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Molecular identification and environmental screening for members of the *Vibrionaceae* family

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Trabalho efetuado sob a orientação de:

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2022

Declaração de autoria de trabalho / Statement of Authorship

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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.

I hereby declare to be the author of this work, which is original and unpublished. Authors and works consulted are properly cited in the text and included in the reference list.

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Inês Filipa Cabral Leal

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Abstract

Bacteria of the *Vibrio* genus are one of the most abundant and diverse prokaryotes in aquatic environments, comprising well-known opportunistic pathogens that infect human and marine animals, and are responsible for human foodborne diseases and disease outbreaks in aquaculture systems. Currently, the risk of infection by *Vibrio* is rapidly increasing because of climate change effect on the ocean and thus characterization of novel strains and the development of technologies for their early detection are essential for management and to minimize their impact. In this study, seven marine *Vibrio* isolates from infected sole (*Solea senegalensis*) and their bathing waters were characterised using microbiological, biochemical, and molecular techniques. Colony morphology, growth, and activity of putative virulence-secreted enzymes were determined under optimal and stress conditions (modified pH and salinity). All isolates had different colony morphologies and sequencing of 16S rRNA fragment (> 1400 bp) revealed they are likely non-described strains. All isolates grew at 22ºC, but only the isolates of *V. parahaemolyticus* and *V. harveyi*, which are potential human pathogens, grew at 37 ºC. All isolates are antibiotic sensitive although Amikacin was ineffective. Proteolytic and hydrolytic assays revealed that *V. parahaemolyticus* and the two *V. harveyi* isolates had different enzyme activities at 22 and 37 ºC. Culture under decreased salinity (0.75% NaCl) and modified pH (7.8 and 8.2) caused a significant ($p < 0.05$) increase in their growth and enzymatic activity and revealed the capacity for environmental adaptability. A multiplex PCR (5-plex) targeting different *Vibrio* species was developed but was inefficient for use with environmental samples. Our results highlight that a large variety of *Vibrio* spp. remains to be characterised and indicated that culture conditions modify growth and most likely virulence.

Keywords: *Vibrio*, pathogens, microbiology characterization, biochemical activity, molecular detection

Resumo alargado

A família *Vibrionaceae* inclui diversos géneros extremamente importantes nos ecossistemas marinhos, entre eles o género *Vibrio*. Este género é caraterizado por bactérias Gram-negativas móveis (devido à presença de um flagelo polar), anaeróbias facultativas e quimiorganotróficas, que existem na forma livre na coluna de água, nos sedimentos ou em associação com organismos aquáticos através do estabelecimento de relações de comensalismo. As bactérias do género *Vibrio* têm um papel essencial no equilíbrio do ecossistema marinho e na recirculação do carbono orgânico. Em consequência do seu curto tempo de duplicação (geralmente 10-20 minutos), as espécies de *Vibrio* crescem a uma vasta gama de temperaturas, pH e salinidade. De entre as mais de 100 espécies de *Vibrio* já identificadas, apenas uma parte constitui perigo para o ecossistema marinho e para a saúde humana através do consumo de peixe e mariscos crus ou pouco cozinhados, sendo responsáveis por surtos de infeções (vibrioses) com impacto na Saúde Pública. As espécies de *Vibrio* podem ser agentes patogénicos primários, causando geralmente infeção mesmo em hospedeiros saudáveis, ou patogénicos oportunistas. Neste caso, algumas espécies que fazem parte da microbiota dos organismos saudáveis poderão encontrar as condições de crescimento ideais em caso de diminuição da imunidade do hospedeiro ou disbiose, levando a uma possível infeção. Em aquacultura são responsáveis por diversos surtos e elevadas perdas económicas, afetando particularmente as fases larvares dos organismos marinhos. Existem pelo menos 12 espécies classificadas como agentes patogénicos humanos, nomeadamente *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus* e *V. cholerae*. Estas espécies poderão infetar o hospedeiro através da via oral ou por contacto com a pele. Infeções por estas espécies de *Vibrio* são extremamente rápidas e os sintomas variam em função do agente infecioso, a via de entrada e condição imunitária do hospedeiro. A sua capacidade infeciosa advém de fatores de virulência como as estruturas de adesão, mobilidade ou produção de enzimas que causam lesões no hospedeiro e permitem a sua proliferação. Alterações das características físico-químicas do meio aquático como temperatura, salinidade e pH, causadas pelas alterações climáticas, têm um papel importante na dinâmica das interações patogénico-hospedeiro-ambiente. Estas alterações têm sido observadas ao longo das últimas décadas e prevê-se que sejam ainda mais proeminentes no futuro, com drásticas modificações no ecossistema marinho. As bactérias e a sua interação com o hospedeiro são modificadas pelas condições ambientais, com particular ênfase nos membros do género *Vibrio* onde o aquecimento dos oceanos e mudanças de salinidade e pH favorecem o crescimento das estirpes patogénicas.

Este trabalho teve como objetivo a identificação e caraterização de sete isolados correspondentes a cinco possíveis espécies de *Vibrio,* isoladas a partir da água dos sistemas de aquacultura e do fígado de linguado (*Solea senegalensis)* infetado cultivado em sistemas de recirculação de água. Os isolados de *Vibrio* caracterizados incluem cinco potenciais estirpes patogénicas (*V. parahaemolyticus*, duas estirpes de *V. harveyi* e duas de *V. ichthyoenteri*) e dois isolados não patogénicos (*V. renipiscarius* e *V. alfacsensis*). Após a confirmação da pureza dos isolados e a sua morfologia em Thiosulfate Citrate Bile Salts Sucrose (TCBS), os mesmos foram identificados através da amplificação do gene *16S rRNA* e comparação das sequências nucleotídicas com a base de dados do *National Center for Biotechnology Information* (NCBI). O crescimento em TCBS, a sua cinética de crescimento e produção de enzimas proteolíticas e hidrolíticas (esterásica e lipolítica) relacionadas com o seu potencial de virulência foram analisados a 22 (temperatura de referência das zonas costeiras) e 37 ºC (temperatura de referência do corpo humano no início de uma infeção). O efeito das condições de cultura, nomeadamente a diminuição em 25% da salinidade e o pH (8.2- referência do meio marino; 7.8- diminuição de 0.4 do pH marinho prevista) na cinética de crescimento e atividade enzimática dos isolados também foi analisada para prever, em condições laboratoriais, qual o impacto das alterações climáticas nos oceanos no crescimento e potencial virulência dos isolados. Por fim, foi desenvolvida uma ferramenta molecular com base na amplificação do DNA genómico por PCR para detetar simultaneamente as cinco espécies de *Vibrio* (5-plex) em amostras ambientais.

A amplificação do gene *16S rRNA* resultou em fragmentos com cerca de 1465 pares de bases cuja sequência de nucleótidos foi comparada com a base de dados do NCBI. Os resultados indicam que os sete isolados pertencem ao género *Vibrio*. No entanto, a percentagem de identidade obtida para cada isolado indica que poderá tratar-se de novas estirpes ainda não caraterizadas. Todos os isolados crescem a 22 ºC, mas só os isolados de *V. parahaemolyticus* (Vpa) e *V. harveyi* (Vha1 e Vha2) crescem a 37ºC. Todos os isolados demonstram sensibilidade a todos os antibióticos testados, exceto para a amicacina, cuja suscetibilidade se restringiu aos isolados *V. renipiscarius* e *V. ichthyoenteri* 2. Ensaios enzimáticos realizados em placas de agar suplementadas com os substratos específicos (gelatina, Tween 20 e Tween 80) demostraram que só os isolados *V. parahaemolyticus* e *V. harveyi* apresentaram atividade enzimática a 22 e 37 ºC, observando-se um aumento significativo do crescimento dos três isolados a 37ºC, tal como um aumento da atividade proteolítica e esterásica do isolado *V. harveyi* 1. Alterações das condições de cultura afetaram a cinética de crescimento dos isolados de forma distinta. Com a diminuição da salinidade (0.75% NaCl) observou-se um aumento significativo ($p < 0.05$) do

crescimento dos isolados *V. alfacsensis* e *V. ichthyoenteri*. Quando cultivados a pH mais baixo apenas o crescimento dos isolados *V. alfacsensis*, *V. renipiscarius*, *V. ichthyoenteri* 1 e *V. harveyi* 1 diminuiu de forma significativa ($p < 0.05$). Os três isolados com atividade enzimática a 22 ºC (*V. parahaemolyticus*, *V. harveyi* 1 e *V. harveyi* 2) também demonstraram que a influência das condições de cultura afeta diferencialmente as espécies e estirpes. A atividade esterásica foi estimulada pela diminuição da salinidade, diminuindo com a sucessiva redução do pH. O isolado *V. alfacsensis*, que não apresentou qualquer atividade enzimática a 22 e 37 ºC, adquiriu atividade esterásica com o decréscimo de 25% da salinidade. Apenas a atividade proteolítica do isolado *V. parahaemolyticus* foi afetada pelo pH, atingindo o seu maior índice a pH 8.2. A atividade lipolítica dos dois isolados de *V. harveyi* foi inibida com a diminuição da salinidade e aumento do pH.

Para a sua deteção molecular, os *primers* desenvolvidos para as cinco espécies de *Vibrio* alvo são eficientes e específicos quando quantidades equivalentes dos diferentes DNAs genómicos são utilizados. Para testar a eficiência do PCR-multiplex (mPCR) desenvolvido na deteção de *Vibrio* spp. foram utilizadas amostras de mexilhão recolhidos da Ria Formosa segundo as indicações propostas na ISO 21872-1:2017 através de um duplo enriquecimento em meio de cultura apropriado para favorecer o crescimento das espécies de *Vibrio*. Uma amostra de cada enriquecimento incubada em TCBS indicou a presença de diversas colónias características das espécies de *Vibrio*, mas nenhuma correspondia à morfologia típica das espécies alvo deste estudo. Nas amostras por PCR usando individualmente os *primers* específicos para cada espécie foi detetado com base na análise molecular a presença de DNA de *V. parahaemolyticus* e *V. harveyi*, mas quando se aplicou o mPCR apenas se detetou *V. harveyi*. Isto sugere que poderá ter ocorrido alguma interferência na reação ou no microbioma do hospedeiro que inibiu a amplificação da deteção de *V. parahaemolyticus* ou então que a abundância de *V. parahaemolyticus* é muito inferior à de *V. harveyi*.

Em resumo, sete novos isolados de *Vibrio* spp. (dos quais 4 representam duas estirpes das espécies *V. harveyi* e *V. ichthyoenteri*) foram identificados e caracterizados pela primeira vez. Caracterização microbiológica, molecular e bioquímica indica que os isolados de *V. parahaemolyticus* e *V. harveyi* podem representar potenciais agentes patogénicos humanos, pois são os únicos com capacidade de crescer a 37ºC. O aumento da temperatura favorece o seu crescimento e a atividade enzimática (proteolítica e hidrolítica) associada à sua virulência é alterada com a temperatura e modificação das condições ambientais. O impacto distinto das alterações nas condições de cultura entre os diferentes isolados indica que estes conseguem adaptar-se a diferentes condições, observando-se uma tendência para o aumento do crescimento

e atividade enzimática com a diminuição da salinidade, mas o efeito oposto aquando da diminuição do pH. Mais ainda, as modificações nas condições de cultura podem estimular a atividade enzimática de enzimas associadas à virulência em isolados inicialmente não patogénicos, modificando desta forma a sua interação com o hospedeiro. No futuro, a sequenciação do genoma dos sete isolados será um passo fundamental para entender a sua biologia e outros testes mais detalhados deverão ser aplicados para verificar o efeito das alterações do meio de cultura na dinâmica destes isolados, tal como a realização de ensaios de infeção nos organismos vivos para ter uma perceção da sua dinâmica e relação com os hospedeiros nas condições mais próximas das ambientais.

Palavras-chave: *Vibrio*, agentes patogénicos, caraterização microbiológica, atividade bioquímica, deteção molecular

Content

List of Tables

List of Figures

List of Abbreviations

APS – Ammonium Persulphate

APW – Alkaline Peptone Water

bp – Base pair

CDC – Centers for Disease Control and Prevention

CEN – Committee of European Normalization

CFU – Colony Forming Units

CLSI – Clinical and Laboratory Standards Institute

CO_2 – Carbon dioxide

DNA – Deoxyribonucleic Acid

dNTP – Deoxynucleotide triphosphate

DT – Duplication time

EC – European Commission

EMA – Ethidium bromide monoazide

EtOH – Ethanol

EPS – Extracellular products

EUCAST – European Committee on Antimicrobial Susceptibility Testing

FAO – Food and Agriculture Organization of the United Nations

FISH – Fluorescence In Situ Hybridization

gDNA – Genomic DNA

GW – Global warming

IPCC – International Panel for Climate Change

ISO – International Organization for Standardization

MA – Marine agar

MH – Muller-Hinton

MLSA – Multilocus sequence analysis

MPN – Most Probable Number

mPCR – Multiplex PCR

NaCl – Sodium chloride

NCBI – National Center for Biotechnology Information

PCR – Polymerase Chain Reaction

PMA – Propidium monoazide

qPCR – Quantitative Polymerase Chain Reaction

rtPCR – Real-Time Polymerase Chain Reaction

QS – Quorum sensing

RAS – Recirculating aquaculture system

RNA – Ribonucleic Acid

rRNA – Ribosomal Ribonucleic Acid

RTE – Ready-to-eat food

TAE – Tris-acetate-EDTA

TBE – Tris-borate-EDTA

TCBS – Thiosulfate Citrate Bile and Sucrose

TDH – Thermostable Direct Haemolysin

TLH – Thermolabile Haemolysin

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

UV – Ultraviolet

VBNC – Viable But Not Culturable

VFDB – Virulence Factor Database

WGS – Whole genome sequencing

WHO – World Health Organization

Chapter 1: Introduction

1.1. The bacteria of the Vibrio genus

1.1.1. The bacteria Vibrio spp.

The *Vibrionaceae* family englobes at least nine genera and more than 190 bacterial species, including the *Vibrio* genus which is one of the most important bacterial genera that are naturally present in estuarine, freshwater, and marine environments (Aagesen, Phuvasate, Su, & Häse, 2018; Ali et al., 2020; T. Balbi et al., 2013; Bienfang et al., 2011; Bunpa, Sermwittayawong, & Vuddhakul, 2016; Charles, Trancart, Oden, & Houssin, 2020; Grimes, 2020; Jiang et al., 2021). This genus comprises more than 100 species of Gram-negative, rod-shaped bacteria, usually curved and non-spore-forming (Figure 1.1) (Aagesen et al., 2018; Ali et al., 2020; Baker-Austin et al., 2018; T. Balbi et al., 2013; Bienfang et al., 2011; Bunpa et al., 2016; Canesi & Pruzzo, 2016; Charles et al., 2020; Jiang et al., 2021; Thompson, Iida, & Swings, 2004; Triñanes & Martinez-Urtaza, 2021). The members of this genus belong to the *Gammaproteobacteria* class, the most diverse class of motile Gram-negative bacteria (due to the presence of a polar flagellum), and are typically facultative anaerobes and chemoorganotrophic bacteria (Aagesen et al., 2018; Ali et al., 2020; T. Balbi et al., 2013; Bienfang et al., 2011; Bunpa et al., 2016; Charles et al., 2020; Sampaio, Silva, Poeta, & Aonofriesei, 2022). The *Vibrio* spp. are metabolically complex heterotrophic bacteria capable of swimming freely in the water column, living in sediments and abiotic surfaces, or in association with living organisms (Canesi & Pruzzo, 2016; Gregory & Boyd, 2021; Montánchez & Kaberdin, 2020; Sampaio et al., 2022; Schärer, Savioz, Cernela, Saegesser, & Stephan, 2011; Thompson et al., 2004). In response to adverse environmental conditions, such as low-nutrient concentration, low temperature, low salinity, or starvation *Vibrio* spp. can form biofilms (aggregates of microorganisms that are frequently embedded in a self-produced matrix) or enter the viable but non-culturable state (VBNC, bacteria that cannot be cultured on routine microbiological media, but remain viable and retain virulence) (Sampaio et al., 2022).

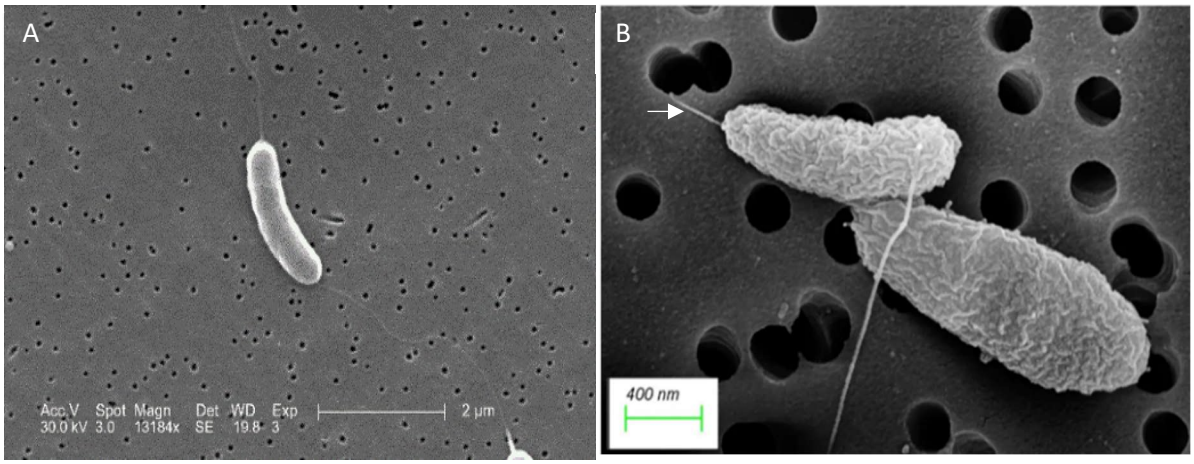


Figure 1.1. Scanning electron microscopy (SEM) images of *Vibrio* spp. SEM images of *V. vulnificus* (A) and *V. parahaemolyticus* (B) showing the rod shape and polar flagellum characteristic of members of this genus. The white arrow indicates the polar flagellum. Image A was retrieved from Public Health Image Library (PHIL) from the CDC, USA, and image B was obtained from the Bioimaging centre at the University of Exeter, UK.

Vibrio species are extremely versatile and grow under a range of growth conditions, possess fast growth rates, and adapt rapidly to favourable environmental conditions (Sampaio et al., 2022). The optimum growth pH range for *Vibrio* spp. is between 8.0 and 8.8, but most species can also grow between pH 6.5 and 9.0. Their optimal growth temperature is between 20 °C and 37 °C, but they can also grow over 40 °C except for some non-pathogenic species. During the warmer seasons, *Vibrio* counts in sea water are high, particularly when the water temperature is between 15 °C and 30 °C (Baker-Austin et al., 2018; Conde, 2019). Members of this genus do not grow under low-temperature conditions (< 15 °C). The optimal water salinity is between 0.5 % to 3 % (NaCl, mV^{-1}) but they can also grow outside the optimum range when nutrient concentrations and water temperatures are favourable for their proliferation (Conde, 2019). No growth occurs in low (0 % NaCl, m V^{-1}) or high salinity conditions (12 % NaCl, m V^{-1}). Most *Vibrio* spp. are oxidase, indole, and citrate positive, and they can reduce nitrate to nitrite, hydrolyse urea and degrade gelatine, starch, lipids, chitin, and alginate (Grimes, 2020; Martin Dworkin & Karl-Heinz Schleifer; Sampaio et al., 2022). Such metabolic diversity and high plasticity and adaptability to different environmental conditions are vital for their high potential to colonize their hosts (Destoumieux-Garzón et al., 2020; Le Roux et al., 2015).

Due to their importance in the aquatic environment, the genome has been sequenced of several members of the *Vibrionaceae* family. The genome of *Vibrio* is organized in two chromosomes of unequal size both containing virulence-encoding genes (Baker-Austin et al., 2018; Pazhani, Chowdhury, & Ramamurthy, 2021) (Figure 1.2). Chromosome I, the largest (~3 Mb), is highly conserved among the *Vibrio* genus and encodes the bacterial vital genes that are responsible for

core cell maintenance and survival, such as replication, transcription, and translation. Chromosome II is smaller and of variable size across species and encodes the phenotype-related genes (Baker-Austin et al., 2018; Gregory & Boyd, 2021). The evolution of the *Vibrio* genus is driven by horizontal gene transfer and recombination of their genetic information is suggested to shape their genomes (Baker-Austin et al., 2018).

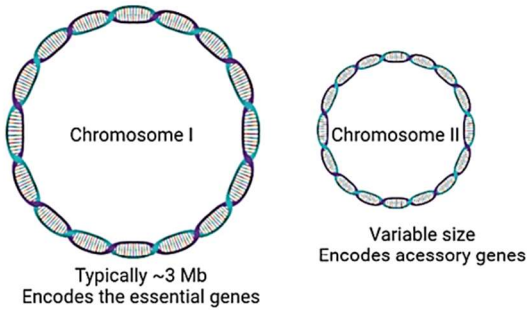


Figure 1.2. Representation of the typical *Vibrio* spp. genome composed by two different-sized chromosomes. Chromosome I is the largest and contains essential genes for survival. Chromosome II is smaller with variable size and encodes accessory genes. Both chromosomes contain virulence-related genes. The image was prepared by the author in BioRender.

The *Vibrio* genus is also highly heterogeneous and polyphyletic (Thompson et al., 2005). At least 16 clades exist based on multilocus sequence analysis (MLSA) (Ragab, Kawato, Nozaki, Kondo, & Hirono, 2022; Sampaio et al., 2022; Tomoo Sawabe, Kita-Tsukamoto, & Thompson, 2007). Some of the major clades are Harveyi, Splendidus, and Cholerae clades, which englobe some of the most important *Vibrio* species in the marine environment and for human health (Sampaio et al., 2022; Tomoo Sawabe et al., 2007; Tarazona, Pérez-Cataluña, et al., 2015) (Figure 1.3).

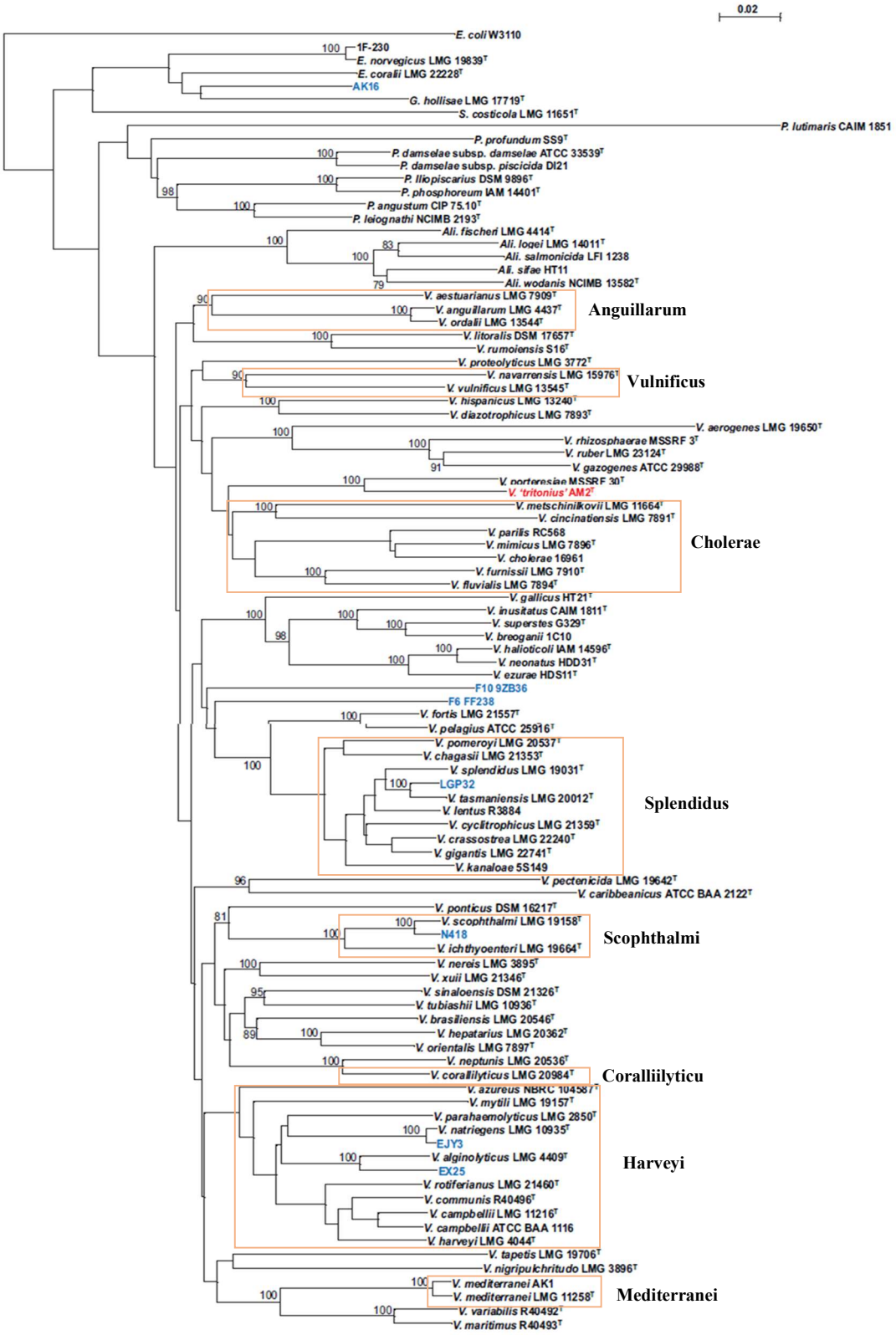


Figure 1.3. Phylogenetic tree of the different clades of the *Vibrio* genus. The *ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, and *topA* gene sequences from 96 taxa were concatenated based on eight gene loci and the tree was reconstructed using the SplitsTree4 program. Different clades highlighted in orange boxes were added to the original tree retrieved from (T. Sawabe et al., 2013).

Within the Harveyi clade are the bacteria *V. harveyi* and *V. parahaemolyticus* which are well-known marine and human pathogens, respectively. This clade is evolutionarily related to the Vulnificus which also includes pathogenic *Vibrio* spp., such as *V. vulnificus*. This bacterium, phenotypically like *V. parahaemolyticus*, causes gastrointestinal diseases through the consumption of contaminated fish and seafood and wound infections and represents 1% of all food-related deaths in the world (Ali et al., 2020).

1.1.2. The importance of Vibrio spp. in the aquatic environment

Members of the *Vibrio* genus are present in freshwater, estuarine, and marine environments (Baker-Austin et al., 2018). They usually prefer brackish warm waters (> 15 ºC) and their abundance is influenced by the water temperature (Baker-Austin et al., 2018). Members of this genus play an important role in the maintenance of the equilibrium of the aquatic systems, particularly in the marine mineral cycles and the geochemistry of the carbon cycle (Figure 1.4) (Grimes, 2020; Jiang et al., 2021). They are also important for the ecology of the marine environment. *Vibrio*'s metabolism in the ocean is focused on carbohydrates, proteins, and lipids, and these bacteria easily hydrolyse sugars and amino acids. They are also capable of hydrolysing collagen which is the most abundant protein in aquatic and terrestrial animal tissues (Grimes, 2020).

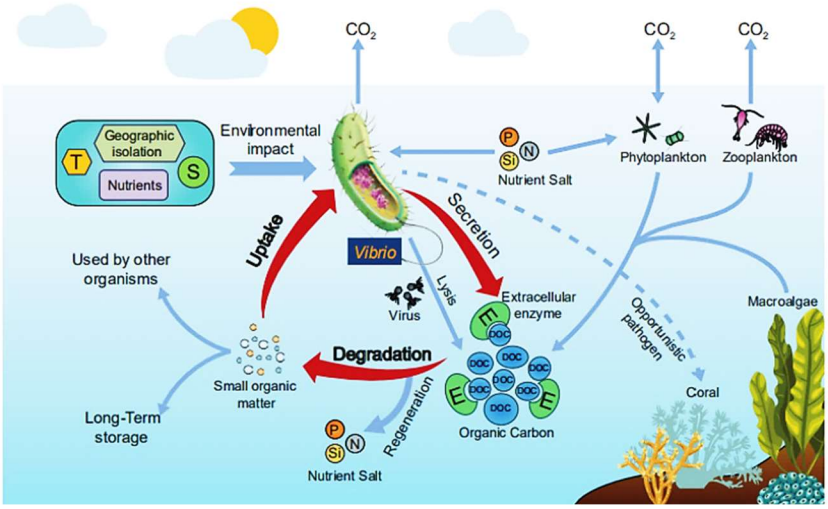


Figure 1.4. Role of *Vibrio* spp. in the carbon cycle in the marine environment. The action of *Vibrio* spp. is influenced by environmental conditions, leading to a higher or lower secretion of extracellular enzymes that degrade organic carbon that can be used by other marine organisms. T- temperature; S- salinity; P- phosphate; N- nitrogen salt; Si- silicate; E- extracellular enzyme; POC- particulate organic carbon; DOC- dissolved organic carbon. The image was obtained from (X. Zhang, Lin, Wang, & Austin, 2018).

Members of the *Vibrio* genus have an extremely rapid proliferation (~10 min) (Grimes, 2020) and account for about 60% of the heterotrophic bacteria associated with aquatic organisms (Sanches-Fernandes, Sá-Correia, & Costa, 2022; Sonia & Lipton, 2012). They are free-living in the water column, but some species also establish mutualistic, commensal, or neutral relationships associated with marine organisms as part of their normal microbiota (Canesi & Pruzzo, 2016; R. A. Costa, Araújo, & Vieira, 2013; Destoumieux-Garzón et al., 2020; Fang, Wolmarans, Kang, Jeong, & Wright, 2015; L. Liu et al., 2016; Sanches-Fernandes et al., 2022; Thompson et al., 2004). Notwithstanding, some bacterial species are classified as a "classic example of indigenous or autochthonous infectious microbes" (Teresa Balbi et al., 2019; Bienfang et al., 2011; Charles et al., 2020; R. A. Costa et al., 2013). *Vibrio* spp. can be primary or opportunistic pathogens capable of infecting healthy or immune-compromised marine organisms that can lead to death causing devastating losses of fish and bivalves in aquaculture, especially during their early larval stages and are the cause of major economic issues (Belkin & Colwell, 2005; Charles et al., 2020; X. Chen et al., 2020; Destoumieux-Garzón et al., 2020; Sanches-Fernandes et al., 2022).

Their contact with humans and impact on human health also comes from their widespread presence in the aquatic environment. Usually, humans have contact with *Vibrio* through the intake of contaminated water, consumption of contaminated seafood, or direct exposure of skin injuries to contaminated waters (Baker-Austin et al., 2018) (Figure 1.5). *V. cholerae* produces a toxin responsible for cholera, an acute intestinal infectious disease that can be deathly in humans and is caused by the consumption of contaminated food or exposure to contaminated water. It can also be transmitted between humans through faeces (which frequently occur in underdeveloped countries) (Bintsis, 2017; Sedas, 2007). Bacterial contamination of marine food products can also occur due to mishandling during transportation, and storage under inappropriate conditions or by cross-contamination with contaminated food products (Ali et al., 2020; Loo et al., 2020; Ndraha, Wong, & Hsiao, 2020). Moreover, bacteria that are already present in refrigerated food products can proliferate when optimal conditions are achieved due to the increase in the storage temperature.

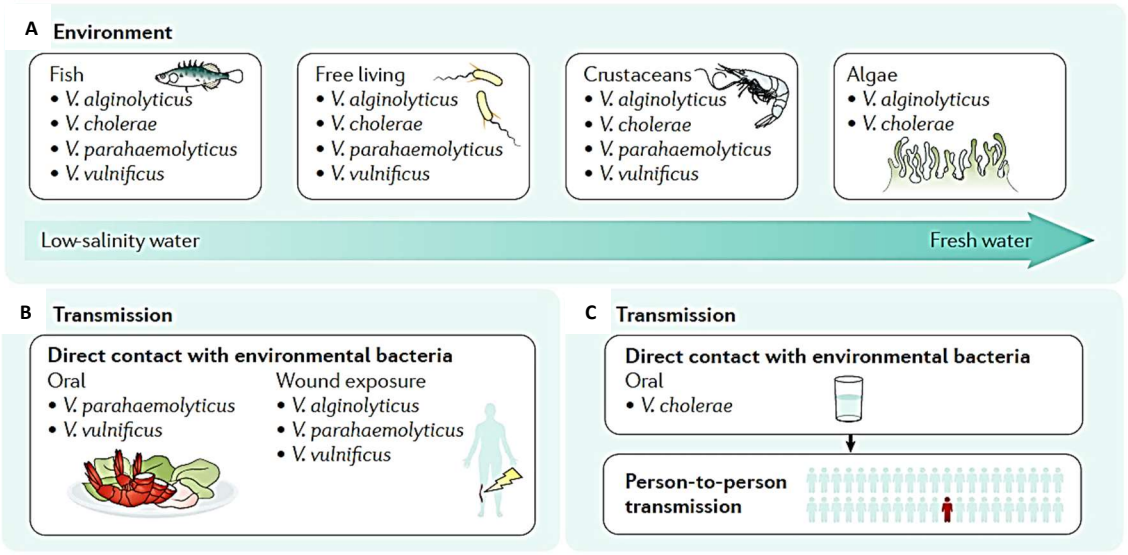


Figure 1.5. Lifestyle and transmission of *Vibrio* spp. A) *Vibrio* spp. can be found freely in the water column or associated with sediments and detritus, plankton, or marine organisms. The route of transmission depends on the species. Contact with non-cholera *Vibrio* spp., such as *V. parahaemolyticus*, *V. vulnificus,* and *V. alginolyticus*, occurs through ingestion of contaminated seafood and exposure to contaminated recreational waters (B), whereas *V. cholerae* can infect humans through contact with contaminated water or transmission between humans (C). Figure obtained from (Baker-Austin et al., 2018).

1.1.3. Pathogenic Vibrio spp.

The *Vibrio* genus contains 12 recognized human pathogens, including *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. alginolyticus*, and *V. cholerae* (Baker-Austin et al., 2018; Belkin & Colwell, 2005; Bienfang et al., 2011; Destoumieux-Garzón et al., 2020; Triñanes & Martinez-Urtaza, 2021) (Table 1.1). The symptoms and severity of each infection are dependent on the pathogen, the route of infection (water, food, or exposure), and the immune condition of the host. Some of these bacteria are not pathogenic for marine organisms, but they persist in wastewater effluents even after depuration, allowing their reintegration into aquatic systems and accumulation in marine organisms. Such dynamic makes them a microbial hazard for humans due to their presence in raw and/or undercooked seafood (Aagesen et al., 2018; Ali et al., 2020; T. Balbi et al., 2013; Destoumieux-Garzón et al., 2020; Schärer et al., 2011; Tack et al., 2019). Some human pathogens are also harmful to marine organisms (e.g., *V. harveyi* and *V. mimicus*) and represent a serious threat to both marine and human life. Of the environmentally and medically relevant *Vibrio* spp., only those relevant for the thesis will be characterized in detail.

Table 1.1. Most important human pathogens of the *Vibrio* genus. At least 12 *Vibrio* spp. can cause human infections. They have different sources of infection and characteristic clinical symptoms. The severity of the infection is responsible for the risk associated with each species. Table obtained from (Baker-Austin et al., 2018).

Species	Source of infection			Route of infection		Clinical manifestations
	Seafood	Sea water	Fresh water	Oral	Wound exposure	
Vibrio cholerae (O1 or O139 strains)	Rarely	Rarely	Yes	Yes	Rarely	Cholera and gastroenteritis; rarely wound infections
Vibrio cholerae (other strains)	Yes	Yes	No	Yes	Yes	Gastroenteritis and wound and ear infections; rarely primary septicaemia
Vibrio parahaemolyticus	Yes	Rarely	No	Yes	Yes	Gastroenteritis and wound infections; rarely sepsis
Vibrio vulnificus	Yes	Yes	No	Yes	Yes	Gastroenteritis, wound infections and sepsis
Vibrio alginolyticus	No	Yes	No	No	Yes	Most commonly ear and wound infections; rarely sepsis
Vibrio fluvialis	No	Yes	No	Yes	Yes	Gastroenteritis; more rarely wound, eye and ear infections
Vibrio hollisae[a]	Yes	Yes	No	Yes	No	Gastroenteritis and wound infections; rarely sepsis
Vibrio mimicus	Rarely	Yes	No	Yes	Yes	Gastroenteritis; more rarely wound, eye and ear infections
Vibrio metschnikovii	No	Yes	No	Probably	No	Gastroenteritis and sepsis

[a]Also known as *Grimontia hollisae*.

1.1.3.1. The human pathogen Vibrio parahaemolyticus

The bacterium *V. parahaemolyticus* is not harmful to marine organisms but when present in the marine environment can accumulate in organisms and become a major human foodborne pathogen. This bacterium was first isolated and described by Fujino and colleagues in 1953 in Japan as the causative agent of a food poisoning outbreak related to the consumption of a local fish product called "shirasu" (Solomakos, Pexara, & Govaris, 2017). It was initially named *Pasteurella parahaemolyticus* until Hugh and Sakauki described the bacterium as a member of the *Vibrio* genus in 1975. The bacterium *V. parahaemolyticus* is a halophilic bacterium with an optimum growth between 2 and 3% NaCl, but with the ability to grow in NaCl concentrations close to 8%. The optimal growth temperature varies between 15 to 44 °C (Solomakos et al., 2017).

This species is one of the most well-studied among the *Vibrio* genus due to its high pathogenicity to humans, causing moderate to severe gastroenteritis through the consumption of raw or undercooked seafood or through contaminated water by contact with open wounds (Aagesen et al., 2018; Baker-Austin et al., 2018; Bienfang et al., 2011). Virulent strains can lyse red blood cells on Wagatsuma blood agar plates (Kanagawa phenomenon, Kp) (Baker-

Austin et al., 2018; Solomakos et al., 2017). This occurrence is associated with the expression of two virulence factors related to their pathogenicity: thermostable direct hemolysin (*tdh*) and TDH-related hemolysin (*trh*) proteins (Baker-Austin et al., 2018; Elbashir et al., 2018; Schärer et al., 2011; Solomakos et al., 2017). Since only 1-2% of the environmental *V. parahaemolyticus* strains carry the *tdh* and *trh* genes, the detection of this gene is a reference indicator of their pathogenicity (Baker-Austin et al., 2018; Solomakos et al., 2017).

The bacterium *V. parahaemolyticus* is associated with almost 35% of reported seafood illnesses (Aagesen et al., 2018; Bienfang et al., 2011) and 44% of the *Vibrio* infections reported in the United States between 1996 and 2020 (CDC, 2021)(Figure 1.6). The symptoms of a *V. parahaemolyticus* infection in humans include headache, nausea, acute abdominal pain, low-grade fever, vomiting, and diarrhoea (Baker-Austin et al., 2018; Solomakos et al., 2017). Infection occurs when the concentration of bacteria is between $2x10^5$ to $3x10^7$ colony-forming units (CFUs) that colonize the intestine and produce toxins that cause cell damage and fluids and electrolytes loss (Solomakos et al., 2017).

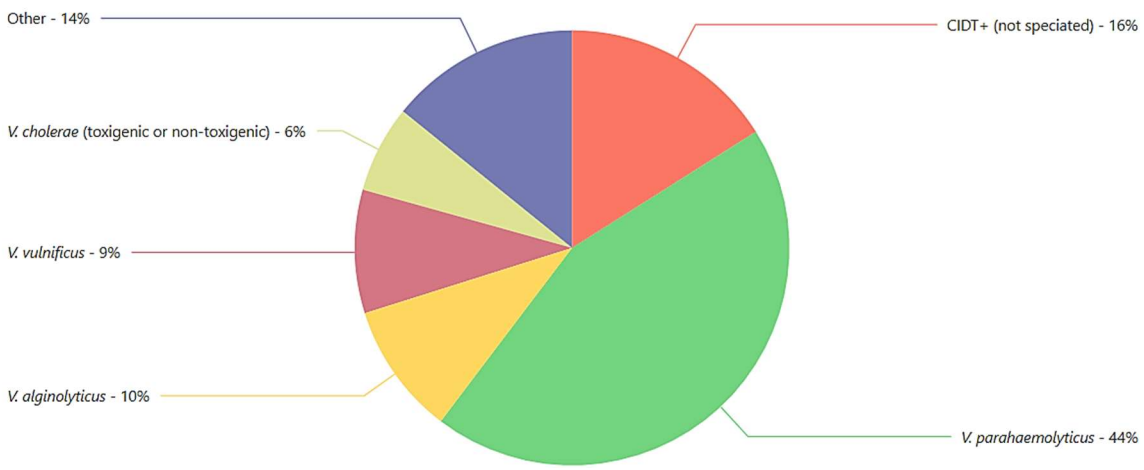


Figure 1.6. The incidence of *V. parahaemolyticus* in *Vibrio* infections between 1996-2020 in the United States. The species *V. parahaemolyticus*, one of the most important human foodborne pathogens is responsible for 44% of all *Vibrio* infections according to the Center for Disease Control and Prevention (CDC). CIDT- Culture-independent diagnostic test. Source: (CDC, 2021).

1.1.4. Marine environmental pathogens

1.1.4.1. Vibrio harveyi

This species was first described in 1936 by Johnson and Shunk as *Achromobacter harveyi*, to refer to a luminous bacterium isolated from the marine environment (F. H. Johnson & Shunk,

1936). This motile Gram-negative bacterium has no spores or capsule and in nutrient seawater agar the colonies are large, flat, highly iridescent, circular, and luminescent. The optimum growth temperature is 35-39 °C but it can also grow at lower temperatures (22-25 °C). The luminescence occurs between 20-40 °C and at pH 7.4-7.8. The bacterium *V. harveyi* has high metabolic versatility and can be found in a free-living state, in commensal or mutualistic associations with several marine organisms, or attached to abiotic surfaces (e.g. rocks) (Montánchez & Kaberdin, 2020). The fate, phenotypic characteristics, and lifestyle of this species are highly regulated by Quorum-Sensing (QS), a density-dependent regulatory mechanism that coordinates the genetic expression and behaviour of bacterial communities (Girard, 2019). Several studies have shown that QS regulates essential cell functions, including the well-known bioluminescence and biofilm formation of this bacterium (Montánchez & Kaberdin, 2020). This cell-cell communication mechanism is also essential for the expression of various virulence factors (Montánchez & Kaberdin, 2020). *V. harveyi* has a very important role in aquaculture systems with a high economic impact on seafood production as it is pathogenic for marine organisms, causing high mortalities and representing a severe threat to the aquaculture industry. Notwithstanding its pathogenic role, some *V. harveyi* strains are non-pathogenic (Montánchez & Kaberdin, 2020). Human infections by the bacterium *V. harveyi* are extremely rare and are caused by the opportunistic behaviour of this bacterium (Montánchez & Kaberdin, 2020). Until today only four cases of human infection have been reported and were directly related to wound infections (Del Gigia-Aguirre, Sánchez-Yebra-Romera, García-Muñoz, & Rodríguez-Maresca, 2017).

1.1.4.2. *Vibrio ichthyoenteri*

Initially isolated in 1971 from the intestinal tract of diseased larval flounder (*Paralichthys olivaceus*), *Vibrio ichthyoenteri* was later identified as the causative agent of intestinal necrosis or bacterial enteritis in these species (Ishimaru, Akagawa-Matsushita, & Muroga, 1996; Tarazona, Ruvira, et al., 2015). This disease was responsible for high mortality rates and huge economical losses of flounders in the ’70s (Ishimaru et al., 1996; Tang et al., 2019). The bacterium *V. ichthyoenteri* (*ichthys*, fish; *enteron*, gut; *ichthyoenteri*, fish gut) is part of the Scophthalmi clade formed by *V. scophthalmi* and is a motile Gram-negative, non-spore-forming short rod with a polar flagellum. It is a facultative anaerobe and non-luminescent, catalase and oxidase positive. The initial biochemical characterization of its possible enzyme activity

revealed that it does not possess gelatinase or lipase activities (Ishimaru et al., 1996). Most strains of *V. ichthyoenteri* can grow between 4 to 30°C and tolerate 1-6% NaCl. Most of the isolates use sucrose as a carbon source and form yellow colonies on TCBS. However, some strains also form green-coloured colonies on TCBS due to the weak production of acid from the fermentation of sucrose (Ishimaru et al., 1996).

V. ichthyoenteri has been the target of several studies (Kim, Cho, & Choi, 2014; Lee et al., 2012) and it has been isolated from the intestine of several teleost fish such as Senegalese sole (*Solea senegalensis*), flounder larvae (*Paralichthys olivaceus*), larval ballan wrasse (*Labrus bergylta*) and goldsinny wrasse (*Ctenolabrus rupestris*) and the kidneys of wild-caught spotted rose snapper (*Lutjanus guttatus*) (Tomoo Sawabe et al., 2007; Tarazona, Pérez-Cataluña, et al., 2015). In 2019, a potential vaccine against the bacterium *V. ichthyoenteri* using a recombinant outer membrane protein T (OmpT) was developed to prevent outbreaks in aquaculture (Tang et al., 2019).

1.1.5. Non-pathogenic Vibrio spp.

Most *Vibrio* spp. are non-pathogenic and are part of the natural microbiota of the marine environment. These species have a huge impact on marine ecosystems and participate in biogeochemical cycles. Among the non-pathogenic species are *V. alfacsensis* and *V. renipiscarius* which were studied in this thesis and are described below. Both bacteria have recently been isolated from fish and only a few studies about their characterization exist.

1.1.5.1. Vibrio alfacsensis

The bacterium *V. alfacsensis* was first isolated in 2012 by Gomez-Gil et al. (Gomez-Gil et al., 2012) from the gills of a healthy sole (*Solea senegalensis*) cultured at the Institut de Recerca I Tecnologia Agroalimentáries (IRTA) in Alfacs bay, Catalonia, Spain. *V. alfacsensis* is oxidase and catalase-positive and a facultative anaerobic bacterium that grows at 0.5 to 7.0% (w/v) salinity (with optimal growth at 2.5-5.0%), has a temperature optimum of 30 to 37 °C and pH 7.0 - 9.0. *V. alfacsensis* possess gelatine enzymatic activity but no esterase or lipase activity. Isolates of this bacteria are characterised by small, yellow, smooth, and circular colonies with complete borders on TCBS agar.

1.1.5.2. Vibrio renipiscarius

In 2015, Tarazona and colleagues (Tarazona, Ruvira, et al., 2015) characterized two strains of Gram-negative bacteria isolated in 2000 from the head kidney of healthy gilthead sea bream (*Sparus aurata*) cultured in Spanish Mediterranean fish farms. Using the 16S rRNA gene and reference genes, they identified two strains closely related to *V. scophthalmi* (98.4% similarity) and *V. ichthyoenteri* (97.2% similarity). The bacterium *V. renipiscarius* (*renes*, the kidneys; *piscarius*, of or belonging to fishing or fish) was characterized as a coccoid to a rod-shaped, motile bacterium. It is a facultative anaerobe capable of fermenting glucose without gas production and reduces nitrate to nitrite. As a member of the *Vibrio* genus, it is oxidase and catalase positive and presents yellow colonies on TCBS. Growth occurs at 4-28 °C with 2-6% NaCl and is inhibited at 37 °C or salinities below 1% NaCl. Biochemical tests showed that this bacterium can use different energy sources (Tarazona, Ruvira, et al., 2015).

1.1.6. Virulence factors and their regulation in Vibrio spp.

The occurrence and severity of vibriosis are complex and depend on the host life stage, immune status, and the influence of the resident microbiota but also on the virulence factors in pathogenic bacteria (Bunpa et al., 2016; Destoumieux-Garzón et al., 2020; Le Roux et al., 2015). The pili (hair-like structures present on the cell surface of prokaryotes) and the flagella (involved in bacterial locomotion) have an important role in the virulence of *Vibrio* spp. by facilitating their attachment to the host's surfaces (Aagesen et al., 2018). Moreover, the presence of non-virulent bacterial species can also potentiate the damages caused by a virulent bacterium (Destoumieux-Garzón et al., 2020; Le Roux et al., 2015). QS controls virulence or virulence-related gene expression (Arunkumar, LewisOscar, Thajuddin, Pugazhendhi, & Nithya, 2020; Girard, 2019; Thompson et al., 2004; Y. Zhang et al., 2018), and also plays an important role in the control of cell density and antimicrobial resistance (Loo et al., 2020; Thompson et al., 2004). Other factors that also contribute to bacterial virulence are bacteriophages, which can act as important virulence transfer agents between bacteria and are responsible for the transformation of non-virulent bacteria into virulent strains (Novriadi, 2016).

Surface protein structures of adhesion, attachment, and persistence, or motile or enzymatic factors produced by the infective agent are also virulence factors (Table 1.2) (Aagesen et al., 2018). Extracellular products (EPSs) such as the lytic enzymes chitinases, proteases,

haemolysins, and metalloproteases that have been isolated from cell-free cultures (supernatants) of several *Vibrio* spp. (Dahanayake, De Silva, Hossain, Shin, & Heo, 2018; Ina-Salwany et al., 2019; X.-H. Zhang & Austin, 2005). These extracellular enzymes are involved in the lysis of cells, and the detection of their encoding genes is widely used to assess the pathogenicity of *Vibrio* spp. in environmental samples, foods, fish, and human clinical samples (Dahanayake et al., 2018). Some of the most important virulence factors are the toxins, haemolysins, and metalloproteases secreted by *Vibrio* spp. (Teresa Balbi et al., 2019; T. Balbi et al., 2013). The expression of the virulence encoding genes is regulated by external stimuli but also by regulatory genes like *toxR*, responsible for pathogenicity modulation of several virulence factors such as haemolysin and cholerae-toxin genes (Okuda et al., 2001; Y. Zhang et al., 2018; Zhou et al., 2022).

Metalloproteases are a large family of zinc-dependent endopeptidases that include 30 enzymes divided into six groups based on their specific substrate (Galvis, Barja, Lemos, & Balado, 2021; Medeiros & Fares Gusmao, 2012). These enzymes impact host cells' functionality and integrity due to the degradation of extracellular matrix components (Medeiros & Fares Gusmao, 2012). Metalloproteases have an important role in the extracellular cleavage and activation process of the *V. cholerae* enterotoxic haemolysin into mature haemolysin (Almuhaideb, Chintapenta, Abbott, Parveen, & Ozbay, 2020). Haemolysin is one of the major pathogenic factors in the *Vibrio* genus (Mizuno, Debnath, & Miyoshi, 2019; X.-H. Zhang & Austin, 2005). The presence of this protein toxin is associated with wound (*V. vulnificus* and *V. alginolyticus*) and gastrointestinal (*V. cholerae*, *V. mimicus*, and *V. parahaemolyticus*) infections because it binds to mammalian erythrocyte membranes causing cell lysis (haemolysis) (Mizuno et al., 2019; X.-H. Zhang & Austin, 2005).

1.1.6.1. The tdh gene

There are two major groups of haemolysins among the *Vibrio* spp. that are subdivided using their thermal stability: thermostable direct haemolysin (TDH) from *V. parahaemolyticus* and other *Vibrio* species and thermolabile haemolysin (TLH) (Mizuno et al., 2019). The *tdh* gene, present on the smaller chromosome, encodes TDH that causes β-haemolysis and contributes partially to the cytotoxicity and enterotoxicity of the pathogen (Y. Zhang et al., 2018). TDH protein is a pore-forming toxin of erythrocytes that also has cytotoxic, cardiotoxic, and enterotoxin activities (Raghunath, 2014). It is present in gastroenteritis-causing *Vibrio* spp. with

minor coding-sequence variations (Baba et al., 1991; Raghunath, 2014; Terai, Shirai, Yoshida, Takeda, & Nishibuchi, 1990), suggesting a transfer of these genes between different species. Amplification of *tdh* has been vastly used for the assessment of *Vibrio* spp. presence and virulence in environmental samples (Mizuno et al., 2019; Raghunath, 2014). The *tdh* gene suffered a modification in *V. harveyi,* and it possesses duplicated haemolysin genes designated *vhhA* and *vhhB*, that share high homology with *tdh* (Conejero & Hedreyda, 2004).

1.1.6.2. The toxR gene

The *toxR* (Cholera toxin transcriptional activator) gene was discovered as the regulator of the cholera toxin operon. It is a widely distributed membrane-localized regulatory protein in the *Vibrionaceae* family, essential in modulating bacterial persistence and virulence (Okuda et al., 2001; Y. Zhang et al., 2018; Zhou et al., 2022). In *V. cholerae*, *toxR* can activate directly or indirectly toxin-producing genes, while in *V. parahaemolyticus* it regulates the expression of thermostable direct haemolysin (*tdh*) (Okuda et al., 2001). It is also important for bacterial survival under adverse conditions by controlling outer membrane proteins and biofilm formation (Y. Zhang et al., 2018). The presence of the *toxR* gene is essential for the expression of several virulence genes and is a putative target for the assessment of *Vibrio* spp. virulence in environmental samples.

Table 1.2. Virulence factors of pathogenic *Vibrio* species. Different *Vibrio* pathogens produce virulence factors with specific targets in the human body and cause damage to the host and enhance pathogen proliferation. Some virulence factors are common across species. Adapted from (Igbinosa & Okoh, 2008).

Causative agent	Factors	Target system
V. Cholerae	Flagellum	Gastrointestinal tract
V. cholerae, V. parahaemolyticus	Adhesins	Gastrointestinal tract
V. Vulnificus	Serum resistance Polysaccharides, acidic	Blood
V. cholerae O1, V. cholerae non-O1, V. mimicus	Enterotoxin cholera toxin	Gastrointestinal tract
V. cholerae O1, V. cholerae non-O1, V. mimicus, V. fluvialis, V. hollisae	Enterotoxin labile toxin or heat-stable toxin	Gastrointestinal tract
V. vulnificus, V. fluvialis, V. damsel, V. cholerae non-O1	Cytolysin labile toxin	Wounds, GI tract
V. cholerae O1, V. cholerae non-O1,V. parahaemolyticus	Cytotoxin shiga	Gastrointestinal tract
V. parahaemolyticus, V. hollisae	Hemolysin (thermostable direct hemolysin)	Gastrointestinal tract
V. alginolyticus, V. vulnificus	Collagenase	Wounds, cutaneous tissues
V. Vulnificus	Protease Siderophore	Cutaneous lesions Blood
V. cholerae O1, V. cholerae non-O1, V. mimicus, V. fluvialis, V. Parahaemolyticus	Mucinase	Gastrointestinal tract

1.2. Vibrio infections in humans

It is estimated that contaminated food causes one in ten people annually to fall ill or die (420 000 deaths, with 25% occurring in children under 5 years) (WHO, 2017). Vibriosis is most frequently caused by the consumption of contaminated seafood and water (oral route) (Baker-Austin et al., 2018; Belkin & Colwell, 2005; Bienfang et al., 2011). Annually in the United States, it is estimated that 80,000 cases of infection (which are more frequent during warmer seasons) and 100 deaths occur and more than half are caused by the bacterium *V. parahaemolyticus* (Baker-Austin et al., 2018; CDC, 2021) (Figure 1.7). In Europe, foodborne outbreaks occur at a rate of 0.95 per 100,000 of the population (Bintsis, 2017). In recent years, a growing incidence of vibriosis has been observed in marine organisms and humans, representing an emerging threat to the marine environment and public health. Moreover, numbers are likely underestimated as most vibriosis cases cause mild gastrointestinal symptoms, which are non-diagnosed (Ho, 2021; Marano et al., 2000; Scallan et al., 2011). Nonetheless, vibriosis poses a serious threat to public health and a global burden especially in underdeveloped countries (Abubakar et al., 2007; Ali et al., 2020; Bienfang et al., 2011; J. Chen, Tang, Liu, Cai, & Bai, 2012; WHO, 2017). In contrast, wound infections caused by *Vibrio* spp. can be easily diagnosed as they originate serious injury (Figure 1.8).

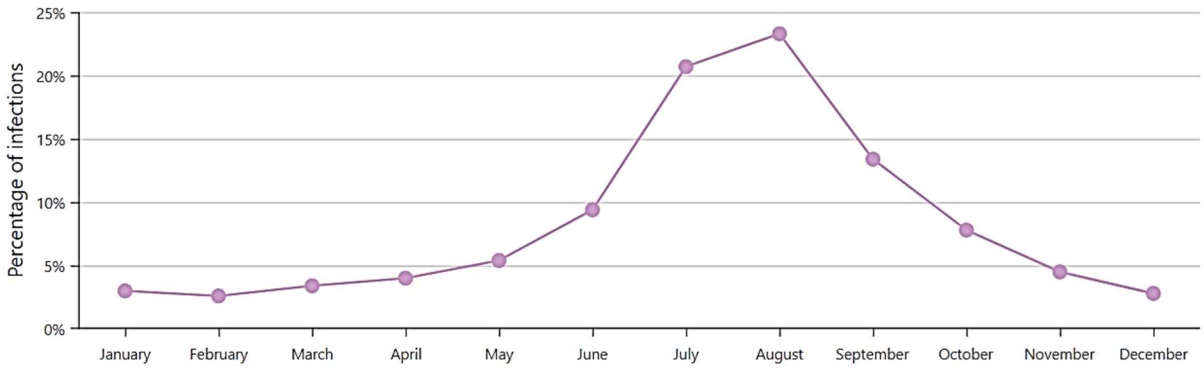


Figure 1.7. Monthly evolution of *Vibrio* infections. The percentage of infections is dependent on the temperature and starts increasing in warmer months. The peak of infection is reached between July and August and then it declines in incidence. Data shown include confirmed cases of vibriosis in the last two decades. Source: (CDC, 2021)

Vibrio infections are classified as either cholera or non-cholera infections and most of the foodborne cases are caused by non-cholera species (Baker-Austin et al., 2018). Cholera is an infectious contagious disease frequent in underdeveloped countries and is transmitted through

drinking water or from person-to-person. Non-cholera-causing *Vibrio* species (including *V. parahaemolyticus*, *V. anguillarum*, *V. metchnikovii*, *Vibrio alginolyticus*, *V. harveyi*, *V. mimicus*, *V. fluvialis*, and *V. vulnificus*) cause vibriosis. The symptoms of infection caused by ingestion vary from self-limiting gastroenteritis to potentially fatal invasive infections or septicaemia (Baker-Austin et al., 2018; Belkin & Colwell, 2005; Helmi, Mukti, Soegianto, & Effendi, 2020; Ho, 2021; Janda, Newton, & Bopp, 2015).

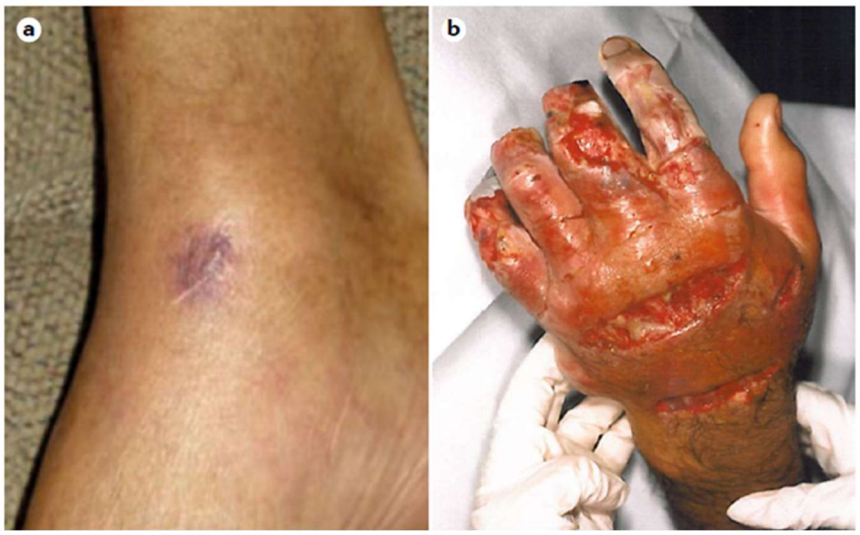


Figure 1.8. Wound infection caused by *V. vulnificus*. a) Simple skin lesions can be the entry route for pathogens to the human body when in contact with contaminated water. In the case of rapid proliferation of the pathogen, patients may develop septicaemia which can be fatal in some cases. b) Skin infection caused by *V. vulnificus* can lead to tissue necrosis and amputation of members. Images were taken from (Baker-Austin et al., 2018).

The risks of contaminated seafood causing illness depend on the product (Ali et al., 2020; Bienfang et al., 2011). Raw and lightly processed molluscs and shellfish fish products are considered high-infection risks (Ali et al., 2020). The bacterium *V. vulnificus* is responsible for serious cases of gastroenteritis, with a hospitalization rate of 90%, whereas the bacterium *V. parahaemolyticus* is the most common non-cholerae pathogen (Baker-Austin et al., 2018; Ho, 2021; Janda et al., 2015). The treatment of vibriosis includes rehydration and in some cases antibiotics, such as (i) a combination of a third-generation cephalosporin (e.g., ceftazidime, cefotaxime) and tetracycline or analogues (e.g., doxycycline), or (ii) a fluoroquinolone (e.g., levofloxacin, ciprofloxacin) (Ho, 2021).

1.3. Vibriosis in aquaculture

In 2018, the aquaculture industry was responsible for the production of 179 million tonnes of fish worldwide (Figure 1.9) and fish and bivalves were the greatest aquaculture food products in the globe (FAO, 2020a, 2020b). Vibriosis outbreaks occur all over the world and it is one of the major causes of severe mortality in fish and shellfish aquaculture systems and is responsible for billion-dollar losses (B. Austin, 2007; Dubert et al., 2016; Ina-Salwany et al., 2019; Loo et al., 2020; Novriadi, 2016).

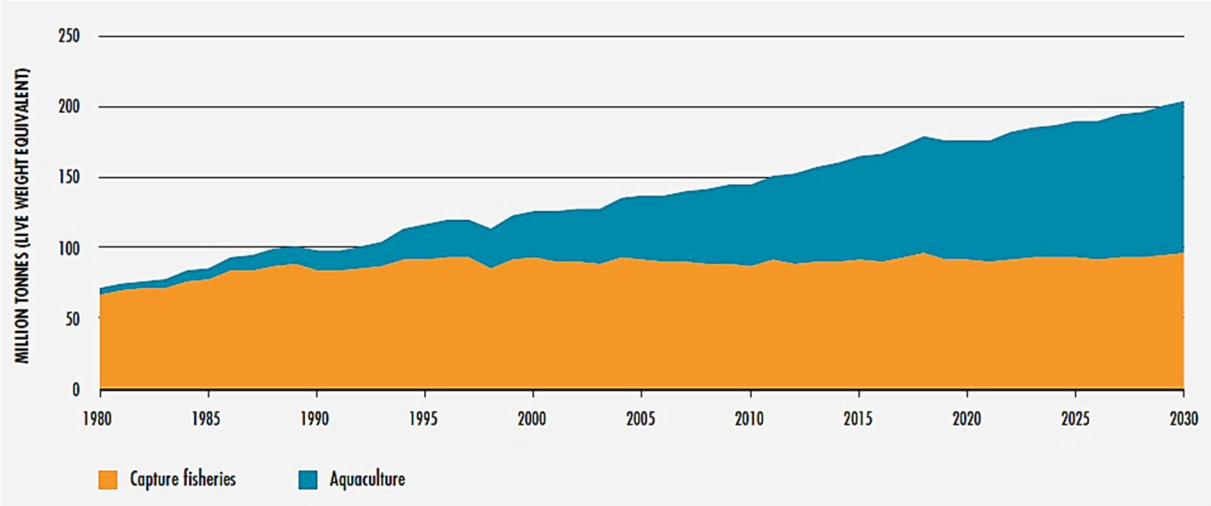


Figure 1.9. World capture fisheries and aquaculture production between 1980-2030. Aquaculture production has been increasing exponentially in the last decades. Obtained from (FAO, 2020b).

Vibriosis outbreaks are triggered by sudden changes in water temperature (normally at the end of the winter and beginning of the summer), poor water quality, fluctuations in salinity, and the animal's condition (Charles et al., 2020; Ina-Salwany et al., 2019). To date, it is known that aquaculture systems are a source of more than 30% of the species currently classified as *Vibrio* genus (Tarazona, Ruvira, et al., 2015). This is because aquaculture conditions enhance the proliferation of pathogenic *Vibrio*, which can also be transferred and contaminate the natural environment as primary or opportunistic pathogens (Canesi & Pruzzo, 2016; Ina-Salwany et al., 2019; Novriadi, 2016). The increase in disease outbreaks negatively impacts the economic value of fisheries and aquaculture since there is (i) higher mortality, slower growth, or lower biological productivity, which reduces capture, (ii) alteration of the original characteristics of the marine product, and (iii) increased risk for human health (Table 1.3) (Lafferty et al., 2015).

Table 1.3. *Vibrio* disease outbreaks in aquaculture systems and their clinical and socioeconomic impact. Adapted from (Novriadi, 2016).

Country	Agent	Losses and other impacts
China	*V. fluvialis*	>US$ 120M annual losses between 1990-1992
Indonesia	Luminescent *Vibrio*	>US$ 100 M in 1991 at shrimp hatcheries
Thailand	*V. harveyi*	Mass mortalities in *P. monodon*
Ecuador	*V. harveyi*	Mass mortalities in *P. monodon*
Japan	*V. carchariae*	Mass mortalities in Japanese abalone *Haliotis diversicolor*
India	*V. parahaemolyticus* *V. alginolyticus* *V. anguillarum* *V. vulnificus*	Poor growth, lethargic movements, red discoloration, and mortality in *Penaeus monodon*
West coast of North America	*V. tubiashii*	Reduce bivalve shellfish larval and seed production. Estimated 59% loss in 2007 production.
India	*V. harveyi*	Tail rot, erythema, and as white patches on the body of seahorses, *Hippocampus kuda*
Italy	*V. alginolyticus* *V. anguillarum* *V. harveyi* *V. ordalii* *V. salmonicida* *V. vulnificus*	Mass mortalities in bivalves farm located in Mar Piccolo in Taranto
Tunisia	*V. parahaemolyticus*	Darkened body colour, white nodular skin lesion, and sudden death with haemorrhages in the skeletal muscle of European Seabass
Egypt	*V. anguillarum* *V. alginolyticus* *V. ordalii* *V. harveyi*	Red spot on the ventral and lateral area; Swollen and dark skin lesions, necrosis, haemorrhagic areas, exophthalmia, and ulcers on the skin surface 50% mortality in Seabass and Seabream
Mexico	*V. parahaemolyticus*	Acute Hepatopancreatic Necrosis Disease (AHPND) in *L. vannamei* includes empty gut, anorexia, lethargy, expanded chromatophores, and pale HP with discoloration

The bacteria *V. parahaemolyticus, V. alginolyticus, V. harveyi, V. owensii,* and *V. campbelli* are the major marine pathogens (Ina-Salwany et al., 2019) and the primary transmission route of *Vibrio* spp. is the skin, gills, and gastrointestinal tract of marine organisms in contaminated water (Dahanayake et al., 2018; Ina-Salwany et al., 2019; Ndraha et al., 2020; Novoslavskij et al., 2016). The initial signs of *Vibrio* infection in fish are usually external infections and when not treated can progress to systemic infections (Ina-Salwany et al., 2019). In fish, vibriosis usually causes lethargy, tissue and appendage necrosis, slower growth, slower metamorphosis, body malformations, bioluminescence, muscle opacity, and melanisation. Externally, fish show

skin depigmentation, red necrotic lesions in the abdominal muscle, and erythema at the base of the fins, around the vent, and within the mouth (Novriadi, 2016) (Figure 1.10). Oysters and other bivalves exhibit a very distinct pattern when infected and this includes bacterial swarming around the velum, loss of larval motility, soft tissue necrosis, and rapid mortality (within 24h) (Canesi & Pruzzo, 2016).

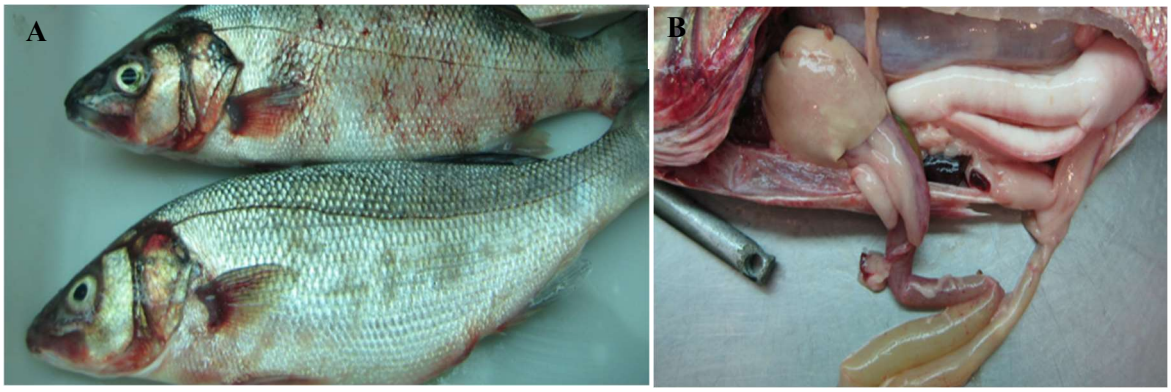


Figure 1.10. Clinical signs of a *Vibrio* infection in the European sea bass (*Dicentrarchus labrax*). A) Skin lesions characterized by red spots on the skin and erythema at the base of fins, operculum, and mouth. **B)** Internal lesions showing intestinal fluid and internal bleeding. Images obtained from (PharmaQ).

1.4. Current challenges to control vibriosis

1.4.1. Antibiotic resistance

Antibiotics are broadly used to manage or treat bacterial diseases in humans as well as in veterinary medicine, but antibiotic resistance is a growing public health threat (WHO, 2014). *Vibrio* spp. are usually susceptible to most antibiotics for veterinary and human purposes, however, several reports document an increase in antimicrobial resistance and their ineffectiveness against *Vibrio* spp. due to the accumulation of antibiotics in wastewater and their generalised use in aquaculture (Loo et al., 2020). *V. cholerae* is resistant to many antibiotics that are commonly used to treat diarrhoeal and other bacterial infections and antimicrobial resistance has evolved rapidly and spread across the globe in the last few decades (Das, Verma, Kumar, Ghosh, & Ramamurthy, 2020). *V. cholerae* and other *Vibrio* genomes are rich in mobile genetic elements linked to resistance genes and may contribute to the transmission of resistance traits to other bacterial pathogens. The rapid dissemination of resistance genes is a major threat to public health. Recent works showed that *Vibrio* spp. has developed resistance to ampicillin, chloramphenicol, tetracycline, streptomycin, kanamycin, trimethoprim, and carbapenems (Loo et al., 2020).

1.4.2. Water pollution and climate changes

Host-pathogen interactions are highly vulnerable to environmental change (Burge et al., 2014; Triñanes & Martinez-Urtaza, 2021). The strong connection between coastal populations and the ocean has a negative impact on the marine environment (Bienfang et al., 2011). Some pathogens occur naturally in the sea, but many others are introduced by domestic, hospital, agricultural, and industrial waste discharges and can infect marine organisms (Bienfang et al., 2011; Cruz, Rocha, & Mateus, 2015). An added factor is global warming and climate change, which modify the physical and chemical properties of the oceans, with repercussions on marine organisms. According to the European Environment Agency, the rise in sea surface water temperature is one of the major physical consequences of climate change (Le Roux et al., 2015) and is associated with a decrease in salinity, pH, oxygen, and nutrient concentrations (Burge et al., 2014; Edwards, Reid, & Gorick, 2011; Vezzulli, Pezzati, Brettar, Höfle, & Pruzzo, 2015) (Figure 1.11), which affect growth, proliferation, and virulence of marine pathogens (Belkin & Colwell, 2005; Bienfang et al., 2011). Among the environmental factors that influence the distribution and dynamics of *Vibrio* spp. the rise in seawater temperature and changes in salinity are major drivers of disease outbreaks (Froelich & Daines, 2020; Le Roux et al., 2015; Montánchez & Kaberdin, 2020; Triñanes & Martinez-Urtaza, 2021; Vezzulli et al., 2015; X. Wang, Liu, Liang, Sun, & Zhang, 2020).

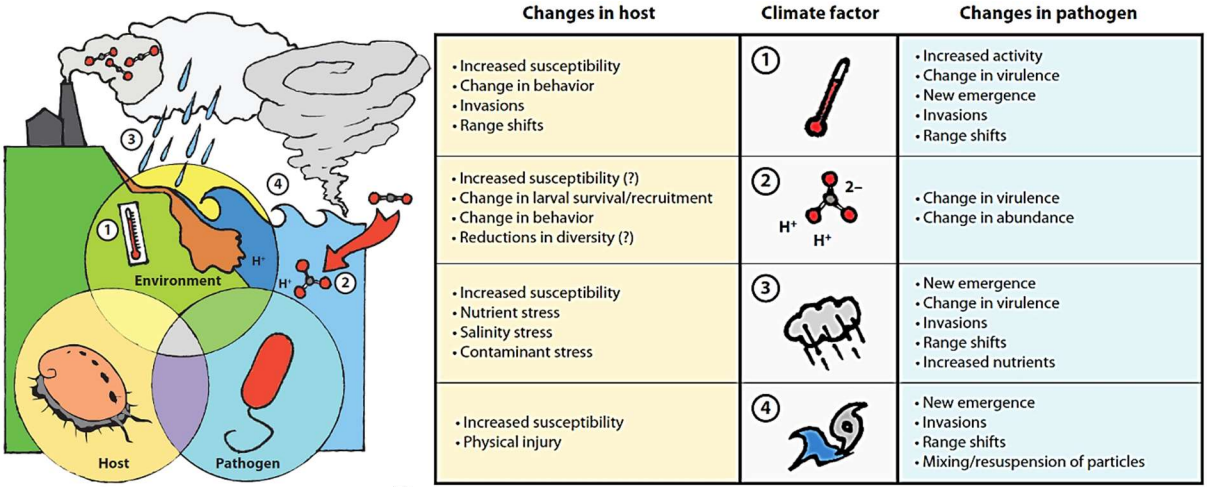


Figure 1.11. Effect of climate change on host-pathogen-environment relationships. Changes in the temperature, CO_2 concentration, pluviosity and salinity, and pH affect not only marine pathogens but their interaction with the host and the environment. The synergetic action of environmental changes on the host and pathogens increases the risk for human health due to the increase in disease outbreaks. Figure retrieved from (Burge et al., 2014).

1.4.2.1. Effect of temperature increase

The thermodependency of *Vibrio* spp. is a serious concern as the bacterial proliferation and expression of virulence factors are exacerbated in warmer water (Baker-Austin et al., 2018; Teresa Balbi et al., 2019; Destoumieux-Garzón et al., 2020; Ferchichi, St-Hilaire, Ouarda, & Lévesque, 2021; Le Roux et al., 2015; Semenza et al., 2017). This is a particular concern as 71% of the world's coastlines are getting warmer (Ferchichi et al., 2021; Triñanes & Martinez-Urtaza, 2021). In contrast, lower temperatures usually induce viable but nonculturable phenotypes (VBNC), usually stimulated by low nutrient availability, low salinity, or ultraviolet (UV) radiation (Le Roux et al., 2015). There is a considerable number of studies that have positively correlated the concentration of *Vibrio* spp. with higher water temperatures, suggesting that the risk of outbreaks caused by pathogenic or opportunistic pathogens will increase (Ferchichi et al., 2021; C. N. Johnson, 2015; Triñanes & Martinez-Urtaza, 2021; Vezzulli et al., 2016). The concentration of the bacterium *V. parahaemolyticus* has been positively correlated with temperature in the northern Gulf of Mexico, United States, France, India, and Germany, including in oysters and sediments (C. N. Johnson, 2015). This trend was already observed over the last few decades in European coastal waters, where the temperature increased 4-7 times faster than the global ocean and was associated with outbreaks of *Vibrio*-associated human illnesses (Le Roux et al., 2015). Higher temperatures were also positively correlated with a stronger expression of virulence-related genes and pathogen range expansion (Cohen et al., 2018; Urmersbach et al., 2015).

1.4.2.2. Effect of changes in salinity

Increased ice melt in the coldest regions of the planet, associated with an alteration in rain has contributed to reduced salinity of marine coastal waters (Triñanes & Martinez-Urtaza, 2021). *Vibrio* species are halophilic and halotolerant and grow normally at salinities < 2.5% NaCl (Gregory & Boyd, 2021; Vezzulli et al., 2015). Although, a detailed assessment of how salinity affects the growth, survival, and virulence of *Vibrio* species is not well-established (Gregory & Boyd, 2021). Salinity effects are very complex and may be associated with other environmental factors, making it difficult to understand the relationship between salinity and *Vibrio* spp. in the environment (C. N. Johnson, 2015). Studies of *V. harveyi* infections in different hosts showed contradictory results concerning salinity on growth and virulence, while in *V. parahaemolyticus* salinity was positively correlated (C. N. Johnson, 2015; Le Roux et al., 2015). When analysing

the influence of salinity on *Vibrio* abundance in Lake Pontchartrain (Louisiana, US), no correlation was found (Nigro, Hou, Vithanage, Fujioka, & Steward, 2011). A study using *V. cholerae* O139 to assess the effect of salinity on its growth and adhesion showed that growth was delayed, and adhesion was reduced at very low or high NaCl concentrations (Sung, Chang, & Lan, 2004). All these studies suggest that the influence of salinity on *Vibrio* spp. depends on the characteristics of the pathogen and the host immune state (C. N. Johnson, 2015; Nigro et al., 2011).

1.4.2.3. Effect of nutrient load

Climate change has a direct effect on nutrient availability in the ocean but the role of nutrients on the survival and growth of marine primary and opportunistic pathogens remains unclear (Belkin & Colwell, 2005). Although, a higher nutrient concentration is already associated with a higher abundance and virulence of *Vibrio* spp. (D. H. Zhao et al., 2009). Nutrient requirements regulate the survival, distribution, diversity, and virulence of bacteria (X. Chen et al., 2020). Shifts in the *Vibrio* community driven by ammonium, nitrite, and phosphate, and modifications in these nutrients between different geographical locations lead to different *Vibrio* species in different regions (X. Chen et al., 2020; Nigro et al., 2011).

1.4.2.4. Effect of decreased pH

The ocean absorbs about 30% of the carbon dioxide (CO_2) that is released into the atmosphere, with consequent acidification of the ocean surface water. It is predicted that ocean surface pH will decrease to values between 8.05 and 7.75 by the end of the 21st century, affecting aquatic organisms, including bacteria, and the marine ecosystem (IPCC, 2022). Members of the *Vibrio* genus grow in a high range of pHs, but each species has an optimal pH that determines its growth and virulence. Modifications in *Vibrio* abundance and characteristics caused by pH changes are controversial. In India, a positive correlation existed between pH and *V. cholerae*, but the opposite was detected with the same species from Japan (C. N. Johnson, 2015), suggesting that the influence of pH is species and site-dependent (C. N. Johnson, 2015; N. Mohamad et al., 2019). A study using *V. cholerae* O139 to assess the effects of pH on its growth and adhesion showed that growth was not affected by pH, but adhesion was pH dependent (Sung et al., 2004). Other studies suggested that only a dramatic pH decrease down to 5.5 will

have a negative effect on *Vibrio* spp. virulence (Prayitno & Latchford, 1995; Y. Wang & Gu, 2005).

1.5. Monitoring of marine pathogenic bacteria

Pathogen monitoring is essential to assess the risk level for humans and marine organisms. This includes the use of sensitive methods to detect pathogens even at low levels to predict and minimize future risks. The presence of infectious microbes in coastal waters and food is usually assessed using standard microbial indicators (Belkin & Colwell, 2005; Bienfang et al., 2011) but molecular methods based on the analysis of genomic DNA and integrated approaches combining classical culture-based and molecular-based technics are becoming popular.

1.5.1. Microbiology culture-based methods

Traditional detection methods rely on the enrichment and subsequent cultivation of the target pathogens in general, selective, and differential media to provide a "presumptive identification" of the pathogens (Abubakar et al., 2007; Adzitey, Huda, & Ali, 2013; Belkin & Colwell, 2005; J. Chen et al., 2012; Tantillo, Fontanarosa, Di Pinto, & Musti, 2004). Owing to their characteristics, species of the *Vibrio* genus grow on general media such as Marine Agar (MA) and Tryptic Soy Agar (TSA), used for bacterial recovery and maintenance, and selective media such as Thiosulfate-Citrate Bile Salts Sucrose agar (TCBS) to isolate *Vibrio* spp. (Baker-Austin et al., 2018; Belkin & Colwell, 2005; Janda et al., 2015; Thompson et al., 2004). TCBS provides the salts and pH necessary for the growth of both human and marine *Vibrio* pathogens. This medium is also differential through the fermentation of sucrose and associated acidification, which is indicated by a colour modification in the medium (Belkin & Colwell, 2005). TCBS is used for clinical and environmental identification of *Vibrio* spp., but some alkaline-tolerant enteric bacteria require subsequent confirmation by other methods (Belkin & Colwell, 2005). Presumptive *Vibrio* colonies are usually subcultured to a general medium like TSA and subjected to microscopy and biochemical analysis, such as Gram-staining, motility, oxidase and indole tests, or biochemical galleries (e.g., REMEL, API 20E) (Abubakar et al., 2007; Baker-Austin et al., 2018; Belkin & Colwell, 2005; Janda et al., 2015; Tantillo et al., 2004). The quantification of pathogen abundance using culture methods can be inferred from the number of Colony-forming Units (CFUs) using a solid medium or by Most Probable Number (MPN)

dilutions of environmental samples (Belkin & Colwell, 2005). Each method is chosen based on the type of sample analysed.

Culture-based methods have several advantages as they require less training and are cost-effective (186). They also allow the preservation of the pathogen in the laboratory for further experiments (Belkin & Colwell, 2005). However, these traditional techniques are time-consuming (usually require several days) and have a low throughput due to their reliance on the ability of bacteria to grow in a culture medium (Abubakar et al., 2007; Baker-Austin et al., 2018; Belkin & Colwell, 2005; Charles et al., 2020; J. Chen et al., 2012; Destoumieux-Garzón et al., 2020; Tantillo et al., 2004). Metabolic requirements, temperature, and generation time can also be an obstacle to plate detection methods as most pathogens are very difficult to cultivate or cannot grow under laboratory conditions (Belkin & Colwell, 2005). Bacteria can remain in their viable but non-culturable state (VBNC) in response to stressful conditions or be present in very low numbers that impede detection and may lead to false results and increase the threat to public health (Abubakar et al., 2007; Belkin & Colwell, 2005; Tantillo et al., 2004).

1.5.2. Nucleic-acid-based methods

The development of molecular techniques provides a significant advance for the identification of clinical and environmental pathogens in their environment (Abubakar et al., 2007; Belkin & Colwell, 2005; J. Chen et al., 2012; Tantillo et al., 2004). Molecular methods can discriminate between closely related pathogenic and non-pathogenic bacteria due to genotype differences and are commonly used for the characterization of *Vibrio* spp. (Baker-Austin et al., 2018; Belkin & Colwell, 2005). These approaches allow faster, more sensitive and specific detection of pathogens, and avoid ambiguous identification of phenotypically similar bacteria (Adzitey et al., 2013). They also allow the detection of VBNC bacteria that are not identified through conventional microbiology methods (Binsztein et al., 2004; Conde, 2019). For nucleic-acid-based methods the identification of molecular signatures characteristic of each species based on genome information is necessary. The discrimination based on the nucleotide variation of gene sequences among different species is usually accomplished by fluorescent *in situ* hybridization (FISH), polymerase chain reaction (PCR), and multiplex PCR (Belkin & Colwell, 2005; Conde, 2019). Whole genome sequencing (WGS) of the pathogen provides the high molecular resolution needed to investigate bacterial evolution and population structure, contributing to understand the great diversity among the *Vibrio* genus and to the identification

of critical pathogens and species that may be a threat to public health (Janecko, Bloomfield, Palau, & Mather, 2021).

The amplification of the 16S ribosomal RNA (16S rRNA) gene is one of the most common techniques used to detect pathogenic bacteria but does not distinguish between highly related species (Destoumieux-Garzón et al., 2020; Tantillo et al., 2004). The design of species-specific primers targeting specific gene regions that are highly variable between species is a better approach for pathogen detection. Molecular tests based on *tdh*, *gyrB*, or *toxR* genes are commonly used for *Vibrio* spp. detection (Tantillo et al., 2004). An alternative approach to conventional PCR techniques is quantitative or real-time PCR (rtPCR or qPCR) which allows the amplification and quantification of pathogen abundance in samples (Abubakar et al., 2007; Belkin & Colwell, 2005; J. Chen et al., 2012; Conde, 2019). The development of real-time PCR is TaqMan PCR, which uses fluorescent nucleic-acid probes complementary to an internal segment of the target DNA (Hoy, 2013). Multiplex-PCR (mPCR) allows the simultaneous detection of several pathogens within a single reaction targeting different species-specific sequences, saving time and resources (Abubakar et al., 2007; Belkin & Colwell, 2005; J. Chen et al., 2012; Hoy, 2013). This technique is based on the production of different-sized amplicons and enables the differentiation of DNA sequences (Adzitey et al., 2013). However, the complexity of the mPCR may lead to misidentification and the inhibition of some primers (Adzitey et al., 2013). This problem can be overcome by quantitative mPCR with TaqMan probes that allow the detection of different targets with amplicons of very similar sizes (Linck, Krüger, & Reineke, 2017). These PCR approaches are applied to detect viable and dead *Vibrio* spp. in fish and seafood (Conde, 2019). The reaction of several specific primers allows the differentiation of individual amplicons by size or by fluorescent labelling with species-specific probes (Belkin & Colwell, 2005). This technique has evolved in the last few years and provides a better understanding of the bacterial community in samples (Abubakar et al., 2007; J. Chen et al., 2012).

Despite the advantages of molecular approaches, the cost of the material necessary to implement them is high compared to traditional microbiology. Due to the high sensitivity of PCR, any contamination of the sample can lead to misleading results and the sample matrix such as salts and culture media can interfere with or inhibit the PCR reactions (Adzitey et al., 2013; Conde, 2019). In addition, the detection of VBNC bacteria does not allow the discrimination between viable and dead bacteria limiting the use of PCR for pathogen monitoring in food and environmental samples (Conde, 2019). The techniques tested to

overcome this problem include reverse transcriptase PCR and the use of ethidium bromide monoazide (EMA) and propidium monoazide (PMA) that can successfully detect both live and dead *Vibrio* spp. (S. Wang & Levin, 2006; Yoon, Moon, Choi, Ryu, & Lee, 2019; S. Zhao, Zhang, Li, Han, & Kan, 2021). Dead cells have damaged membranes that allow the penetration of EMA and PMA and the covalent binding to DNA, whereas viable cells do not allow this (S. Wang & Levin, 2006; Yoon et al., 2019; S. Zhao et al., 2021).

1.5.3. Integrative microbiology and molecular approaches

An integrative approach of culture-based and molecular-based methods are currently the best pathogen detection method for food and environmental samples and is also recommended by the *International Standard for Microbiology of the food chain- Horizontal method* for the determination of *Vibrio* spp. (ISO 21872-1:2017) (Conde, 2019; Destoumieux-Garzón et al., 2020; ISO, 2017). The combination of m-PCR and enrichment procedures is a consistent and effective technique for the analysis of foodborne pathogens (J. Chen et al., 2012). The pre-enrichment step allows the growth of bacteria present at low concentrations in food samples and overcomes the inhibitory effects of glycogen, and organic phenolic compounds, present in the food and which affect PCR efficiency (Belkin & Colwell, 2005; J. Chen et al., 2012). The most recent method to detect *Vibrio* spp. is ISO 21872-1:2017, a standard protocol based on a two-step enrichment of the samples in alkaline peptone water (APW), an optimal medium for *Vibrio* growth. Briefly, the samples are homogenised and incubated in APW for six hours, followed by a second enrichment in APW for 18 hours. From each enriched culture, one sample is inoculated on TCBS and a second selective medium for the microbiological identification of presumptive *Vibrio* spp. (ISO, 2017). The identity of the presumptive *Vibrio* colonies is then confirmed by PCR after the extraction of the genomic DNA (gDNA).

1.6. Objective

Bacteria of the *Vibrio* genus are ubiquitous in aquatic systems and pathogenic *Vibrio* species are a major threat to marine organisms and humans and a major public health burden. Incidence of vibriosis in humans and disease outbreaks in aquatic systems is increasing and with the changes in the oceans, some members of the *Vibrio* genus represent a growing risk. Characterization of novel *Vibrio* strains and the development of efficient methods for their detection are essential to identify and apply control measures and to facilitate their early detection to minimize future risks. This project aims to identify, characterize, compare, and assess the potential impact of the predicted changes in the ocean environment (pH, temperature, and salinity) on seven novel bacterial isolates representative of five different putative pathogenic (*V. parahaemolyticus*, *V. harveyi,* and *V. ichthyoenteri*) and non-pathogenic (*V. renipiscarius* and *V. alfacsensis*) species of *Vibrio* using classical microbiology, biochemical and molecular assays. Briefly:

a) Microbiological assays were applied to characterize the morphology, growth kinetics, and antibiotic susceptibility of the seven *Vibrio* isolates.

b) Biochemical assays were used to assess the presence and activity of potential virulence-related enzymes (proteolytic and hydrolytic activities).

c) Molecular methods were used to establish the identity of the isolates and to develop an mPCR molecular tool to facilitate the rapid detection and discrimination of five *Vibrio* species in seafood and environmental samples.

Chapter 2: Materials and methods

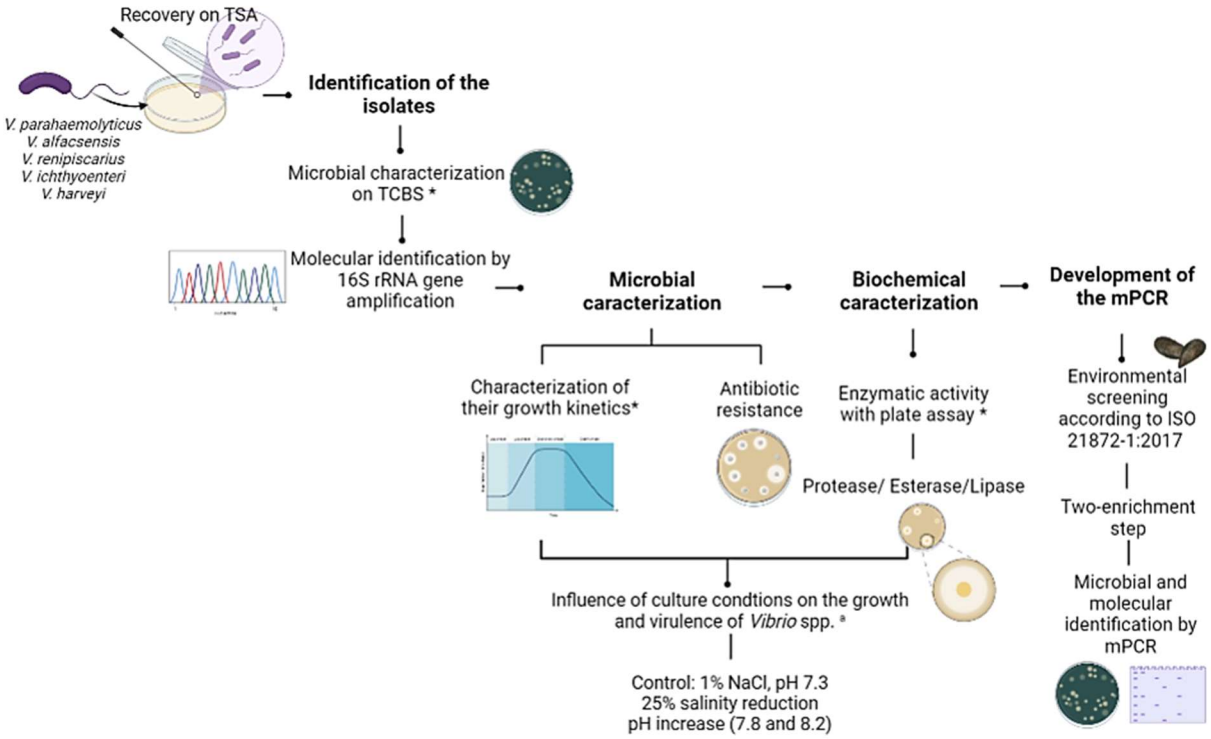


Figure 2.1. Schematic representation of the methodology used in this study. * The growth and enzymatic assays were performed to analyse the potential pathogenicity of the isolates to marine organisms (22 ºC) and humans (37 ºC). The figure was made by the author in BioRender.

2.1. Bacterial isolates maintenance and storage

Six marine isolates presumably belonging to the *Vibrio* genus were isolated from the tank water and the liver of infected Senegalese sole (*Solea senegalensis*) cultivated indoors in a Recirculating Aquaculture System (RAS). The isolates were kindly provided by Dr. Manuel Manchado (Puerto de Santa Maria, Cadiz, Spain) (Table 2.1). The putative *Vibrio* spp. isolates included one isolate of the bacterium *V. parahaemolyticus* (Vpa), *V. ichthyoenteri* (Vic), *V. alfacsensis* (Val), and *V. renipiscarius* (Vre), and two isolates of the bacterium *V. harveyi* (Vha1 and Vha2). The bacterium *V. parahaemolyticus* is a major human food-borne pathogen (Solomakos et al., 2017), whereas *V. harveyi* and *V. ichthyoenteri* constitute two main marine pathogens (Ishimaru et al., 1996; F. H. Johnson & Shunk, 1936; Tarazona, Ruvira, et al., 2015). The bacteria *V. alfacsensis* and *V. renipiscarius* are poorly studied species and are a normal part of marine organism microbiota (Gomez-Gil et al., 2012; Tarazona, Ruvira, et al., 2015).

The isolates were transported to CCMAR seeded on Marine Agar (MA) plates and recovered on Tryptic Soy Agar (TSA) (MP Biomedicals, USA) supplemented with 1% NaCl (Sigma-Aldrich, Spain) (TSA/1% NaCl) (pH 7.3 ± 0.2) and grown at 22 °C for 48 hours (R. Costa, Amorim, Araujo, & Vieira, 2013; Hernández-Cabanyero et al., 2020). The average temperature of 22 ºC was used as a reference temperature of the ocean water. The isolates were subsequently incubated on Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) at 22 °C for 48 hours to analyse for the presence of contaminates and to characterise colony morphology under a stereomicroscope (Farida Hikmawati, 2019). The bacterium isolates were inoculated in Tryptic Soy Broth (TSB) (MP Biomedicals, USA) supplemented with 1% NaCl (TSB/ 1% NaCl, regular pH of 7.3 ± 0.2) and incubated at 22 °C overnight (~16h) with shaking at 150 rpm. Cultures were centrifuged and the bacteria pellets were used for the preparation of 20% glycerol stocks for storing at -80ºC.

Table 2.1. The seven putative marine *Vibrio* spp. isolates analysed in this study. The bacterial isolates were kindly provided by Dr. Manuel Manchado and were either isolated from Senegalese sole liver or the fish tank water under RAS conditions.

Putative species names	Abbreviation used	Isolate origin
V. parahaemolyticus	Vpa	Tissue: Liver
V. renipiscarius	Vre	Tank water
V. alfacsensis	Val	Tank water
V. ichthyoenteri	Vic1 Vic2	Tank water
V. harveyi	Vha1 Vha2	Tissue: Liver

2.2. Molecular analysis of the marine Vibrio spp.

2.2.1. Genomic DNA extraction

Genomic DNA (gDNA) was extracted using an optimized version of the GES method described by Pitcher et al. 1989 for their application to the development of species-specific primers. Initially, the isolates were incubated in TSB/1% NaCl (pH 7.3 ± 0.2) at 22 °C, overnight (16 ± 2 hours) at 150 rpm. The cultures were then centrifuged for 10 minutes (min) at 13,000 rpm to

obtain the bacterial pellet. The pellet was resuspended in 100 µL of TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8), where 500 µL of GES (60 g of Guanidium Thiocyanate; 20 ml of 0,5 M EDTA pH 8; 5 mL of 10% Sarkosyl, and distilled water for a final 100 mL solution) and 6 µL of RNase (10 mg/mL) (Sigma, Spain) were added. The RNase treatment step was included as preliminary results revealed high contamination with rRNA. The reaction was incubated for 10 minutes (min) at room temperature before the addition of 250 µL 10M Ammonia Acetate to precipitate the proteins. After homogenization by gentle inversion of the tubes and subsequent incubation on ice for 10 min, 500 µL of chloroform/isoamyl alcohol (24:1) solution was added to remove the proteins from the gDNA. The reaction was centrifuged at 13000 rpm, 10 minutes at 4°C after homogenization by gentle inversion of the tubes. The supernatant of the two-phase solution was collected into a new Eppendorf tube and supplemented with 400 µL of cold isopropanol to precipitate the gDNA. The mixture was vortexed and centrifuged at 13000 rpm, 5 min at 4ºC, and the precipitated genomic DNA was washed with 400 µL of cold 70% ethanol three times and centrifuged between the washes at 13000 rpm, 5 min at 4°C. The precipitated gDNA pellet was air-dried for 10 min to allow ethanol evaporation and resuspended in 50 µL of water purified using a MilliQ system (Millipore, USA). The quality and concentration of the extracted gDNA were measured using a NanoDrop (ThermoFisher Scientific, USA) and its integrity was assessed by evaluating gDNA (5 µL, at an initial concentration of 50 ng/µL) by 1% agarose/1x Tris-Acetate-EDTA (TAE: 40 mM Tris-base, 20 mM Acetic acid, 1 mM EDTA) gel electrophoresis.

2.2.2. Polymerase Chain reaction (PCR)

2.2.2.1 Amplification of 16S rRNA gene

To confirm the identity of the *Vibrio* isolates, the 16S rRNA housekeeping gene was amplified by qualitative-PCR using the universal 27F and 1492R primers (Table 2.2). The reactions were prepared with 2 µL of gDNA (50 ng/µL); 2 µL 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); 0.125 µL of DreamTaq polymerase 5 U (Thermofisher, USA) for a final volume of 20 µL. The thermocycle conditions consisted of 95 °C for 3 min, 35x (95 °C for 30 seconds (sec), 59 °C for 30 sec, 72 °C for 90 sec), and a final extension at 72 °C for 10 min. The success of each reaction was analysed by 1% agarose/1x TAE gel electrophoresis and was observed using a transilluminator and Image Lab Software GelDoc XR+ (Bio-Rad, USA) to detect the

amplicons of the expected size. All amplicons were sequenced using the Sanger method at the CCMAR facilities using an automated sequencer Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems, USA) and the BigDye®Terminatorv3.1 kit. Their identity was confirmed by analysing their nucleotide sequences against the NCBI database (https://blast.ncbi.nlm.nih.gov) using the BLASTn algorithm to retrieve similar nucleotide sequences.

2.2.2.2. Amplification of virulence-related species-specific genes

2.2.2.2.1. Primer design

Species-specific primers to amplify the virulence-related genes *toxR* (tox) and *tdh* (hae) were designed for the five *Vibrio* species (Table 2.2). The selection of the target genes was based on their relevance to the virulent profile of *Vibrio* spp. as described in the published bibliography and the Virulence Factors of Pathogenic Bacteria (VFPB) database (Ceccarelli, Hasan, Huq, & Colwell, 2013; Pascual, Macián, Arahal, Garay, & Pujalte, 2010; VFDB, 2022). For primer design, the nucleotide sequences for each *Vibrio* isolate were retrieved from the VFPB or were obtained from the NCBI or Ensembl (https://bacteria.ensembl.org/Multi/Tools/Blast) databases using the orthologue sequence from other species. To identify regions within the sequences that are specific to each species the sequences were aligned using the MUSCLE alignment software available from the AliView platform (Larsson, 2014), and primers were manually designed taking into consideration the species unique regions. Primer selection was guided by specific criteria: length, 19-22 base pairs (bp); a similar G+C content (> 50%); an annealing temperature of 58-64 ºC; an amplicon size of 100 to 250 bp to allow for their use for qPCR analysis. The primer sequences are not reported in the thesis for confidentiality reasons pending the publication of this study.

2.2.2.2.2. Qualitative PCR

For the optimization and validation of the species-specific primers, 15 µL reactions were performed using 1 µL of the target gDNA (50 ng/µL); 1.5 µL 10xDreamTaq Buffer; 0.3 µL of 20 mM dNTPs; 0.3 µL of each primer (10 mM); 0.06 µL of DreamTaq polymerase (5 U). To assess the optimal annealing temperature, a gradient of temperatures (56º to 60 °C) was tested. The thermocycle used consisted of 95°C for 3 min, 34x (95 °C for 20 sec, 56-60 °C for 20 sec,

72 °C for 20 sec), and 72 °C for 5 min. The success of each PCR reaction was analysed using 2.5% agarose/1xTAE gel electrophoresis and the gels were visualized using Image Lab Software GelDoc XR+, and the amplicons were sequenced to confirm their identity as was described above. An annealing temperature of 57 °C was chosen as optimal for all primer pairs. The specificity of each primer pair was tested on the gDNA of several bacteria available in the lab. The reaction mix and thermocycling were the same as described above and the amplicons were analysed on 2.5% agarose/1xTAE gel electrophoresis.

Table 2.2. List of the primers used in this study. The universal primers for the amplification of the 16S rRNA gene were obtained from the bibliography. The species-specific primers were designed during this work and their sequence is not included since a publication is pending.

Target	Target gene	Primer name	Amplicon Size (bp)	Optimal temperature (ºC)	Reference
All bacteria	*16S rRNA*	16S_27F 16S_1492R	1400	59	(Lane, 1991)
V. parahaemolyticus	*toxR*	Vpa.toxF Vpa.toxR	130	57	This study
V. alfacsensis	*toxR*	Val.toxF Val.toxR	196	57	This study
V. renipiscarius	*Haemolysin (tdh)*	Vre.haeF Vre.haeR	248	57	This study
V. ichthyoenteri	*Haemolysin (tdh)*	Vic.haeF Vic.haeR	168	57	This study
V. harveyi	*Haemolysin (vhhb)*	Vha_vhhbF Vha_vhhbR	180	57	This study

2.2.2.2.3. Quantitative PCR (qPCR)

The efficiency of species-specific primer pairs was evaluated by quantitative PCR (qPCR) using the standard curve amplification method. For the construction of the standard curve, the sequence and confirmed species-specific amplicons were used as the DNA template. The amplicons were extracted and purified from the agarose gel using a GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, USA) following the manufacturer's instructions. The

concentration and quality were assessed using a NanoDrop (ThermoFisher Scientific). To construct the standard curve for each primer pair, nine serial dilutions from 1 ng/µL to 10 ag/µL of each PCR product were tested (to identify the detection limit). The reactions were performed for a final volume of 10 µL with 5 µL of SsoFast EvaGreen Supermix (Bio-Rad, Portugal); 0.3 µL of 10 mM forward and reverse species-specific primers; 2.4 µL of purified MiliQ water and 2 µL of PCR product. The reactions were performed in duplicate in a 96-well microplate (Axygen Scientific) and the PCR reactions were run on a CFX Connect™ Real-Time PCR Detection System using the following cycle: 2 min at 95 °C; 40 cycles of (5 sec at 95 °C, 30 sec at 57 °C, 10 sec at 72 °C). Data were analysed using the Bio-Rad CFX Maestro software (Bio-Rad). A gradient of temperature from 60º to 95 °C with a temperature increase of 0.5 °C every 10 sec was added to identify possible contaminations and the presence of non-specific products. The detection limit of each pair of primers was established and the cycle threshold (Ct) values superior to 29 were chosen as the upper cut-off and indicated low concentrations of DNA in the samples (WVDL).

2.2.2.2.4. Multiplex-PCR (mPCR)

An mPCR was developed to detect 5 different gDNAs of the target *Vibrio* spp. A mixture of all gDNAs of all *Vibrio* isolates (10 µL of each target gDNA at 50 ng/µL in a final volume of 50 µL) and all primer pairs (5 µL of each primer at an initial concentration of 10 mM in a final volume of 50 µL) was prepared. To determine the optimal performance conditions four different reactions (N, M1 to M3) varying in the concentrations of primer pairs and gDNA were tested: the normal reaction (N) contained 4 µM of each primer pair and 5 ng/µL of each target gDNA, M1 reaction contained the 2x the gDNA concentration, M2 reaction contained twice the concentration of primers, and M3 reaction contained two times the concentration of primer and gDNA. All reactions were performed at 57 °C for a final volume of 20 µL. The reaction M2 was selected as the best approach because it allowed the amplification of all target species and used fewer primers and gDNA. The optimal M2 reaction contained: 2 µL 10xDreamTaq Buffer; 0.4 µL of 20 mM dNTPs; 4 µL of the primer mixture, 0.1 µL of DreamTaq polymerase 5 U and 2 µL of the gDNA mixture. The thermocycle conditions were: 95 °C for 3 min, 35x (95 °C for 20 sec, 57 °C for 20 sec, and 72 °C for 20 sec), and a final extension at 72 °C for 5 min. The detection limit of the mPCR was also assessed using different dilutions of the initial gDNA mixture from 1:2 (5 ng/uL), 1:4 (2.5 ng/uL), 1:8 (1.25 ng/uL), and 1:10 (1 ng/uL). The

success of the amplification was initially assessed by2.5% agarose gel/1x TAE electrophoresis and subsequently by 10% polyacrylamide/1x Tris-Borate-EDTA (TBE) gel electrophoresis, and the results were visualized using Image Lab Software GelDoc XR+.

2.2.2.3. Gel electrophoresis

2.2.2.3.1. Agarose gel electrophoresis

For the analysis of the amplicons of the qualitative-PCRs for the16S rRNA and virulence genes, different agarose gels were prepared. For the 16S amplicons, with an expected size of 1465 bp, a 1% agarose gel was prepared. For the analysis of the species-specific amplicons, between 130 bp and 248 bp, 2.5% agarose gels were prepared to allow the migration and separation of smaller fragments. For the visualization of the nucleic acids, 10 µL of GreenSafe (1:10) (NZY Tech, Portugal) were added to 50 mL of agarose on 1x TAE buffer. Agarose gels were run at 90 V for 30 ± 5 min and two different ladders: NZYTech Ladder III (10000-200 bp) and NZYTech Ladder V (1000-100 bp) (NZYTech, Portugal) were used as a reference for amplicon size. Gels were visualized under UV light using Image Lab Software GelDoc XR+ (BioRad, USA) and digital images were taken.

2.2.2.3.2. Polyacrylamide gel electrophoresis

The similar-sized amplicons were separated using high-resolution polyacrylamide gel electrophoresis (Lonza). Ten percent polyacrylamide gels with 1mm thickness were prepared by mixing 6.6 mL of deionized water, 3 mL of 40% Acrylamide (Biorad, USA), 2.4 mL of 5x TBE (0.13 M Tris/ 45 mM Boric acid/ 2.5 mM EDTA) buffer, 200 µL of freshly prepared 10% Ammonium Persulphate (APS), and 10 µL of TEMED (N, N, N′, N′-Tetramethylethylenediamine) with constant agitation. After the addition of TEMED to allow polymerization, the solution was quickly pipetted into vertical glass supports and was let to solidify at room temperature. The polyacrylamide gels were run using vertical support at a constant voltage of 16 mA. The gels ran for 90 ± 15 min or until a clear separation of the ladder was observed. For the verification of the size of each amplicon, the NZYTech Ladder V was used. After running, the gels were incubated with a solution (10:1) of 1x TBE with GreenSafe (NZYTech, Portugal) to stain the nucleic acids and were visualized under UV light using the Image Lab Software GelDoc XR+ (BioRad, USA) and digital images were taken.

2.3. Microbiological characterization

2.3.1. Bacterial growth curves

The growth rate of all isolates was characterized at 22 °C. For the analysis, 1 µL loop of each bacterium cultivated on TSA/1% NaCl (pH 7.3 ± 0.2) was inoculated in 5 mL TSB/1% NaCl (pH 7.3 ± 0.2) at 22 °C, overnight (16 ± 2 h) at 150 rpm. After preliminary results showing an extensive lag phase for some of the isolates, different initial dilutions of each bacterium on TSB/1% NaCl were used to achieve an initial OD_{600nm} value between 0.01- 0.02. For the isolates *V. parahaemolyticus* and *V. harveyi,* the overnight cultures were diluted 1:5 in the culture medium, whereas the other isolates were diluted 1:2. Twenty microliters of each dilution were inoculated into 180µL TSB/1% NaCl in a sterile 96-well microplate with a lid (Sarsted, Germany) and triplicate wells (n= 3) were performed as technical replicates. Growth was monitored every hour by reading the OD_{600nm} on a Synergy Neo2 Hybrid Multi-Mode Multiplate Reader (Biotech, USA) for a total of nine hours. Before each reading, the microplate was automatically agitated for 10 seconds at an amplitude of 3 millimetres inside the microplate reader. Growth curves were constructed using the mean OD_{600nm} value for each *Vibrio* spp. culture subtracted from the geometric mean of the 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 duplication time (DT) was calculated according to (Bioquest, 2020).

To understand their likely growth in a human host, the growth curve of each isolate was also characterized at 37 °C, to mimic the average temperature of the human body. To characterize the impact of the changes in the environment on bacterial performance, the effect of changes in salinity (0.75%) and pH (7.8 and 8.2) according to the predictions of the International Panel for Climate Change (IPCC) (IPCC, 2022) for the marine environment were also analysed. Bacterial growth was characterized in TSB/1% NaCl with a modified pH: pH 8.2 (normal pH of the Ria Formosa) and pH 7.8 (the predicted 0.4 pH decrease). To simulate lower salinity conditions we decreased the optimal NaCl concentration of the medium to 0.75% NaCl. For all the conditions, a reference growth curve of each isolate incubated at 22 ºC was performed simultaneously to allow for direct comparisons.

2.3.2. Colony morphology on TCBS

The morphology of each isolate was analysed and assessed on TCBS plates. One colony of each isolate inoculated on TSA/1% NaCl was seeded on TCBS plates, followed by incubation at 22 ºC for 48 h. After the initial observation of pure cultures, based on the colonies' colour, the morphology of isolated colonies was analysed under an Olympus SZ-ST stereo microscope (Olympus, Japan) considering the size, colour, surface, border, and elevation of the colony (Farida Hikmawati, 2019). The effect of 37 °C on the colony morphology was also assessed and each isolate was incubated for 24 h following the standard protocol used for the detection of *Vibrio* spp. in environmental samples (Kriem et al., 2015).

2.3.3. Antibiotic susceptibility

The widespread use of antibiotics is an important source of contaminants to aquatic systems by the release of wastewater from the aquaculture industry or by the direct use of antibiotics in open systems, affecting both aquatic and terrestrial animals (Novriadi, 2016). The antimicrobials approved in Europe for aquaculture include amoxicillin, florfenicol, flumequine, sarafloxacin, sulphadiazine-trimethoprim, oxolinic acid, and oxytetracycline (Elmahdi, DaSilva, & Parveen, 2016; Loo et al., 2020; Stevens et al., 2014).

Different antibiotics that are commonly used in aquaculture systems and the clinical treatment of vibriosis as recommended by the Disease Control and Prevention (CDC) were tested (Adesiyan, Bisi-Johnson, Ogunfowokan, & Okoh, 2021; Deng et al., 2020; Kitiyodom, Khemtong, Wongtavatchai, & Chuanchuen, 2010; Letchumanan et al., 2015; Nurliyana Mohamad et al., 2019; Onohuean, Okoh, & Nwodo, 2022; Páll et al., 2021). The conventional disk diffusion technique (Kirby-Bauer test) (Bauer, Kirby, Sherris, & Turck, 1966) was used following the Clinical and Laboratory Standards Institute (CLSI) and EUCAST guidelines (CLSI; CLSI; EUCAST). An overnight culture on TSA/1% NaCl of the *Vibrio* isolates was resuspended in 2 mL of Phosphate Buffered Saline (PBS) and adjusted to the equivalent of 0.5 McFarland standard (10^6 CFU/mL). Sterile cotton-wool swab sticks were dipped in the solution and evenly streaked onto Mueller-Hinton (MH) agar plates supplemented with 1% NaCl (121). The antibiotic disks were carefully dispensed onto the plate and incubated at (i) 35 °C for 18 ± 2h for the bacteria *V. parahaemolyticus* and *V. harveyi* (both isolates) and (ii) 22 °C for 48 ± 2h for the bacteria *V. ichthyoenteri*, *V. alfacsensis,* and *V. renipiscarius*. The inhibition zones were measured in millimetres and interpreted as Resistant (R), dose-dependent/Intermediate (I),

and Susceptible (S) according to the standards recommended by CLSI M45 (94). The antibiotic used were Imipenem (IMI- 10 µg), Ciprofloxacin (CIP- 5 µg), Amikacin (Ak- 30 µg), Florfenicol (FFC- 30 µg), Piperacillin-Tazobactam (TZP- 30+6), Chloramphenicol (C- 30 µg). All antibiotics were purchased from Oxoid (Oxoid Limited, UK). Three technical replicates were performed on different plates.

2.4. Biochemical characterization

2.4.1. Enzymatic activity

The enzymatic activity of the *Vibrio* isolates was characterized to understand their potential virulence for marine organisms. For that, the culture media used, and the assay conditions were previously optimized for the screening of the proteolytic and hydrolytic (esterase and lipase) activities of the isolates which are enzymes associated with virulence. The assays were performed at 22 °C on TSA/1% NaCl (pH 7.3 ± 0.2) supplemented with (i) 1% Tween 20 to analyse the esterase activity, (ii) 0.1% Tween 80 to test for lipase activity, and (iii) 1% gelatine to analyse the protease activity (Table 7.1, Supplementary Material 1). For the protocol optimization, two strategies were used: (i) inoculating single colonies freshly obtained from TSA/1% NaCl plates or (ii) using sterile disks impregnated with cell-free or culture supernatants. The plates were evenly divided, and colonies and disks were seeded with the aid of a sterile loop and sterile tweezers, respectively. For the cell-free assay, the bacterial supernatants were obtained by centrifugation of 2 mL of TSB/1% NaCl overnight cultures at 12,000 x g for 10 min and filtered through a 0.22 µL filter (Fisherbrand) (D. Liu et al., 2015; Salamone, Nicosia, Ghersi, & Tagliavia, 2019). The sterile blank disks (Oxoid) were inoculated with 10 µL and immediately transferred to the plates. After an initial assessment of the enzymatic activity, the second approach with cell-free cultures was discarded for lack of positive results and the assay proceeded with the colonies. After 48 h of incubation, the plates were photographed with a digital camera, and the area of the halo was calculated using the ImageJ program. All isolates were tested in triplicates as technical replicates.

To analyse the effect of the temperature on enzyme production and virulence, similar experiments were carried out at 37 ºC for the isolates, *V. parahaemolyticus* and *V. harveyi*, that grow at this temperature. The effect of changes in temperature, salinity, and pH on the enzymatic profile selected based on the findings of IPCC (IPCC, 2022) was also analysed by modifying the pH of the optimal TSA/1% NaCl medium to pH of 7.8 and 8.2 and by decreasing

medium salinity to 0.75% NaCl and incubated at 22ºC. The Enzymatic Index for each bacterium was calculated using the formula described in (Florencio, Couri, & Farinas, 2012; Ortiz Lechuga, Isela, & Arevalo, 2016) and compared between all the conditions tested.

2.5. Screening for Vibrio spp. in environmental samples

The current method of reference for the detection of *Vibrio* spp. in food and environmental samples is described in ISO 21872-1:2017 and was used to screen members of the *Vibrio* genus in the bivalve samples collected from the environment. The method used is based on a two-step enrichment with Alkaline Peptone Water (APW) at pH 8.6 to allow the proliferation of *Vibrio* spp. and their isolation in TCBS medium (Figure 2.2). Slight modifications were performed to optimize the protocol for sample type and samples from each enrichment of the liquid culture were collected for genomic DNA extraction to confirm the identity of the grown bacteria.

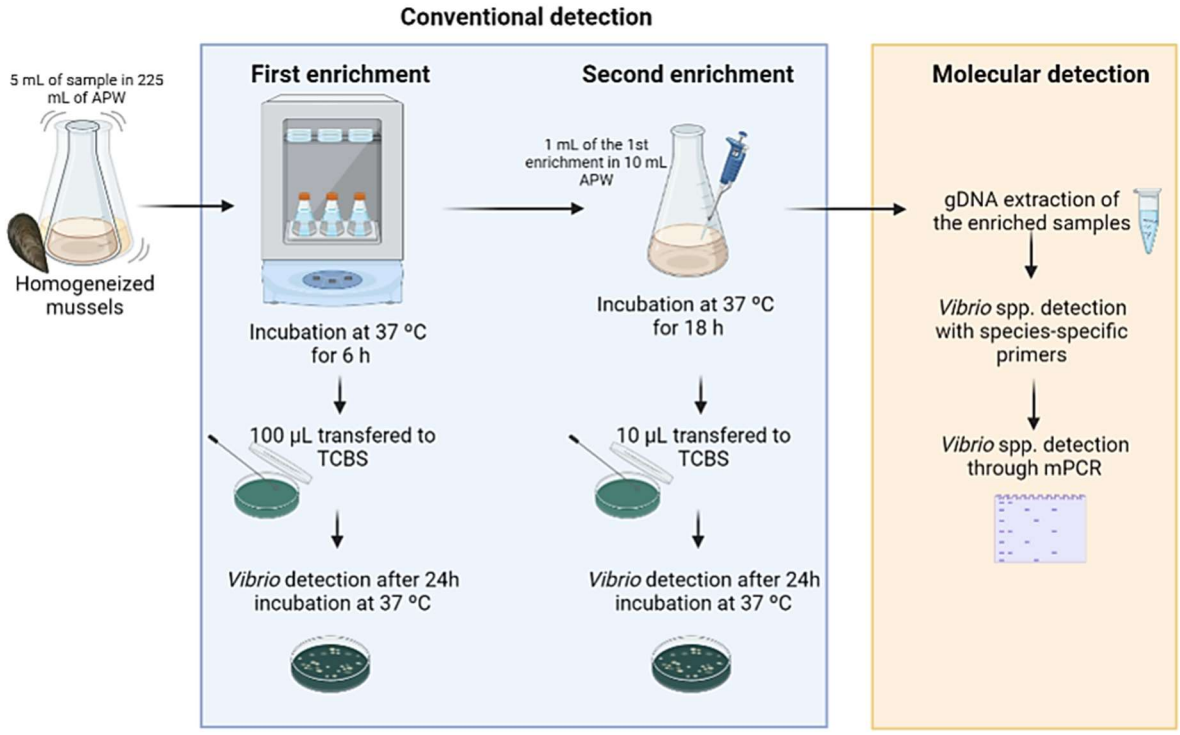


Figure 2.2. Schematic representation of the method used for the environmental screening of *Vibrio* spp. according to ISO 21872-1:2017. Samples from the two enrichment steps were used for molecular analysis. Diagram was built by the author in BioRender.

2.5.1. Sample collection and processing

Four specimens of the bivalve Mediterranean mussel (*Mytilus galloprovincialis*) (3 - 4 cm in length) collected from Praia de Faro (Portugal) in June 2022 were used for the screening of *Vibrio* spp. and to test the applicability of the mPCR developed on environmental samples. Briefly, the mussels were collected during the low tide and transported live to CCMAR in plastic bags filled with environmental water, where they were analysed on the same day. On arrival, the mussel shells were cleaned and disinfected with 70% EtOH to avoid external contamination and the animals were opened with a sterile blade under sterile conditions. The soft body of each mussel was collected and weight (5 ± 0.5 g) and was transferred to UV-sterile plastic bags with APW using the proportion of 1:10 mg/mL. The mussels were then manually homogenised until the complete dissociation of the tissue (ISO, 2017).

2.5.2. Culture enrichment and plate screening

For the first selective enrichment, each complete homogenate (soft tissue + APW) was transferred to sterile 100 mL Erlenmeyer and incubated at 37 °C for 6 hours, 150 rpm (samples 3A - 3D). Posteriorly, the second enrichment was prepared by transferring 1 mL of the first enrichment to a sterile Falcon tube containing 10 mL of sterile APW that was afterwards incubated at 37 °C for 24 hours, 150 rpm (samples 4A - 4D).

To examine the presence of *Vibrio* spp. in each mussel after each enrichment step, 100 µL of the first enrichment and 10 µL of the second one was spread on TCBS (n=2/culture) and incubated at 37 °C for 24 h to verify the presence of typical colonies of the five species under study.

2.5.3. Molecular analysis by multiplex PCR

Two millilitres of each enriched culture were centrifuged for 5 min at 13,000 rpm and the pellet was used for the DNA extraction following the GES method as described in section 2.1. Before the mPCR, and to access for the potential influence of the organism microbiome on the PCR, the gDNA extracted from each enriched culture at 50 ng/µL was screened for the human pathogen *V. parahaemolyticus* and the marine pathogen *V. harveyi* in the collected mussels. The PCR reactions and thermocycler used were the same as described in section 2.2.2.2.2. with the gDNA of each enriched sample as a template. The same gDNA at 50 ng/µL was used for

the mPCR and the mPCR reaction contained: 2 µL 10xDreamTaq Buffer; 0.4 µL of 20 mM dNTPs; 4 µL of the primer mixture, 0.1 µL of DreamTaq polymerase 5 U and 2 µL of gDNA. The thermocycle conditions were: 95 °C for 3 min, 35x (95 °C for 20 sec, 57 °C for 20 sec, and 72 °C for 20 sec), and a final extension at 72 °C for 5 min. As a positive control, a mixture of the gDNA of one isolate of each species was used in the reaction. The success of the amplification was initially assessed in a 2.5% agarose gel/1x TAE electrophoresis and subsequently run on a 10% polyacrylamide/1x TBE gel electrophoresis. Both gels were visualized using Image Lab Software GelDoc XR+.

2.6. Data analysis and statistics

All graphics and statistical analyses were performed in the program GraphPad Prism version 9 for Mac OS X (USA). Triplicates (n=3) biological replicates for each assay were performed and a One-way ANOVA analysis with multiple comparisons was used to detect statistically significant differences in the growth rate of each isolate and the influence of the culture conditions on growth and enzymatic activity. T-tests were used to compare the growth rate and enzymatic activity at 22 and 37 ºC of all isolates.

Chapter 3: Results

3.1. Characterization of marine Vibrio isolates

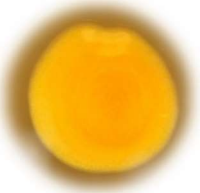
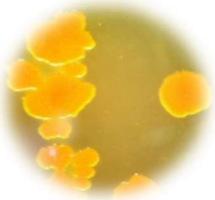
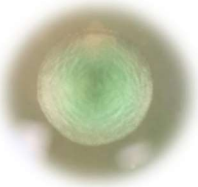
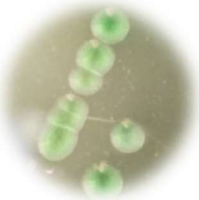
3.1.1. Colony morphology on TCBS plates

The TCBS medium is the ideal medium that favours the growth of *Vibrio* species because of the high concentrations the Thiosulfate-Citrate, bile, and alkaline pH which inhibits the growth of *Enterobacteria* and Gram-positive bacteria. The initial assessment of the purity of the *Vibrio* culture was performed based on the colour of the colonies, which is associated with their capacity to metabolize sucrose. Most of the marine colony isolates were characterized by their yellow colour, a result of the sucrose fermentation that produces acid and changed the TCBS pH indicator to yellow. The exception was the putative *V. ichthyoenteri* isolate (Figure 3.1) where two different coloured colonies (yellow- sucrose fermenters, and green- non-sucrose fermenters) were obtained, suggesting that the isolate contained two different strains, and they were designated *V. ichthyoenteri* 1 and *V. ichthyoenteri* 2. The marine *V. parahaemolyticu*s isolate is also a non-sucrose fermenter. The *V. alfacsensis* and *V. harveyi* 2 isolates showed the highest sucrose fermentation capacity and consequently the highest production of acid which resulted in a complete alteration of the original plate colour from dark green to yellow. The remaining sucrose-fermenter isolates only showed an alteration of colour around the colonies.

Each isolate had a characteristic shape, size, surface pattern, and halo morphology (Table 3.1). All colonies of the same isolate showed medium-sized colonies (estimated to be between 2 - 4 mm) with no significant differences between them. The isolates *V. parahaemolyticus*, *V. renipiscarius*, and *V. alfacsensis* have slightly larger colonies when compared to the other strains.

The isolates *V. renipiscarius* and *V. harveyi* 1 formed irregular colonies. *V. parahaemolyticus* and *V. ichthyoenteri* 1 colonies had concentric layers from the smooth centre to a completely granular light border. The *V. renipiscarius* and *V. alfacsensis* colonies had rough shapes without defined layers on both isolates described before but with the same kind of border. The isolate *V. ichthyoenteri* 2 was characterized by granular colonies with a transparent border and the two *V. harveyi* isolates had a distinct pattern of roughness with a rose-like shape and an irregular transparent border, respectively. Only *V. parahaemolyticus* and *V. ichthyoenteri* 2 had elevated colonies with a convex pattern while the others were flattened. The isolates *V. renipiscarius* and *V. ichthyoenteri* were opaque to light.

Figure 3.1. Digital photographs of representative colonies of the seven isolates on TCBS plates at 22 °C. Plates were incubated for 48h. Images were obtained using two different magnifications (4 x and 1.5 x).

Isolate	4x magnification	1.5 x magnification
Vpa		
Val		
Vre		
Vic1		
Vic2		
Vha1		
Vha2		

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*

Table 3.1. Colony morphology of *Vibrio* spp. on TCBS plates at 22 °C. Colony characteristics were observed after 48 h.

Colony characteristic	Isolate						
	Vpa	**Vre**	**Val**	**Vic 1**	**Vic 2**	**Vha 1**	**Vha 2**
Shape	Circular	Irregular	Circular	Circular	Circular	Irregular	Circular
Size	Medium/large	Medium/large	Medium/large	Medium	Medium	Medium	Medium
Colour	Green with a dark green centre	Yellow/ orange with a darker centre	Yellow with a darker centre	Blueish green with a dark green centre	Yellow/ orange with a darker centre and light border	Yellow/ orange with a darker centre	Yellow/ orange with a darker centre
Surface	Concentric layers from the smooth centre to a granular border	Rough appearance from the centre to a granular border	Rough appearance with a granular border	Concentric layers from the smooth centre to a granular border	Granular appearance	Rough appearance (Concentric rose shape)	Rough appearance (Concentric rose shape)
Halo	Lighter green	Dark yellow	Alteration of the medium colour to dark yellow	Lighter green	Dark yellow	Dark yellow	Alteration of the medium colour to dark yellow
Elevation	Convex	Flattened	Flattened	Convex	Flattened	Flattened	Flattened
Border	Granular appearance	Lighter yellow with a granular appearance	Lighter yellow with a granular appearance	Incomplete transparent border	Transparent border	Irregular Transparent border	Irregular Transparent border
Transparency	Translucent	Opaque	Translucent	Opaque	Opaque	Translucent	Translucent

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*

3.1.2. Effect of temperature on colony morphology

At 37 °C growth was only observed for the marine isolates *V. parahaemolyticus* and the two presumptive strains of *V. harveyi* (Vha1 and Vha2) (Figure 3.2). The morphological characteristics (shape, size, surface, and halo) of each isolate were analysed (Table 3.2) and compared with the bacterial morphology at 22 ºC. At 37ºC, the morphology of the three isolates was modified and the isolates *V. harveyi* 1 and *V. harveyi* 2 suffered the most profound modifications.

Isolate	4x magnification	1.5 x magnification
Vpa		
Vha1		
Vha2		

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*

Figure 3.2. Digital photographs of the representative colonies of the isolates on TCBS plates at 37 °C. Each isolate was incubated for 24 h. Images were obtained using two different magnifications (4 x and 1.5 x).

The colony shape of isolate *V. harveyi* 1 changed from irregular to circular, the colony surface from rough to granular, and the transparency of the colonies was only restricted to the border. The shape of isolate *V. harveyi* 2 shifted from circular to irregular and the yellow halo that altered the entire culture plate at 22 ºC was reduced to only a small halo. All the morphological characteristics of isolate *V. parahaemolyticus* were maintained except that the colony elevation lost the convex profile observed at 22ºC.

Shape	Circular	Circular	Irregular
Size	Medium	Medium	Medium
Colour	Green with a dark green centre	Yellow with a darker centre	Yellow
Surface	Rough appearance with slight concentric layers with granular border	Granular uniform appearance	Rough appearance (Concentric rose-shape lines)
Halo	Lighter green	Dark yellow	Dark yellow
Elevation	Flattened	Flattened	Flattened
Border	Complete, Transparent with a granular appearance	Incomplete, Transparent with granular appearance	Complete, Transparent with granular appearance
Transparency	Translucent	Translucent borders	Translucent

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*

3.1.3. Molecular identification of the marine Vibrio isolates

Bacterial identification was performed by the amplification of a fragment of ~1400 bp of the 16S rRNA gene. The alignment of the obtained sequences with the homologues deposited in the NCBI database confirmed that all isolates in this study are members of the *Vibrio* genus. The study species shared between 95%- 98% nucleotide sequence identity with the orthologues deposited in the database suggesting that they may represent novel strains (Table 3.3; Table 7.2, Supplementary material 2). The nucleotide sequence alignment of the two isolates of the *V. harveyi* and *V. ichthyoenteri* retrieved different strains and the alignment of the sequenced 16S rRNA fragments revealed that they are 99.13% and 95.79% identical, respectively, confirming that they may represent different strains of the same species as suggested by the morphological assessment performed previously.

Table 3.3. Nucleotide BLAST results of the 16S rRNA sequence of the studied marine *Vibrio* spp. The first BLAST hit is represented with the description of the most similar orthologue, the statistical e-value, the percentage of sequence similarity, and the accession number.

Isolate	Description	e-value	% identity	Accession number

Vre	*Vibrio renipiscarius* strain DCR 1-4-2 16S ribosomal RNA, partial sequence	0.0	98.23	NR_135891.1
Vpa	*Vibrio parahaemolyticus* strain MC32 16S ribosomal RNA gene, partial sequence	0.0	98.61	MT534026.1
Vha 1	*Vibrio harveyi* strain 2010V-1024 chromosome 1	0.0	98.33	CP051122.1
Vha 2	*Vibrio harveyi* strain MFB03 16S ribosomal RNA gene, partial sequence	0.0	98.46	MT605241.1
Vic1	*Vibrio* sp. HGLP-44 16S ribosomal RNA gene, partial sequence	0.0	95.89	KX001857.1
Vic 2	*Vibrio* sp. V170 16S ribosomal RNA gene, partial sequence	0.0	97.38	DQ146979.1
Val	*Vibrio alfacsensis* strain Y831 16S ribosomal RNA gene, partial sequence	0.0	99.14	MK452736.1

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*

3.2. Microbiological characterization of the marine Vibrio isolates

3.2.1. Growth curves

All seven *Vibrio* isolates grew at 22 ºC (Figure 3.3 A). Most of the isolates had a small lag phase that lasted approximately 1 hour, except for *V. renipiscarius* and *V. ichthyoenteri* 2 isolates which lasted for approximately three hours. After nine hours of growth, the *V. parahaemolyticus* isolate reached the highest optical density value (close to 1) during the stationary phase, whereas the isolate *V. ichthyoenteri* 2 showed the lowest (~0.09). However, the isolate *V. parahaemolyticus* showed the shortest exponential phase whereas *V. ichthyoenteri* 2 had exponential growth over 9 hours. After reaching the stationary phase, the OD_{600nm} remained stable with no decline in the cell number or cell death observed for all isolates.

At 37 ºC only the isolates *V. parahaemolyticus*, *V. harveyi* 1, and *V. harveyi* 2 were able to grow, with the temperature increase modifying their growth performance (Figure 3.3 B). When compared to 22ºC, all isolates have a shorter lag phase, followed by a short exponential growth phase of 3 hours. The isolate *V. parahaemolyticus* had the highest optical density (> 1) at 37 ºC, higher than that observed at 22 ºC (~1). On the other hand, the optical density of both *V. harveyi* isolates was reduced at higher temperatures (~0.3). For the three isolates, the temperature increase led to a decreased length of the exponential phase, but cell density was maintained at both temperatures, suggesting that despite the shorter exponential phase at 37 ºC, the isolates reached maximum growth at both temperatures.

A

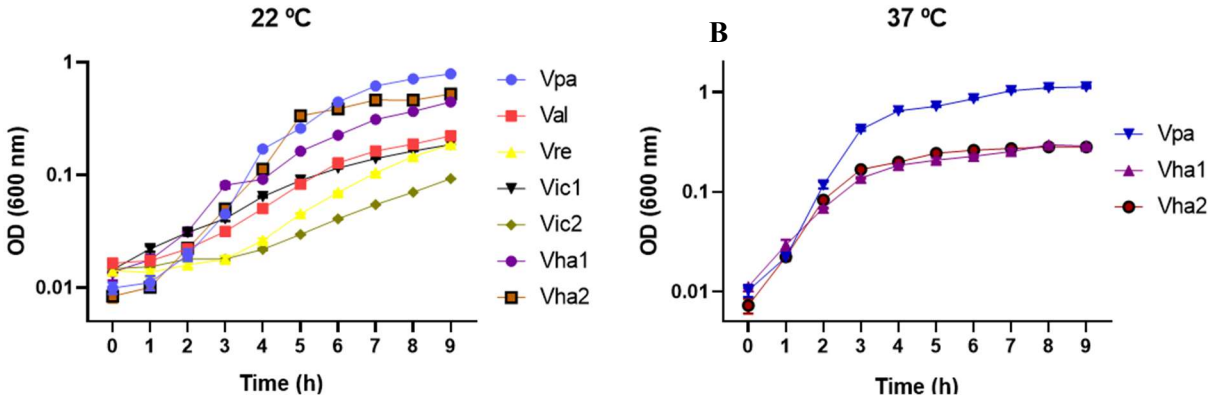


Figure 3.3. Growth curves of the *Vibrio* isolates at 22ºC (A) and 37ºC (B). Each curve was constructed using the mean values of three replicates/time points. The OD_{600nm} values were transformed to a semilogarithmic phase. All isolates are represented by their abbreviations and the curves have different colours to facilitate identification.

Analysis of their growth kinetics allowed the calculation of specific growth rates and duplication time (Table 3.4).

Table 3.4. Growth kinetics of *Vibrio* spp. at 22ºC and 37ºC. Growth Rates (μ) and duplication Time (DT) were calculated at 22 ºC and 37 ºC. The values represent mean ± SEM (n=3). A T-test was performed to detect statistically significant differences between the conditions (* $p < 0.05$).

Isolate	22 ºC		37 ºC	
	Specific growth rate (h^{-1})	Duplication time (h)	Specific growth rate (h^{-1})	Duplication time (h)
Vpa	0.80 ± 0.04	0.87 ± 0.04	1.29 ± 0.06*	0.54 ± 0.03
Val	0.45 ± 0.02	1.55 ± 0.07	-	-
Vre	0.46 ± 0.03	1.52 ± 0.09	-	-
Vic 1	0.36 ± 0.03	1.92 ± 0.15	-	-
Vic 2	0.29 ± 0.01	2.40 ± 0.06	-	-
Vha 1	0.51 ± 0.04	1.36 ± 0.10	0.84 ± 0.03*	0.83 ± 0.03
Vha 2	0.77 ± 0.03	0.90 ± 0.04	1.23 ± 0.16*	0.57 ± 0.07

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*;

* Statistically significant ($p < 0.05$) compared to 22 ºC.

The isolate *V. parahaemolyticus* (0.803 ± 0.040 h^{-1}) and *V. harveyi* 2 (0.769 ± 0.030 h^{-1}) had the highest growth rates. The isolates *V. ichthyoenteri* 1 (0.362 ± 0.029 h^{-1}) and *V. ichthyoenteri* 2 (0.289 ± 0.007 h^{-1}) possessed the slowest growth. At 37 ºC (Table 3.4) a statistically significant increase ($p < 0.05$) in the growth rate was observed. The isolates *V.*

parahaemolyticus, *V. harveyi* 1, and *V. harveyi* 2 reached a growth rate of 1.288 ± 0.061 h^{-1}, 0.839 ± 0.028 h^{-1}, and 1.233 ± 0.158 h^{-1}, respectively, suggesting that an increase in the incubation temperature is positively associated with an increase in bacteria growth rate.

3.3. Antibiotic susceptibility

Antibiotic resistance of the isolates *V. parahaemolyticus* and *V. harveyi* was performed according to the EUCAST and CLSI standards. However, only the bacterium *V. parahaemolyticus* is formally included in both protocols for human pathogens. The antibiotic susceptibility of the remaining isolates, unable to grow at 35 ºC, was performed at 22 ºC and the halos were analysed according to the standard procedures (Table 3.5).

Table 3.5. Antibiotic susceptibility of *Vibrio* isolates according to the CLSI and EUCAST standards. Analysis of the Vpa, Vha 1, and Vha 2 isolates was performed at 35 °C and the others at 22 °C. The isolates were characterized as susceptible (S), with intermediate resistance (I) or resistance (R).

Isolate	Antibiotic					
	TZP (30+6 µg/µL)	**FFC*** (30 µg/µL)	**CIP** (5 µg/µL)	**AK** (30 µg/µL)	**C** (30 µg/µL)	**IMI** (10 µg/µL)
Vpa	S	S	S	I	S	S
Val	S	S	S	I	S	S
Vre	S	S	S	S	S	S
Vic 1	S	S	S	I	S	S
Vic 2	S	S	S	S	S	S
Vha 1	S	S	S	I	S	S
Vha 2	S	S	S	I	S	S

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*
Imipenem (IMI), Ciprofloxacin (CIP), Amikacin (Ak), Florfenicol (FFC), Piperacillin-Tazobactam (TZP), Chloramphenicol (C).
*Reference values based on Chloramphenicol resistance.

All the marine *Vibrio* isolates tested were susceptible (100%) (S) to piperacillin-tazobactam (TZP- 30+6), chloramphenicol (C- 30 µg), imipenem (IMI- 10 µg), ciprofloxacin (CIP- 5 µg), and florfenicol (FFC- 30 µg). Only the isolates *V. renipiscarius* and *V. ichthyoenteri* 2 (28.57%) are susceptible to amikacin (Ak- 30 µg), and the remaining isolates (*V. parahaemolyticus*, V. *alfacsensis*, *V. ichthyoenteri* 1, *V. harveyi* 1, and *V. harveyi* 2) had intermediate resistant (I).

The susceptibility of the two isolates of the bacterium *V. ichthyoenteri* was similar for all the antibiotics tested except for amikacin (83.33 %), to which the isolate *V. ichthyoenteri* 1 was susceptible, and *V. ichthyoenteri* 2 showed intermediate susceptibility. No differences were observed between the two isolates of the bacterium *V. harveyi* (Vha1 and Vha2).

3.4. Biochemical characterization

3.4.1. Secreted enzyme activity

The enzymatic activity of the isolates was characterized by screening for proteolytic (protease) and hydrolytic (lipase and esterase) activities, which are related to potential virulence. After 48 hours of incubation, only the isolates *V. parahaemolyticus*, *V. harveyi* 1, and *V. harveyi* 2 showed positive results for at least one assay (Figure 3.4). The remaining isolates did not show any enzymatic activity at 22 ºC, suggesting that they may not be harmful to the marine organisms.

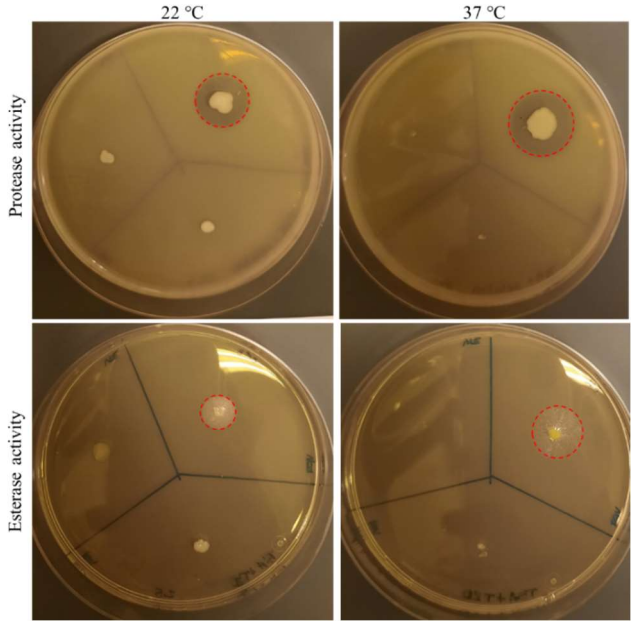


Figure 3.4. Photographs of the enzyme activity of the isolate *V. parahaemolyticus* at 22 ºC and 37 ºC. Images were taken using a digital camera. Protease and esterase activity was observed at both temperatures and for both activities an increase of the degradation halo was observed and is demarked by the red dashed circle.

At 22 ºC the isolate *V. parahaemolyticus* showed the highest protease (5.215 ± 0.323) and esterase (12.352 ± 0.201) activities, followed by the isolate *V. harveyi* 1 (protease activity = 3.475 ± 0.569; esterase activity = 7.273 ± 0.512) (Figure 3.5). The lipase assay only showed

positive results for the *V. harveyi* isolates, *V. harveyi* 2 (2.255 ± 0.364), and *V. harveyi* 1 (2.128 ± 0.687).

3.4.2. Influence of temperature on enzyme activity

At 37 ºC, the enzymatic activity of the three isolates was modified, suggesting that temperature changes modulate the activity of putative virulence-related enzymes (Figure 3.5).

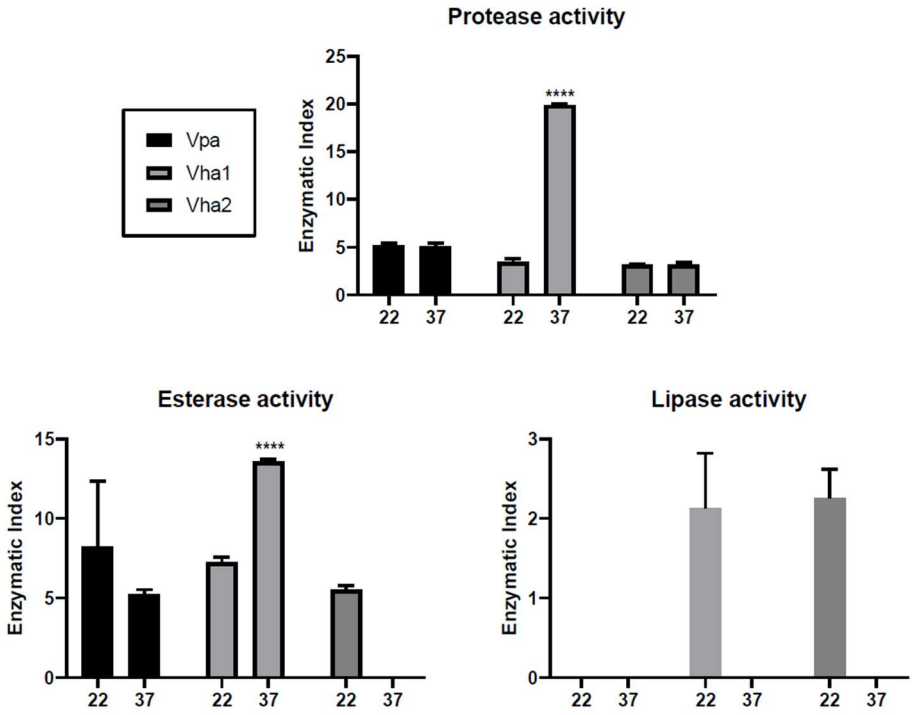


Figure 3.5. Enzymatic index of *Vibrio* isolates at 22 ºC and 37 ºC. Each assay was performed in triplicate. No lipase activity was detected for Vpa, Vha1, and Vha2 at 22ºC. A T-test was performed to detect statistically significant differences between the conditions (* $p < 0.05$).

The esterase activity was the most affected by the temperature increase and a decrease of the enzymatic index of the isolate *V. parahaemolyticus* (5.230 ± 0.512) and a statistically significant ($p < 0.05$) increase of *V. harveyi* 1 activity (13.569 ± 0.308) were observed. Moreover, the highest temperature inhibited the esterase activity of *V. harveyi* 2. The protease activity of isolate *V. harveyi* 1 (19.93 ± 0.22) was significantly ($p < 0.05$) stimulated by the temperature increase (Table 3.6). At 37 ºC the lipase activity of the *V. harveyi* isolates was inhibited. No activity was observed for *V. parahaemolyticus* at 22 and 37 ºC, suggesting that this isolate may not produce and secrete lipolytic enzymes.

Table 3.6. Enzymatic index of the isolates after incubation at 22 and 37 ºC. Enzymatic Index was determined using the dimension of the colonies and their respective activity halos. A students T-test was used to detect significant differences (* $p < 0.05$).

Isolate	Protease activity		Esterase activity		Lipase activity	
	22 °C	37 °C	22 °C	37 °C	22 °C	37 °C
Vpa	5.22 ± 0.32	5.11 ± 0.60	12.35 ± 0.20	5.23 ± 0.51	na	na
Vha 1	3.48 ± 0.57	19.93 ± 0.22*	7.27 ± 0.51	13.57 ± 0.31*	2.13 ± 0.69	na
Vha 2	3.14 ± 0.16	3.19 ± 0.37	5.53 ± 0.42	na	2.26 ± 0.36	na

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*

* Statistically significant ($p < 0.05$); na- no enzyme activity

3.5. Effect of modified culture conditions on marine Vibrio spp. growth and enzymatic activity

3.5.1. Effect on bacteria growth

The effect of the decreased salinity (25% decrease, from 1% to 0.75% final concentration) or increased pH (control pH 7.3 to pH 7.8 and pH 8.2) on growth kinetics of the seven isolates in the culture medium was analysed at 22 ºC (Figure 3.6). Overall, the changes in the culture medium composition modified the bacterial growth profile in a species-specific manner. This suggests that bacteria of the *Vibrio* genus show different susceptibilities. With decreasing salinity and increasing pH, growth of the isolates *V. parahaemolyticus*, *V. renipiscarius,* and *V. alfacsensis* were induced, resulting in the absence of the typical lag phase. A drop of 25% in salinity and an increase in pH to 8.2 resulted in very similar growth curves, which was also observed for the other isolates. The *V. renipiscarius* and *V. ichthyoenteri* isolates had a shorter exponential phase at pH 7.8 when compared to the control, reaching the stationary phase before the control reached the exponential phase. This shortening of the exponential phase was observed at all conditions for *V. harveyi* isolates. At medium pH 7.8, isolate *V. harveyi* 2 also showed a distinct curve with a decrease of the culture's optical density after two hours in the stationary phase, suggesting that the culture entered cell death.

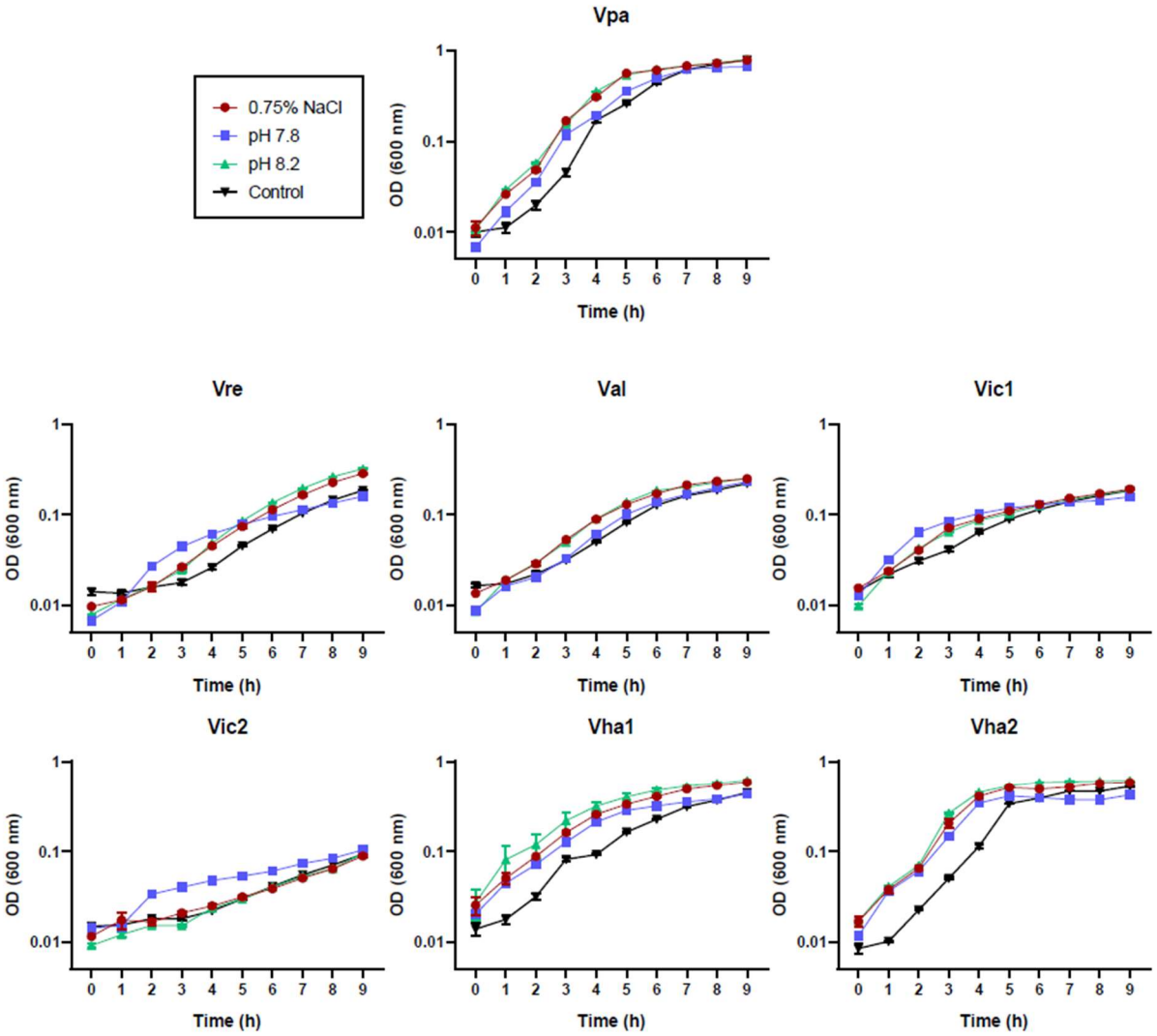


Figure 3.6. Effect of modified culture conditions on growth of the *Vibrio* isolates at 22ºC. Curves were constructed using the mean values of three replicates/per time point. The OD_{600nm} values were transformed to a semilogarithmic scale. The different conditions tested are indicated by different coloured lines. Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi.*

The growth kinetics for each isolate (specific growth rate and duplication time) are described in Table 3.7. Changes in the growth rate were observed for some isolates with both strains of *V. harveyi* and *V. ichthyoenteri* isolates responding differently. The decrease in culture medium salinity and the increase in pH did not cause any significant alteration ($p > 0.05$) in the growth rates of *V. parahaemolyticus* and *V. harveyi* 2 isolates when compared to the control conditions suggesting that these bacteria may be more resilient to the changes in culture conditions. In contrast, isolates *V. alfacsensis* and *V. ichthyoenteri* 1 were highly susceptible and the changes in the growth conditions significantly ($p < 0.05$) increased their growth rate (Val- 0.75% NaCl: 0.50 ± 0.01, pH 7.8: 0.55 ± 0.04, pH 8.2: 0.55±0.02; Vic1- 0.75% NaCl: 0.48 ± 0.01, pH 7.8: 0.81 ± 0.05, pH 8.2: 0.73 ± 0.04). The growth rate of isolate *V. renipiscarius* also significantly

increased ($p < 0.05$) with an increase in pH (pH 7.8 and pH 8.2). At pH 8.2 the growth of the isolated *V. harveyi* 1 was significantly ($p < 0.05$) stimulated (0.72 ± 0.01) compared to the control and pH 7.8. The conditions of decreased salinity in the culture medium (0.75% NaCl) only significantly ($p < 0.05$) inhibited the growth of the isolate *V. ichthyoenteri* 2 (which is the opposite of what was observed for the isolate *V. ichthyoenteri* 1), suggesting that this bacterium is sensitive to changes in NaCl concentration and that the two *V. ichthyoenteri* isolates responded differently to salinity.

Table 3.7. Growth kinetics of the *Vibrio* isolates cultured under modified medium conditions. Growth Rates (μ) and duplication Time (DT) at 22ºC are represented. Values represented Mean ± SEM (n=3). One-way ANOVA was performed to detect statistically significant differences (* $p < 0.05$). only biological replicates were performed in this analysis.

Isolate	Control (1% NaCl; pH 7.3±0.2)		0.75% NaCl		pH 7.8		pH 8.2	
	μ (h^{-1})	DT (h)	μ (h^{-1})	DT (h)	μ (h^{-1})	DT (h)	μ (h^{-1})	DT (h)
Vpa	0.80 ±0.04	0.87 ±0.04	0.86 ±0.06	0.81 ±0.05	0.87 ±0.04	0.80 ±0.03	0.87 ±0.02	0.80 ±0.02
Val	0.45 ±0.02	1.55 ±0.07	0.50±0.01*	1.38 ±0.04	0.55±0.04*	1.27 ±0.10	0.55±0.02*	1.27 ±0.04
Vre	0.46 ±0.03	1.52 ±0.09	0.50 ±0.03	1.40 ±0.08	0.75±0.01*	0.92 ±0.01	0.63±0.04*	1.11 ±0.07
Vic 1	0.36 ±0.03	1.92 ±0.15	0.48±0.01*	1.44 ±0.03	0.81±0.05*	0.86 ±0.05	0.73±0.04*	0.95 ±0.05
Vic 2	0.29 ±0.01	2.40 ±0.06	0.25±0.02*	2.83 ±0.27	0.26 ±0.00	2.68 ±0.03	0.27 ±0.01	2.54 ±0.06
Vha 1	0.51 ±0.04	1.36 ±0.10	0.62 ±0.10	1.13 ±0.19	0.60 ±0.01	1.16 ±0.01	0.72±0.01*	0.96 ±0.02
Vha 2	0.77 ±0.03	0.90 ±0.04	0.81 ±0.03	0.86 ±0.03	0.81 ±0.01	0.85 ±0.01	0.88 ±0.02	0.79 ±0.02

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*

* Statistically significant ($p < 0.05$) compared to control condition.

When comparing the higher pH 8.2 (reference pH of the ocean) with control (pH 7.3) and pH 7.8, simulating the effect of a drastic and moderate seawater pH decrease, respectively, a negative correlation between decreased pH and growth was observed.

3.5.2. Effect on enzyme activity

The decrease in culture medium salinity and increase in pH modified the enzymatic activity of all isolates (Figure 3.7). No enzyme activity was observed for the isolates *V. renipiscarius* and *V. ichthyoenteri*.

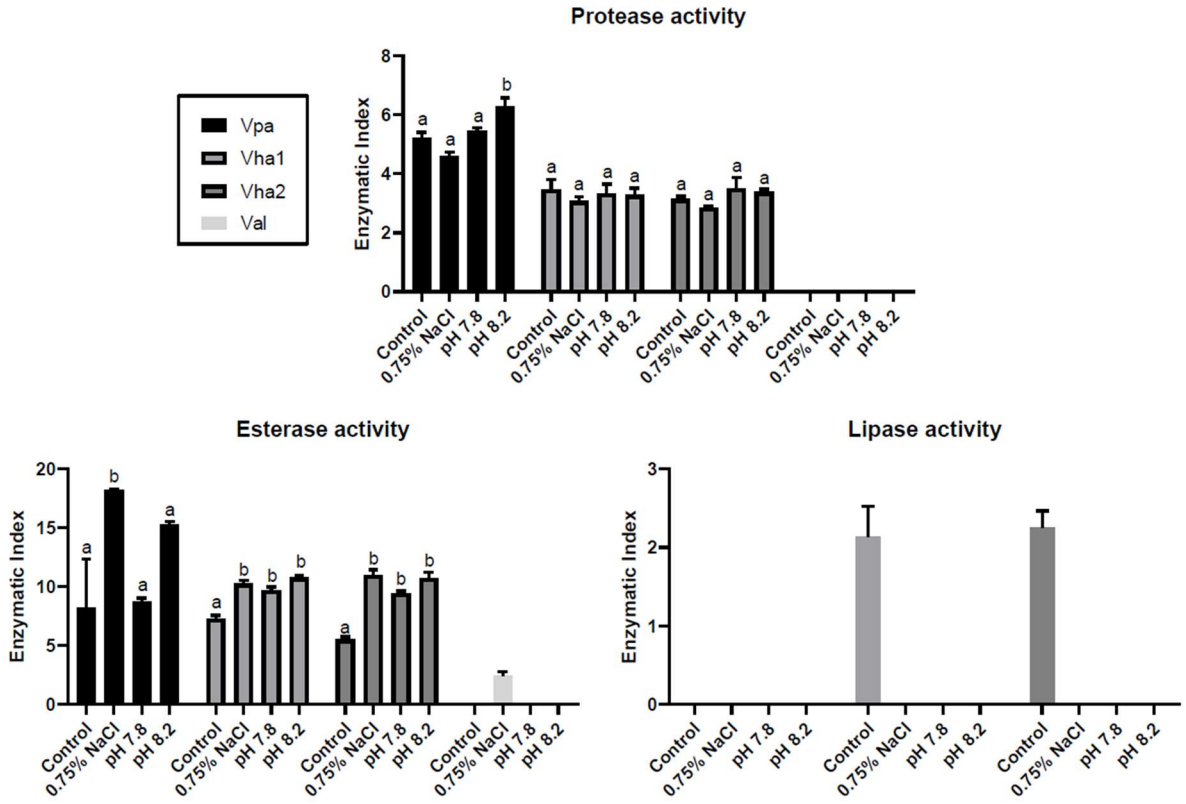


Figure 3.7. Effect of modified medium culture conditions on the enzyme activity of the *Vibrio* isolates at 22ºC. The enzymatic index was calculated using the mean value of three technical replicates. One-way ANOVA was performed to detect statistically significant differences indicated by different letters ($p < 0.05$): Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vha- *V. harveyi.*

The lipase activity characteristic of both *V. harveyi* isolates at 22 ºC was inhibited when the culture conditions were modified. The esterase activity of all isolates was affected by at least one condition. For *V. parahaemolyticus* isolates a statistically significant ($p < 0.05$) increase in the esterase activity (18.24 ± 0.13) was observed with a salinity decrease. In contrast, a decrease in salinity and higher pH increased significantly ($p < 0.05$) the esterase activity of both *V. harveyi* isolates. The *V. alfacsensis* isolate had no enzyme activity under the control conditions (22 ºC) but when cultured at lower salinity the esterase activity (2.40 ± 0.39) was activated. Protease activity did not modify compared to the control condition except for the isolate *V. parahaemolyticus* that showed a significant ($p < 0.05$) increase at pH 8.2 (6.30 ± 0.48) (Table 3.8).

Table 3.8. Enzymatic index of the isolates under modified culture conditions at 22 ºC for 48h. The Enzymatic Index was determined using the dimension of the colonies and their respective halos of activity. One-way ANOVA

analysis was performed to detect significant differences ($p < 0.05$). The isolates Vpa and Vha did not show lipase activity.

Isolate	Protease activity				Esterase activity			
	Control (1% NaCl; pH 7.3	**0.75% NaCl**	**pH 7.8**	**pH 8.2**	**Control** (1% NaCl; pH 7.3	**0.75% NaCl**	**pH 7.8**	**pH 8.2**
Vpa	5.22 ± 0.32	4.61 ± 0.22	5.46 ± 0.20	6.30 ± 0.48*	12.35 ± 0.20	18.24 ± 0.13*	8.72 ± 0.52*	15.25 ± 0.55
Val	na	na	na	na	na	2.40 ± 0.39	na	na
Vha 1	3.48 ± 0.57	3.08 ± 0.27	3.34 ± 0.53	3.29 ± 0.39	7.27 ± 0.51	10.27 ± 0.43*	9.84 ± 0.60*	10.80 ± 0.28*
Vha 2	3.15 ± 0.16	2.85 ± 0.12	3.52 ± 0.65	3.38 ± 0.16	5.53 ± 0.42	11.00 ± 0.78*	9.36 ± 0.40*	10.75 ± 0.89*

Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi*

* Statistically significant ($p < 0.05$) compared to 22 ºC; na- no enzyme activity

3.6. Molecular characterization of marine Vibrio isolates

3.6.1. Development of a multiplex PCR for Vibrio detection in environmental samples

3.6.1.1. Primer specificity

Specific primers to amplify virulence-related genes were designed and qualitative PCR using gDNA of different bacteria revealed that they were specific for each species, as no amplified products were obtained in the presence of other bacteria gDNA (Figure 3.8). The exception was for the Vic.hae primer where a PCR product was obtained for a non-target species (*Pseudoalteromonas* sp, Figure 3.8 F) but subsequent sequencing of the amplicon revealed that the initial identification of the non-target species was misidentified and was revealed to be a strain of *V. ichthyoenteri*.

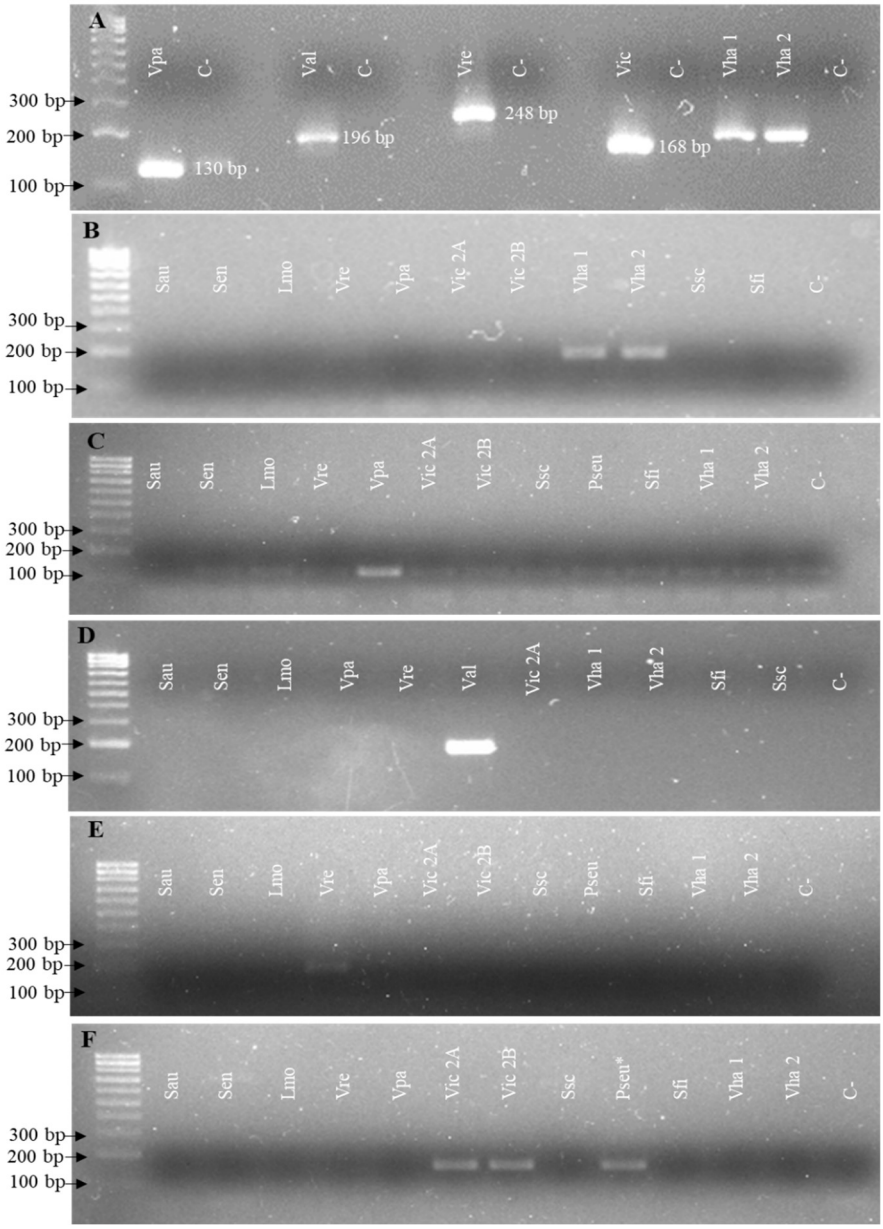


Figure 3.8. Agarose gel electrophoresis of the amplified PCR products was obtained during the optimization and validation reactions of the species-specific primers. Samples were run through gel electrophoresis on a 2.5% agarose/1x TAE gel. **A)** PCR amplicons obtained during the optimization of the species-specific primers on the species-specific gDNA; **B-F)** PCR amplicons obtained from the assessment of the specificity of each primer pair from Vha.vhhb, Vpa.tox, Val.tox, Vre.hae to Vic.hae on gDNA of several bacteria available in the lab. Abbreviations used: Sau- *Staphylococcus aureus*; Sen- *Salmonella enterica*; Lmo- *Listeria monocytogenes*; Ssc- *Shewanella schlegeliana*; Pse- *Pseudoalteromonas* sp.; Sfi- *Shewanella fidelis*; Vpa- *V. parahaemolyticus*; Val- *V. alfacsensis*; Vre- *V. renipiscarius*; Vic- *V. ichthyoenteri*; Vha- *V. harveyi.* * Posterior identified as *V. ichthyoenteri*.

3.6.1.2. Primer efficiency and reaction detection limit

The qPCR analysis performed revealed that all primer pairs had an efficiency > 85%, the lowest efficiency considered as good for the optimal performance of the primers (Bio-Rad) (Table 3.9;

Figure 7.1, Appendix 3). The performance of melting curves allowed the confirmation of single peaks for the amplification products confirming the specificity of each primer pair, and the absence of contamination (Figure 7.2, Appendix 3). Through the Ct values identified for amplification for each dilution tested, the limit of detectable gDNA was 1 fg/µL for primer pair Vpa.tox and 10 fg/µL for the remaining primer pairs.

Table 3.9. Q-PCR analysis of the species-specific primers. The efficiency, detection limit, and specificity of each primer pair were assessed through qPCR using serial gDNA dilutions from an initial concentration of 1 ng/µL to 10 ag/µL.

Specific primer	Target species	Efficiency (%)	Detection limit (µL)	Specificity
Vpa.tox	*V. parahaemolyticus*	86.7	1 fg/µL	Specific
Val.tox	*V. alfacsensis*	89	10 fg/µL	Specific
Vre.hae	*V. renipiscarius*	85.3	10 fg/µL	Specific
Vic.hae	*V. ichthyoenteri*	93.2	10 fg/µL	Specific
Vha.vhhb	*V. harveyi*	92.5	10 fg/µL	Specific

3.6.1.3. Development of multiplex PCR

For the development and optimization of the mPCR reaction conditions, the effect of the concentration of the primer mix and the gDNA mix (N, M1, M2, and M3) was tested. The reaction M2 was selected because it enabled the amplification of all target genes with the lowest gDNA concentration (Figure 3.9 A). The polyacrylamide gel electrophoresis showed that the mPCR successfully amplified the target gDNA of the five target species using the optimized reaction (M2), demonstrating that this technique worked when a combination of the gDNA of the target species was present at a similar concentration (Figure 3.9 B).

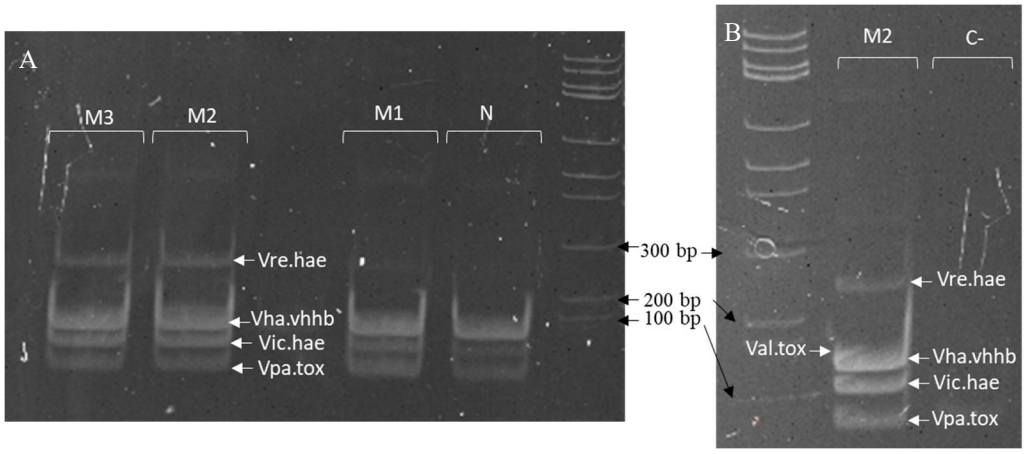


Figure 3.9. Polyacrylamide gel electrophoresis of the optimization and validation of the multiplex PCR amplicons. A) mPCR amplicons obtained during the optimization of the mPCR. The optimization conditions were initially only performed for the amplification of 4-plex PCR because at the time we were still waiting for the confirmation of the Val isolate B) mPCR amplicons of the 5-plex PCR using the optimal reaction M2. N- 4 µM of each primer + 5 µg/µL gDNA; M1- 4 µM of each primer + 10 µg/µL gDNA; M2- 8 µM of each primer + 5 µg/µL gDNA; M3- 8 µM of each primer + 10 µg/µL gDNA. N- normal; M- modified; C^- - negative control.

The detection limit of the mPCR was analysed to understand the lowest concentration of gDNA that it was possible to detect. From the four dilutions tested, only the lowest gDNA concentration tested (0.325 µg/µL of each gDNA) did not allow the amplification of the five expected products (Figure 3.10). The highest dilution where all amplicons were observed was 1:8, corresponding to a concentration of 0.75 µg/µL of each gDNA template. When compared to the results obtained with the q-PCR, the detection limit of each primer pair allowed the amplification of the target genes at lower concentrations than with the mPCR.

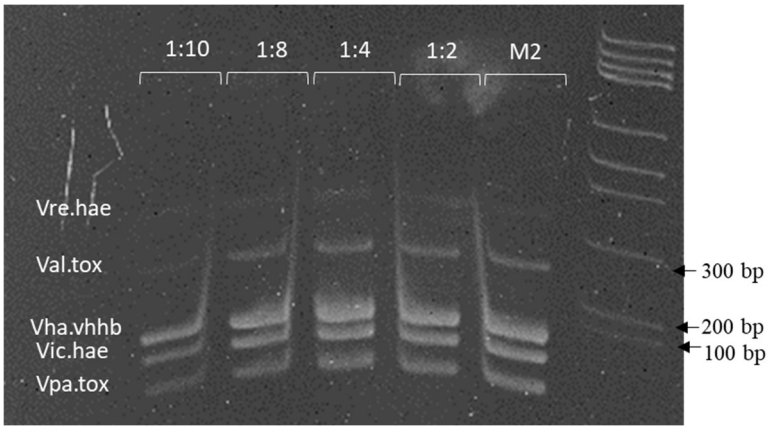


Figure 3.10. Polyacrylamide gel electrophoresis of the PCR amplicons for the detection limit of the multiplex-PCR (5-plex) reaction. Products were analysed on a 1x TBE 10% polyacrylamide gel. Serial dilutions from the initial gDNA mix used in M2 were performed: 1:2 (5 µg/µL) to 1:10 (0.325 µg/µL). All fragments were amplified in all dilutions except at gDNA 0.325 µg/µL, where Vre.hae primers failed.

3.7. Screening for the presence of Vibrio spp. in marine bivalve samples

To test the efficacy of the developed mPCR to screen *Vibrio* spp. present in environmental sampled wild mussels (*Mytilus galloprovincialis*) from the Ria Formosa were analysed. The approach used followed the guidelines described in ISO 21872-1:2017 for their microbiological and molecular detection/identification based on a two-enrichment step in an alkaline medium (Alkaline Peptone Water).

3.7.1. Microbiological identification of Vibrio spp.

After the first and second enrichment, one sample of each mussel (n=4/ enrichment step) was incubated on TCBS plates to assess if *Vibrio* spp. were detected. All plates showed a high abundance of bacteria which may represent putative *Vibrio*-like colonies, but observation of the colony morphology revealed that none of them showed the characteristic colonies previously described for the five target *Vibrio* species of this study. For this reason, no further steps were performed for the colony analysis.

3.7.2. Molecular identification

The gDNA extracted from the enriched cultures of the mussels was used to detect the five species of *Vibrio* by applying the mPCR developed. To assess if any of the study species of *Vibrio* were present an initial assessment was performed using standard qualitative-PCR to detect *V. parahaemolyticus* and *V. harveyi*, which are the two main pathogenic *Vibrio* spp. analysed in this study and of major public health concern. Both reactions confirmed the presence of gDNA from both species (Figure 3.11).

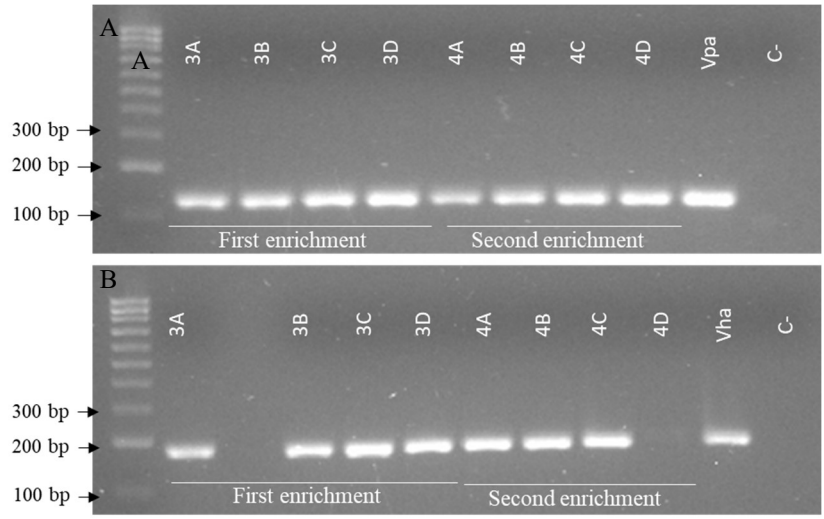


Figure 3.11. Agarose gel electrophoresis of the screening for the presence of *V. parahaemolyticus* (A) and *V. harveyi* (B) on the enriched culture media (two-step enrichment) from the 4 mussels collected from Praia de Faro (Ria Formosa) according to the ISO 21872-1:2017. The presence of amplicons was assessed through a 1x TAE 2.5% agarose gel electrophoresis. 3A-3D: first enrichment samples; 4A-4D: second enrichment samples.

Despite the detection of *V. parahaemolyticus* and *V. harveyi* in all samples, only *V. harveyi* was detected by the mPCR reaction (Figure 3.12) and no amplicons of *V. parahaemolyticus* or other *Vibrio* spp. were observed. This suggests that the mPCR was not effective and the primer efficiency may have been affected by the normal microbiome of the mussel and so needs to be further optimized. Another explanation may be that the concentration of *V. parahaemolyticus* in the sample was very low and this difficulted its detection as the detection limit of the individual primer pair allowed the amplification of the target genes at lower concentrations when compared to the mPCR, as previously shown. Nonetheless, the primers developed were able to detect the presence of *Vibrio* spp. in the environmental samples.

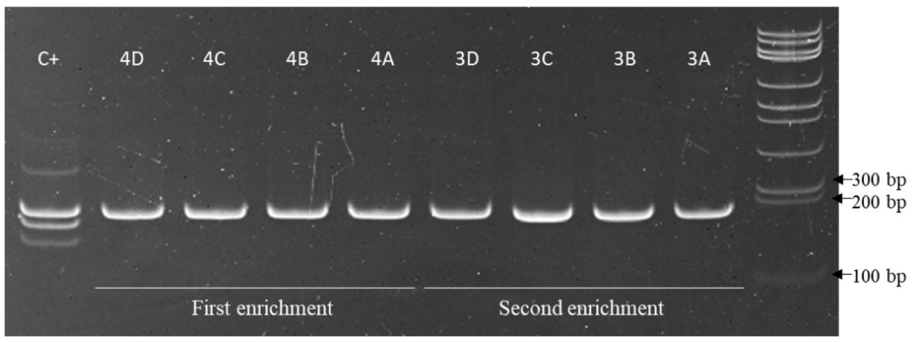


Figure 3.12. Polyacrylamide gel electrophoresis of the mPCR amplicons on the gDNA of the 4 mussels collected from Praia de Faro (Ria Formosa) after a two-step enrichment according to ISO 21872-1:2017. The presence of amplicons was assessed through a 10% polyacrylamide/1x TBE gel electrophoresis. 3A-3D: first enrichment samples; 4A-4D: second enrichment samples.

Chapter 4: Discussion

The bacteria of the genus *Vibrio* are a large and ubiquitous group of microorganisms in the aquatic environment, but only a few species are pathogenic and a threat to marine organisms and humans, causing mass mortalities in the aquatic ecosystems or foodborne disease. In this study, seven novel marine *Vibrio* isolates collected from diseased Senegalese sole or RAS seawater were characterized. The *Vibrio* isolates likely represent novel bacterial strains, with two different strains of *V. harveyi* and *V. ichthyoenteri* identified. The results revealed that the marine environment is rich in *Vibrio* spp. and that many genera remain to be characterised and they may be a threat to aquaculture sustainability and human health and that modified culture conditions changed growth and the activity of putative virulence enzymes (Table 4.1).

Table 4.1. Summary of the results obtained in this study on the characterization of the isolates. The potential pathogenicity of each isolate to marine and human organisms was assessed based on their growth and enzymatic activity at 22 (marine organisms) and 37 ºC (humans).

Isolate	Antibiotic susceptibility	Possible pathogenicity		Influence of culture conditions	
		Marine organisms	Humans	Lower salinity	Lower pH
Vpa	Intermediate to amikacin	+	+	= growth ↑ esterase activity	=growth ↓ esterase and protease activity
Val	Intermediate to amikacin	-	-	↑ growth Appearance of esterase activity	↓ growth
Vre	Susceptible to all antibiotics	-	-	= growth	↓ growth
Vic 1	Intermediate to amikacin	-	-	↑ growth	↓ growth
Vic 2	Susceptible to all antibiotics	-	-	↓ growth	= growth
Vha 1	Intermediate to amikacin	+	+	= growth ↑ esterase activity	↓ growth ↓ esterase activity
Vha 2	Intermediate to amikacin	+	+	= growth ↑ esterase activity	= growth ↓ esterase activity

Vpa- V. parahaemolyticus; Val- V. alfacsensis; Vre- V. renipiscarius; Vic- V. ichthyoenteri; Vha- V. harveyi

To assess the effect of the predicted future changes in salinity and pH of the ocean, a 25% decrease in NaCl concentration was tested. However, as the normal pH of the culture media used is 7.3, lower than the ocean pH (8.2), the pH of the media was increased, and the modified media was compared to the control. The effect of possible acidification of the ocean was analysed using pH 8.2 as a reference and the data was analysed inversely.

4.1. The marine Vibrio isolates

Molecular identification of the seven *Vibrio* isolates was performed through the amplification of a fragment of the 16S rRNA gene and analysis of the sequences available on the database confirmed their identity as members of the *Vibrio* genus. It has been previously suggested that the 97 % 16S rRNA gene sequence can be used as the threshold for differentiating two distinct prokaryote species towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity (Edgar, 2018). The complete 16S rRNA gene sequences of the seven isolates have not been fully characterized but the amplified sequence fragments (> 1400 bp) share 95.8% - 99.14% of nucleotide sequence identity with the orthologue species suggesting that some of the strains characterized in this study may represent novel marine *Vibrio* (Edgar, 2018). Following this hypothesis, all isolates were briefly characterized and their whole genome sequencing is currently being performed to provide the answer based on genetic differences.

Characterization in TCBS, a *Vibrio* spp. selective medium, revealed that the isolate *V. parahaemolyticus* was typified by dark green colonies, a characteristic that was shared among the other *V. parahaemolyticus* strains described (Solomakos et al., 2017). The remaining isolates were characterized by yellow colonies resulting from their capacity to degrade sucrose and the consequent production of acid. The bacteria *V. alfacsensis*, *V. renipiscarius, V. ichthyoenteri*, and *V. harveyi* described in the literature are non-sucrose fermentative suggesting that the novel marine isolates shared similar biochemical properties (Gomez-Gil et al., 2012; Ishimaru et al., 1996; F. H. Johnson & Shunk, 1936; Tarazona, Ruvira, et al., 2015).

The growth kinetics of all isolates at 22 ºC (average temperature of the marine waters) were in accordance with the usual microbial growth curves (Conde, 2019; Martinez, Megli, & Taylor, 2010). The isolates identified as human and marine pathogens, *V. parahaemolyticus* and *V. harveyi*, respectively, have the highest growth rates when compared with the other isolates, potentially associated with their pathogenicity and capacity to rapidly adapt and grow under

different conditions. A longer lag phase of the *V. renipiscarius* and *V. ichthyoenteri* 2 isolates may be the result of lower adaptation skills. In addition, isolate *V. ichthyoenteri* 2 did not reach the stationary phase, unlike the other bacteria, suggesting that it is a slow grower. Only the isolates *V. parahaemolyticus* and *V. harveyi* were able to grow at 37ºC, which is considered the average temperature of the human body at the beginning of an infection. The capacity of *V. parahaemolyticus* and *V. harveyi* to grow at 37 ºC had been already described because of their pathogenicity to humans (F. H. Johnson & Shunk, 1936; Montánchez & Kaberdin, 2020; Solomakos et al., 2017). The absence of growth of the other isolates was expected based on literature where both species are described as non-human pathogens (Ishimaru et al., 1996; Tarazona, Ruvira, et al., 2015). Several strains of *V. alfacsensis*, also described as a non-human pathogen, can grow at 37 ºC. However, other strains are not able to grow at this temperature, possibly due to their genetic variation (Gomez-Gil et al., 2012).

The abuse of antibiotics and the release of antimicrobial residues from clinical, agriculture, and aquaculture facilities into the environment contributes to a global public health concern associated with the increased antibiotic resistance of pathogenic bacteria such as *Vibrio* spp. The susceptibility of *Vibrio* spp. to antibiotics depended on the species, the isolation site, and the strain (Adesiyan et al., 2021; Deng et al., 2020; Kitiyodom et al., 2010; Letchumanan et al., 2015; Nurliyana Mohamad et al., 2019; Onohuean et al., 2022; Páll et al., 2021). The susceptibility of the *Vibrio* isolates to antibiotics commonly used in aquaculture and by humans were tested and all *Vibrio* isolates were found to be susceptible to the piperacillin-tazobactam, chloramphenicol, imipenem, ciprofloxacin, and florfenicol antibiotics. The exception was amikacin, where only two of the novel *Vibrio* isolates, *V. renipiscarius* and *V. ichthyoenteri* 2, showed susceptibility. Previously, *Vibrio* susceptibility to ciprofloxacin, chloramphenicol, imipenem, and florfenicol has been demonstrated (Adesiyan et al., 2021; Deng et al., 2020; Kitiyodom et al., 2010; Letchumanan et al., 2015; Nurliyana Mohamad et al., 2019; Onohuean et al., 2022; Ottaviani et al., 2001; Páll et al., 2021). However, *Vibrio* spp. susceptibility to different classes of antibiotics is dependent on the species and geographical location of the pathogen (Adesiyan et al., 2021; Elmahdi et al., 2016; Ottaviani et al., 2001). Whereas *V. parahaemolyticus* isolated from oysters in the United States was susceptible to chloramphenicol, the same species isolated from water samples in the same country showed intermediate resistance (Elmahdi et al., 2016). *V. parahaemolyticus* and *V. harveyi* isolated from Southwestern Nigerian rivers heavily impacted by human activities had lower susceptibility to chloramphenicol when compared to other *Vibrio* species (Adesiyan et al.,

2021; Onohuean et al., 2022). Resistance to amikacin is uncertain since some authors have shown that *Vibrio* spp. have high resistance to amikacin, while others showed the opposite, with 100% susceptibility (Adesiyan et al., 2021; Letchumanan et al., 2015; Páll et al., 2021). The antibiotic resistance of *Vibrio* spp. might also be affected by environmental factors such as salinity.

Bacteria are extremely diverse, and many species and strains of *Vibrio* have been described to date (Baker-Austin et al., 2018). Characterization of different strains belonging to the same species may help to understand how culture conditions affect each species and the variability between species and strains. In this study, two novel strains of *V. ichthyoenteri* and *V. harveyi* were characterized, based on their distinct behaviours which may be related to small modifications in their genomes, potentially associated with environmental adaptation. The nucleotide sequence alignment of the two isolates of *V. harveyi* and the two isolates of *V. ichthyoenteri* revealed that the 16S rRNA gene fragments amplified were 99.13% and 95.79% identical, respectively, confirming that they are most likely different strains of the same species (Edgar, 2018).

However, the two *V. ichthyoenteri* isolates shared a lower identity and had greater morphological differences. Both isolates showed distinct behaviours concerning sucrose fermentation. The lower fermentative capacity of the *V. ichthyoenteri* 1 isolate has already been described for this species (Ishimaru et al., 1996). Most of the *V. ichthyoenteri* described in the literature are sucrose fermenters and only a few show a very weak acid production that resulted in green colonies (Ishimaru et al., 1996). The two *V. ichthyoenteri* isolates also showed differences in antibiotic susceptibility, with both being susceptible to all antibiotics tested, except for the *V. ichthyoenteri* 2 isolate which had intermediate resistance to amikacin.

The isolates of *V. harveyi* were able to ferment sucrose, but colonies had different morphologies. While *V. harveyi* 1 was characterized by irregular colonies, *V. harveyi* 2 was regular and circular. Such difference may be the result of their distinct adaptation to TCBS, a highly complex and selective medium. In addition, the acid production due to sucrose metabolism of the isolate *V. harveyi* 1 was restricted to a small halo around the colonies, while in *V. harveyi* 2 a complete transformation of the culture medium pH, indicated that sucrose may be the ideal carbon source for the isolate *V. harveyi* 2. This observation might also be explained by the better adaptation of *V. harveyi* 2 to the TCBS selective medium. No differences in antibiotic resistance were observed between the two *V. harveyi* isolates.

4.2. The novel marine V. parahaemolyticus and V. harveyi are likely human pathogens and temperature increases modify phenotype and enzyme activity

Of the seven isolates, only the marine *V. parahaemolyticus* and the two *V. harveyi* isolates (Vha1 and Vha2) grew at 37 ºC, suggesting that even though they were isolated from a fish liver they, are likely to grow and proliferate in the human body after consumption of contaminated fish.

At 37 ºC the normal phenotype of the isolates at 22ºC was modified. The temperature rise impacted the elevation of the *V. parahaemolyticus* colonies and altered the morphology of both *V. harveyi* isolates in a different way, suggesting that they adapted differently to temperature increase. The shape of *V. harveyi* 1 colonies was modified from irregular to circular, with the opposite being observed for the *V. harveyi* 2 isolate. The alteration of the *V. harveyi* 1 colonies from transparent to opaque suggests that temperature favoured biomass growth as reflected by the significant increase in the growth kinetics at this temperature. Increased growth rates at 37ºC were also observed for *V. harveyi* 2 and *V. parahaemolyticus,* but this did not modify the colony transparency. However, the elevation of the *V. parahaemolyticus* colonies changed from convex to flattened, indicating a decrease in the biomass of the colonies, opposite to the observed growth rate at 37 ºC. Sucrose metabolism was also influenced by the temperature increase, with a reduction in sucrose fermentation of *V. harveyi* 2 and the formation of a small halo around the colonies in contrast to what was described at 22ºC. As the optimal growth temperature of marine *V. harveyi* is between 35 - 39 ºC, the observation of morphological modifications between 22 and 37 ºC was expected as a form of adaptation to more appropriate growth conditions (F. H. Johnson & Shunk, 1936). In contrast, *V. parahaemolyticus* (a well-known human pathogen) also showed optimal growth temperatures at 37ºC, but no changes in colony morphology were found, suggesting that this bacterium may be more resilient to temperature changes and is better adapted to infect humans as only very few *V. harveyi* infections in humans have been reported (Del Gigia-Aguirre et al., 2017; Montánchez & Kaberdin, 2020).

Protease and hydrolase enzymes are produced by several microorganisms (Ortiz Lechuga et al., 2016). The enzymatic activity of the isolates in the present study was characterized by screening their proteolytic (protease) and hydrolytic (lipase and esterase) activities, which are related to their potential virulence when infecting a host. Only the isolates *V. parahaemolyticus* and *V.*

harveyi showed positive results at 22ºC and 37ºC which is in line with their pathogenicity in marine organisms and humans (F. H. Johnson & Shunk, 1936; Solomakos et al., 2017). However, enzyme activity was modified with temperature, suggesting that the isolates may use different virulence enzymes at different temperatures. At 37 ºC the esterase activity of the isolate *V. parahaemolyticus* decreased significantly ($p < 0.05$), whereas the activity of *V. harveyi* 1 significantly increased but was inhibited in the isolate *V. harveyi* 2. Only the protease activity of isolate *V. harveyi* 1 was significantly ($p < 0.05$) stimulated by the temperature increase. At 37 ºC the lipase activity for the isolates *V. harveyi* was repressed. The protein profile of the enzymes is currently being analysed by zymography and has revealed that growth temperatures modified the proteolytic protein profile of the isolates (data not shown).

4.3. Modified culture conditions impact growth and enzymatic activity

The effect of climate change on the marine environment are being analysed to understand their evolution in the future and to develop strategies to mitigate or prevent its impact on ecosystems. Ocean warming and ocean pollution have a negative impact on marine life including the microbiota which has an important role in maintaining marine ecosystems (Grimes, 2020; Jiang et al., 2021). Host-pathogen interactions are highly vulnerable to environmental changes, and modifications in water temperature, pH, and salinity have been related to pathogenic microbe outbreaks (Burge et al., 2014; Froelich & Daines, 2020; Le Roux et al., 2015; Montánchez & Kaberdin, 2020; Semenza et al., 2017; Triñanes & Martinez-Urtaza, 2021; Vezzulli et al., 2015). Bacteria of the *Vibrio* genus are highly tolerant to environmental change and can grow at a wide range of temperatures, pH, and salinity, and are useful models to assess the effect of climate change on marine microbiota and their interaction with the marine ecosystem (Froelich & Daines, 2020; Le Roux et al., 2015; Montánchez & Kaberdin, 2020; Sampaio et al., 2022; Semenza et al., 2017; Triñanes & Martinez-Urtaza, 2021; Vezzulli et al., 2015). *Vibrio* spp. distribution and proliferation in the marine environment are changing with the changes in the marine environment. The rise in seawater temperature and salinity affects the growth and distribution of *Vibrio* in the marine environment (Burge et al., 2014; Ferchichi et al., 2021; Montánchez & Kaberdin, 2020; Triñanes & Martinez-Urtaza, 2021; Vezzulli et al., 2016). While temperature increases are considered to be beneficial for *Vibrio* growth and increase the incidence of *Vibrio*-associated disease outbreaks, the role of salinity is still poorly understood and depends on the species analysed (Burge et al., 2014; Le Roux et al., 2015; Montánchez &

Kaberdin, 2020; Triñanes & Martinez-Urtaza, 2021; X. Wang et al., 2020). The acidification of seawater also influences the growth and virulence of *Vibrio* spp. and analysis of the effects of pH decrease on the interaction between *V. harveyi* and penaeid prawn larvae showed that only at very low pH was the virulence of the bacterium affected (Montánchez & Kaberdin, 2020; Prayitno & Latchford, 1995).

In our study, modifications of culture conditions altered differently the growth kinetics of the isolates, suggesting that different strains of the same species are more susceptible than others. Whereas some changes favoured bacterial growth and were closer to optimal by decreasing the lag phase of the isolates, others reduced the duration of the exponential phase and consequently anticipated the onset of the stationary phase. This is in line with what was previously described for other *Vibrio* strains and members of the *Vibrio* genus, which usually have a very short duplication time, adapting easily to environmental changes (Froelich & Daines, 2020). The isolates *V. parahaemolyticus* and *V. harveyi* 2 were the most resilient and no change in growth was observed under modified salinity and pH, suggesting that they are highly tolerant to environmental change. In contrast, the isolates *V. alfacsensis* and *V. ichthyoenteri* 1 were significantly ($p < 0.05$) affected and the growth rate increased. The growth rate of isolate *V. renipiscarius* also increased significantly ($p < 0.05$) with a pH increase, whereas the rate of *V. harveyi* 1 growth only increased at pH 8.2 contrasting with the tendency of the other isolates. *V. ichthyoenteri* 2 was the only isolate that was sensitive to modified salinity and pH, which negatively affected its growth.

Modified culture conditions also affected the enzyme activity of all isolates analysed suggesting that this may have an impact on the isolate's virulence. Whereas the protease and esterase activity suffered changes, the lipase activity was completely inhibited when salinity and pH were modified. Only the isolate *V. parahaemolyticus* suffered a significant ($p < 0.05$) alteration in the protease activity that increased at pH 8.2, indicating that pH (pH 8.0 ± 0.2) induced the release of enzymes capable of degrading compounds like gelatine. On the other hand, the esterase activity of all isolates significantly ($p < 0.05$) changed, showing that the activity of this group of enzymes was more dependent on the growth conditions. The esterase activity of the *V. parahaemolyticus* isolate increased at lower salinity but decreased at pH 7.8. The activity of both *V. harveyi* isolates increased with all modifications. This suggests that each isolate is differently affected by the culture conditions, probably due to the type of enzymes produced or their ability to respond to different environmental stressors. The *V. alfacsensis* isolate, which

showed no enzymatic activity at control conditions (22 ºC), had esterase activity at lower salinity suggesting that it has the potential to become pathogenic for marine organisms.

Overall, we observed that a 25% reduction in salinity promoted the growth rate and enzymatic activity of some isolates but had no significant effect on others. Similar results were observed when the pH was increased, showing that the effects of climate change are likely to be complex as different parameters influence species physiology differently. The results obtained in the present study are coherent with other published studies showing that a decrease in salinity is likely to promote the growth and potential virulence of *Vibrio* spp. as their ideal growth occurs at salinities below 25% NaCl (Froelich & Daines, 2020). On the other hand, our study shows for the first time that lower pH will negatively impact the growth of all isolates, except *V. ichthyoenteri* 2, and the enzymatic activity of *V. parahaemolyticus* and *V. harveyi* isolates. Other tests should be performed to better understand the impact of culture conditions on bacteria growth and virulence because, although physicochemical conditions were changed optimal media (TSA and TSB) were used and environmental changes when bacteria are living in the water column or in association with other organisms may be different. However, the complexity of the *Vibrio*-host interaction can only provide a partial understanding of the effect of global warming on the evolution of disease outbreaks caused by *Vibrio* spp. as these dynamics are regulated by a range of biotic and abiotic factors.

4.4. Development of a molecular tool to detect Vibrio spp.

The molecular identification of pathogens is essential to complement their detection by classical methods because of their reliability, speed, and ease of execution. DNA-based technologies are more precise and reliable and thus several molecular techniques have been developed to detect pathogenic bacteria in clinical practices, food, water, and environmental samples (Abubakar et al., 2007; Belkin & Colwell, 2005; J. Chen et al., 2012; Tantillo et al., 2004). The development of specific primers for the detection of target pathogens allows more certain results when compared to the amplification of the 16S rRNA gene (Destoumieux-Garzón et al., 2020). In the present study, five specific primers were successfully developed for the detection of *V. parahaemolyticus, V. alfacsensis, V. renipiscarius, V. ichthyoenteri*, and *V. harveyi,* and an mPCR was developed for the simultaneous detection of several strains in food or environmental samples.

As each country consider a different concentration of *Vibrio* spp. as potentially hazardous for human health, food microbiology laboratories usually assess the presence/absence of a pathogen using standard protocols provided by the European Commission (EC) legislation for the isolation of *Vibrio* spp. from seafood as established in Portugal (Jorge, 2019). Among the 15 methods validated by the Committee of European Normalization (CEN) the ISO/TS 21872 (Conde, 2019; Hartnell et al., 2019) is commonly used. This standard method is divided into two parts and is based on classic microbiology culture methods, complemented with biochemical tests for the confirmation of the identity of the isolates. Whereas ISO/TS 21872–1 provides a detection method for the bacteria *V. cholerae* and *V. parahaemolyticus*, ISO/TS 21872–2 describes a method for the detection of the bacterium *V. vulnificus* (Conde, 2019; Hartnell et al., 2019; ISO, 2017).

In our study enriched cultures from mussels collected from their natural environment were used. Mussels are sessile filter feeders and are good risk indicators for the marine environment. Due to their water filtering capacity, they tend to accumulate particles (pollutants, microbes, plastics, etc) in suspension in the water column (Teresa Balbi et al., 2019; T. Balbi et al., 2013). Members of the *Vibrio* genus are commonly isolated from bivalves as they establish both mutualist and parasitic relations with bivalves (Teresa Balbi et al., 2019; T. Balbi et al., 2013; Destoumieux-Garzón et al., 2020). The gDNA amplification revealed the presence of both *V. parahaemolyticus* and *V. harveyi* in the samples. However, when the same samples were analysed through mPCR only the bacterium *V. harveyi* was detected. Multiplex PCR is a complex technique that depends on adequate primer development and the possible interaction between the different primers and non-target DNA (Hoy, 2013). Despite the initial assessment of the primer specificity and the optimization of the reaction with target gDNA, the application of the technique was not optimal for environmental samples. This may be a result of the interference of the natural microbiome of the samples with the efficiency of the mPCR or may be due to the low concentration of gDNA of *V. parahaemolyticus* present in the samples which favoured the amplification of *V. harveyi*. The mPCR is currently being optimized to understand the reasons for the selective failure.

Chapter 5: Conclusion and Future Perspectives

5.1. Conclusion

The present study identified and characterised for the first time seven novel *Vibrio* isolates obtained from diseased fish (pathogens) and the marine environment (non-pathogens): one isolate of *V. parahaemolyticus*, *V. alfacsensis*, and *V. renipiscarius* and two different isolates of the marine pathogens *V. harveyi* and *V. ichthyoenteri*. Both isolates of *V. harveyi* and *V. ichthyoenteri* were phenotypically and biochemically distinct, showing genetic differences that help explain the observed differences. All isolates grow at 22 ºC with distinct growth kinetics, but only the isolates *V. parahaemolyticus* and *V. harveyi* grow at 37 ºC. Analysis of the growth kinetics and production of virulence-related enzymes suggested that the isolates *V. parahaemolyticus* and *V. harveyi* (both strains) may have the ability to grow and cause potential damage in both marine and human organisms, thus representing a potential threat. The remaining isolates did not show any evidence of potential risk. The modification of the culture conditions (salinity and pH) had a species-specific effect on the different *Vibrio* studied. Whereas *V. parahaemolyticus* was less affected by the pH and salinity variations, both *V. harveyi* isolates responded differently to the modifications. In addition, a non-pathogenic species, *V. alfacsensis*, acquired esterase activity with the salinity modification, suggesting that salinity may be involved in the potential virulence activity of this bacterium. The influence on the growth and enzyme production of the isolates indicates that pathogen dynamics in the environment may change according to the abiotic conditions further supporting the complex relationship between the abiotic factors and *Vibrio* growth and potential virulence. An mPCR developed detected 5 different *Vibrio* spp. under laboratory conditions but failed with environmental samples and it appears that the microbiome and genomic DNA concentration in the samples may interfere with primer efficiency. Our results highlight the large variety of *Vibrio* spp. that remain to be characterised and indicate, based on modified growth and virulence under changed culture conditions, the potential impact on *Vibrio* of changes in the environment caused by climate change.

5.2. Future perspectives

This work provided preliminary data about the potential pathogenicity of seven novel isolates obtained from aquaculture. More assays targeting their virulence activity should be performed to confirm their pathogenicity to both marine and human organisms. The complete genome sequencing of the seven strains is being sequenced to fully characterize the isolates and confirm that they correspond to novel strains that have not been reported. With the protein predicted gene and identification and comparisons of the different virulence gene families will be performed to better understand and compare their pathogenicity and identify the virulence-like genes that are specific to marine organisms and the genes that are specifically activated in humans. Genomic data will also enable the identification and characterization of the genetic differences responsible for the variability observed among the two isolates of *V. harveyi* and *V. ichthyoenteri* isolates, to better understand the different responses of the distinct isolates to the same culture conditions.

Following the results of this study and the hypothesis that culture conditions affect *Vibrio* spp. in a species-specific manner, the predicted variations caused by climate change on the marine environment should be assessed by exposing the *Vibrio* isolates to a range of salinity and pH to better understand the bacterial behaviour in conditions like the ocean. Considering the results, a rapid proliferation of the isolates would be observed at lower salinities and higher pH. The enzymatic activity should also be analysed for these isolates through similar tests used in this thesis and new and more specific ones, to verify the effect of culture conditions on the isolates' virulence. The exposure of *Vibrio* isolates to healthy and infected marine organisms, such as bivalves, to a range of salinity and pH may also be a better approach to understand the modifications in *Vibrio* dynamics in association with aquatic organisms.

We hypothesize that the host's microbiome may interfere with the primer's efficiency when applying the mPCR, but to test this hypothesis the effect of different hosts' microbiome should be tested. Briefly, the gDNA of different hosts contaminated with *Vibrio* spp. should be tested using the mPCR to understand if the inhibition is host specific. The primers' efficiency using different concentrations of the host and *Vibrio's* gDNA should also be tested to understand the better ratio for the optimal functioning of the primers.

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Supplementary material

Supplementary material 1

Table 7.1. Modified TSA/1% NaCl supplemented with specific substrates for the analysis of the isolates' enzymatic activity. The culture media were prepared based on the type of enzymes analysed and interpreted according to the bibliography (R. Costa et al., 2013).

Enzyme	Substrate	Preparation of supplemented medium (400mL)	Incubation of plates	Interpretation of results
Protease	Gelatine	400mL of base medium was supplemented with 4g of gelatine (1%) before autoclave	Plates submerged with a saturated solution of ammonium sulphate	Transparent halo around the colonies reflects the protease activity
Lipase	Tween80	400mL of autoclaved base medium was supplemented with 0.4 mL (0.1%) of Tween80 previously filtrated	No incubation needed	Precipitation of Tween80 around the colonies denotes lipase activity
Esterase	Tween20	400mL of base medium was supplemented with 4mL of Tween20 (1%) before autoclave	No incubation needed	Precipitation of Tween20 around the colonies denotes esterase activity

Supplementary material 2

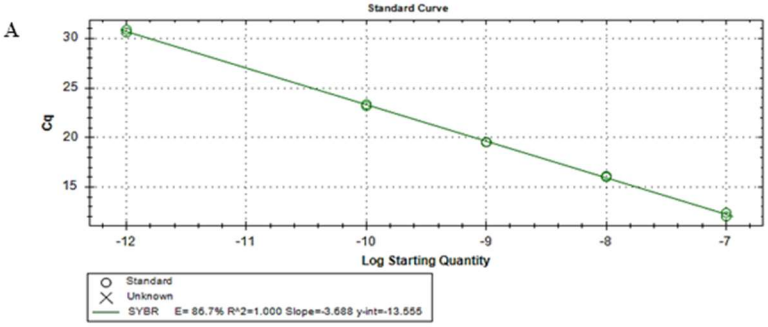
Table 7.2. Nucleotide BLAST results of the 16S rRNA sequence of *Vibrio* spp. The first five BLAST hits are represented and include the description of the most similar orthologue, the statistical e-value, the percentage of sequence similarity, and the accession number are also indicated. The isolates are represented by their abbreviations.

Isolate	Description	e-value	% identity	Accession number
Vre	*Vibrio renipiscarius* strain DCR 1-4-2 16S ribosomal RNA, partial sequence	0.0	98.23	NR_135891.1
	Vibrio renipiscarius partial 16S rRNA gene, strain DCR 1-4-12	0.0	98.14	HG931126.1
	Uncultured *Vibrio* sp. clone O 16S ribosomal RNA gene, partial sequence	0.0	98.14	KC169768.1
	Uncultured bacterium clone OTU66 16S ribosomal RNA gene, partial sequence	0.0	98.05	KC120631.1
	Vibrio renipiscarius strain KTVR 16S ribosomal RNA gene, partial sequence	0.0	98.21	OK001798.1
Vpa	*Vibrio parahaemolyticus* strain MC32 16S ribosomal RNA gene, partial sequence	0.0	98.61	MT534026.1
	Vibrio parahaemolyticus strain VC005 16S ribosomal RNA gene, partial sequence	0.0	98.61	MT534020.1
	Vibrio parahaemolyticus strain VC004 16S ribosomal RNA gene, partial sequence	0.0	98.61	MT534019.1
	Vibrio parahaemolyticus strain VC002 16S ribosomal RNA gene, partial sequence	0.0	98.61	MT534018.1
	Vibrio parahaemolyticus strain VC001 16S ribosomal RNA gene, partial sequence	0.0	98.61	MT534017.1
Vha 1	*Vibrio harveyi* strain 2010V-1024 chromosome 1	0.0	98.33	CP051122.1
	Vibrio harveyi strain 2011V-1164 chromosome 1, complete sequence	0.0	98.33	CP035693.1
	Vibrio harveyi strain WAB2194 16S ribosomal RNA gene, partial sequence	0.0	98.33	MH169327.1
	Vibrio harveyi V1614 gene for 16S ribosomal RNA, partial sequence	0.0	98.33	LC369706.1
	Vibrio harveyi strain FDAARGOS_107 chromosome 2, complete sequence	0.0	98.33	CP014039.2
Vha 2	*Vibrio harveyi* strain MFB03 16S ribosomal RNA gene, partial sequence	0.0	98.46	MT605241.1
	Vibrio harveyi strain 2010V-1024 chromosome 2	0.0	98.46	CP051123.1
	Vibrio harveyi strain 2010V-1024 chromosome 1	0.0	98.46	CP051122.1
	Vibrio harveyi strain 2011V-1164 chromosome 1, complete sequence	0.0	98.46	CP035693.1
	Vibrio harveyi strain 2011V-1164 chromosome 2, complete sequence	0.0	98.46	CP035692.1

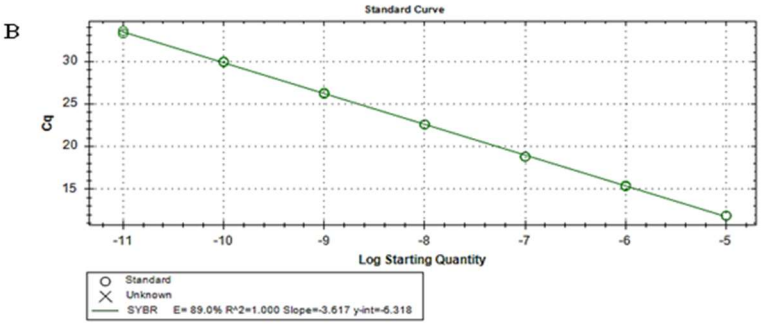
Vic 1	*Vibrio* sp. HGLP-44 16S ribosomal RNA gene, partial sequence	0.0	95.89	KX001857.1
	Vibrio scophthalmi strain VS-05 chromosome 1, complete sequence	0.0	95.98	CP016414.1
	Vibrio scophthalmi strain VS-12 chromosome 1, complete sequence	0.0	95.98	CP016307.1
	Vibrio ichthyoenteri partial 16S rRNA gene, strain CECT8171	0.0	95.98	HG931133.1
	Vibrio scophthalmi partial 16S rRNA gene, strain CECT5969	0.0	95.98	HG931131.1
Vic 2	*Vibrio* sp. V170 16S ribosomal RNA gene, partial sequence	0.0	97.38	DQ146979.1
	Vibrio ichthyoenteri partial 16S rRNA gene, isolate DAQ 2-1-1A	0.0	97.30	HG931122.1
	Vibrio ichthyoenteri partial 16S rRNA gene, isolate DCR 1-4-11	0.0	97.13	HG931124.1
	Vibrio ichthyoenteri partial 16S rRNA gene, isolate DAQ 2-1-1B	0.0	97.13	HG931123.1
	Vibrio scophthalmi strain VS-05 chromosome 1, complete sequence	0.0	97.13	CP016414.1
Val	*Vibrio alfacsensis* strain Y831 16S ribosomal RNA gene, partial sequence	0.0	99.14	MK452736.1
	Vibrio alfacsensis 04Ya108 DNA, chromosome 1, complete sequence	0.0	99.14	AP024165.1
	Vibrio alfacsensis 04Ya249 DNA, chromosome 2, complete sequence	0.0	99.14	AP019850.1
	Vibrio alfacsensis 04Ya249 DNA, chromosome 1, complete sequence	0.0	99.14	AP019849.1
	Vibrio sp. B1REV9 genome assembly, chromosome: 2	0.0	99.14	HG992746.1

Supplementary material 3

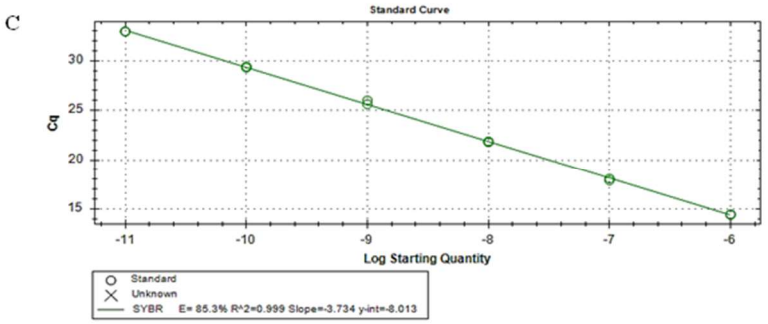
A



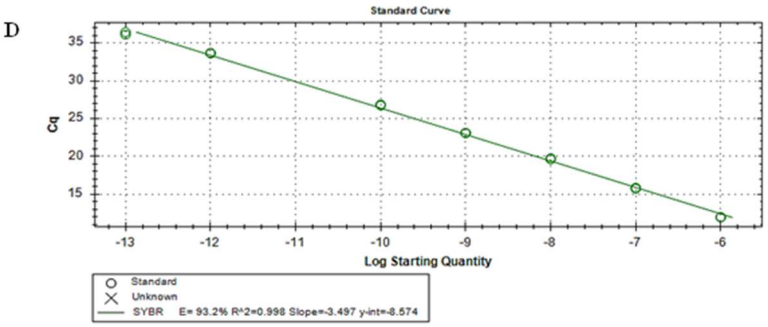
B



C



D



E

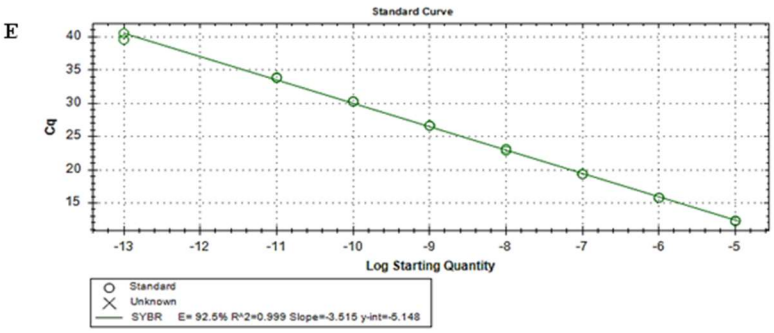


Figure 7.1. Q-PCR standard curves of each species-specific primer pair. The standard curves were performed using nine decimal serial dilutions to analyse the efficiency of each primer pair. A- Vpa.tox; B- Val.tox; C- Vre.hae; D- Vic.hae; E- Vha.vhhb.

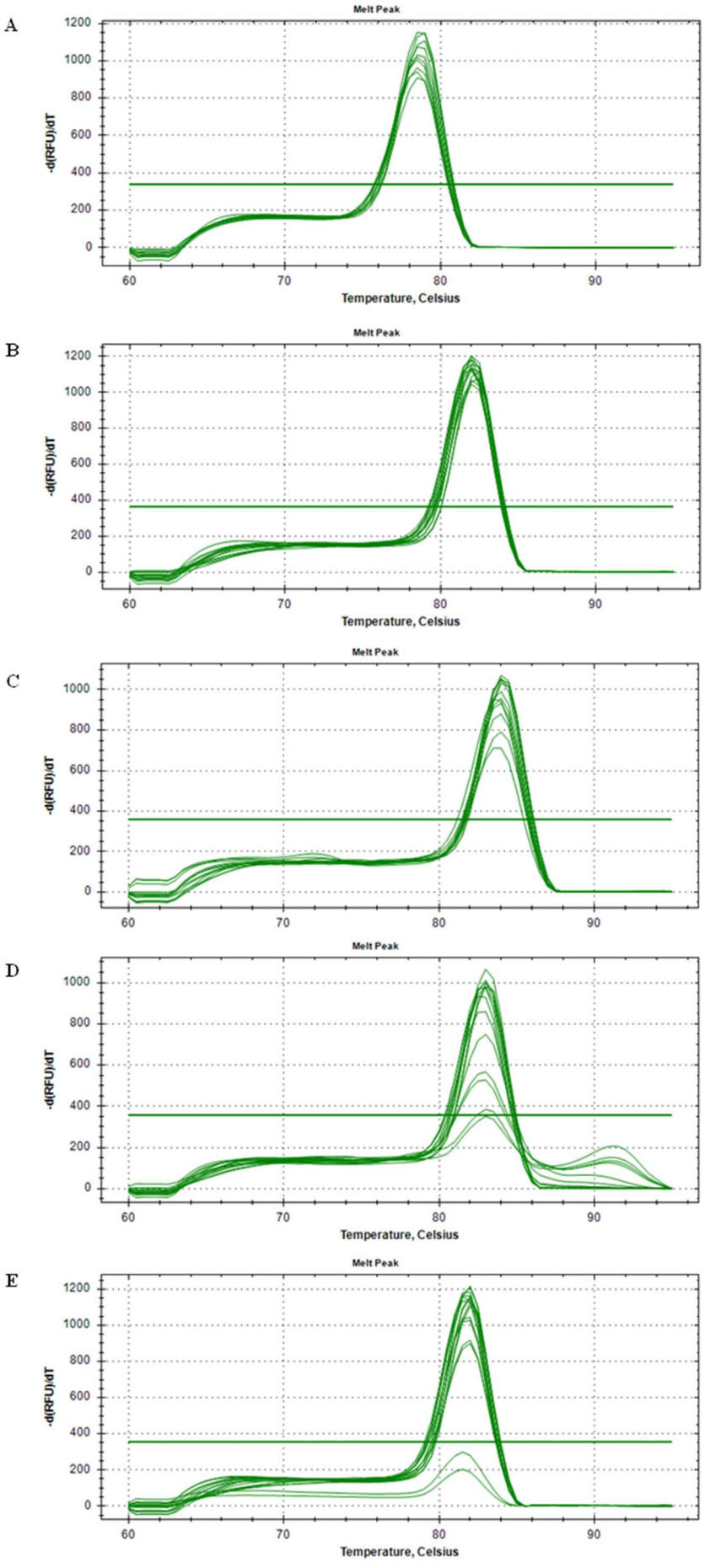

A
Melt Peak
-d(RFU)/dT
Temperature, Celsius
B
Melt Peak
-d(RFU)/dT
Temperature, Celsius
C
Melt Peak
-d(RFU)/dT
D
Melt Peak
-d(RFU)/dT
Temperature, Celsius
E
Melt Peak
-d(RFU)/dT
Temperature, Celsius

Figure 7.2. Melt peak of each species-specific primer pair. and an additional step was added to the thermocycler to analyse the melting curve of each primer for the assessment of possible cross-amplifications. A- Vpa.tox; B- Val.tox; C- Vre.hae; D- Vic.hae; E- Vha.vhhb.