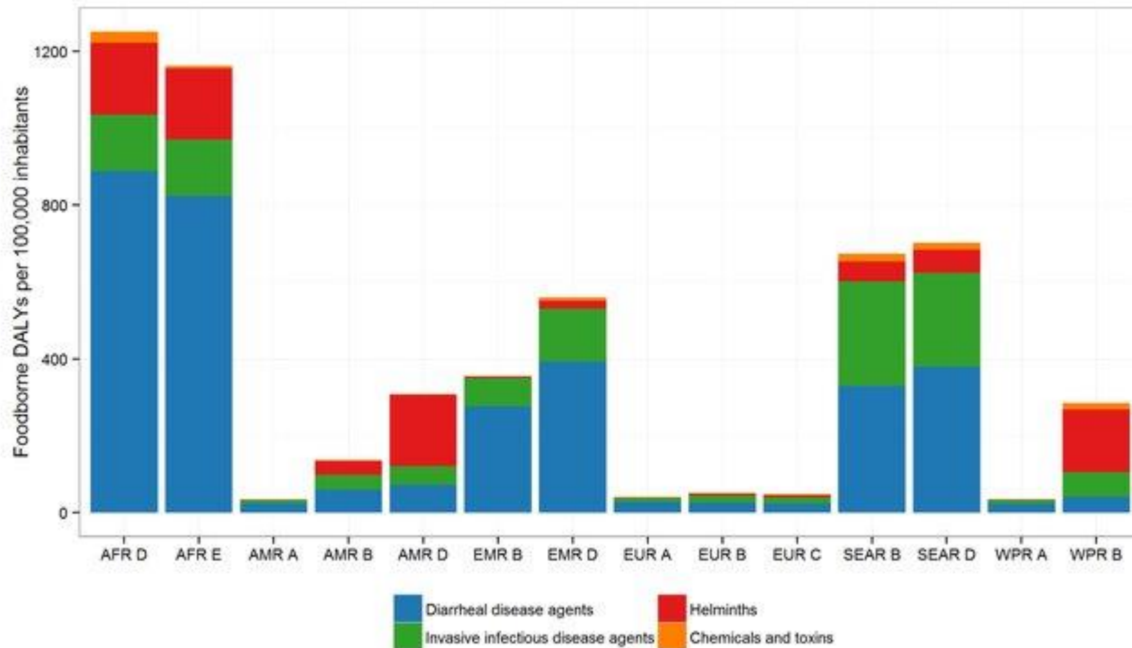

Figure: 1. 1. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. The height of the bar shows the burden of foodborne disease per 100,000 inhabitants and the colours represent the causative agent. Obtained from (Havelaar et al., 2015).

One of the main vectors of foodborne illnesses are marine seafood products that are collected or harvested from contaminated marine environments without quality control of their quality and safety for human consumption. This illness commonly arises from the consumption of contaminated molluscan bivalves and various other types of shellfish (Etheridge, 2010; Johnson & Schantz, 2017). Bivalves, such as mussels, clams, oysters, and scallops are a tasty, healthy, and rich source of protein and other nutrients and are popular gastronomic delicacies in many countries worldwide. However, the related illnesses due to the ingestion of pathogens and toxin-contaminated bivalves can result in serious and potentially life-threatening health complications (Ansdell, 2019; CDC, 2022).

1.2. The bivalves

1.2.1. Bivalves are rich and healthy foods for humans

Bivalves are molluscs, one of the most diverse animal phyla. They are sessile, water filter-feeders, and mostly marine organisms and play a critical role in aquatic ecosystems providing multiple services ((Suheriyanto et al., 2024; Von Cosel & Gofas, 2019). In addition to playing a significant

ecological role, they are also an important seafood for human consumption (Robledo et al., 2019). Bivalves are recognized as a rich protein source, and this nutritional profile renders them a favorable option for individuals aiming to boost their protein intake (Suplicy, 2020) and their consumption provides the nine essential amino acids in humans that are crucial for processes such as muscle repair and growth (Almonacid et al., 2015; Suplicy, 2020). Bivalves have been found to contain more nutrients than fish and meat and to be a rich source of important omega-3 fatty acids (Table 1.1), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are vital for brain and cardiovascular disease prevention as well as infant development (Willer & Aldridge, 2019a;Weichselbaum et al., 2013).

Table: 1. 1. Nutritional value, and environmental impact of different food sources. Data on nutritional and environmental impacts of common animal foodstuffs were obtained from (Willer & Aldridge, 2020).

	MEAT (beef)	POULTRY (chicken)	FISH (tilapia)	BIVALVE
NUTRITIONAL VALUE				
Protein (mg $kcal^{-1}$)	98	121	205	150
Omega 3 (mg $kcal^{-1}$)	0.5	0.7	1.9	4.8
Vitamin B12 (ng $kcal^{-1}$)	10	4	15	126
ENVIRONMENTAL IMPACT				
Land use (ha per t protein)	50	3	7.5	0
Greenhouse gas emissions (tCO_2 per t unit)	337.2	42.3	40.7	11.1
Freshwater use (m^3 per kg protein)	112.5	34.3	15.9	0
Eutrophication potential (kg P per t protein)	180	40	82	-148

*ha per t= hectares per tonne; mg $kcal^{-1}$= milli gram per kilocalorie; m^3= meter cube per kilogram; kg p per t= kilogram of phosphorus per protein yield

1.2.2. The impact of bivalve aquaculture on the food industry

The bivalve aquaculture sector has experienced rapid expansion because of the increasing demand for bivalve consumption worldwide (Figure 1.2). Bivalve molluscs are extensively farmed across Europe, North America, China, and Chile and China produces 35% of the global production. In the last two decades, the bivalve industry has almost doubled in dimension and reached 17.7 million tons with a market value valued at 29.8 billion dollars (FAO, 2022).

Bivalve aquaculture is environmentally friendly and unlike other food production systems feeding is not required or renewal of water. Greenhouse gas emissions are 30 times lower than emissions from livestock farming and 4 times lower than fish aquaculture (Table 1.1) (Willer et al., 2020).

Moreover, mollusc culture also holds considerable potential to bolster global food security and by growing under monitored environmental conditions can mitigate risks and ensure safe and good quality food products for consumers. In addition, bivalve aquaculture when well managed not only addresses food security concerns but also yields additional benefits such as providing habitats for other organisms, mitigating eutrophication, and enhancing nutrient cycling (Zhao et al., 2022). This sector has demonstrated positive environmental impacts, nutritional value, and health benefits, resulting in substantial growth and economic value (Campanati et al., 2022). Bivalve aquaculture plays a pivotal role in nutrient dynamics, serving as a significant source of nutrients like phosphate, which influences riverine fluxes and ecosystem processes (R. Li et al., 2016). Its potential to augment nitrogen removal in coastal estuaries underscores its importance in ecosystem health and functioning (Carmichael et al., 2012). In developing countries, aquaculture practices are poorly developed and concentrated on the production of freshwater fish (Theuerkauf et al., 2021). Although bivalve farming lags behind fish aquaculture growth (Figure 1.2), it is a promising avenue for providing affordable, safe, and nutritious food sustainably, especially in regions where food insecurity is high (Fitzer et al., 2018; Willer & Aldridge, 2019b).

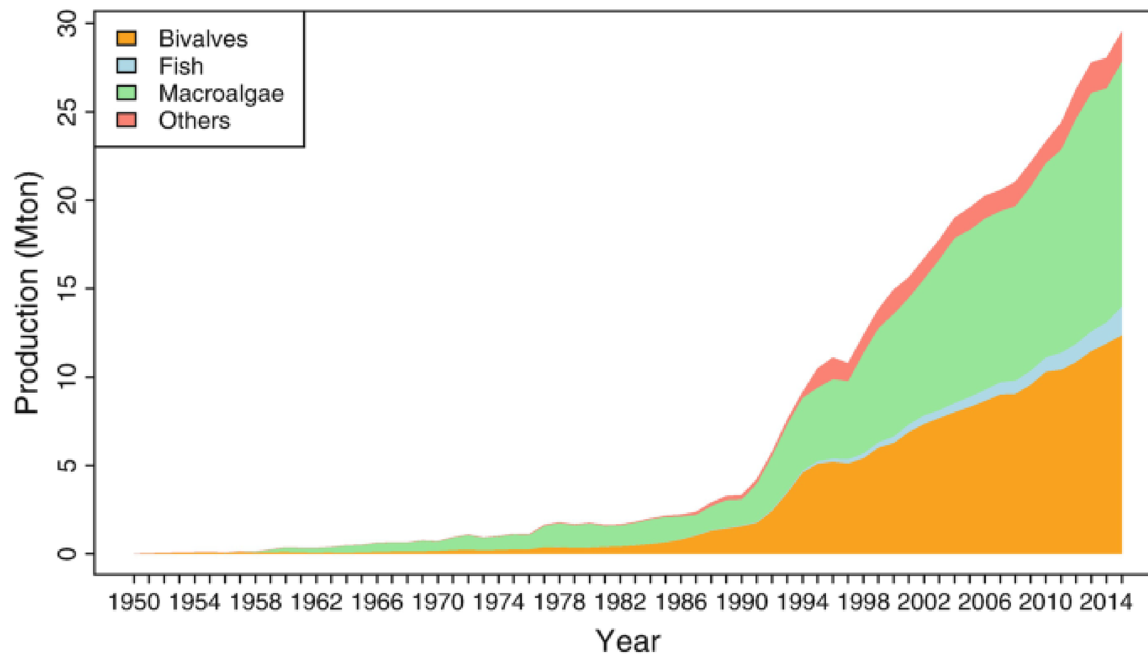

Figure: 1. 2. Global production of marine bivalves. The graph illustrates the trend of bivalve production from 1950 to 2014 showing a continuous increase in production. Obtained from (Wijsman et al., 2019).

1.2.3. Risks associated with bivalve consumption

Because they are filter feeders, bivalves can accumulate and biomagnify waterborne contaminants contained in the aquatic environment (Martinez-Albores et al., 2020). They can accumulate both natural and human-made contaminants, which can pose notable risks to public health (Mansfield, 2011). While bivalves contribute positively to the ecosystem by grazing on natural phytoplankton (Smith et al., 2018), the presence of pathogenic marine organisms and other pollutants in the marine environment can be biomagnified in bivalve soft tissues raises concerns regarding food safety and human health (Zhou et al., 2020; Gamarro et al., 2020). Furthermore, outbreaks of microbial diseases frequently affect aquaculture and reduce productivity (Cherian et al., 2023).

1.3. Pathogenic microorganisms

Consuming food contaminated with pathogenic bacteria, viruses, and parasites can lead to foodborne illnesses (Toyofuku, 2024). More than 250 foodborne illnesses have been identified, with different causes and modes of transmission (World Bank, 2018). The impact of these diseases is notably greater in low- and middle-income countries, attributed to factors such as limited food safety awareness, inadequate personal hygiene, improper food handling, and insufficient food storage practices (WHO, 2015; World Bank, 2018). Bacteria are responsible for two-thirds of the global human foodborne illnesses (Abebe et al., 2020) and bacterial diseases in marine environments pose a significant barrier to the advancement and sustainability of aquaculture, significantly affecting the socioeconomic status of fisherfolk in developing nations across the globe (Hegde et al., 2023). Bivalves have the potential to accumulate and harbour harmful pathogenic microorganisms, which can lead to symptoms such as diarrhea and vomiting if ingested. The rising incidence of seafood-related illnesses highlights the need for a thorough understanding of associated risks and the development of appropriate strategies for diagnosis and management of the underlying causes (Pepi & Focardi, 2021). Below I will focus on bacteria of the genus Vibrio which are the topic of this thesis and contain some of the most pathogenic bacteria strains that affect marine life and can be transmitted to humans and cause outbreaks of seafood poisoning.

1.3.1. The bacteria of the *Vibrio* genus

1.3.2. General characterization, diversity, and pathogenicity

Vibrio genus members are characterized as being curved or straight gram-negative rods, exhibiting facultative anaerobic behaviour, lacking spores, and displaying motility. Typically, they possess a single polar flagellum when cultivated in a liquid medium. *Vibrio* species commonly demonstrate oxidase and catalase activity and ferment glucose without gas production (Kaysner et al., 2004; Haifa-Haryani et al., 2023).) While most ferment sucrose, there are exceptions within the genus, making sucrose fermentation a notable phenotypic trait for differentiation purposes (Desmarchelier & Reichelt, 1984; Lopez-Joven et al., 2011).

Bacteria of the *Vibrio* genus are commonly found in aquatic and marine environments and are part of the natural microbiota of marine organisms (Baker-Austin et al., 2018a; Sampaio et al., 2022a). *Vibrio* species are known for their high diversity with 149 species described and non-pathogenic and opportunistic pathogens have been identified. Some species, such as *V. cholerae*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus*, are significant human pathogens, causing diseases like cholera, acute enteritis, and wound infection (Hackbusch et al., 2020). Infections caused by *Vibrio* are designated vibriosis. On the other hand, many *Vibrio* species are non-pathogenic and are natural constituents of freshwater, estuarine, and marine environments(Brumfield et al., 2021), exhibiting variations in their biochemical and genetic characteristics (Thompson et al., 2005).

Consuming contaminated seafood, especially when raw or undercooked, poses a significant risk of transmitting pathogenic *Vibrio* species, particularly among individuals with underlying health conditions and compromised immune systems (CDC, 2023). Of the over 149 described species of *Vibrio*, 12 are known to cause infections in humans (Baker-Austin et al., 2018b). The major human diseases resulting from pathogenic *Vibrio* bacteria are divided into two groups: cholera and non-cholera infections. *V. cholerae* is responsible for cholera, a severe diarrheal disease. Non-cholera *Vibrio* species like *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus* lead to acute enteritis, bacterial diarrhea, wound infection, and ear infection (Morris, 2013; West, 1989). Figure 1.3 highlights the risks of *Vibrio* to human health either by consumption of contaminated foods or open wounds.

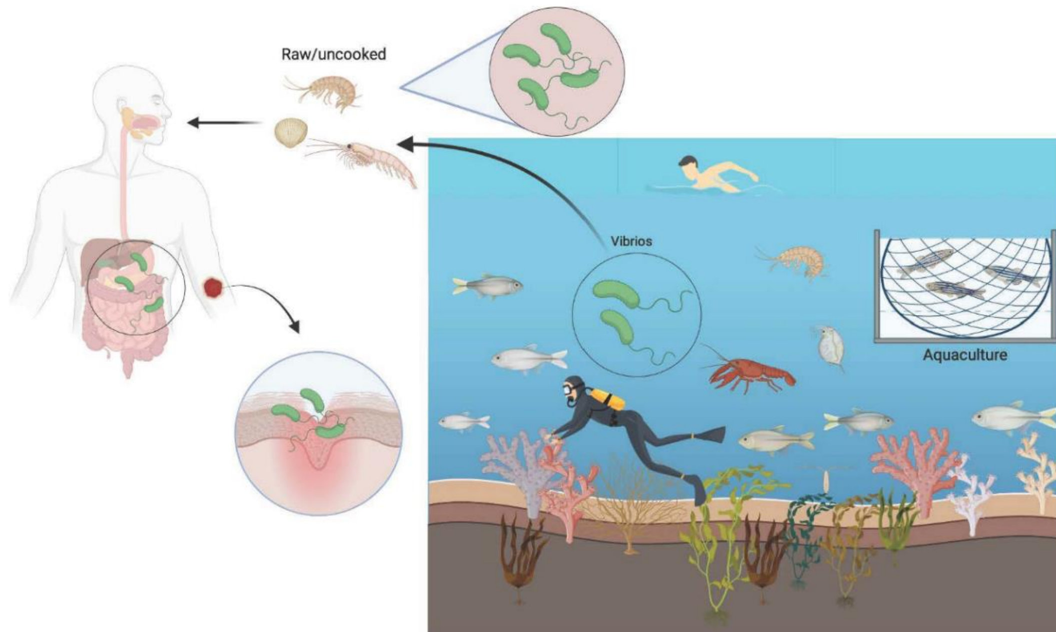

Figure: 1. 3. Life Strategies and Risks of *Vibrio*. The picture demonstrates how *Vibrio* species infect humans through the consumption of contaminated seafood. Obtained from (Sampaio et al., 2022b).

In aquaculture production, diseases are a major financial burden, with the World Bank estimating annual losses of around US$3 billion, with *Vibrio* infections being a significant factor in these economic losses (Sanches-Fernandes et al., 2022a). Several *Vibrio* species are considered pathogens or opportunistic pathogens in aquaculture, causing diseases in reared finfish, shellfish, and shrimp (Manchanayake et al., 2023). Some of the frequently encountered pathogens are *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyi*, and *V. vulnificus*, which are also human pathogens. These species are known to be associated with vibriosis outbreaks resulting in substantial production and economic losses (Manchanayake et al., 2023; Sanches-Fernandes et al., 2022b). Figure 1.4 summarizes major marine and clinical *Vibrio* pathogens.

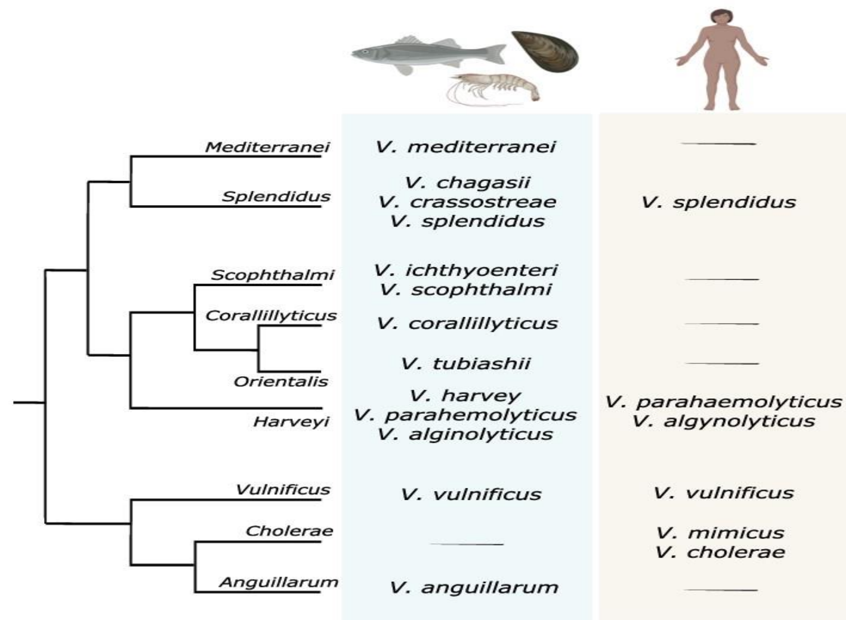

Figure: 1. 4. Illustration of major marine and clinical Vibrio pathogens. Species within the different clades are represented. Obtained from (Cardoso et al., 2024).

The virulence factors of *Vibrio* species determine their pathogenicity (Darshanee Ruwandeepika et al., 2012a). These factors are necessary for the bacteria to destroy and infect the host while escaping the host's immune system (Darshanee Ruwandeepika et al., 2012a). Understanding virulence factors in *Vibrio* species offers a valuable understanding of their pathogenicity and can contribute to the development of more focused treatment options and prevention measures. Therefore, to comprehend the pathogenic potential of Vibrio and develop effective solutions, a thorough analysis of virulence factors is essential. The main virulence factors of *Vibrio* species are discussed in this thesis, with a focus on their functions and significance in bacterial infections.

1.3.3. Virulence factors of the *Vibrio* genus

Certain pathogenic *Vibrio* species have virulence characteristics that allow them to infect humans and aquatic organisms (Sechi et al., 2000a). The most common species that infect humans include *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, and *V. mimicus* (Kim et al., 2015). Virulence factors include adhesion factors, capsules, polysaccharides and cytotoxins, (Huang et al., 2021). The emergence of novel pathogenic strains of the *Vibrio* genus has been proposed due to the distribution of virulence genes from *V. cholerae* among other *Vibrio* species and DNA transfer mechanisms. Horizontal gene transfer such as conjugation, transformation, or

transposition of external DNA and incorporation into their chromosomal DNA is relatively common in *Vibrio* (Sechi et al., 2000b). Moreover, the discovery of virulence factors in *Vibrio'*s that have not previously been identified emphasizes the dynamic nature of virulence factor evolution within the *Vibrio* genus (Ray et al., 2016).

Numerous studies on the pathogenesis and virulence regulation of *Vibrios* in the *Harveyi* clade (Figure 1.4) have shown how complex virulence factors interact in pathogenic *Vibrio* species (Darshanee Ruwandeepika et al., 2012b). Studies have revealed the presence of virulence genes similar to those found in *V. cholerae* and *V. parahaemolyticus* across various species, including *V. alginolyticus*, indicating shared molecular mechanisms (Xie et al., 2005a). While *V. cholerae* is recognized for its production of cholera toxin and toxin-co-regulated pilus, other *Vibrio* species like *V. parahaemolyticus*, *V. alaginolyticus*, *V. vulnificus*, and *V. mimicus* exhibit distinct sets of virulence factors, implying variations in their pathogenic strategies (Hung et al., 2005; Vora et al., 2005). In *V. alginolyticus*, various virulence factors contribute to its pathogenicity including LuxS quorum-sensing system that is crucial for biofilm formation and motility (Ye et al., 2008. (Gu et al., 2019). Studies on the prevalence of virulence genes and pathogenicity islands in environmental *Vibrio* strains give insight into the genetic diversity and potential pathogenicity of previously known non-pathogenic *Vibrio* isolates (Gennari et al., 2012).
Additionally, the presence of *trh* gene in *V. alginolyticus*, analogous to *V. parahaemolyticus* orthologue, underscores the potential for toxin production in these species (Oberbeckmann et al., 2011). Notably, various *Vibrio* strains have been found to produce toxins capable of inducing cytoskeletal-dependent changes, indicating their pathogenic ability (Barbieri et al., 1999). Moreover, the alkaline serine protease (*Asp*) has been implicated in *V. alginolyticus* virulence, affecting processes of motility and biofilm formation (Hao et al., 2015).

To comprehend the virulence mechanisms and toxin synthesis of non-cholera *Vibrio* species, particularly *V. parahaemolyticus* and *V. alginolyticus*, it is imperative to explore the presence of essential genes and factors influencing their pathogenicity. Amongst the different pathogenic *Vibrios* described, in this thesis attention is devoted to *V. parahaemolyticus* and *V. alginolyticus*. Both species are genetically very similar and are easily confused during diagnosis (Figure 1.4). They are both members of the *Harveyi* clade and are pathogenic for both humans and marine organisms.

1.3.4. The bacteria Vibrio parahaemolyticus

The bacteria *V. parahaemolyticus* demonstrates a preference for saline environments, notably in marine and estuarine settings, particularly in warmer regions (Rezny & Evans, 2020). Its impact on global gastroenteritis cases is substantial, and attributed to the ingestion of undercooked or raw seafood, with shellfish such as mussels, oysters, crabs, and various crustaceans serve as the most common vectors (Broberg et al., 2011; CDC, 2013). Its prevalence in water, shellfish, and other marine habitats underlines its ubiquitous nature (Rezny & Evans, 2020). This bacterium has been identified across different continents, spanning North and South America, Europe, Africa, and Asia, with *vibriosis* outbreaks documented worldwide (Broberg et al., 2011; CDC, 2013). A particularly important pandemic variant is the O3:K6 serotype, responsible for significant outbreaks in Asia since 1996, and subsequently spreading to other continents (Broberg et al., 2011).

Its pathogenicity is strengthened by a range of virulence factors, including thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*), which play pivotal roles in virulence. These virulence factors can trigger acute gastroenteritis, marked by symptoms such as diarrhoea, abdominal cramps, nausea, vomiting, headaches, fever, and chills in humans when they are infected (Broberg et al., 2011; Wong et al., 2000). The onset of these symptoms typically occurs within a period of 4 to 96 hours post-exposure (Wong et al., 2000). However, it is worth noting that not all strains of *V. parahaemolyticus* are pathogenic; some are environmental and pose no threat of disease to humans (Broberg et al., 2011). Figure 1.5 illustrates the major virulence factors identified in *V. parahaemolyticus*.

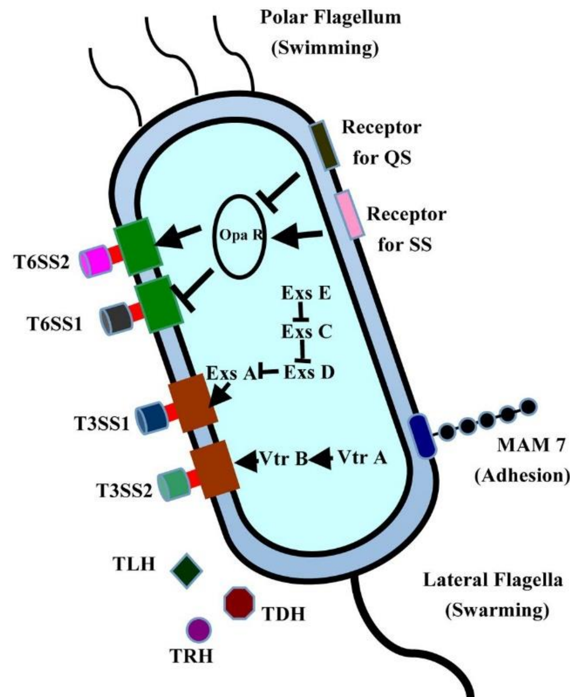

Figure: 1. 5. Virulence Associated Factors of *V. parahaemolyticus*. The picture illustrates the different virulence factors; like, type-6 secretion systems (T6SS), type-3 secretion system (T3SS), adhesion, motility, haemolytic related factors (*tdh*, *tlh*, *trh*), Quorum sensing (QS), and extra cellular enzyme. Obtained from (Wang et al., 2015).

1.3.5. The bacteria Vibrio alginolyticus

The bacterium *V. alginolyticus* is frequently found in estuarine and marine habitats. It is well-known for causing disease in a variety of marine organisms, such as fish, shellfish, and crustaceans (Abd El Tawab et al., 2018; Liang et al., 2012). Furthermore, *V. alginolyticus* poses a threat to human health, as exposure to contaminated water can result in ear and wound infections (Yin et al., 2022). This bacterium is a ubiquitous and opportunistic marine pathogen that can induce vibriosis in aquatic species, resulting in large economic losses for aquaculture (Peng et al., 2019). Research has demonstrated how crucial it is to comprehend the genetic and phenotypic traits of *V. alginolyticus* strains that are isolated from aquaculture and marine habitats (George et al., 2005; Lafisca et al., 2008). To evaluate the possible effects of *V. alginolyticus* strains on shellfish health and aquaculture operations, enzymatic characterization has been carried out (Lafisca et al., 2008 (Ina-Salwany et al., 2015). Understanding the genetic diversity, pathogenic mechanisms, and antibiotic resistance profiles of *V. alginolyticus* is crucial for developing effective control and management strategies. Additionally, risk identification, regular monitoring, and regulating the

harvesting habits and processing of seafood are crucial to reduce contamination risks. These measures aim to ensure the safety of seafood consumption and reduce the incidence of vibriosis.

1.4. Tetrodotoxin and *Vibrio* species

Many marine organisms, such as pufferfish, gastropods, and bivalves, contain a potent neurotoxin known as tetrodotoxin (TTX) (Abal et al., 2017). This toxin is known for ablating nerve impulses by blocking sodium ion channels, but leaving potassium ion permeability unaffected (Jal & Khora, 2015). It has been reported that TTX can be found in European bivalves (Gerssen et al., 2018; A. D. Turner et al., 2015; Vlamis et al., 2015). Given that TTX is a highly poisonous molecule that may cause severe intoxication and even fatalities in humans, the discovery of TTX in bivalves has sparked worries about food safety and contamination (Bane et al., 2014; Lago et al., 2015). Detection of TTX in bivalves is linked to the possible involvement of some marine microbes, especially *Vibrio* species, in the toxin's synthesis pathway (Bäuerl et al., 2014; Mansson et al., 2011). The production of TTX has been associated with the presence of the bacterium *V. alginolyticus* (Bäuerl et al., 2014; A. D. Turner et al., 2018). Studies have revealed that *V. alginolyticus* may carry TTX-secreting genes, suggesting a possible connection between these bacteria and TTX presence in marine environmental samples (Bäuerl et al., 2014). Therefore, regulatory efforts to reduce the hazards of TTX contamination in seafood are reflected in the introduction of detection limits for the chemicals in live bivalves (Katikou, 2023).

1.5 Factors affecting Vibrio distribution

1.5.1. Climate change

Over recent decades, climate change has increasingly complicated global issues, notably exacerbating foodborne diseases by influencing the occurrence, potency, and resilience of medically significant microorganisms (Duchenne-Moutien & Neetoo, 2021). The Intergovernmental Panel on Climate Change (IPCC,) reports that climate change has caused a substantial impact on terrestrial, freshwater, coastal, and open ocean ecosystems, leading to increased public health concern from increased infectious diseases (IPCC, 2023).

Elevated temperatures resulting from climate stressors indirectly impact human health by modifying the ocean ecosystem and fostering favourable conditions for bacterial diseases like *Vibrio* spp.(Dermawan et al., 2022). The prevalence and distribution of *Vibrio* species are significantly influenced by rising sea surface temperatures, alterations in salinity, and shifts in

oceanic patterns due to climate change, impacting their presence, frequency, and intensity (Archer et al., 2023; Deeb et al., 2018; Hoffman et al., 2023; Vezzulli et al., 2016; Archer et al., 2023). These environments typically contain elevated levels of dissolved organic carbon, which is essential for the growth of *Vibrio* spp. (Archer et al., 2023; Deeb et al., 2018). Salinity also affects the distribution of *Vibrio* species, with *V. parahaemolyticus* and *V. alginolyticus* favoring higher salinities while *V. vulnificus* favors more moderate salinities (Ruiz-Cayuso et al., 2021). The rise in *Vibrio* bacteria resulting from climate change has contributed to a rise in *Vibrio*-associated human illnesses, including wound infections, gastroenteritis, ear infections, and sepsis (Archer et al., 2023). Alongside temperature shifts, emerging factors like droughts and even dust emissions, and wind patterns also play pivotal roles in influencing infections associated with *Vibrio* spp (Duchenne-Moutien & Neetoo, 2021).

1.5.2 Increasing drug resistance

The growing resistance of *Vibrio* spp to commonly used drugs represents a significant Public Health threat, given their role in diseases such as cholera, gastroenteritis, and wound infections, frequently spread through the consumption of contaminated seafood. Multiple studies have highlighted the emergence of antibiotic-resistant *Vibri*o strains, and the challenges in effectively treating and the spreading of vibriosis including those resistant to multiple drugs (MDR)(Das et al., 2020) reducing the efficacy of existing treatment methods for infections caused by these bacteria.

The rise of antimicrobial resistance of *Vibrios* can be attributed to various factors, such as horizontal gene transfer and the uptake of resistance genes from the surrounding environment (Dutta, Kumar, et al., 2021). Within the genomes of drug-resistant *Vibrios*, mobile genetic elements play a crucial role as they serve as significant vehicles for carrying antimicrobial resistance factors (Dutta, Kumar, et al., 2021). Using antibiotics inappropriately in aquaculture and healthcare, as well as the spread of resistance genes through mobile genetic elements, exacerbates this problem. For the bacteria *V. parahaemolyticus* there is considerable prevalence of antibiotic resistance, notably against ampicillin, cefazolin, and penicillin (Tan et al., 2020). A significant proportion of isolated strains, 85.71%, were resistant to multiple antibiotics when various seafood types were evaluated in Malaysia (Tan et al., 2020). In a study conducted in 2023 to assess the distribution of antimicrobial resistance of 110 *vibrio* isolates using the disc-diffusion method,

multidrug resistance (MDR) was noted in 8.18% of the isolates, and 21.82% isolates were resistant to two classes of antimicrobials (Hirshfeld et al., 2023). Ampicillin, penicillin, and tetracycline are the most commonly found antibiotic resistance profiles that examined antibiotic resistance in *V. parahaemolyticus* and *V. vulnificus* (Elmahdi et al., 2016). *V. alginolyticus* has developed resistance to antibiotics, particularly quinolones used in aquaculture, by altering its metabolism in response to ofloxacin stress, resulting in increased fatty acid synthesis and disruptions of the pyruvate cycle (Yin et al., 2022). MDR strains of *V. alginolyticus* have also been observed, exhibiting resistance to a spectrum of antibiotics such as ampicillin, chloramphenicol, tetracycline, and cefotaxime (Liu et al., 2019).

As shown in Figure 1.6, antimicrobials target a diversity of active sites in bacteria, affecting processes such as bacterial adhesion, biofilm formation, DNA replication, and the function of essential proteins. In response, bacteria employ a variety of resistance mechanisms, including increased antibiotic efflux, enhanced biofilm formation, reduced uptake of antibiotics, and modification of target sites. Thus, thorough testing using phenotype and genotype screening is required to comprehend the occurrence of antibiotic resistance in *Vibrio* isolates. With these approaches, the resistance patterns in isolates can be thoroughly understood by identifying both known and emerging resistance mechanisms. Understanding this is essential to control the effects of antibiotic resistance in aquaculture and public health.

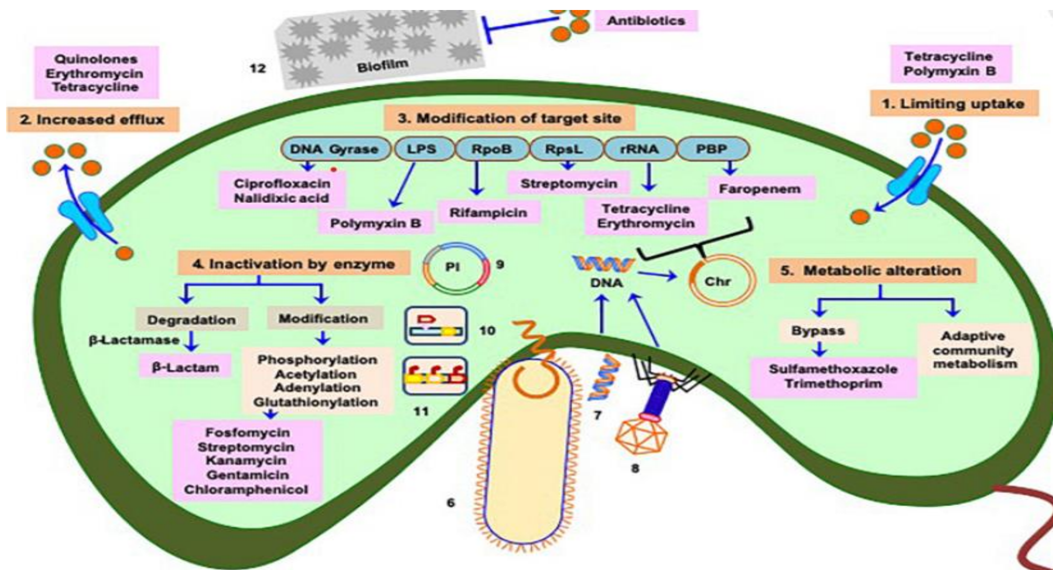

Figure: 1. 6. The target site of selected antimicrobials on a bacterial cell. The image demonstrates the important target sites of antimicrobials for vibriosis. The interaction of antibiotics is highlighted in light purple with the target sites in light blue. The mechanism of action is shown by the orange colour. Obtained from (Dutta, Kumar, et al., 2021)

1.6. Current methods to screen and diagnose for the presence of *Vibrio* spp.

In the literature, a range of detection methods spanning from conventional approaches to more sophisticated techniques have been documented. However, each method comes with its own set of strengths and weaknesses. Therefore, there is a constant need for improvement and the development of techniques that offer high sensitivity, specificity, affordability, and ease of obtaining consistent and accurate diagnoses. Conventional approaches entail preliminary microbiological identification based on colony morphology and metabolic characteristics such as their sucrose fermenting capacity on thiosulfate-citrate-bile salts-sucrose (TCBS) agar, followed by confirmation using classical biochemical tests such as the oxidase reaction and nitrate reduction. The International standardization organization (ISO) and Food and Drug Administration Bacteriological Analytical Manual (FDA-BAM) methods, encompassing enumeration and detection steps for *Vibrio* pathogen detection (Messelhäusser et al., 2010; Wu et al., 2019).

Molecular methods based on DNA amplification have also been employed and are more rapid than the classical microbiological approaches. Methods such as PCR-based test enable quick detection of *Vibrio* species in various samples (Loo et al., 2022a; Messelhäusser et al., 2010). But the most commonly used is the combination of the microbiological and molecular approaches and a straightforward procedure for rapid identification of *Vibrio* from aquatic samples involves enriching samples in alkaline peptone water, selecting yellow colonies, and confirming through PCR and biochemical tests (Desmarchelier & Reichelt, 1984; Wu et al., 2019). Using PCR, particular target genes have been found in the literature to detect and differentiate *Vibrio* species including *V. alginolyticus* and *V. parahaemolyticus*. The virulence genes *toxR* and *tdh* are among those chosen to detect *V. parahaemolyticus* (B. Liu et al., 2012) and the DNA gyrase gene, *gyrB*, has been reported to be specific to *V. alginolyticus* and can be used as a molecular marker to differentiate this species from closely related Vibrio species such as *V. parahaemolyticus*.

The recent development of sequencing technologies and the decrease in the cost associated with whole genome sequencing (WGS) serves as a pivotal tool in the molecular analysis of bacteria including *Vibrio* species, offering unparalleled resolution in studying bacterial evolution and population dynamics. The use of this technology provides a comprehensive understanding of the diversity of *Vibrio* species based on genome analysis and can also detect multiple *Vibrio* species within individual samples and across diverse sample sets (Brown et al., 2019; Janecko et al., 2021).

WGS also serves as a critical tool in identifying antimicrobial resistance and virulence genes. Researchers have been exploring the whole genome sequence of *Vibrio* species to enhance understanding of its antibiotic resistance profile, which is vital for effective treatment and surveillance of infections caused by *Vibrio* species (Letchumanan et al., 2016).

WGS can be used to identify and type *Vibrio* species, enabling comparisons with conventional microbiological, biochemical, and rapid molecular typing techniques. This facilitates more precise species identification and enhances comprehension of genomic diversity among *Vibrio* species. (Greig et al., 2018; Mevada et al., 2023).-This understanding is crucial for formulating effective diagnostic targets and public health interventions to address the impact of *Vibrio*-related illnesses, including those transmitted through contaminated food. Such insights into the evolution of *Vibrio* species can strengthen the capacity to protect public health and respond proactively to outbreaks of vibriosis. Figure 1.7 illustrates the application of testing methods for the screening and diagnosis of *Vibrio* species from conventional culture methods to more advanced methodologies including DNA amplification based on PCR and hybridization techniques (microarrays), immunoassay approaches, and rapid screening methods.

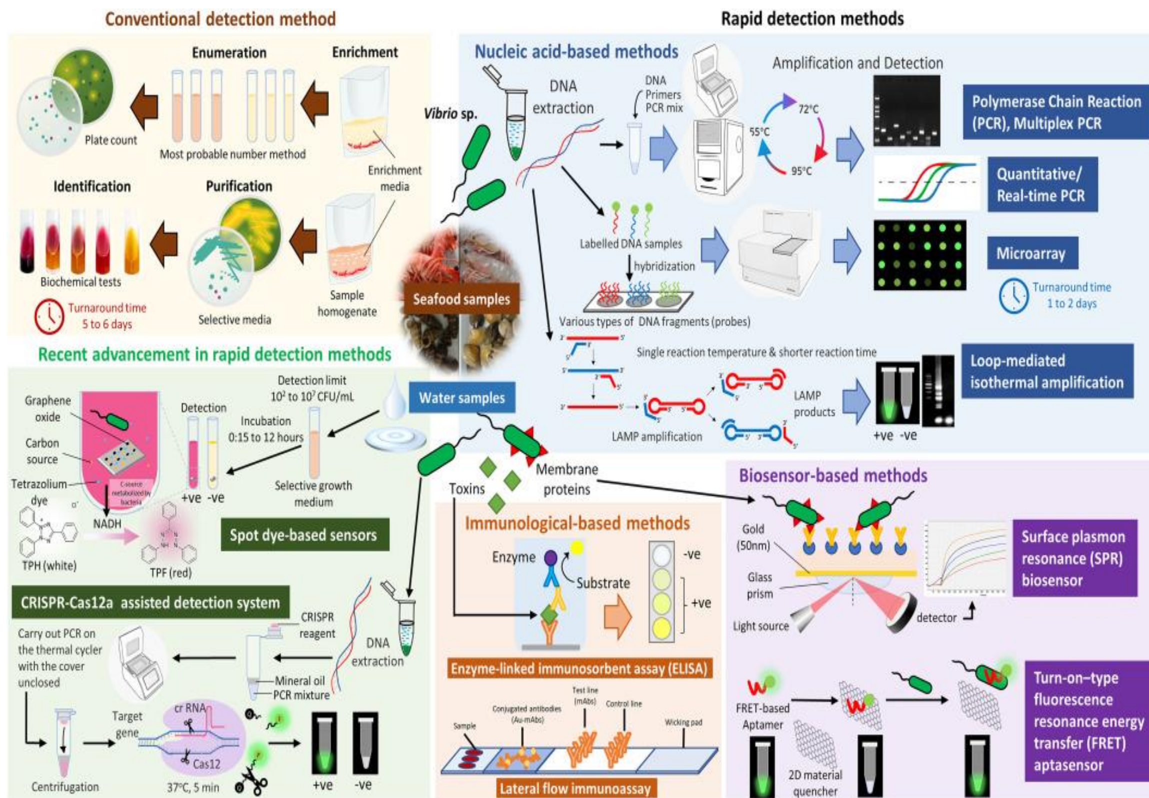

Figure: 1. 7. Illustration of screening methods for *Vibrio* species. The figure summarizes conventional detection methods (plate method and biochemical), Rapid detection methods (multiplex PCR, Quantitative PCR, Microarray, loop-mediated isothermal amplification (LAMP)), and detection with immunoassays like ELISA. Obtained from (Loo et al., 2022b).

1.7. Objective

The objective of this study was to isolate and characterize *Vibrio* spp from seawater and seafood samples collected from the Namibe coastline, Angola to identify potential pathogenic isolates that may pose a risk of foodborne illnesses to humans. Samples analysed were from a field expedition and were analysed in this thesis project after preliminary selection to enrich *Vibrio* spp in mussels and seawater samples collected from the site of mussel collection. The species of mussels analysed were the Brown mussel (*Perna perna*) and the work involved the application of molecular (PCR and genome sequencing), microbiological, and biochemical techniques to characterize the isolates and identify and compare their potential virulence factors and antibiotic-resistant genes.

More specifically the objectives were:

- Isolate *Vibrio* spp using classical microbiology techniques on selective media
- Molecular characterization of isolates using DNA amplification techniques
- Characterize the *Vibrio* spp isolates based on their biochemical properties and microbiological characteristics
- Quantify the presence of the neurotoxin the tetrodotoxin (TTX) in bivalve samples related to *Vibrio* spp presence using LC- HRMS high-resolution mass spectroscopy technique.
- Analyse the genome sequence from *Vibrio* spp candidate isolates to confirm the species and identify genes responsible for virulence and resistance to antibiotics.

The work developed in this thesis was in the scope of the HealthyBi4Namibe project which aims to develop technological tools for the identification and mitigation of biological and non-biological risks associated with the consumption of bivalves in the Namibe region of Angola.

2. Materials and methods

A schematic representation of the methods applied in this thesis and used to isolate and characterize *Vibrio* spp in samples collected from the environment (bivalves and seawater) is represented in Figure 2.1. The methods applied are organized into five sections: 1) Molecular (PCR) and Bioinformatic analysis, 2) Microbiology methods, 3) Biochemical assays, 4) Toxicology analysis, and 5) Genome sequencing and Bioinformatics.

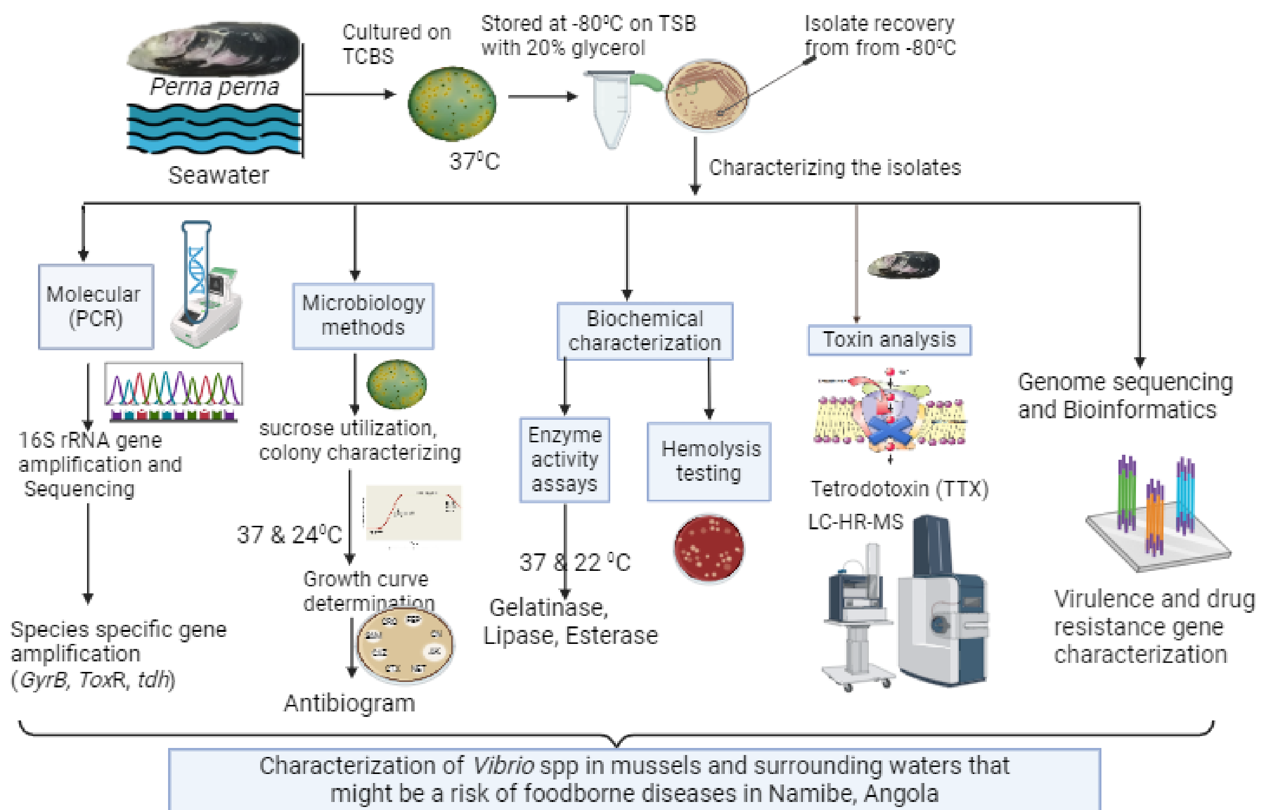

Figure: 2. 1. Schematic diagram of the research methodology employed in this study. The graph is drafted using Bio-render software to show the tasks and methodologies employed in this study.

2.5. Sample collection, isolate recovery, and nomenclature

Samples were collected from Praia das Conchas (Pco) in Namibe (Angola) which is a site located in the capital city of Mocâmedes that is used by the local population to collect bivalve mussels for commercialization in the local markets (Figure 2.2). Two liters of environmental seawater and approximately 3 Kg of mussels were hand-picked for analysis during low tide and were collected

and transported in ice boxes at 4ºC to the laboratories of the University of Namibe (UNINBE) for isolation of microorganisms by a research team from the Centre of Marine Sciences (CCMAR).

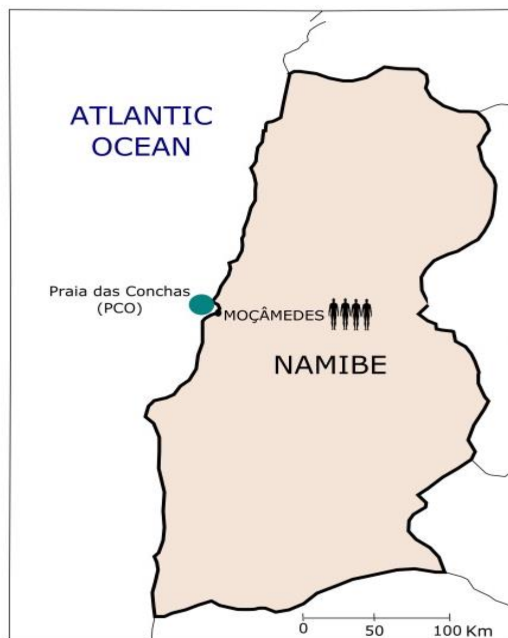

Figure: 2. 2. Specific sampling point in Namibe, Angola. As indicated in the dot-point, the isolates in this study were obtained from mussels and seawater samples collected in the Praia das conchas (Pco), an area where people collect bivalves for consumption.

For sample collection mussels were initially divided into two groups according to the shell phenotype and presumptive species identification SA group (*Semimytilus algosus*) and PP group (*Perna perna*), however subsequent molecular analysis by group members revealed that the samples of bivalves were all *Perna perna*. To isolate *Vibrio spp*, Thiosulfate Citrate Bile Salt (TCBS) agar (VWR, Portugal), a selective and differential medium for *Vibrio* species was used. For the seawater samples, two liters were filtered through a 0.22 µm filter and half of the filter was plated on a TCBS agar plate and incubated at 37 ºC for 24h. To isolate the bacteria in the mussel tissues, the ISO 21872:1-2017 standard protocol was followed. Several colonies were isolated through successive subcultures and preserved at -80°C in Tryptic Soy Broth (TSB) with 1% NaCl and 20% glycerol. For this study, the isolates stored at -80°C were revived by inoculating them on Tryptic Soy Agar (TSA) containing 1% NaCl and incubating overnight at 37°C. Following the successful recovery of each isolate, a single colony was sub-cultured overnight at 37°C on TCBS, to obtain the isolates for subsequent characterization. The nomenclature adopted was:

site/origin/selective media/plate/subculture. Pco or P indicates sample origin- Praia das Conchas, SA or PP indicates mussel samples, A indicates water sample, T-2 or T-4 indicates TCBS, A1, A2, B1, and B2 represent the different subcultures.

2.6. Molecular and bioinformatic analysis

2.6.1. Genomic DNA (gDNA) extraction for PCR

From the bacterial isolates genomic DNA (gDNA) was extracted using the optimized GES method as described by (Pitcher et al., 1989). Initially, a single colony of each isolate was inoculated into 5 mL of Tryptic Soy Broth (TSB) with 1% NaCl (pH 7.3 ± 0.2) and incubated overnight at 22°C (for 16 ± 2 hours) at 150 rpm. Once sufficient turbidity indicating bacterial growth was observed, 2 mL of the inoculum was transferred to a 2 mL Eppendorf tube and centrifuged for 10 minutes at 5000 rpm (Eppendorf 5415R centrifuge). The supernatant was then discarded to obtain the bacterial pellet. The pellet was resuspended in 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 6 µL of RNase (10 mg/mL) (Sigma, Spain). Subsequently, 500 µL of GES solution (comprising 60 g of Guanidium Thiocyanate, 20 ml of 0.5 M EDTA pH 8, 5 mL of 10% Sarkosyl, and distilled water to a final volume of 100 mL) (Sigma, Spain) was added. After incubating the mixture at room temperature for 10 minutes, 250 µL of 10M Ammonium Acetate was added to precipitate the proteins. Following gentle inversion to homogenize, the tubes were incubated on ice for 10 minutes before adding 500 µL of chloroform/isoamyl alcohol (24:1) solution to remove the proteins from the gDNA. After gentle mixing by inversion, the reaction was centrifuged at 13000 rpm for 10 minutes at 4°C. The supernatant from the two-phase solution was then transferred to a new Eppendorf tube, and 400 µL of cold isopropanol was added to precipitate the gDNA. After incubating on ice for 10 minutes and centrifuging at 13000 rpm for 5 minutes at 4°C (Eppendorf 5415R), the precipitated gDNA pellet was washed three times with 400 µL of cold 70% ethanol, centrifuging each time at 13000 rpm for 5 minutes at 4°C. The pellet was air-dried to remove ethanol and then resuspended in 50 µL of Milli-Q water (Millipore, USA). After the vortex resuspended the pellet, the suspension was incubated on ice for 1 hour. The quality and concentration of the extracted gDNA were assessed using a NanoDrop (ThermoFisher Scientific, USA), while its integrity was evaluated by running the gDNA on a 0,8 % agarose gel electrophoresis (with 5 µL of gDNA and 5 µL of 6X loading buffer).

2.6.2. Genomic DNA extraction for genome sequencing

Genomic DNA for genome sequencing was extracted using a Qiagen kit (DNeasy Blood and Tissue Kit) following a quick start protocol. A single colony of each isolate was inoculated into 5 mL of Tryptic Soy Broth (TSB) with 1% NaCl (pH 7.3 ± 0.2) at 37°C and incubated overnight (16 ± 2 hours) at 150 rpm. Two mL of the pre-inoculum was centrifuged for 5 minutes at 8000 rpm (Eppendorf 5415R), to obtain the bacterial pellet. After discarding the supernatant, the pellet was resuspended by adding 180 µL of Alkaline Tissue Lysing Buffer (ATL) and 4 µL of RNase to each tube. The mixture was vortexed and incubated at room temperature for 10 minutes. Following the incubation, 200 µL of AL buffer was added and vortexed. Subsequently, 200 µL of absolute cold ethanol was added and vortexed. The mixture was pipetted and transferred to a column assembled into a 2 mL collection tube and centrifuged at 8000 rpm (Eppendorf 5415R) for 1 minute. The column was then transferred into a new collection tube, and 500 µL of AW1 solution was added and centrifuged at 8000 rpm for 1 minute (Eppendorf 5415R). Next, the column was transferred into another centrifuge tube, and 500 µL of AW2 solution was added and centrifuged) for 3 minutes at 14000 rpm, (Eppendorf 5415R. Finally, the column was inserted into a 1.5 µL microcentrifuge tube, and 50 µL of eluting buffer (TNS-HCL) was added to each tube and centrifuged at 8000 rpm for 2 minutes (Eppendorf 5415R). The concentration of the extracted genomic DNA was measured using a NanoDrop (ThermoFisher Scientific, USA). The quality of the DNA was assessed by observing the ratio of maximum absorbance at 260nm/280nm and 260nm/230nm, while its integrity was evaluated by running the genomic DNA on a 0,8 % agarose gel electrophoresis (with 2 µL of genomic DNA and 5 µL of 6X loading buffer).

2.6.3. Amplification of 16S rRNA gene

16S rRNA housekeeping gene amplification was conducted using qualitative PCR with universal primers of 16S rRNA gene to identify the species based on DNA amplification. The primers are, 16S rRNA gene forward (CCCAGATGGGATTAGCTTGT) and 16S rRNA gene reverse (TCTGGACCGTGTCTCAGTTC) both with 5' to 3' orientation (Lane, 1991). The reaction mixture was prepared following a previously optimized protocol in our laboratory. Initially, a master mix was prepared in a separate PCR tube by sequentially combining 14.375 µl of nuclease-free water, 2 µL of 10x DreamTaq Buffer (Thermofisher, USA), 0.5 µL of 20 mM dNTPs (Thermofisher, USA), 0.5 µL of each primer (initial concentration of 10 mM), and 0.125 µL of DreamTaq polymerase 5 U (Thermofisher, USA) per reaction. After thorough mixing, 18 µL of

the mixture was transferred to each separate PCR tube, followed by the addition of 2 µL of gDNA (50 ng/µL) to each tube, resulting in a final reaction volume of 20 µL. Subsequently, PCR was performed with the following thermocycler conditions: 95 °C for 3 minutes (initial denaturation), followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 59 °C for 30 seconds, and extension at 72 °C for 90 seconds, with a final extension step at 72 °C for 10 minutes. The PCR products were then analysed by electrophoresis on a 1% agarose gel in 1x TAE buffer. Amplicons of the expected size (1400bp) were visualized using a transilluminator and Bio-Rad's Image Lab Software GelDoc XR+ by comparing them with DNA ladder III (NZYTech, Portugal). Upon confirmation of the expected amplicon size and reaction intensity, 5 µL of each PCR product was sent for sequencing according to the Sanger method with an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, USA) and the BigDye Terminator v3.1 kit at the CCMAR Molecular Biology platform (https://ccmar.ualg.pt/en/our-services). The resulting nucleotide sequences were analysed to determine bacterial identity by searching against the public National Centre for Biotechnology Information (NCBI) database, employing the BLASTn algorithm to retrieve and compare them with similar nucleotide sequences deposited in the database.

2.6.4. Species-specific gene amplification

To further substantiate the molecular identification of the isolates and differentiate between *V. alginolyticus* and *V. parahaemolyticus* species-specific gene amplification was performed, and three pairs of primers (Table 1) were tested: two pairs were custom-designed for *V. parahaemolyticus* targeting *tox-R* and *tdh* genes (obtained from (Leal, 2022) while the third pair targeted the *gyrB* gene that is suggested to be specific for *V. alginolyticus* and was obtained from (S. Zhou et al., 2007). The *V. parahaemolyticus* were already optimized (Leal, 2022) but to optimize the PCR conditions for *gyrB* the primers were initially tested at two annealing temperatures, 58°C and 62°C, using genomic DNA from presumptive *V. alginolyticus* and *V. parahaemolyticus* isolates and 58ºC was found to be the optimal temperature based on the single reaction product with a high intensity obtained. The PCR reaction followed a pre-established protocol. Initially, a master mix was prepared in a separate PCR tube by sequentially combining 11.54 µL of nuclease-free water, 1.5 µL of 10x DreamTaq Buffer (Thermofisher, USA), 0.3 µL of 20 mM dNTPs (Thermofisher, USA), 0.3 µL of each primer (initial concentration of 10 mM), and 0.06 µL of DreamTaq polymerase 5 U (Thermofisher, USA) per reaction. After thorough mixing, 14 µL of the master mix was dispensed into individual PCR tubes, followed by the addition of 1

μL of gDNA (50 ng/μL) to each tube, resulting in a final reaction volume of 15 μL. The PCR was conducted with the following thermocycler conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58° for 30 seconds, extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 min. The resulting PCR products were analysed by electrophoresis on a 2% agarose gel in 1x TAE buffer. Amplicons of the expected size were visualized using a transilluminator and Image Lab Software GelDoc XR+ (Bio-Rad, USA) by comparing them with DNA ladder V (NZYTech, Portugal).

Table: 2. 1. List of primers used with the respective size of amplified DNA fragments and optimal annealing temperatures.

Target	**Target gene**	**Primer name**	**Amplicon size (bp)**	**Optimal temperature (^{0}C)**	**Reference**
All bacteria	16S rRNA	16S_27F 16S_1492R	1400	59	(Lane, 1991)
V. alginolyticus	*gyrB*	GyrB-F GyrB-R	338	58	(S. Zhou et al., 2007)
V. parahaemolyticus/ V. alginolyticus	*toxR*	Vpa.toxF Vpa.toxR	130	57	(Leal, 2022)
V. parahaemolyticus/ V. alginolyticus	*Tdh*	Vpa.haeF Vpa.haeR	236	57	(Leal, 2022)

2.6.5. Agarose gel electrophoresis

The success of all PCR reactions and the bacteria's genomic DNA was analysed through agarose gel electrophoresis. To ensure optimal separation and detection of bands, varying gel concentrations were employed based on the expected gene fragment size. A 1% agarose gel was utilized for 16S rRNA amplicons with an expected size of 1465 bp. Species-specific amplicons < 350 bp were analysed on a 2 % agarose gel. To visualize nucleic acids, 10 µL of GreenSafe (1:10) from NZY Tech, Portugal, was added to 50 mL of agarose in 1x TAE buffer. Gel electrophoresis was conducted at 90 V for 30 ± 5 minutes, with two different ladders (NZYTech Ladder III for 10000-200 bp and NZYTech Ladder V for 1000-100 bp) serving as reference standards for amplicon size interpretation. UV light exposure and visualization were carried out using the Image Lab Software GelDoc XR+ from BioRad, USA.

2.6.6. Sequence analysis and phylogeny

The identity of the bacterial isolates was confirmed by analysing their 16S nucleotide sequences against the NCBI database (https://blast.ncbi.nlm.nih.gov) using the BLASTn algorithm. Sequence identity was established by retrieving the most significant hits with the lowest e-values. To better characterize the identity of the bacteria isolates, the 16S sequence fragments obtained were compared with other *Vibrio* spp sequences from reference strains with genomes available in the NCBI database. The nucleotide sequences from the isolates and those retrieved from the database were aligned in ClustalW program available from Aliview and the sequence alignment was edited to obtain a block of nucleotide sequences of similar size (510bp) that was used for the phylogenetic analysis. The phylogenetic tree was constructed using the Maximum Likelihood Method in the ATGC platform (http://www.atgc-montpellier.fr/) using the PhyML software with the HKY85 substitution model and 100 bootstrap replicates were used. The tree was displayed in Figtree and edited in the Inkscape program.

2.6.7. Bacteria whole Genome sequencing

The genome sequence of 3 candidate isolates that were selected based on their distinct microbiological profiles was outsourced (BMKGene company, Germany) applying Illumina Novaseq PE150 sequencing following the principle of sequencing by synthesis with 100x /sample (100x coverage). Genome annotation was also performed by the company using their standard protocols and databases such as Nr, Uniprot, COG, KEGG, CAZyme, PHI, and CARD. The virulence factors were extracted by comparing predicted proteins from protein databases with the list of major virulence factors in the Virulence factors of pathogenic bacteria databases.

2.7. Microbiology procedures

2.7.1. Colony characterization

Bacterial colonies cultured on TCBS media were examined for detailed characterization based on colony parameters and their ability to utilize sucrose as a carbon source. Parameters such as size, shape, surface texture, halo type, elevation, border definition, and transparency were considered during the initial assessment. Colonies exhibiting distinct characteristics were selected for further analysis. Under a stereo microscope, (Olympus, Japan) with a magnification power of four times (4X), the selected colonies were carefully examined. This microscopy approach was for detailed

observation of colony morphology and characteristics. By employing this higher level of magnification, finer details of colony structure and features could be recognized, aiding in the accurate characterization of the bacterial isolates.

2.7.2. Bacterial growth kinetics

To analyse the growth kinetics of the isolates, a single colony was cultured overnight in 5 mL of TSB/1%NaCl at 37°C. A 1/5 dilution was prepared by transferring 100 µL of pre-inoculum into 400 µL of TSB/1%NaCl. From this dilution, 20 µL was added to each well of a 96-well microplate containing 180 µL of TSB/1%NaCl, resulting in a final 1/50 dilution. All preparations were performed in triplicate to assess well-to-well variability. For the negative control, 200 µL of TSB/1%NaCl was added to triplicate wells to monitor any effects on optical density due to solvent or unforeseen factors. To determine the initial colony forming units (CFU) before the growth curve analysis, a serial dilution ranging from 10^-1 to 10^-6 was prepared, and 10 µL of each dilution was plated onto TSA/1%NaCl media in triplicate. Plates were then incubated at 37°C overnight, and CFU per mL was calculated, considering the dilution factor. Growth was monitored every hour by measuring the optical density at 600nm using a Synergy Neo2 Hybrid Multi-Mode Microplate Reader at 37°C for 18 hours (Biotek, USA). Before each reading, the microplate was agitated for 10 seconds at an amplitude of 3 millimeters. Growth curves were generated using the difference in OD between isolates and the mean of negative controls (n=3). The specific growth rate (µ) was calculated from the average slope of the exponential growth phase of each isolate on a semilogarithmic scale, and the doubling time (DT) was calculated using the formula ln(2)/µ, where ln denotes the natural logarithm (Bioquest, 2020).

2.7.3. Antibiotic susceptibility test

Antibiotic susceptibility testing was conducted following the guidelines outlined by the Clinical Laboratory Standards Institute (CLSI) and through a review of pertinent literature. This testing aimed to evaluate the susceptibility and resistance patterns of *Vibrio* isolates to a range of antibiotics commonly utilized both in aquaculture and clinical settings for managing Vibrio-related illnesses. The chosen antibiotics are amoxicillin (30 µg), azithromycin (15 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), Florfenicol (30 µg), tetracycline (30 µg) (Table 2.2). A suspension of a single colony of freshly grown isolates in 1X phosphate-buffered saline (PBS) equivalent to a 0.5 McFarland standard was prepared and sterile cotton-wool swab sticks were immersed in this

solution and evenly streaked onto Mueller-Hinton (MH) agar plates supplemented with 1% NaCl to ensure uniform distribution of the bacterial culture on the agar surface. The plates were allowed to stand for 15 minutes to facilitate the adherence of the bacterial culture. Following this, antibiotic disks corresponding to the selected antibiotics were carefully placed onto the streaked MH plates. Chloramphenicol antibiotic disks were from Liofilchem, Italy, while the rest were from Oxoid, United Kingdom (OXOID, UK). To promote optimal diffusion and interaction between the antibiotics and the bacterial culture, an additional 15-minute incubation period was observed before initiating the incubation process. The plates were then incubated at 37°C for 18 ± 2 hours. After the completion of the incubation period, the zones of inhibition surrounding each antibiotic disk were meticulously measured in millimetres using a ruler. These measurements were then interpreted according to the specific breakpoints outlined in the CLSI guidelines, allowing for the classification of isolates as susceptible, intermediate, or resistant to each antibiotic tested.

Table: 2. 2. List of the antibiotics used with their respective class and target site. (Saikia & Chetia, 2024)

Antibiotic type	Class	Mechanism of action (target site)
Amoxicillin/clavulanic acid	Penicillin	Cell wall inhibition
Azithromycin	Macrolides	Protein synthesis inhibition
Chloramphenicol	Amphenicol	Binds to 50S ribosomal sub-unit
Tetracycline	Tetracyclines	Inhibits DNA replication
Ciprofloxacin	Fluoroquinolone antibiotic	Binds to DNA gyrase
Florfenicol	A synthetic analogue of thiamphenicol	Protein synthesis inhibition

2.8. Biochemical assays

Several assays were performed to characterize the activity of haemolytic and proteolytic enzymes potentially related to their virulence.

2.8.1. Haemolytic activity assay

A single isolated colony was inoculated using a sterile inoculating loop on Columbia Blood Agar Plates (VWR, Portugal). This type of medium also serves to characterize the type of cell haemolysis: alpha haemolytic activity- results in a darker formation around the colony; beta

haemolytic activity- results in lysis in the erythrocytes around the colony; and gamma haemolytic activity- the absence of haemolytic activity. Plates were prepared with a fresh streak of the bacteria isolates obtained from TSA/1% NaCl plates and incubated for approximately 16h at 37 ℃ and the results were observed.

2.8.2. Proteolytic and Hydrolytic assays

For the proteolytic and hydrolytic enzymatic activities, TSA/1% NaCl was supplemented with 1% gelatine, 0.1% Tween 80, and 1% Tween 20 for proteolytic, lipolytic and esterase, respectively. For proteolytic activity testing, 4 g of gelatine was dissolved with TSA/1% NaCl (400mL final volume) and autoclaved. An isolated colony of fresh culture from TSA/1%NaCl was taken using a sterile inoculating loop and transferred on a TSA/1% NaCl containing 1% gelatine and incubated overnight at 37^0C. After overnight incubation, a saturated solution of ammonium sulphate was added to each plate to observe protein precipitation. For the lipase activity, 0.4 mL of previously filtered Tween-80 was added to 400 mL of TSA/1% NaCl. For the esterase activity, 4 mL of Tween-20 was added to 400 mL of TSA/1% NaCl. Like the proteolytic activity, an isolated colony of each isolate from a fresh culture on TSA/1% NaCl was taken and transferred to each media (without spreading) and incubated overnight at 37^0C. Each plate was evaluated for possible hydrolytic activity (lipase and esterase) by observing a precipitation/halo around the colony. Digital images were taken from all the plates to measure the area of the hallo using ImageJ open software and determine the enzyme activity index. For all three enzymatic assays, three technical replicates and three biological replicates were performed, and all isolates were tested on a separate plate to assess the variation due to random error and to eliminate the potential interference of the quality of the media in the assay.

2.9. Toxicology analysis

Because the production of neurotoxins has been associated with the presence of *Vibrio* spp, especially *V. algynolyticus* (Bacchiocchi et al., 2021a), the collected samples were analysed for the presence of the Tetrodotoxin (TTX) on the mussel body tissues. The sample extraction was done according to the method used by (Lage et al., 2023) which was applied to determine tissue accumulation of TTX and analogues in trumpet shell *Charonia lampas*. The mussel samples were homogenized using an Ultra-Turrax (T25 easy clean digital, IKA 107-Werke GmbH & Co.KG, Germany) until it reached a pasty consistency. Then, to 2 grams of the homogenized sample, 2 mL

of 1% acetic acid, was added and the mixture was homogenized for 3 minutes and next was incubated for 5 minutes in boiling water, with the lids of the tubes slightly unscrewed to release pressure. Subsequently, the tubes were transferred to a cold water bath containing ice for cooling. Once cooled, the sample undergoes homogenization using a rotating automatic homogenizer for 3 minutes. Following homogenization, the samples were centrifuged for 10 minutes at 4500 rpm at 15°C ((Mega Star 600 R, VWR, Avantor, USA). The resulting supernatant was carefully pipetted into a plastic tube and 5 µL of 25% ammonia was added, and the mixture homogenized manually. The ENVI-CARB cartridge (Supelclean, Supelco, Sigma-Aldrich, Germany), assembled into a vacuum system with two rubbers, was prepared by cleaning with 3 mL of an aqueous solution containing 20% acetonitrile and 0.25% acetic acid at a speed of 6 mL/min, discarding the cleaning solution afterward. Subsequently, 5 mL of 0.025% aqueous ammonia solution was passed through the cartridge at the same speed and discarded. Following this, 500 µL of the extracted sample was added to the cartridge and allowed to filter at the same speed, followed by the addition of 700 µL of Milli-Q water to the cartridge and subsequent filtering. A plastic tube was secured at the bottom of the cartridge in the vacuum system, and 2 mL of an aqueous solution containing 20% acetonitrile and 0.25% acetic acid was added to the cartridge and filtered at the specified speed. Finally, 200 µL of the filtered sample is diluted with 600 µL of acetonitrile and stored at -20°C until analysis until analysis by liquid chromatography- high-resolution mass spectroscopy (LC-HRMS). The LC-HRMS run was carried out by a lab member.

2.10. Data analysis

To assess the significance of temperature on the growth kinetics and enzyme activity of isolates and the difference between isolates, a two-factor ANOVA was applied. Statistical significance was considered when $p < 0.05$ with a 95% confidence limit Statistical analyses and graphics were generated using Excel and Origin-18 software. To determine the growth rates of the isolates and assess enzyme activity, each test was conducted using three technical replicates (n=3) and repeated on three different days to minimize random errors. The averages of the replicates were then calculated and used for further analysis.

R software was used to compare the virulence genes across different isolates by generating a heatmap. This approach allowed for clear and concise visualization of the variations and

similarities in virulence gene profiles, making it easier to identify patterns and draw meaningful conclusions from the data.

3. Result and discussion

3.1. Microbiological characterization

3.1.1. Colony isolation and initial characterization on TCBS agar

Like other bacterial strains, colony characterization of *Vibrio* on a specific and differential media provides useful phenotypic information to initially characterize the bacterial isolates. On TCBS agar, *Vibrio* colonies typically manifest as either yellowish or greenish in appearance based on their sucrose fermentation capabilities. When *Vibrio* isolates ferment sucrose, the resulting colonies appear yellow due to the acidification of the medium, causing pH-dependent indicators bromothymol blue and thymol blue to shift to a yellow colour. The appearance of yellowish colonies on TCBS agar indicated that all examined isolates in this investigation were capable of fermenting sucrose. On the other hand, differences in their colony appearance and abilities to ferment sucrose were noted.

As seen in Figure 3.1, the isolates can be categorized into three groups according to how well they ferment sucrose on TCBS agar: high, moderate, and poor fermenters. This indicates a high ability for sucrose fermentation, the isolates P-PP-T-4-A1 and P-PP-T-4-A2 showed widespread yellowing throughout the entire agar plate. As an indication of a reduced capacity for sucrose fermentation, isolates P-SA-T-2-A1, P-SA-T-2-B1, and P-SA-T-2-B2 instead caused localized yellowing close to their growth sites. The two isolates that remained, Pco-A-T1 and Pco-A-T3-A1, showed intermediate yellowing patterns and a moderate degree of fermentation. Based on the colony characteristics, the isolates may consist of three or more distinct *Vibrio* species or strains.

Although *Vibrio* spp can be classified based on its ability to use sucrose as a carbon source, the capacity of fermentation may not only vary between different species but also among different strains of the same species (Abushattal et al., 2020). Another study also suggested that there is no distinction in the ability of *Vibrio* strains to ferment sucrose on TCBS (Serratore et al., 1999) as typically sucrose non-ferments *Vibrio* spp, like *V. parahaemolyticus* and *V. vulnificus* can acquire the gene responsible for sucrose utilization through horizontal gene transfer (Hammerl et al., 2021).

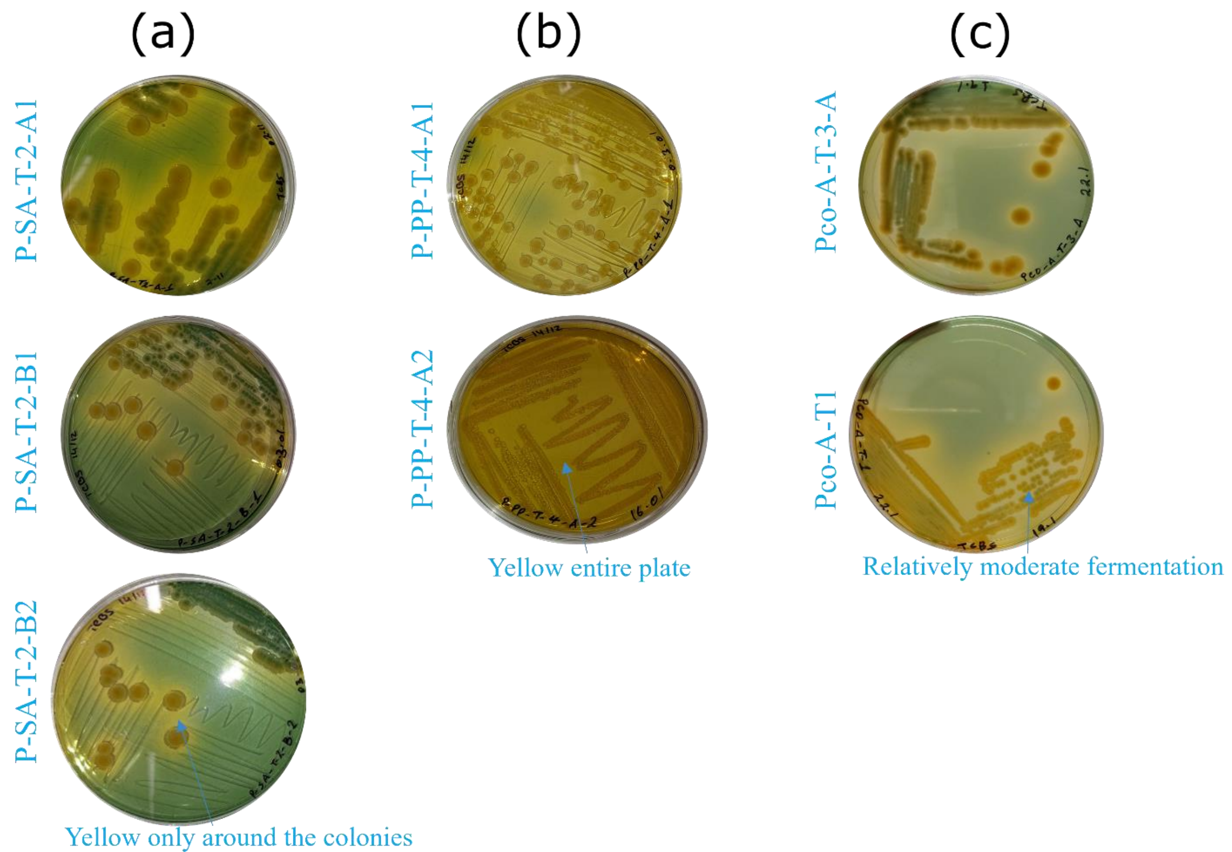

Figure: 3. 1. Digital images of the isolates on TCBS agar plates at 37 ^{0}C after 24 hours. The images demonstrate the sucrose fermentation capacity of each bacterial isolate; **(a)**, indicates the three isolates from one mussel sample (P-SA-T2), **(b)** two isolates from the other mussel sample (P-PP-T4), and **(c),** indicates the other two isolates from the surrounding water sample (Pco-A).

In addition to their biochemical, various *Vibrio* species can be distinguished from one another phenotypically based on the shape of their colonies. Table 3.1. summarizes the characteristics of sucrose-fermenting *Vibrio* species, which include medium to high colony sizes, no elevation (flat), and a circular yellow appearance, as observed in all colonies (Kourany, 1983; Letchumanan et al., 2014).

The isolates P-PP-T-4-A1 and P-PP-T-4-A2 were identified by their opaque colonies that had a translucent border in the surroundings. The colonies of the remaining isolates, on the other hand, were completely opaque. In addition, the isolates P-SA-T-2-A1, P-SA-T-2-B1, and P-SA-T-2-B2 showed comparatively larger to medium-sized colonies on TCBS agar. The sizes of the colonies in the other isolates ranged from medium to tiny. Together with their biochemical characteristics,

these phenotypic variations offer important information for differentiating and classifying *Vibrio* species and further suggest that we are in the presence of different strains.

Table: 3. 1. Summary of colony characteristics of isolates on TCBS agar plate at 37 ^{0}C after 24 hours. The colonies were characterized both with the naked eye and 4X microscopic magnification.

Isolate	Colour	Size	Shape	Texture	Halo	Elevation	Transparency
P-SA-T-2-A-1	Yellow	Large/Medium	Circular	Smooth	Yellow around the colony	Flattened	Opaque
P-SA-T-2-B-1	Yellow	Large/ Medium	Circular	Smooth	Yellow around the colony	Flattened	Opaque
P-SA-T-2-B2	Yellow	Large/Medium	Circular	Smooth	Yellow around the colony	Flattened	Opaque
P-PP-T-4-A-1	Yellow	Medium/Small	Circular	Smooth	yellow entire plate	Flattened	Opaque centre, transparent border
P-PP-T4-A-2	Yellow	Medium/Small	Circular	Smooth	yellow entire plate	Flattened	Opaque centre, transparent border
PCO-A-T-1	Yellow	Medium/Small	Circular	Smooth	Yellow around the colony	Flattened	Opaque
PCO-A-T-3-A	Yellow	Medium/Small	Circular	Smooth	Yellow around the colony	Flattened	Opaque

3.2. Molecular characterization

After the initial characterization of the isolates using standard microbiological techniques, molecular techniques were performed to characterize their identity and verify the species and potential strains. This involved amplifying, sequencing, and analyzing the 16S rRNA gene to accurately identify the *Vibrio* species.

3.2.1. 16S rRNA gene amplification and sequence analysis

Amplifying the 16S rRNA gene using universal primers and analysing the sequences was done with the objective of species identification as this specific region of the bacterial genome allows for the taxonomic classification of the bacteria. A search against the NCBI database revealed that four isolates; P-SA-T-2-B2, P-PP-T-4-A-1, P-PP-T-4-A2, and Pco-A-T-1 corresponded to either *V. alginolyticus* or *V. parahaemolyticus*. Additionally, the isolate P-SA-T-2-A1 was found to

correspond to *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*. The isolate P-SA-T-2-B1 corresponded to *V. alginolyticus*, *V. parahaemolyticus*, and *V. azureus*. Lastly, the isolate Pco-A-T-3-A corresponded to *V. alginolyticus*, *V. parahaemolyticus*, and *V. natriegens.* All the isolates corresponded to *V. alginolyticus* in common.

As shown in Table 3.2, three representative nucleotide BLAST hits from the NCBI database highlight similar levels of percent identity and other parameters, leading to inconsistent identification. This suggests that short sequences of the 16S rRNA gene may not be effective at differentiating between these closely related *Vibrio* species. The multiple sequence alignment analysis of 510 base pairs of 16S rRNA gene sequence including reference strains of *V. parahaemolyticus* and *V. alginolyticus* shows almost similar base sequences except a single nucleotide change in one of the *V. parahaemolyticus* reference strains (Supplementary figure 1). This revealed that the determination of species identity using 16S rRNA gene sequencing was challenging, especially when dealing with short segments. As encountered in this research and supported by other researchers, distinguishing between closely related *Vibrio* species like *V. alginolyticus*, *V. parahaemolyticus*, *V. diabolicus*, and *V. harvveyi* can be challenging and misleading. Researchers have not only underscored the limitation of the 16S rRNA gene as a genetic marker, especially when it comes to closely related strains due to less variability of the region but also emphasized the need for alternative tools that overcome this limitation (Bartoš et al., 2024). Hoffmann et al also explained that although 16S rRNA is a valuable marker, its applicability beyond the genus level is highly limited with good resolution mainly at the genus level, but not at the species or strain level (Hoffmann et al., 2010). The difficulty in accurately distinguishing between these species emphasizes the need for more precise methods of determination or longer gene sequences.

Table: 3. 2. Top 3 blast hits of 16S ribosomal RNA gene sequence of isolates showing similar sequences in the NCBI database. For ease of data presentation, only sample hits are taken. Except for the first three isolates, with less than 100% identity, the rest showed similar hits with inconsistent corresponding to *Vibrio* species.

Isolate code	**Top 3 similar strains**	E-value	%Identity	Accession
P-SA-T-2-A1	*V. alginolyticus*	0.0	99.80	MF996868.1
	V. harveyi	0.0	99.80	MF996867.1
	V. parahaemolyticus	0.0	99.80	MF996867.1
P-SA-T-2-B1	*V. alginolyticus*	0.0	100	CP054700.1

	V. alginolyticus	0.0	100	LC550089.1
	V. azureus strain	0.0	100	MT588841.1
P-SA-T-2-B2	*V. alginolyticus*	0.0	100	KU845389.1
	V. alginolyticus	0.0	100	KU845386.1
	V. parahaemolyticus	0.0	100	KU845385.1
P-PP-T-4-A-1	*V. alginolyticus*	0.0	100	OR785723.1
	V. parahaemolyticus	0.0	100	CP119301.1
	V. parahaemolyticus	0.0	100	CP133891.1
P-PP-T-4-A2	*V. alginolyticus*	0.0	100	KU845389.1
	V. alginolyticus	0.0	100	KU845386.1
	V. parahaemolyticus	0.0	100	KU845385.1
Pco-A-T-1	*V. parahaemolyticus* s	0.0	100	CP074415.1
	V. alginolyticus	0.0	100	CP054700.1
	V. alginolyticus	0.0	100	LC550089.1
Pco-A-T-3-A	*V. parahaemolyticus*	0.0	100	CP127847.1
	V. alginolyticus	0.0	100	CP142837.1
	V. natriegens	0.0	100	CP072782.1

3.2.2. Species-specific gene amplification

The genes *toxR*, *tdh*, and *gyrB* are a few examples of specific gene targets that can be used to identify and discriminate *Vibrio* species. *toxR* and *tdh* primers were selected specifically for the detection of *V. parahaemolyticus*, whereas the *gyrB* gene primer was selected because of its expected specificity to *V. alginolyticus*. The *toxR* and *tdh* primers amplified a DNA sequence in three isolates but were predicted to be *V. alginolyticus* based on the gyrB result, as shown in Figure 3.2. Previous research findings also underlined, when relying on toxR gene for the specific detection of *V. parahaemolyticus*, cross-reactivity with other *Vibrio* species might be observed due to the conserved nature of this gene among other strains of *Vibrio* species (Chakraborty et al., 2006; Najwa et al., 2015). Another study highlighted that *tdh* gene is a specific marker for the rapid detection of *V. parahaemolyticus* (S. Bin Park & Zhang, 2024), while other similar studies stressed this gene is also shared by other *Vibrio* species and might pose a false positive detection of *V. parahaemolyticus* (Baba et al., 1991; Yu et al., 2022). This indicates that it is still challenging to distinguish between at least the two species using these primer sets. However, *gyrB* primer later verified this result by precisely identifying each isolate as *V. alginolyticus*. *V. parahaemolyticus*,

which was genuinely included as a negative control, was not amplified by the primer confirming the specificity of our result as it does not seem to cross-react with *V. parahaemolyticus*, and most likely specific for the detection of *V. alginolyticus*. Thus, it is most likely that all of the isolates correspond to different strains of *V. alginolyticus* based on thorough characterization of the isolates.

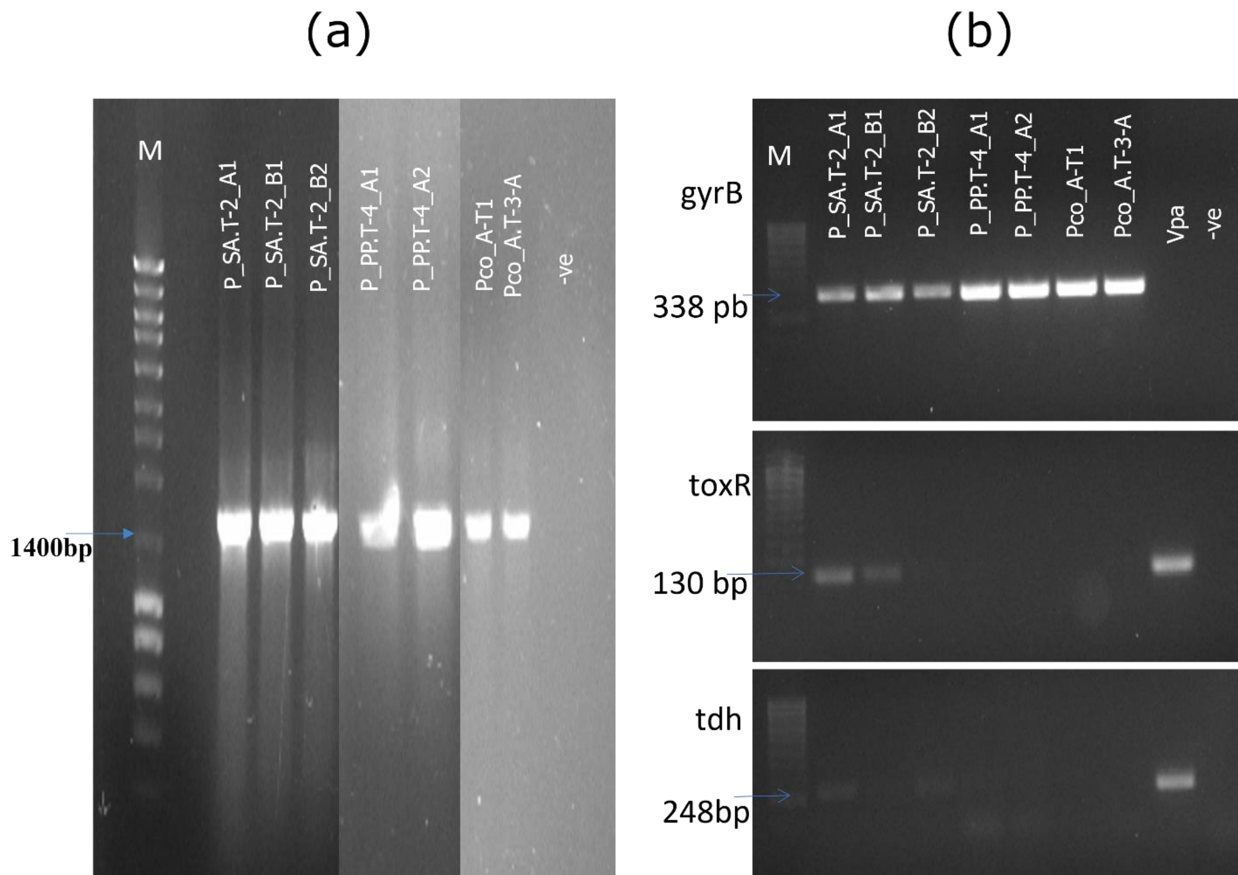

Figure: 3. 2. Gel electrophoresis of the success of the PCR amplifications using specific genes. (a): 16S rRNA gene amplification, photomontage summarising the results of the PCRs carried out: (b): species-specific gene amplification using three pairs of primers. The arrow indicates the size of the amplicon/fragment of the DNA marker/ladder that corresponds to the size amplified in the sample. Vpa was used as the positive control. -ve represents the negative control reaction (no genomic DNA included).

3.2.3. Growth curve and kinetics of isolates

A growth curve analysis was conducted to explore differences in the growth kinetics of bacterial isolates under two distinct temperature conditions: 37°C, representing the optimal body temperature for human hosts, and 24°C, representing the representative temperature range of marine organisms such as bivalves. This experiment aimed to assess the rate of bacterial multiplication and its potential implications for pathogenicity in both humans and the marine environment. For an illustration of bacterial growth profile, the curve was constructed using the log-transformed OD against time.

All the isolates successfully grow at both 37°C and 24°C, suggesting the capability of these isolates at a wide range of temperatures. Comparative studies also reported that most *Vibrio* species can grow at various temperatures though it can vary across different species and strains (Farid & Larsen, 1981a; Y. W. Kim et al., 2012; Miles et al., 1997). The growth behaviours of the isolates exhibited notable disparities between temperatures of 37°C and 24°C, as illustrated in Figure 3.2. At 24°C, all the isolates never reached the final stationary phase within the 18 hours of incubation which may be attributed to their slower nutrient utilization at this specific temperature due to a relatively lower growth rate compared to at 37°C.

Among the seven isolates, three displayed a unique growth characteristic at 24°C, marked by three distinct phases: an initial exponential phase, followed by a short stationary phase, and then another clear exponential phase. These unique phases might occur due to various factors, like a switch in nutrient utilization due to a depilated first choice energy source (Baker et al., 1983), change in pH, occurrence of metabolic byproducts (Goh et al., 2002), quorum sensing, and so on. A study by (Hammer & Bassler, 2003) explained that quorum sensing can lead to a temporary pause in growth (stationary phase) followed by a resumption of growth once a critical population density or environmental condition is met.

This experiment revealed at least four distinct growth behaviours or kinetics among the seven isolates (Figure 3.3). Notably, three isolates; P-SA-T-2-A1, P-SA-T-2-B1, and P-SA-T-2-B2, highlighted in black, red, and blue respectively, demonstrated remarkably similar growth behaviours, with their growth kinetics lines nearly overlapping across all growth phases. This suggests a potential relatedness among these isolates. This similarity was further documented by their corresponding colony morphology characteristics, sucrose utilization status on TCB agar medium, and enzyme activity index.

Similarly, isolates designated as P-PP-T-4-A1 and P-PP-T-4-A2, identified in green and purple respectively, exhibited closely resembling growth patterns, implying potential similarity. This was supported by their growth behaviour on TCBS agar medium, as illustrated in Figure 3.1. Conversely, the pair of isolates, Pco-AT1 and Pco-A-T-3A, sampled from surrounding water, displayed distinct growth behaviours and kinetics both among themselves and compared to other isolates suggesting they might be distinct *vibrio* isolates. In this study, the *V. parahaemolyticus* (VPA) strain, which had been initially characterized by another group in the laboratory and used here as a control for comparison, exhibited a distinctive growth pattern when analysed alongside the new isolates. This growth pattern was notably different from those observed in the new isolates at both tested temperatures.

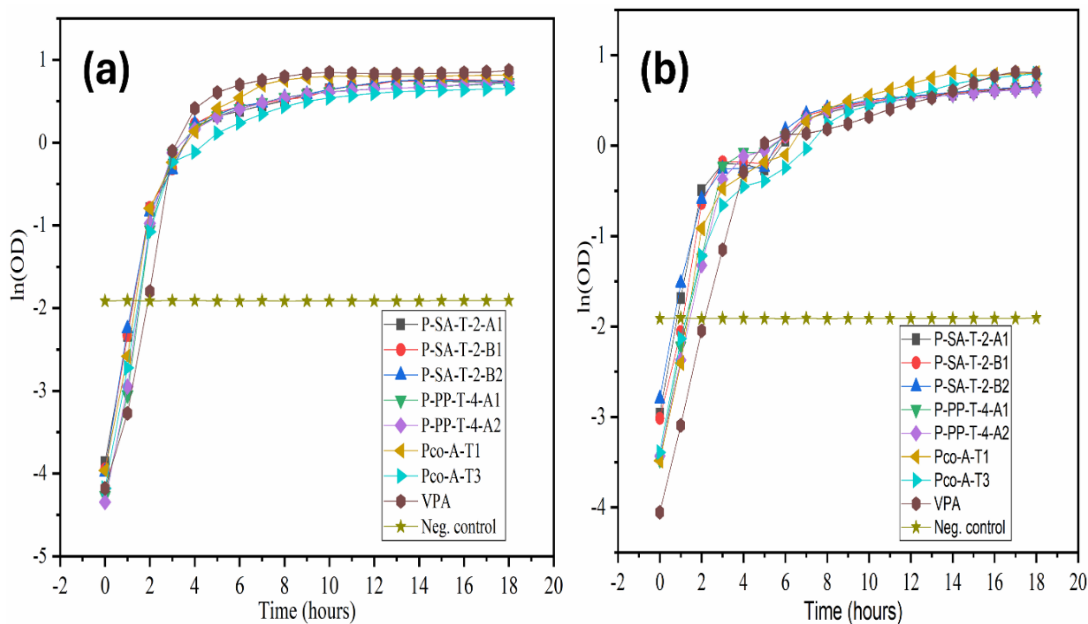

Figure: 3. 3. Log-transformed growth patterns of isolates in TSB; (a); 37 ^{0}C; (b); 24 ^{0}C. The change in optical density (OD at 600 nm) was measured using Synergy Neo2 Hybrid Multi-Mode Microplate Reader, (Biotek, USA). The graphs are constructed using the log-transformed (natural logarithm/ln) mean of the triplicate OD of each isolate.

Specific growth rates and duplication of each isolate are calculated by considering the clear exponential phase in the bacterial growth phase determination. As summarized in Table 3.3, it is evident that across all isolates, there was a substantial increase in growth rates per hour when incubated at 37°C compared to 24°C. This difference was statistically significant, with a p-value of less than 0.01 and a 95% confidence level. At the same temperature level, a statistically

significant difference was not observed between isolates. This has been previously reported for this species where it has been shown that *V. alginolyticus* and *V. parahaemolyticus* were better isolated at 37°C than any of the temperature levels including 22°C which supports the current study (Tall et al., 2013). A study conducted by Farid and Larson on the growth of *V. alginolyticus* with temperatures ranging from 5-42°C, reported extraordinary flexibility of *V. alginolyticus* with the highest growth between 30-37 °C(Farid & Larsen, 1981b). Gu *et al* also reported similar findings explaining that *V. alginolyticus* can survive a wide range of temperatures; 22 to 42°C (Gu et al., 2016). The average duplication time of *Vibrio* species varies based on genetic characteristics, available nutrients, environmental factors, and other factors. At the optimal human body temperature of 37°C, all isolates exhibited rapid duplication, completing the process between 25 to 28 minutes while at 24°C duplication required 34 to 46 minutes. A similar study on the generation time of 30 *V. parahaemolyticus* and *V. alginolyticus* species reported a doubling time of 12 to 25 minutes at 37°C, (Ulitzur, 1974) which suggests growth kinetics may vary between different strains of the same isolate.

A study done by Josef Hoff *et al* and Weinstock *et al* reported a slower duplication time for *V. parahaemolyticus* and *V. alginolyticus*, 12 -14 minutes, and 10 minutes doubling time by another species, *V. natriegens* (Hoff et al., 2020; Weinstock et al., 2016). Another study also reported a lower generation time for *V. alginolyticus,* 14 minutes on average at 37°, which is a lower doubling time than the isolates in the current study (Li et al., 2021). This variation may be due to various reasons, like, differences in strain, nutrition, initial number of isolates, the estimation method, and other factors. Among the isolates, *V. parahaemolyticus* demonstrated the slowest growth rate per hour at 24°C, growing at 0.903 per hour. Following closely behind was Pco-A-T1, which also exhibited a relatively lower growth rate compared to the other isolates, with a growth rate of 0.998 per hour.

Table: 3. 3. The average growth rate and duplication time of isolates at 37 ^{0}C and 24 ^{0}C. The growth rate of each isolate was calculated using a log-transformed slop of the exponential phase and duplication time was calculated using the formula; Duplication time= ln (2)/growth rate; where '**ln**' is the natural logarithm.

Isolate code	**37 ^{0}C**		**24 ^{0}C**	
	Growth rate/hour	Duplication time (hours)	Growth rate/hour	Duplication time (hours)
P-SA-T2-A1	1.52±0.02	0.60 ±0.01	1.17 ±0.05	0.59±0.02
P-SA-T2-B1	1.55±0.04	0.45 ±0.01	1.14 ±0.05	0.61±0.03
P-SA-T2-B2	1.51±0.10	0.46 ± 0.03	1.08 ±0.03	0.64±0.01
P-PP-T-4-A1	1.55±0.09	0.45 ± 0.03	1.04 ±0.08	0.67±0.055
P-PP-T-4-A2	1.60±0.13	0.44 ± 0.04	1.00 ± 0.03	0.70±0.02
Pco-A-T1	1.56±0.03	0.44 ± 0.01	1.23 ± 0.06	0.57±0.03
Pco-A-T3-A	1.51±0.07	0.46 ± 0.02	1.06 ± 0.12	0.66±0.07
VPA	1.54±0.06	0.45 ± 0.02	0.90± 0.05	0.77±0.50

3.2.4. Haemolysis testing on BAP

Vibrio species are reported to show haemolysis activity, including *V. alginolyticus* and *V. parahaemolyticus* (Gargouti et al., 2015) which is among a substantial virulence factor contributing to their pathogenicity. The haemolytic activity is mainly attributed to the production of haemolysin toxin which is encoded by the gene, especially *hlyA* and *tdh*, and responsible for the lysis activity in mammalian erythrocytes (Gargouti et al., 2015). In the present study, all the isolates exhibited alpha haemolysis (Figure 3.3), visually characterized by a distinct greenish discoloration around the colonies. This manifestation signifies a partial lysis of erythrocytes, suggestive of a significant role in the pathogenicity of the bacteria, contributing to the initiation or progression of disease within the host organism.

Although the specific type of haemolysis was not detailed, Gargouti et al., 2015) documented the occurrence of haemolysis in *V. alginolyticus* through a haemolysis activity assay. Their study indicated that *V. alginolyticus* exhibits haemolytic activity, suggesting the production of haemolysins or other factors capable of lysing red blood cells. This observation is consistent with the general understanding that haemolysis is a notable feature of various *Vibrio* species.

Another research on '*Comparative haemolytic Activity of V. parahaemolyticus and Related Vibrios'* reported that *V. parahaemolyticus* showed all three types of haemolysis activity types (alpha, beta, gamma) where each type of haemolysis varied on the blood group and source of blood used (ox, monkey, sheep, human) (Twedt et al., 1970). Although there is clear evidence that some *Vibrio* species have different types of haemolysis both in vitro and in vivo, the exact mechanism of haemolysis is not established, unlike haemolytic activities in other bacterial species.

In a recent study we employed species-specific PCR to analyse seven Vibrio isolates and found that the tdh gene was present in only three of these isolates (P-SA-T-2-A1, P-SA-T-2-B1, and P-SA-T-2-B2). Despite this, all isolates exhibited partial lysis of erythrocytes when tested on blood agar plates, a result coherent with haemolysis. This discrepancy suggests that the observed haemolytic activity might not be solely attributed to the tdh gene. Instead, it implies that other haemolytic virulence factors could be at play in the haemolytic process. The partial haemolysis on blood agar plates points to the potential involvement of additional or alternative virulence determinants beyond the tdh gene, which may contribute to the haemolytic phenotype observed in these Vibrio species. Further investigation into other haemolysin genes or virulence factors may provide a clearer understanding of the mechanisms underlying this haemolytic activity.

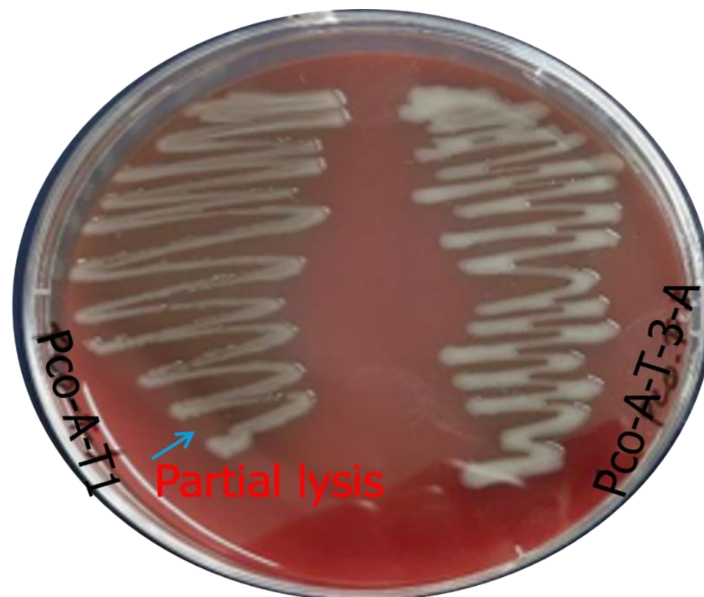

Figure: 3. 4. A digital photograph of representative haemolysis characteristics of isolates on blood agar plate (magnification of 4.67 pixel/mm). The arrow indicates a greenish discoloration around the colonies showing partial haemolysis of erythrocytes (alpha haemolysis). Only two isolates (Pco-A-T1 and Pco-A-T-3-A) that were collected from the environmental water are represented as the others exhibit a similar haemolytic pattern. The magnification is calculated by dividing the pixel of the image by the diameter of the petri dish (90 milli meter)`

3.2.5. Antimicrobial susceptibility

The antibiotic disks used for the present study were chosen based on their current applicability in treating *Vibrio* infections. The antibiotics were Amoxicillin in combination with clavulanic acid, azithromycin, ciprofloxacin, chloramphenicol, florfenicol, and tetracycline. When tested against the isolates, the antibiotics showed a variety of inhibitory zones, as shown in Figure 3.4. Depending on the defined cutoff values for each antibiotic, it is possible to evaluate these results and determine whether an isolate is classed as sensitive, intermediate, or resistant. Standard-setting bodies establish these cutoff values and offer instructions for reliable and consistent susceptibility data interpretation. Among these organizations are the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI. The varied zones of inhibition highlight how crucial it is to apply these standardized criteria to precisely assess how well antibiotics work against *Vibrio* isolates. As summarized in Table 3.4, seven of the isolates tested in the current study were susceptible to all six antibiotics at 37°C, suggesting the potential of these agents in effectively treating *Vibrio* infections potentially caused by these isolates.

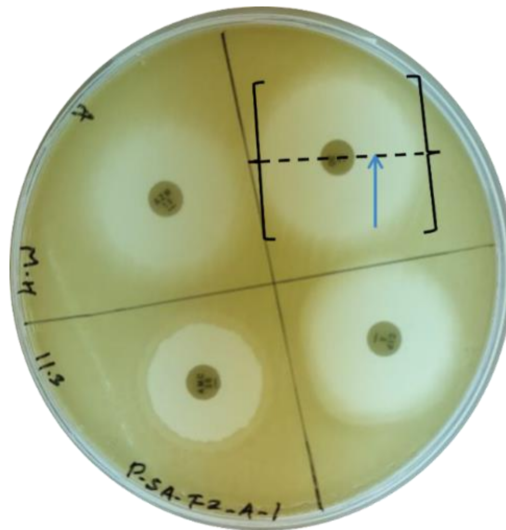

Figure: 3. 5. Digital image of a representative disk diffusion method antimicrobial susceptibility testing (magnification of 5.89 pixel/mm). The arrow indicates the zone of inhibition of bacterial growth. The magnification is calculated by dividing the pixel of the image by the diameter of the petri dish (90 milli meter)

Research by Eun-Gyoung *et al* and Chia W. Tan1 *et al* reported 100% susceptibility of *Vibrio* species for Amoxicillin–clavulanic acid which agrees with the findings in our research. On the other hand, the same research revealed that 11.9, 3.7%, and 0.5% of the isolates were resistant to tetracycline, chloramphenicol, and ciprofloxacin respectively which is different from our results

(Oh et al., 2011; Tan et al., 2017). This discrepancy suggests that variations in strain types, geographical locations, or antimicrobial practices may contribute to the observed differences in antibiotic resistance profiles between the studies. Such variations highlight the importance of considering local factors and strain-specific characteristics when evaluating antimicrobial resistance in Vibrio species. As reported by Gxalo et al., 2021b, unlike the expected efficacy of Amoxicillin-clavulanic acid to most *Vibrio* species, *V. mimicus*, *V. vulnificus*, and *V. fluvialis* were resistant to this antibiotic, 72.5%, 97.3%, and 90.2% respectively. Other studies also revealed 100% susceptibility of *Vibrio* species for chloramphenicol and tetracycline which is a similar finding to our study (Pandey et al., 2023).

Table: 3. 4. Type of antibiotics with the average zone of inhibition in milli meter (mm). The test was performed in triplicates and the average was taken as a zone of inhibition and compared against the CLSI and EUCAST antimicrobial susceptibility break-point for the classification.

Isolates	AMC		AZY		CIP		C	S/I/R	FFC	S/I/R	TE	S/I/R
P-SA-T-2-A1	19.50± 0.50	S	20.70± 0.58	S	21.20± 0.29	S	24.80± 0.29	S	26.80± 0.29	S	21.00± 1.00	S
P-PP-T-4-A1	19.30± 0.58	S	23.70± 0.58	S	26.70± 0.58	S	23.80± 0.29	S	24.70± 0.58	S	20.30± 0.58	S
Pco-A-T1	20.70± 1.15	S	23.30± 0.58	S	22.00± 1.00	S	26.80± 0.29	S	27.00±1.00	S	22.70± 1.15	S
Pco-A-T-3-A	18.70± 0.58	S	20.30± 0.58	S	25.70± 0.58	S	26.70± 0.58	S	24.80± 0.29	S	18.30± 0.58	S
VPA	23.00± 1.00	S	21.70± 0.58	S	22.70± 0.58	S	25.30± 0.58	S	25.20± 0.29	S	21.67 ± 0.58	S
Breakpoint (mm)	≥18		≥16		≥21		≥18		≥20		≥15	

* S= Susceptible: I= Intermediate: R= Resistant
* AMC= Amoxicillin/Clavulanic; AZY= Azithromycin: CIP= Ciprofloxacin:C= Chloramphenicol: FFC= Florfenicol

Research findings indicate significant variability in the antimicrobial susceptibility of *Vibrio* species for specified antimicrobials. Some studies report 100% resistance (Pandey et al., 2023) to certain antimicrobials, while others report no resistance at all which shows an inconsistent efficacy of antimicrobials even within the same species. This variation might be due to various factors, including differences in antimicrobial usage across different study sites and between countries, variations in bacterial strains, environmental factors influencing susceptibility, and horizontal gene transfer between strains (Deng et al., 2020a; Dutta, Kaushik, et al., 2021; Haque et al., 2023a; Onohuean et al., 2022). These inconsistent reports between different researchers make it

challenging for the establishment of clear conclusions about the efficacy of specific antibiotics against certain bacterial isolates and the susceptibility of specific bacterial isolates to certain antimicrobials. This variation highlights the need for standardized antibiotic utilization in patient care and by stakeholders with a global focus.

3.3. Enzyme activity testing

3.3.1. Proteolytic activity

Gelatinases are examples of extracellular enzymes some *Vibrio* species produce that may hydrolyse proteins, including gelatine. Gelatinases are metalloproteinases with the capacity to hydrolyse collagen and other extracellular matrix constituents. Certain *Vibrio* species have been shown to secrete gelatinase-active enzymes, which may increase their pathogenicity and virulence (Deane et al., 1986; Vu et al., 2017). These enzymes are known for their crucial significance in the virulence and survival strategies of *Vibrio* species (Chimalapati et al., 2020; Miyoshi, 2013). To account for the ideal body temperature for humans and the temperature of the marine environment, isolates were examined for the activity of secreted enzymes using plate assay procedures at two different temperature levels, 37 ^{0}C and 22 ^{0}C, respectively (Figure 3.6). At both temperatures, all isolates exhibited proteolytic activity, with 37 ^{0}C showing the highest activity with a $p\text{-value} < 0.01$ and a 95% confidence level. However, statistically significance difference was not observed between the isolates at the same temperature. According to a study by (De Silva et al., 2019), gelatinase activities were was observed in *V. parahaemolyticus, V. alginolyticus*, and *V. diabolicus*.

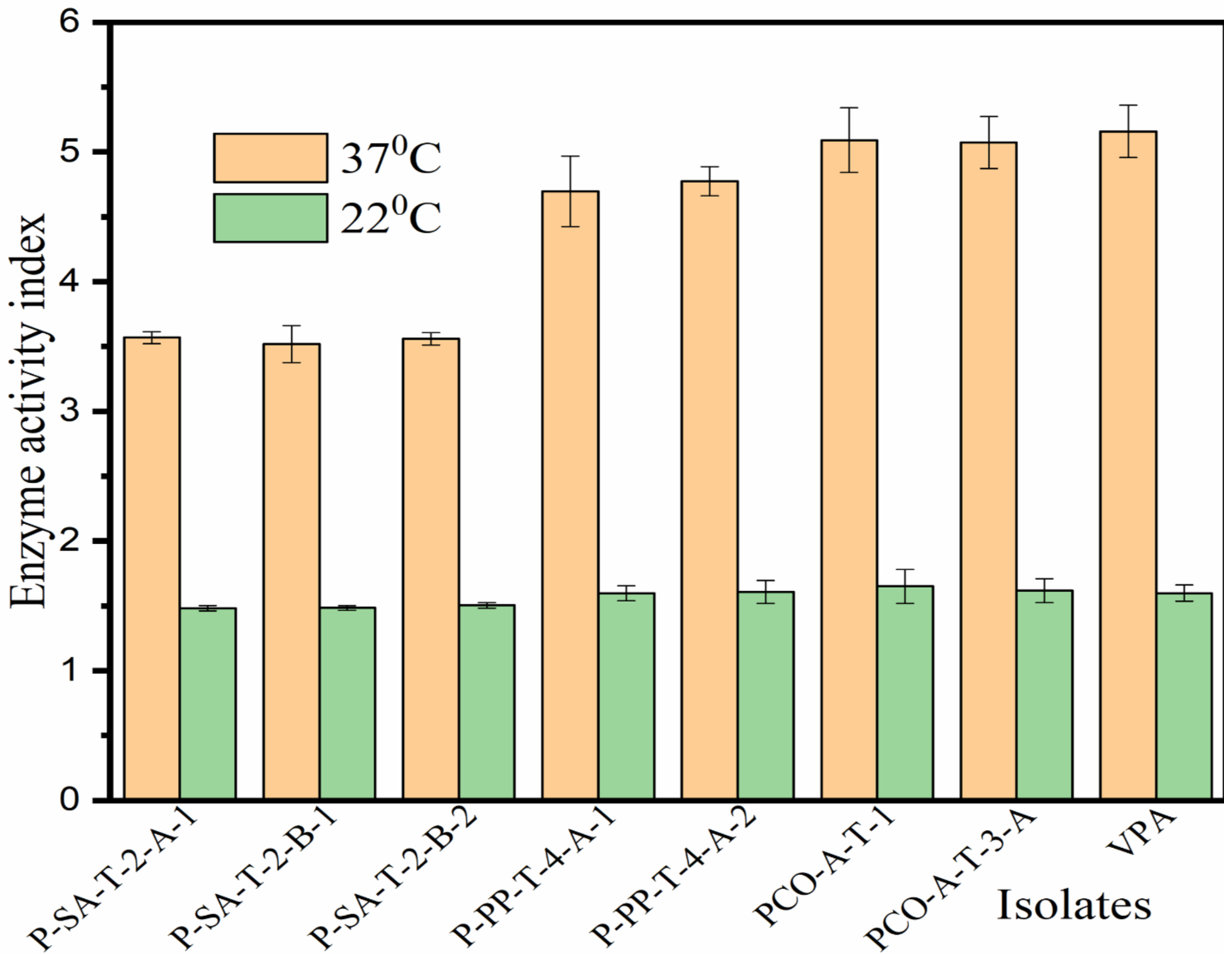

Figure: 3. 6. Average gelatinase activity index of isolates, (n=3) at 37^0C and 22^0C. The Vertical-axis shows the enzyme index and the Horizontal-axis list of isolates The enzymatic index was calculated by dividing the size of the total halo area (colony size + halo size) by colony size. The area is measured using Image J software.

3.3.2. Lipase and esterase activities

Together with other virulence factors, lipase, and esterase enzymes provide a complex strategy that increases the capacity of *Vibrio* species to infect their hosts and cause disease. In this study, the activity of lipase and esterase enzymes were determined using TSA supplemented with tween-80 (0.5%) and tween-20 (1%), respectively. All isolates showed both lipase and esterase activity at 22 ^{0}C with the control bacteria, *V. parahaemolyticus* was the exception that did not show any lipase activity. No isolate showed lipase and esterase activity at 37 ^{0}C. Four isolates, P-PP-T-4-A1, P-PP-T-4-A1, Pco-A-T1, and Pco-A-T-3-A showed relatively higher esterase activity than the rest of the isolates, while lipase activity was relatively higher in only in two of the isolates, Pco-A-T1, and Pco-A-T1. PA showed the highest esterase activity, followed by Pco-A-T-3-A (Table

3.5). Since the hydrolysis was colourless, it was not possible to measure the area of enzyme hydrolysis in our study or capture it in digital images to compute the enzyme index. As a result, we choose to report the data as positive or negative with a relative activity level as presented in Table 3.5. This restriction emphasizes the necessity of using various approaches or developing new strategies if quantitative measurements are needed in subsequent research.

Table: 3. 5. Qualitative lipase and esterase activity of isolates with a relative activity level. The activities were visually estimated at 37 ^{0}C and 22 ^{0}C using plate assay method.

	Enzyme activity			
	Lipase		**Esterase**	
Isolate code	**37 ^{0}C**	**22 ^{0}C**	**37 ^{0}C**	**22 ^{0}C**
P-SA-T-2-A1	-	+	-	+
P-SA-T-2-B1	-	+	-	+
P-SA-T-2-B2	-	+	-	+
P-PP-T-4-A1	-	+	-	++
P-PP-T-4-A2	-	+	-	++
Pco-A-T1	-	++	-	++
Pco-A-T-3-A	-	++	-	+++
VPA	-	-	-	++++

* **+ = Activity present :** ++= Moderate activity present: +++= higher activity: ++++= very high activity present: - **= Enzyme activity not present**

The reason for the negative lipase and esterase activity in the test at 37℃ could be the absence of expression of the genes encoding these enzymes in plate assay techniques, suggesting that expression of this enzyme may be temperature dependent. Using the plate test method at room temperature, it was discovered that *V. parahaemolyticus* and *V. alginolyticus* secreted measurable quantities of lipase and esterase, according to a study on the extracellular secretion of pathogenic enzymes by Manilal et al., 2010. This result is in line with our investigation, which found that lipase and esterase activities were positive at 22°C.

3.4. Tetrodotoxin (TTX) detection

Since the presence of TTX has been reported from samples where *Vibrio* species have been found, the mussel samples were examined for the presence of this toxin using a high-resolution mass spectrometry detection method. The LC-HRMS method made use of a recognized TTX standard with an 8.1-minute retention time. Figure 3.7 shows a sample chromatogram of interest with TTX standard and corresponding retention time. According to the analysis, TTX levels were either completely absent from the mussel samples or present in amounts below the method's limit of detection (LOD), at least when it came to the same analogue form as the standard used for detection. In a previous study using mussel samples collected in Italy, it was reported that 3% of mussel samples from the year 2018 (99 samples) and 14% of those from 2019 (58 samples) contained measurable levels of TTX (Bacchiocchi et al., 2021b). The European Food Safety Authority (EFSA) recommends a maximum TTX level of 44 µg/kg for mussels and oysters. However, due to the lack of up-to-date and reliable data, this guideline indicates that consumers who consume large portions of 400 g or more might occasionally face concerns regarding TTX levels ((CONTAM) et al., 2017). In our study and in order to have a better representation of the contamination in the mussel samples in the sampling area a larger number of samples should be analyzed in the future to have a better assessment of the potential bioaccumulation of this toxin in mussel and other marine organisms.

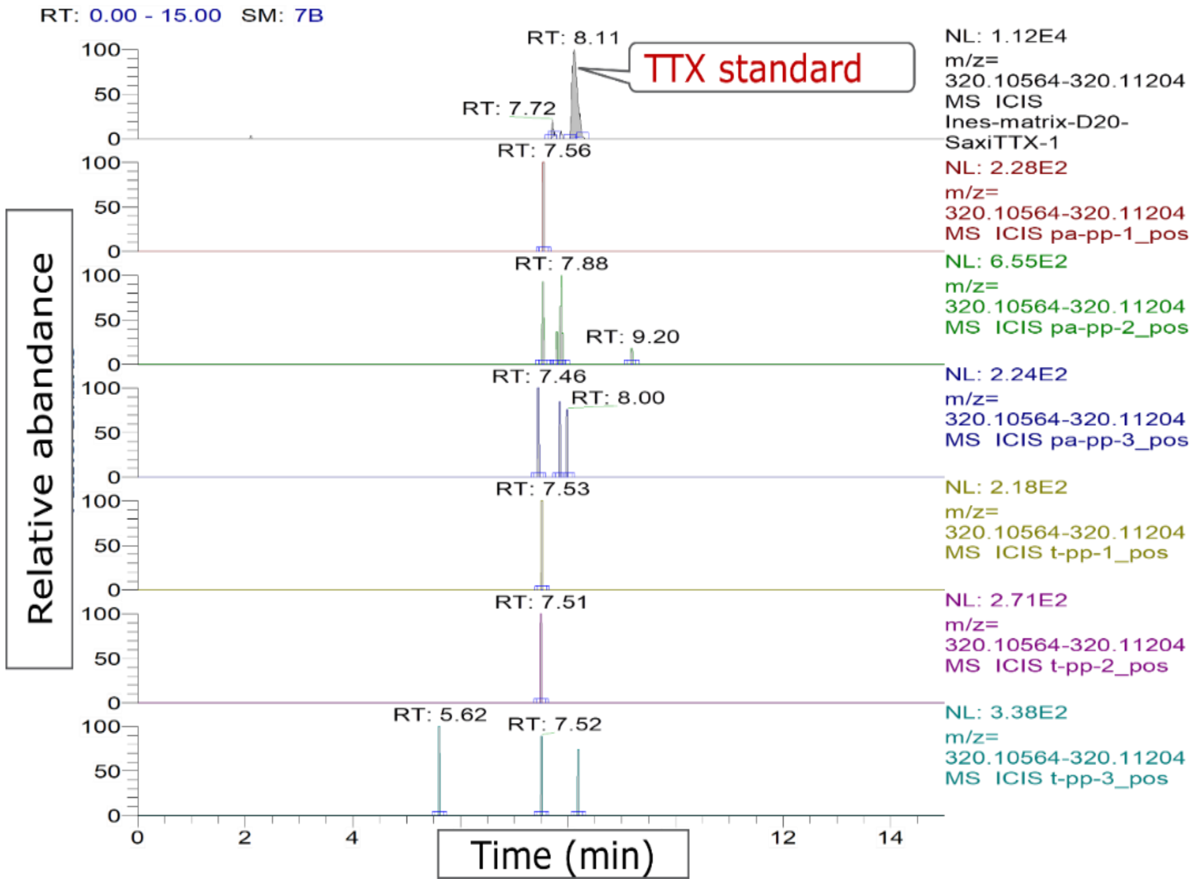

Figure: 3. 7. Sample chromatogram picture of the tested sample with respective retention time. Numbers from 0 to 1000 on the vertical axis show the relative mass-to-charge ratio (m/z) and on the horizontal axis the retention time from 0 to 14. The analyte of interest has a retention time of 8.1 minutes.

3.5. Genome sequence analysis

After the general characterization of all the isolates using basic microbiological characterization, PCR (16s rRNA and species-specific gene amplification), enzymatic activity, and toxin analysis (TTX), three isolates Pco-A-T1, P-PP-T-4-A1, and P-SA-T-2-A1, the first isolated from the water and the two other isolated from mussel tissues were selected for whole genome sequence for a well comprehensive analysis and to confirm their identity. This genome analysis enhances our understanding of the genomic diversity, virulence, and antibiotic resistance of *Vibrio* species.

3.5.1. General genome annotation

Based on the genome assembly results, the isolates showed different genome lengths across the three isolates (Table 3.6). Specifically, Pco-A-T1, P-PP-T-4-A1, and P-SA-T-2-A1 genome sizes were 4,995,277 bp, 5,335,012 bp, and 5,150,191 bp, respectively. All three isolates had a guanine-cytosine (GC) content of at least 44%. The isolates differed in terms of assembly metrics, with P-SA-T-2-A1 having 37 contigs, P-PP-T-4-A1 having 44 contigs, and Pco-A-T1 having 39 contigs. The number of predicted coding genes is also distinct and revealed that P-PP-T-4-A1 has the highest (4,813) and P-SA-T-2-A1 the lowest (P-SA-T-2-A1) These differences in the overall features of the genome annotation imply that the isolates may be different *Vibrio* species. The higher N50 value (represents the size of the largest contig that has at least 50% the bases of the assembly) suggesting higher assembly continuity.

Table: 3. 6. General genome annotation of the isolates comparing the three isolates. The table presents the most significant parameters showing the quality of assembly and for the rough comparison of the genome of the isolates.

Parameter	Isolates		
	P-SA-T-2-A1	P-PP-T-4-A1	Pco-A-T1
Genome length (bp)	4,995,277	5,335,012	5,150,191
GC content	44.83%	44.64%	44.56%
N50 (bp)	1,036,580	333,907	316,925
Contig number	37	44	39
Number of Predicted coding genes (bp)	4,479	4,813	4,616
Length of coding genes (bp)	4,343,274	4,645,356	4,467,468

3.5.2. Species homologous identification

The distribution of homologous species is predicted in Figure 3.8. This confirmed that the three isolates were *Vibrio* species but that the isolate P-SA-T-2-A1 is likely to be *V. diabolicus* (28.67%) while the remaining two isolates, P-PP-T-4-A1 and Pco-A-T1 share 34.92% and 29.91%, are likely to be *V. alginolyticus* confirming the results from the preliminary molecular analysis using the 16S rRNA and species-specific gene amplification. Because of their different levels of homology as well as their unique behavioural and genotypic traits, these two isolates are therefore likely to represent different strains of *V. alginolyticus*. These results are in agreement with the specie-specific amplifications because the isolate P-SA-T-2-A1 shows amplification for all the three genes tested suggesting it may be a different species/strain when compared with the other isolates, while only *gyrB* gene was amplified from P-PP-T-4-A1 and Pco-A-T1 (Figure 3.2).

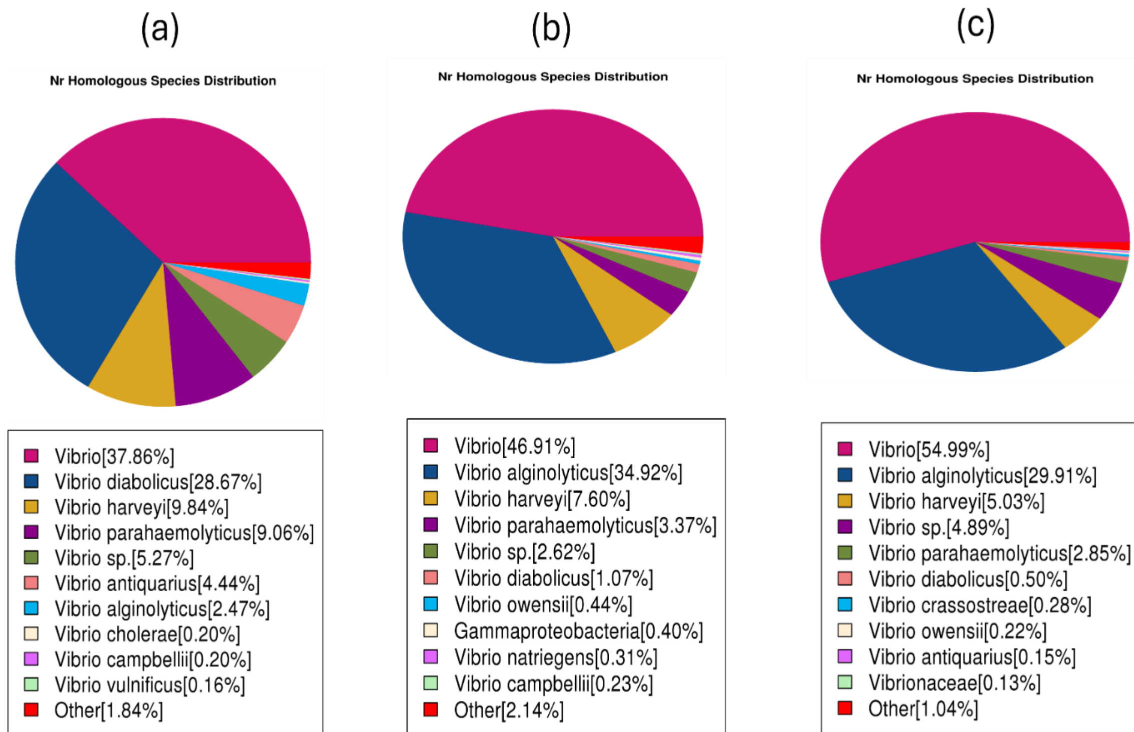

Figure: 3. 8. Annotation of the genes identified in the genome of the 3 isolates. Indicating the relative contribution to the annotation of multiple *Vibrio* species. These results are obtained by comparing all the predicted proteins identified in the genomes of the three isolates against the NCBI Non-Redundant (nr) database. (a); P-SA-T-2-A1: (b); P-PP-T-4-A1: (c); Pco-A-T1.

Because of its virulence and antibiotic resistance, the marine bacteria *V. diabolicus* has been highlighted as a possible hazard to public health (Nava-Soberanes et al., 2021). *V. diabolicus* belongs to the *Harveyi* clade and is genetically very similar to *V. alginolyticus* (Nava-Soberanes et al., 2021; J. W. Turner et al., 2018). *V. diabolicus*, *V. alginolyticus* and *V. antiquarius* had genome similarities of more than 97% (J. W. Turner et al., 2018b), indicating that phenotypic characterization and short sequence DNA fragment amplification are not reliable methods for differentiating between these closely related species.

3.5.3. Virulence factor prediction

A brief overview of the different virulence factors found and the differences between the two different species and strains is provided. A total of 128 virulence factors, listed in the Virulence Factors Database (VFDB) were predicted in the genome annotation of this study. Of these, 67 virulence factors were common to all the three isolates. In the two *V. alginolyticus* species, 105 virulence factors were predicted in each genome, while in *V. diabolicus*, 92 virulence-associated factors were found. Of the 128 predicted virulence factors, 17 were associated with adherence and biofilm formation, 49 with effector delivery systems, 50 with flagella, 4 with exotoxins, 2 with biofilm formation and quorum sensing, and the remaining 6 were related to other virulence functions. As illustrated in Figure 3.9, of the 65 virulence genes that varied between isolates, 37 were attributed to type-III and type-VI effecter delivery systems.

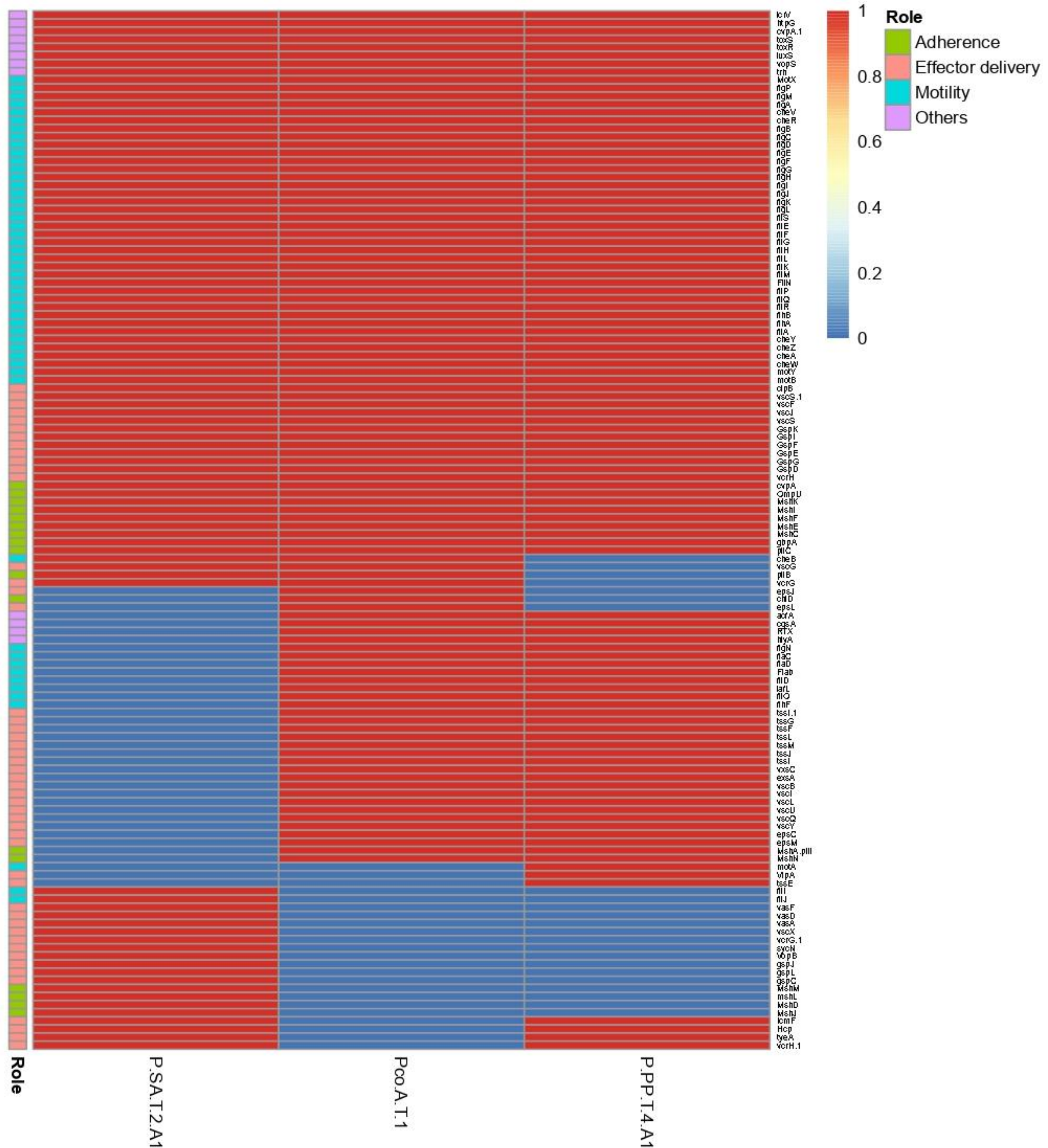

Figure: 3. 9. Heatmap showing the distribution of virulence factor across the three *Vibrio* species. The red label shows the presence of the virulence factor while the blue label shows their absence. The different colours indicate the role of the virulence factors.

3.5.3.1. Virulence factors related to adherence

Chitinase plays a crucial role in the pathogenicity of bacteria by aiding in host attachment and tissue penetration (Defoirdt et al., 2010). The gene *chiD*, which codes for chitinase is only detected in one of *V. alginolyticus* (Pco-A-T1). Virulence factor chitinase has been detected in *Vibrio* isolates, including those from the *Harveyi* clade.(Ruwandeepika et al., 2010). The other virulence factor *pilB* (type IV pilus), which plays a crucial role in motility, adhesion, biofilm formation, and pathogenesis (Dye & Yang, 2020) was detected in one of the *V. alginolyticus* (Pco-A-T1) and also in *V. diabolicus*. The proteins *MshA* and *MshN*, both play a crucial role in bacterial adhesion, colonization, biofilm formation, and virulence (Jonson et al., 1994) and *OmpU* (Outer Membrane Protein U), mediating adhesion of *Vibrio* spp. to host cells (X. Liu et al., 2015). These genes were only identified in the two *V. alginolyticus* species. Studies have reported that *MshA* and *MshN* affect surface attachment and biofilm formation in *Vibrio* cholerae by contributing to pilus extension and retraction (Deng et al., 2020b; Hughes et al., 2022; Wickramanayake et al., 2020). Additionally, these two virulence factors are associated with the bacteria's survival in aquatic environments by upregulating adhesin production (Deng et al., 2020b; Stauder et al., 2010; Wickramanayake et al., 2020). A study by (D.-K. Park et al., 2006) addresses the role of *OmpU* in several pathogenicity-related activities in *Vibrio* species, including bile acid production, resistance to antimicrobial peptides, organic acid tolerance, biofilm development, and adhesion to host cells. Other adherence-related virulence genes, such as *MshJ*, *MshL*, and *MshM* were only predicted in *V. diabolicus* and they are associated with the assembly and function of the mannose-sensitive hemagglutinin (MSHA) pilus (Hughes, 2022) which are essential for biofilm formation and surface attachment (Erken et al., 2015; Hughes et al., 2022).

3.5.3.2. Effector delivery system-related

Effector delivery mechanisms, essential to the pathogenicity of bacteria, alter host cell activities by injecting effector proteins into host cells or the surrounding environment. According to (Miller et al., 2019), the Type VI secretion system (T6SS) and the Type III secretion system (T3SS) are the two most common effector delivery systems in *Vibrio* spp. These systems play a crucial role in coordinating bacterial behaviour and pathogenicity, as evidenced by their strong relationship to quorum sensing in a variety of *Vibrio* species (Ball et al., 2017). Forty nine effector delivery system virulence genes were detected in at least one of the isolates, and only 12 were consistently present

across all three isolates. Among the total effector delivery system virulence genes, 17 were unique to the two isolates of *V. alginolyticus*. Of these unique genes, 10 were associated with the Type III secretion system (T3SS), and the remaining 7 were related to the Type VI secretion system (T6SS). Distinct effector delivery system virulence factors were identified between the two isolates of *V. alginolyticus*. In the isolate P-PP-T-4-A1, six unique virulence genes were detected. Conversely, the isolate Pco-A-T1 possessed three unique virulence genes. Furthermore, in *V. diabolicus*, ten unique effector delivery system virulence factors were detected that were absent in the other two isolates, as illustrated in Figure 3.9.

3.5.3.3. Virulence factor related to the flagella

In *Vibrio* species, flagella are crucial for movement and have key roles in adhesion, biofilm formation, and the initiation of the immune response (Canellas et al., 2021). Understanding *Vibrio* pathogenicity and determining treatment targets depend on these virulence characteristics. Of 50 predicted flagella-related virulence genes, only 12 were found to differ across the three isolates and were selected for comparison. Of these eight were exclusive to the two isolates of *V. alginolyticus* and two were specific to the *V. diabolicus* isolate. The only virulence gene associated with flagella that separated the two isolates of *V. alginolyticus* was motA, which was exclusive to isolate P-PP-T-4-A1. For bacterial motility, flagellar rotation, adhesion, and biofilm formation, the MotA protein is a critical part of the flagellar motor of *Vibrio* species (Echazarreta & Klose, 2019; Yorimitsu & Homma, 2001).

3.5.3.4. Other major virulence factors

The other important Vibrio virulence factors, acfA (Accessory Colonization Factor A), *cqsA* (Cholera Quorum-Sensing Autoinducer Synthase A), *RTX* (Repeats in Toxin), and *hlyA* (Hemolysin A), were only detected in the two *V. alginolyticus* isolates. Hemolysin A, in *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticu*s increases bacterial pathogenicity by causing cell lysis and tissue damage facilitating infections and disease progression (Falco et al., 2023; Fullner et al., 2002). The genome annotation of all three isolates in this study revealed the presence of toxR. ToxR, was initially identified as the cholera toxin operon regulatory gene, has been demonstrated to control the expression of several genes in *Vibrio* species, impacting disease and environmental adaptation (Hubbard et al., 2016). PCR amplification using a *toxR* primer intended for *V. parahaemolyticus* only detected the *toxR* gene in *V. diabolicus*, despite genome annotation

revealing its existence in all three isolates. This implies that the *toxR* gene shows notable sequence variations across various *Vibrio* species. None of the three isolates had the *tdh* gene, which codes for the thermostable direct haemolysin. But, other important cytolytic genes were detected in the two *V. alginolyticus* isolates, including haemolysin A (*hylA*), thermolabile haemolysin (*trh*), and RTX toxin. On the other hand, *V. diabolicus* was found to contain only the *trh* gene.

During phenotypic haemolytic activity testing, partial haemolysis was seen in all isolates, which is probably correlated with the presence of these haemolysis-related virulence factors. In *V. diabolicus*, the *tdh* gene was amplified by PCR in this work, despite the fact this gene is associated with *V. parahaemolyticus* as a major virulence factor. Nevertheless, not a single isolate, including *V. diabolicus* was found to contain the *tdh* gene found in its genome annotation. There are multiple explanations for the disparity that exists between the positive PCR result and its lack in the genome annotation. These include the lack of specificity of the primers used, the presence of other gene fragments with similar primer binding regions, and the presence of the *tdh* gene on extrachromosomal DNA acquired through horizontal gene transfer. The inconsistency between the PCR detection and genome annotation of the *tdh* gene highlights the need for further investigation into primer specificity and consider other potential species-specific gene fragments.
Thermostable haemolytic gene, *tdh* was not detected in all three isolates, but, other genes responsible for cytolysis and erythrolysin like thermolabile hemolysin (*trh*), haemolysisn A (*hylA*), and *RTX* toxin are detected in the two *V. alginolyticus* isolates while in *V. diabolicus*, only *trh* was detected. The presence of these haemolytic-related virulence factors may be correlated with the presence of partial haemolysis in all the isolates in the phenotypic haemolytic activity testing in this study.

3.5.3.5. Tetrodotoxin responsible genes

According to the literature, Non-Ribosomal Peptide Synthetase (*NRPS*) and hybrid Polyketide Synthase (*PKS*) genes play crucial roles in the biosynthesis of tetrodotoxin (TTX) in various bacteria, including Vibrio alginolyticus (Bacchiocchi et al., 2021c). However, in our study, these genes were not identified in the annotated protein databases of our Vibrio isolates. Given that some *V. alginolyticus* genomes contain TTX biosynthesis genes, we attempted to detect TTX in mussel samples using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HR-MS) and searched for these genes in our *Vibrio* isolates through genome analysis. Our results showed that neither TTX was detected in the mussel samples nor were the *NRPS* and *PKS* genes found in the sequenced genomes of the Vibrio isolates. These results highlight the genetic variability within *V. alginolyticus* populations and the need for further research to understand the distribution of TTX biosynthetic pathways in marine environments.

3.3.1. Antibiotic resistance genes

The Comprehensive Antibiotic Resistance Database (CARD) was utilized to compare the predicted genes for antibiotic resistance and compared with the disc diffusion assays. Antibiotic resistance genes in all three isolates (Pco-A-T1, P-PP-T4-A1, and P-SA-T2-A1). Table 3.7 summarizes the list of resistance genes identified in this study with their corresponding antibiotics and resistance mechanisms. The CARB-19 gene confers resistance to penam antibiotics via antibiotic inactivation across all isolates. The *parE* (E. coli) gene and *PBP3* (*H. influenzae*) gene were responsible for resistance to fluoroquinolone and a range of β-lactam antibiotics (carbapenem, cephalosporin, monobactam, penam, and cephamycin), respectively, through target alteration resistance mechanism. The *AdeF* gene which was present in multiple copies in each isolate, and the *tet(35)* gene confer resistance to tetracycline and fluoroquinolone through antibiotic efflux mechanisms. The CRP gene was also found in all three isolates and showed resistance to penam, macrolide, and fluoroquinolone by antibiotic efflux mechanism. These findings indicate a significant potential for multidrug resistance in these strains.

In this study, phenotypic determination of antibiotic resistance was conducted using six commonly prescribed antibiotics for the treatment of vibriosis (Table 2.2). No resistance was observed in any of the isolates based on the disk diffusion method. However, genomic analysis predicted the presence of resistance genes against tetracycline and fluoroquinolone. This

discrepancy between the phenotypic results and the genomic predictions could be attributed to several factors. One possibility is that the resistance genes identified in the genomic analysis were not expressed or are expressed at very low levels under the test conditions, leading to a lack of detectable phenotypic resistance. Additionally, the resistance mechanisms might require specific environmental conditions which might not be present in the disk diffusion method. Another factor could be that the resistance genes are present but not functional due to regulatory issues or mutations (Haque et al., 2023b)

Table: 3. 7. Antibiotic-resistant genes and mechanisms of resistance. The antimicrobial resistance genes are predicted by comparing by annotating the predicted proteins with the CARD comprehensively updated database.

Isolate	Protein	Gene ID	Antibiotic	Resistance mechanism
Pco-A-T1	CARB-19	GE000522	Penam	antibiotic inactivation
	parE (*E. coli*)	GE002608	fluoroquinolone	antibiotic target alteration
	PBP3 (*H .influenzae*)	GE002588	Carbapenem, cephalosporin, monobactam, penam, cephamycin	antibiotic target alteration
	AdeF	GE000516	Tetracycline, fluoroquinolone	antibiotic efflux
	AdeF	GE001221	Tetracycline, fluoroquinolone	antibiotic efflux
	tet(35)	GE002120	tetracycline	antibiotic efflux
	CRP	GE003921	penam; macrolide, fluoroquinolone	antibiotic efflux
P-PP-T4-A1	CARB-19	GE001210	Penam	antibiotic inactivation
	parE (*E. coli*)	GE003403	Fluoroquinolone	antibiotic target alteration
	PBP3 (*H. influenzae*)	GE003787	Carbapenem, cephalosporin, monobactam, penam, cephamycin	antibiotic target alteration
	tet(35)	GE001363	Tetracycline	antibiotic efflux
	adeF	GE001204	Tetracycline, fluoroquinolone	antibiotic efflux
	adeF	GE002844	Tetracycline, fluoroquinolone	antibiotic efflux
	adeF	GE000060	Tetracycline, fluoroquinolone	antibiotic efflux
	CRP	GE003889	Penam, macrolide, fluoroquinolone	antibiotic efflux
P -SA-T2-A1	CARB-19	GE000522	Penam	antibiotic inactivation
	parE (*E. coli*)	GE002608	fluoroquinolone	antibiotic target alteration
	PBP3 (*H. influenzae)*	GE002588	Carbapenem, cephalosporin, monobactam, penam;, cephamycin	antibiotic target alteration
	adeF	GE000516	tetracycline antibiotic; fluoroquinolone	antibiotic efflux
	adeF	GE001221	tetracycline; fluoroquinolone	antibiotic efflux
	tet(35)	GE002120	tetracycline	antibiotic efflux
	CRP	GE003921	penam; macrolide antibiotic; fluoroquinolone	antibiotic efflux

*CARB-19= Carbapenemase-19: parE= partitioning enzyme subunit E: PBP3= Penicillin-Binding Protein 3: adeF= Acinetobacter drug efflux F: tet(35)= tetracycline resistance determinant: CRP= cAMP receptor protein

4. Conclusion and Future Perspectives

4.1. Conclusion

This study conducted a detailed characterization of *Vibrio* isolates from mussels and surrounding seawater using microbiological, molecular, biochemical, and toxin analysis techniques. It identified seven *Vibrio* strains that are likely to represent potential threats to the population and be transmitted via the consumption of contaminated specimens. The study shows that the isolates are likely to be distinct however this was not possible to resolve based on 16S rRNA sequencing due to the conserved nature of the gene. All the isolates were able to grow at 24ºC and 37ºC suggesting that they might be able to infect both marine organisms and humans all exhibited alpha haemolysis on blood agar and showed proteolytic and lipolytic activities highlighting their potential virulence. No detectable levels of Tetrodotoxin were found in mussel samples and genes related to the neurotoxin pathway were not detected in the predicted genes. Genomic analysis revealed significant differences in genome size, GC content, and assembly metrics among the isolates, identifying P-SA-T-2-A1 as likely *V. diabolicus* and the other two as strains of *V. alginolyticus*. A total of 128 major virulence factors were identified, with high variability in adherence and effector delivery systems. Antibiotic resistance genes were present, indicating potential multidrug resistance.

4.2. Future perspectives

Future research on *Vibrio* species should prioritize several key areas to enhance our understanding and management of these pathogens. Firstly, developing simple identification techniques targeting the most closely related *Vibrio* species besides 16S rRNA and existing species-specific gene amplification is essential. Investigating the functionality of the identified virulence factors will provide crucial insights into their role in disease processes. Understanding antibiotic resistance more comprehensively including the basis of the discrepancy between phenotypic and genotypic methods of drug resistance determination is crucial. Addressing these areas will not only strengthen our scientific knowledge but also enhance public health strategies and therapeutic approaches to manage *Vibrio* infections effectively.

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Supplementary materials

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Val_NZ_CP098034.1       CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
PCO_SA_T2_A2            CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
PCO_SA_T2_A1            CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
PCO_A_T_3_A             CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
PCO_SA_T_2_B1           CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
PCO_PP_T_4_A_2          CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
PCO_PP_T_4_A1           CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
PCO_A_T_1               CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
PCO-SA-T-2-B-2          CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
Vpa_NC_004603.1         CAGCCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTG
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Val_NZ_CP098034.1       GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
PCO_SA_T2_A2            GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
PCO_SA_T2_A1            GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
PCO_A_T_3_A             GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
PCO_SA_T_2_B1           GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
PCO_PP_T_4_A_2          GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
PCO_PP_T_4_A1           GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
PCO_A_T_1               GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
PCO-SA-T-2-B-2          GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
Vpa_NC_004603.1         GCATTCTGATCCACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAA
                        ************************************************************

Val_NZ_CP098034.1       TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
PCO_SA_T2_A2            TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
PCO_SA_T2_A1            TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
PCO_A_T_3_A             TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
PCO_SA_T_2_B1           TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
PCO_PP_T_4_A_2          TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
PCO_PP_T_4_A1           TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
PCO_A_T_1               TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
PCO-SA-T-2-B-2          TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCCGCCCTCTGTA
Vpa_NC_004603.1         TCCGGACTACGACGCACTTTTTGGGATTCGCTCACTTTCGCAAGTTGGCTGCCCTCTGTA
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Val_NZ_CP098034.1       TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
PCO_SA_T2_A2            TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
PCO_SA_T2_A1            TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
PCO_A_T_3_A             TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
PCO_SA_T_2_B1           TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
PCO_PP_T_4_A_2          TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
PCO_PP_T_4_A1           TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
PCO_A_T_1               TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
PCO-SA-T-2-B-2          TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
Vpa_NC_004603.1         TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCGTC
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Val_NZ_CP098034.1       CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
PCO_SA_T2_A2            CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
PCO_SA_T2_A1            CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
PCO_A_T_3_A             CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
PCO_SA_T_2_B1           CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
PCO_PP_T_4_A_2          CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
PCO_PP_T_4_A1           CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
PCO_A_T_1               CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
PCO-SA-T-2-B-2          CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
Vpa_NC_004603.1         CCCACCTTCCTCCGGTTTATCACCGGCAGTCTCCCTGGAGTTCCCGACATTACTCGCTGG
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Val_NZ_CP098034.1         CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
PCO_SA_T2_A2              CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
PCO_SA_T2_A1              CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
PCO_A_T_3_A               CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
PCO_SA_T_2_B1             CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
PCO_PP_T_4_A_2            CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
PCO_PP_T_4_A1             CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
PCO_A_T_1                 CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
PCO-SA-T-2-B-2            CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
Vpa_NC_004603.1           CAAACAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGC
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Val_NZ_CP098034.1         TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
PCO_SA_T2_A2              TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
PCO_SA_T2_A1              TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
PCO_A_T_3_A               TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
PCO_SA_T_2_B1             TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
PCO_PP_T_4_A_2            TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
PCO_PP_T_4_A1             TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
PCO_A_T_1                 TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
PCO-SA-T-2-B-2            TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
Vpa_NC_004603.1           TGACGACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAA
                          ************************************************************

Val_NZ_CP098034.1         AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
PCO_SA_T2_A2              AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
PCO_SA_T2_A1              AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
PCO_A_T_3_A               AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
PCO_SA_T_2_B1             AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
PCO_PP_T_4_A_2            AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
PCO_PP_T_4_A1             AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
PCO_A_T_1                 AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
PCO-SA-T-2-B-2            AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
Vpa_NC_004603.1           AGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTTGCATCGAAT
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Supplementary figure: 1. Sequence allignment of the isolates of 16S RNA gene sequence. The allignment includes *V. Parahaemolyticus* and *V. Alginolyticus* refference strains, Vpa_NC_004603.1 and Val_NZ_CP098034.1 respectively.