UNIVERSIDADE D
COIMBRA

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MECHANISMS OF ANTIBIOTIC RESISTANCE AND VIRULENCE IN *VIBRIO* SPP.

Dissertação no âmbito do Mestrado em Bioquímica, orientada pela Professora Isabel da Silva Henriques e Doutora Marta Cristina Oliveira Martins Tacão, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Outubro de 2021

Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

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INÊS FILIPA DE OLIVEIRA MONTEIRO MARQUES CAVALEIRO

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Palavras-chave: *Vibrio* spp., resistência a antibióticos, fatores de virulência, formação de biofilmes, atividade enzimática, aquacultura, ambiente estuarino

Resumo:

Vibrio spp. são autóctones de ambientes aquáticos, encontrando-se vastamente disseminadas no ambiente marinho e estuarino, bem como em sistemas de aquacultura. Algumas espécies de *Vibrio* têm sido implicadas como agentes causadores de doenças em animais de aquacultura, levando a elevadas perdas monetárias e taxas de mortalidade. As estirpes patogénicas possuem um vasto manancial de fatores de virulência. Embora espécies de *Vibrio* sejam geralmente suscetíveis à maioria dos antibióticos de uso humano e veterinário, o uso excessivo de antibióticos em hospitais, agricultura e aquacultura levou a um aumento dos níveis da resistência nas espécies deste género. O objetivo deste estudo foi avaliar o possível risco para a saúde humana e animal que espécies de *Vibrio* do estuário da Ria de Aveiro representam. Foi analisada uma coleção de 77 isolados, pertencentes a 11 espécies, previamente obtidos em três campanhas diferentes (Outono, Primavera e Verão) em 26 locais do estuário. A análise de BOX-PCR revelou que os isolados são geneticamente diversos, possivelmente devido à adaptação a condições ambienteis variáveis (e.g. pH, temperatura e salinidade) ao longo do estuário. Os isolados de *Vibrio* da coleção são halotolerantes, com 45% a crescer em meio de cultura suplementado com 7.5% de NaCl. A tolerância ao sal entre os isolados não parece depender dos locais de amostragem ou estação do ano. A suscetibilidade a 8 antibióticos foi avaliada através da análise de 8 compostos e os isolados foram sensíveis à maioria deles, com exceção da amoxicilina para a qual 78% dos isolados apresentaram resistência. Os perfis de resistência a antibióticos não parecem estar associados a locais de amostragem ou espécie. A presença de genes que codificam as β-lactamases TEM, SHV e CTX-M, bem como determinantes de resistência associados a quinolonas (*qnrVC* e *qnrA*) e genes de resistência à tetraciclina (*tetA*, *B*, *C*, *D*, *E* e *M*) foi avaliada. Quatro (5%), nove (11%) e um (2%) isolados possuíam *qnrVC*, bla_{TEM} e *tetB*, respetivamente. bla_{SHV}, bla_{CTX-M}, *qnrA* e *tetA*, *C*, *D*, *E* e *M* não foram detetados. Cinquenta e quatro (70%) dos isolados continham plasmídeos de vários tamanhos, com 6 (7%) deles contendo genes de resistência a antibióticos, enquanto que nenhum integrão de classe 1, 2 e 3 foi detetado. A prevalência de genes associados à virulência (*chiA, vhpA, luxR, flaC, hlyA, tlh,* e $toxR_{VC}$) também foi avaliada. Notavelmente, *chiA*, *luxR* e *tlh* foram detetados em mais de 50% dos isolados, tendo $toxR_{VC}$ e *hlyA* sido detetados exclusivamente em *V. cholerae*. A produção de atividades extracelulares também foi avaliada. Amilase, DNase, lipase e caseinase foram as atividades mais detetadas. Isolados pertencentes a *V. parahaemolyticus*, *V. diabolicus* e *V. alginolyticus* apresentaram mais atividades extracelulares, enquanto que isolados pertencentes a *V. mytili* e *V. mediterranei* não apresentaram nenhuma das atividades pesquisadas. Com base na presença de fatores de virulência e perfis de resistência a antibióticos, 14 isolados de *Vibrio* foram selecionados para avaliar a capacidade de formação de biofilme com duas salinidades diferentes: 1% NaCl e 2.5% NaCl. *V. cholerae* foi consistentemente um produtor de biofilme em ambas as salinidades, enquanto que *V. campbellii* produziu biofilme a 1% NaCl. Assim, este trabalho contribuiu para o conhecimento das características de virulência e suscetibilidade a antibióticos de estirpes estuarinas de *Vibrio*. Estes resultados reforçam a necessidade de estudar e monitorizar rotineiramente a resistência a antibióticos e características de virulência que se podem dispersar no estuário.

Keywords: *Vibrio* spp., antibiotic resistance, virulence factors, biofilm formation, enzymatic activity, aquaculture, estuarine environment.

Abstract: *Vibrio* spp. are autochthonous to aquatic environments, being widely distributed in marine and estuarine environments, as well as in aquaculture systems. Some *Vibrio* species are known to cause infections in aquaculture animals, leading to high monetary losses and mortality rates. Pathogenic strains possess an array of virulence factors. Although *Vibrio* are usually susceptible to most antibiotics of human and veterinary use, the overuse of antibiotics in hospital, agriculture, and aquaculture settings, led to an increase of antibiotic resistance levels among species of this genus. The aim of this study was to assess the possible risk to human and animal health that *Vibrio* species from the estuary Ria de Aveiro represent. A collection of 77 isolates, belonging to 11 species, previously obtained in three different campaigns (Autumn, Spring, and Summer) from 26 sites in the estuary was analyzed. BOX-PCR analysis revealed that the isolates are genetically diverse, possibly reflecting adaptation to variable environmental conditions (e.g., pH, temperature, and salinity) along the estuary. *Vibrio* isolates in the collection are halotolerant, with 45% growing at 7.5% NaCl. Tolerance to salt among isolates did not depend on sampling sites or season. Antibiotic susceptibility was evaluated against 8 compounds and isolates were susceptible to most of them, except to amoxicillin to which 78% of the isolates showed resistance. Antibiotic resistance profiles were not correlated with sampling season or species. The presence of genes encoding β-lactamases TEM, SHV, and CTX-M, quinolone-associated resistance determinants (*qnrVC* and *qnrA*), and tetracycline resistance genes (*tetA*, *B*, *C*, *D*, *E*, and *M*) was inspected. Four (5%), nine (11%), and one (2%) isolates harbored *qnrVC*, bla_{TEM}, and *tetB*, respectively. bla_{SHV}, $bla_{\mathrm{CTX\text{-}M}}$, *qnrA*, and *tetA*, *C*, *D*, *E*, and *M* were not detected. Fifty-four (70%) of the isolates harbored plasmids from various sizes, with 6 (7%) of them also containing ARGs, while no class 1, 2, and 3 integrons were detected. The prevalence of virulence-associated genes (*chiA, vhpA, luxR, flaC, hlyA, tlh,* and $toxR_{VC}$) was also determined. Notably, *chiA*, *luxR*, and *tlh* were detected in more than 50% of the isolates, with $toxR_{VC}$ and *hlyA* being detected exclusively in *V. cholerae*. Production of extracellular activities was also evaluated. Amylase, DNase, lipase, and caseinase were the activities more frequently detected. Isolates affiliated with *V. parahaemolyticus*, *V. diabolicus*, and *V. alginolyticus* showed more extracellular activities, while those affiliated with *V. mytili* and *V. mediterranei* presented no extracellular activity. Based on the presence of virulence factors and antibiotic resistance profiles, 14 *Vibrio* isolates were selected to assess biofilm formation capability at two different salinities: 1% NaCl and 2.5% NaCl. *V. cholerae* was consistently a biofilm producer at both salinities, while *V. campbellii* produced biofilms at 1% NaCl. Thus, this work contributed to the knowledge of virulence characteristics and susceptibility to antibiotics of estuarine *Vibrio* strains. These results reinforce the need to study and routinely monitor antibiotic resistance and virulence traits of environmental isolates that may disperse among other strains in the estuary.

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ABBREVIATIONS

ARGs Antibiotic resistance genes

MGE Mobile genetic elements

ICE Integrative conjugative elements

HGT Horizontal gene transfer

AMR Antimicrobial resistance

CT Cholera toxin

TCP Toxin co-regulated pilus

ACFs accessory colonization factors

LPS Lipopolysaccharide

RTX Repeats-in-toxin

TDH Thermostable direct hemolysin

TRH TDH-related hemolysin

TLH Thermolabile hemolysin

VBNC Viable But Non-Culturable

EU European Union

WWTPs Wastewater treatment plants

MDR Multiple drug resistance

ECPs Extracellular products

QS Quorum sensing

CHO Chinese hamster ovary cell elongation factor

CPS Capsular polysaccharide

EPS Exopolysaccharides

TCS Two-component signal system

HK Sensor histidine kinase

AML Amoxicillin

DO Doxycycline

E Erythromycin

CN Gentamicin

CAZ Ceftazidime

CIP Ciprofloxacin

TE Tetracycline

SXT Trimethoprim-sulfamethoxazole

INTRODUCTION

1. The genus *Vibrio*

The family *Vibrionaceae*, which contains the genera *Vibrio*, *Photobacterium*, *Enterovibrio*, *Salinivibrio*, *Enhydrobacter*, *Listonella*, and *Allomonas*, was proposed in 1965 by Véron (Véron, 1965) and comprises 133 species up to date, with 48 belonging to the genus *Vibrio* (LPSN, 2017; Palit & Nair, 2014). *Vibrio* are gram-negative, rod-shaped bacteria. Their cells are 1.4-2.6 µm in length (Figure 1)(Don et al., 2005) and are motile or rarely non-motile (Osunla & Okoh, 2017).

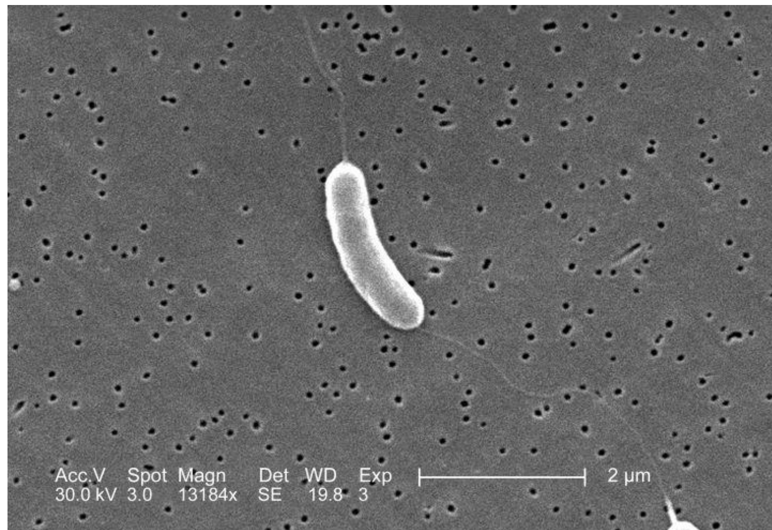


Figure 1 – SEM of *Vibrio vulnificus* bacterium. Photo by Janice Carr, USCDCP, *2016,* Pixnio (https://pixnio.com/science/microscopy-images/vibrio-related-diseases/flagellated-vibrio-vulnificus-bacterium).

All *Vibrio* spp. contain two chromosomes, with chromosome I being larger and coding for most essential functions, such as DNA replication and repair machinery (Zhang et al., 2018). On the other hand, chromosome II' size depends on the species, but is usually smaller, carrying genes acquired by horizontal transfer and encoding mostly accessory proteins (Baker-Austin et al., 2018).

These bacteria withstand a broad range of temperatures (20ºC to 40ºC) and are classified as halophilic or non-halophilic, such as *Vibrio cholerae* and *Vibrio mimicus*, depending on their requirement for NaCl for growth (Wong & Griffin, 2018). Although they prefer alkaline conditions, they can grow between pH 6.5 and 9.0 (Chart, 2012). In addition, members of the *Vibrionaceae* are catalase-positive, facultative anaerobes and oxidase-positive (Percival & Williams, 2013).

Vibrio are autochthonous inhabitants of aquatic environments, being present in marine, brackish, and freshwater environments, where they live as free-living organisms or in commensal relationships with aquatic plants, plankton or invertebrates (Takemura et al., 2014). Copepods, for example, were found to be a reservoir for *V. cholerae* (Mueller et al., 2007). Besides acting as a vehicle for geographic distribution of *Vibrio*, they also act as a carbon source, with these microorganisms being able to break down chitin present in the exoskeleton (Pruzzo et al., 2005), thus promoting the chitin turnover, important for the maintenance of the marine ecosystem (Bassler et al., 1991).

Some members of the genus *Vibrio* are considered to be pathogenic to marine animals, being one of the main causes of death in aquaculture, a vibrant sector that has been growing each year, reaching 82.1 million tons of fish production in 2018 (FAO, 2018). *Vibrio* spp. have been isolated from bivalves, shrimp, fish, live feed (microalgae, rotifers), and seaweed (Vandenberghe et al., 2003), causing vibriosis, a potentially deadly disease that leads to symptoms like lethargy, tissue and appendage necrosis, and body malformations (Cano-Gomez et al., 2009). Although the shrimp aquacultures seem to be the most affected by these bacteria, with mortalities up to 100% (Lee et al., 1996; Liuxy et al., 1996), they are also responsible for mass mortalities in bivalves (Cavallo et al., 2012; Sutton & Garrick, 1993), fish (Liu et al., 2000; Pujalte et al., 2003), and arthropods (Austin et al., 2005). The economic impact in the aquaculture sector is extremely high, with production losses of US$44 billion between 2010 and 2016 in China, Malaysia, Mexico, Thailand, and Vietnam (Valente & Wan, 2021).

On culture medium, *Vibrio* can form two distinct types of colonies, smooth or rugose, as an adaptation to the environment (Jubair et al., 2012; Marsden et al., 2017). They can also be cultured on Alkaline Peptone Water (Letchumanan et al., 2015), Marine Agar, and Thiosulfate Citrate Bile-Salts Sucrose (TCBS), a selective medium widely used for isolation and subculturing of *Vibrio*, where they form yellow or green colonies (Figure 2) depending if they are sucrose-positive or negative, respectively (Percival & Williams, 2013).

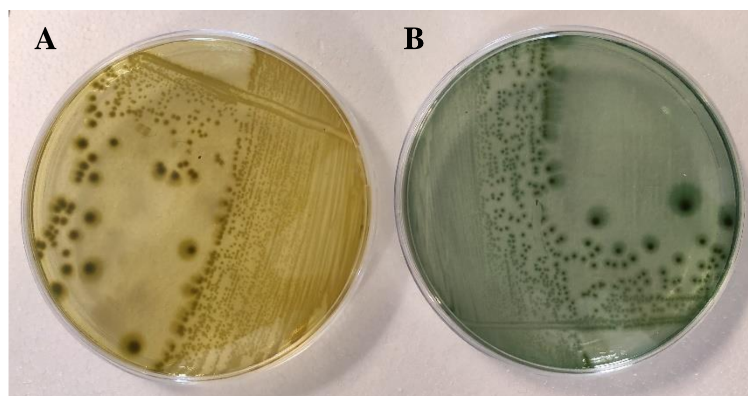


Figure 2 - Colonies of *Vibrio owensii* V9E (A) and *Vibrio parahaemolyticus* P9D (B) on TCBS agar (this study).

2. *Vibrio* natural environment

Vibrio species are widely distributed in aquatic environments, such as marine, brackish estuarine, and freshwater settings, being also present in sediments (Pruzzo et al., 2005). They are less commonly found in plants and algae (with the exception of *Vibrio breoganii* (Corzett et al., 2018)), preferring the water column and sediments (Comeau & Suttle, 2007). These bacteria also concentrate on mollusks, particularly bivalves, where they accumulate and lead to major damages on the host (Destoumieux-Garzón et al., 2020; Dias et al., 2018). Besides bivalves, there also seems to be a high density of *Vibrio* in corals, fish and shrimp (Thompson et al., 2004), being one of the most prevalent microorganisms in aquaculture systems (Novriadi, 2016).

Temperature, salinity, pH, and nutrients concentration are some of the abiotic factors that influence *Vibrio*'s density, whereas biotic factors include the presence of octopodes and other marine organisms with which they are associated through biofilm formation (Takemura et al., 2014). Temperature and salinity are the environmental parameters that more often explain variance in *Vibrio* abundance (Takemura et al., 2014; Zimmerman et al., 2007), with total *Vibrio* correlating positively with increases in temperature even in tropical countries (Wong et al., 2019). Consequently, with the rise of sea surface temperatures in the last few decades, *Vibrio* abundance has increased (Vezzulli et al., 2016). This may present a future problem since, besides their pathogenicity to aquatic animals, some species are pathogenic to humans, which could then lead to more frequent and more extensive *Vibrio* infections outbreaks.

Vibrio species play a crucial role in the aquatic milieu by participating in the turnover of organic matter and making compounds, such as organic carbon and nitrogen, available for other organisms (Neogi et al., 2018). Not only that, but they also have the ability to produce polyunsaturated fatty acids *de novo* for the aquatic food web, which many aquatic organisms cannot (Nichols, 2003).

Vibrio spp. have developed several survival strategies in stress conditions, such as lack of nutrients, lower temperatures, and grazing by predators (Sultana et al., 2018). Biofilm formation, producing a surface-associated aggregated community (Yildiz & Visick, 2009), allows for the accumulation of nutrients and biofilms act as a barrier against chemical compounds such as antibiotics (Beshiru & Igbinosa, 2018). *Vibrio* cells also possess the ability to switch to a dormancy called Viable But Non-Culturable (VBNC) state, granting survival and persistence in the environment (Pruzzo et al., 2005).

3. Virulence properties

With more than 100 species, the genus *Vibrio* contains at least 34 (Table 1) which are associated with diseases in humans and animals, such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, containing virulence factors that are not usually present in environmental strains (Mohamad et al., 2019). However, *Vibrio* genome plasticity and horizontal gene transfer contribute to the growing number of pathogenic strains in the environment (Hackbusch et al., 2020). *Vibrio* spp. are the main cause of human disease due to exposure to aquatic environments and consumption of seafood (Baker-Austin et al., 2018). The bacteria's ability to cause disease depends on many factors, such as environmental stress, physiological conditions and host characteristics (Beceiro et al., 2013). The infection process is overall the same for all pathogenic species: after direct contact with the host, there is attachment and colonization of the cell surface, a process facilitated by the flagellum and a series of enzymes like adhesins and diverse hemagglutinins (Ramamurthy et al., 2020). Then, there is cellular death of the host's cells by the action of hemolysins, cytotoxins and proteases (Janda et al., 1988).

As previously mentioned, *Vibrio* are usually motile, possessing one or more flagella, composed of multiple flagellin subunits, FlaA, -B, -C, -D, -E, and -F (4-6 flagellin proteins depending on the species) (Echazarreta & Klose, 2019). This structure promotes the strain's pathogenicity by acting as adhesins and facilitating the attachment to cell surfaces of the host or to solid surfaces, as per biofilm formation (Duan et al., 2012). Some flagellin proteins seem to play a bigger role when it comes to motility and virulence than others. For example, mutations in *flaC* or *flaB* in *V. vulnificus* significantly decreases motility, adhesion, and cytotoxicity (Kim et al., 2014), while in *Vibrio anguillarum*, mutations in *flaD* and *flaE* strongly decreases virulence (McGee et al., 1996). Other factors also contribute to the strain's virulence, such as *chiA* and *luxR*. *Vibrio* spp. have the ability to degrade chitin, a highly abundant polymer, since they possess chitinases like ChiA, which allow them to survive and persist in marine and estuarine environments (Fennell et al., 2021). *Quorum sensing*, a cell-to-cell communication through detection of small signaling peptides called autoinducers (AIs), is regulated by the *quorum sensing* regulator protein LuxR (Ball et al., 2017). The increase in AI concentration leads to an increase in LuxR production, which positively regulates bioluminescence, siderophore production, and metalloproteases (Pompeani et al., 2008) such as VhpA produced by *Vibrio harveyi*, which lyses proteins such as collagen by hydrolysis (Díaz & Suárez, 2008).

V. cholerae is one of the best-known bacterium of the genus *Vibrio*, causing severe diarrheal diseases. With more than 200 serogroups, only O1 and O139 have been linked to pandemic outbreaks of cholera worldwide (Sultana et al., 2018). Accounts of cholera-like disease have been reported since the era of Hippocrates (Barua, 1992), but in the modern history the first epidemic

outbreak happened in 1817 in India, spreading across Asia (Reidl & Klose, 2002). Six other pandemics have been reported since, with the last one being in the 1960s (Hu et al., 2016). Nowadays, whilst it is rare in developed countries, it is still one of the main causes of mortality in countries with high population densities, reduced sanitation and low access to potable water (Lekshmi et al., 2018). *V. cholerae* serogroup O1 biotype "classical" has shown to be responsible for the fifth and sixth pandemics, while the seventh was due to serogroup O1 biotype "El Tor" (Blake, 2014). Since then, a new serogroup has emerged, O139, and has become endemic to south-east Asia (Albert et al., 1993; Ramamurthy et al., 1993). Non-O1/O139 *V. cholerae* strains are still causative agents of sporadic intestinal and extraintestinal infections (Farmer & Hickman-Brenner, 2006).

V. cholerae is transmitted via oral/fecal route, through consumption of contaminated water, although it can also be transmitted through food (Albert et al., 1997; Rabbani & Greenough, 1999). The virulence of toxigenic *V. cholerae* strains is mainly due to the production of cholera toxin (CT), responsible for the diarrhea characteristic of cholera, and toxin co-regulated pilus (TCP), a pilus required for intestinal colonization (Hackbusch et al., 2020). CT is encoded by the operon *ctx*AB, located on the CTXϕ filamentous phage genome, whereas TCP is encoded by *tcp*A (Baker-Austin et al., 2018). Other virulence factors associated with the CTX phage are zonula occludens toxin (*zot*) (Castillo et al., 2018) and accessory cholera toxin (*ace*) (Trucksis et al., 1993). Non-CTX encoded accessory colonization factors (ACFs), hemagglutinin, outer-membrane porins and the O-antigen of the lipopolysaccharide (LPS), are also involved in the strain's pathogenicity (Pruzzo et al., 2005).

During the infection of the host, in the regulatory cascade, ToxR and TcpP, inner membrane DNA-binding proteins, regulate the expression of ToxT, a transcriptional activator which activates the *ctx* and *tcp* gene clusters (Dorman & Dorman, 2018). On the other hand, ToxR and TcpP are regulons activated by environmental and physiological signals, such as temperature, osmolarity, pH, amino acids, bile (Conner et al., 2016; Raskin et al., 2020), and quorum sensing (Zhu et al., 2001). Non-O1/O139 generally do not produce CT, but instead other factors contribute to their virulence, such as hemolysin A (encoded by the gene *hly*A), heat-stable enterotoxin NAG-ST, and the cytotoxic actin cross-linking RTX (repeats-in-toxin), encoded by the gene *rtx*A (Zago et al., 2017).

V. parahaemolyticus is the leading cause of food-borne illness in many countries, with high prevalence in South East Asia (Janda et al., 2015). This is mainly due to the consumption of raw or undercooked seafood (Raghunath, 2015). *V. parahaemolyticus* causes gastroenteritis in which TDH-related hemolysin (TRH) and thermostable direct hemolysin (TDH) play a crucial role (Thompson et al., 2004). Other virulence factors include adhesins, two type III secretion systems,

T3SS1 and T3SS2, and thermolabile hemolysin (TLH) (Osunla & Okoh, 2017), which causes the lysis of red blood cells by hydrolyzing the phospholipids in the bilayer (Zhang & Austin, 2005). Studies have shown that the majority of the environmental isolates don't present *trh* and/or *tdh*, unlike clinical isolates that usually encode one of the two hemolysin's genes (Robert-Pillot et al., 2004; Yeung & Boor, 2004). On the other hand, the *tlh* gene seems to be present in both environmental and clinical strains of *V. parahaemolyticus* and, therefore, being considered a species-specific marker and used to identify this species (Yáñez et al., 2015).

Like *V. parahaemolyticus*, *V. vulnificus* also presents a high prevalence in the environment and seafood around the world (Tao et al., 2012; Yano et al., 2004). It can cause gastrointestinal-like symptoms as well, but it can also lead to primary septicemia, as well as cause disease via wound infection (Raszl et al., 2016). It has the highest mortality rate of foodborne pathogens, with 1 in 5 people dying from infection (CDC, 2019). Unlike other pathogenic *Vibrio* species, no specific molecular markers have been found to help differentiate pathogenic from non-pathogenic *Vibrio vulnificus* strains (Baker-Austin & Oliver, 2018). *V. vulnificus* possesses an array of putative virulence factors, which includes the presence of a polysaccharide capsule, production of siderophores (Jones, 2014), iron acquisition, proteins involved in invasion and adhesion, and proteases (Jones, 2017).

Vibrio fluvialis, *Vibrio furnissii*, and *Vibrio alginolyticus* are emerging human pathogens, with the first two associated with outbreaks of diarrhea and gastroenteritis (Ramamurthy et al., 2014; Schirmeister et al., 2014), while the latter has been implicated in wound infections (Slifka et al., 2017). *V. fluvialis* and *V. furnissii* are closely-related species and have many virulence factors in common, including a Chinese hamster ovary (CHO) cell elongation factor, CHO-killing factor, cytolysins, cytotoxins, and several enzymes such as hemolysins, proteases, and lipases (Lockwood et al., 1982; Wu et al., 2007). *V. alginolyticus* has shown hemolytic and proteolytic activities, and hemagglutination (Hernández-Robles et al., 2016; Zanetti et al., 2000).

The pathogenicity of virulent *Vibrio* spp. strains has been linked with the secretion of extracellular products such as enzymes. DNases hydrolyze DNA, facilitating the propagation of the bacteria on the host (Al-Wahaibi et al., 2019). Phospholipases cleave the ester bonds of phospholipids, destabilizing the cell membranes and which could lead to hemolysis (Bandana et al., 2018), while lipases play an important role in degrading the lipid membrane of host cells, both aiding host colonization (Jaeger et al., 1994). Gelatinase and caseinase are known to hydrolyze collagen and proteins, respectively (Hossain et al., 2020). Hemolysins are major exotoxins identified in *Vibrio* spp. α-hemolysins lead to partial hemolysis and form pores in the outer membrane, causing an osmotic imbalance and leading to cell death while β-hemolysins, on the other hand, lead to the complete hemolysis of erythrocytes by breaking down cell membranes (Spurbeck & Mobley, 2013).

Table 1 - *Vibrio* species and their occurrence in human and animal specimens. Adapted from Farmer & Hickman-Brenner, 2006

Occurrence in human clinical specimens:[1]

	Intestinal	Extraintestinal
V. alginolyticus	+	++
V. carchariae	-	+
V. cholerae		
Serogroup O1	++++	+
Serogroup Non-O1	++	++
V. cincinnatiensis	-	+
V. damsela	-	++
V. fluvialis	++	-
V. furnissii	++	-
V. hollisae	++	-
V. metschnikovii	-	+
V. mimicus	++	+
V. parahaemolyticus	++++	+
V. vulnificus	+	+++

Species that do not occur in human clinical specimens

V. aesturianus	*V. marinus*	*V. proteolyticus*
V. anguillarum	*V. mediterranei*	*V. salmonicida*
V. campbellii	*V. natriegens*	*V. splendidus*
V. costicola	*V. nereis*	*V. tubiashii*
V. diazotrophicus	*V. nigripulchritudo*	*V. orientalis*
V. fischeri	*V. ordalii*	*V. pelagius*
V. harveyi	*V. gazogenes*	*V. logei*

[1] The symbols +, + +, + + +, and + + + + give the relative frequency of each organism in specimens.

Of all the pathogenic species belonging to the genus *Vibrio*, 22 seem to not occur in clinical specimens, such as *V. anguillarum*, *V. fischeri*, *V. campbellii* and *V. harveyi*. These marine bacteria all belong to the Harveyi clade (Ke et al., 2017) and are associated with vertebrates and invertebrates (Mougin et al., 2020), possessing an array of virulence genes widely distributed among the species (Mohamad et al., 2019), causing vibriosis in aquatic animals.

V. furnissii is an emerging pathogen associated with acute gastroenteritis in humans (Ballal et al., 2017). *V. mediterranei* has been associated with mass mortality of *Pinna nobilis* L. in the Mediterranean (Prado et al., 2020), while *V. harveyi*, *V. owensii*, *V. campbellii*, and *V. parahaemolyticus* affect shrimp populations, causing acute hepatopancreatic necrosis disease (AHPND) (Tang et al., 2020). They are naturally occurring in aquaculture, where they pose a great threat, leading to severe disease outbreaks and millions of losses in this industry (FAO, 2018). A recent article estimated the losses in Thailand through 2010-2017 from shrimp farming to be USD 11.58 billion (Shinn et al., 2018).

3.1 Biofilm formation

As previously mentioned, biofilms are surface attached communities enclosed in a matrix of primarily polysaccharides that promote survival and growth by retaining nutrients and conferring protection from predators and chemical substances like antibiotics. Biofilms are important for *Vibrio* to persist in aquaculture settings, causing high economic losses (Cepas et al., 2019). Biofilms are also important for the colonization process, as well as transmission of virulence/antibiotic resistance genes between strains (Laverty et al., 2020). Biofilm formation depends on cell-to-cell communication, as well as environmental stimuli, including temperature, salinity, nutrient availability, extracellular DNA, and pH (Marsden et al., 2017; Seper et al., 2011; Song et al., 2017).

In the first step the bacterium attaches to a surface and, through flagella-mediated motility, the microcolonies formed move and grow along the attached surface (Guttenplan & Kearns, 2013). Mutations of flagellar genes can result in decreased or non-existent attachment to surfaces (You-Chul et al., 2019). Similar types of pili are used by different *Vibrio* species and they also contribute to cell-surface interactions, with type IV pili playing a more significant role in biofilm formation by *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* (Aagesen & Häse, 2012; Paranjpye & Strom, 2005; Shime-Hattori et al., 2006).

To maintain the biofilm matrix integrity, *Vibrio* produce either capsular polysaccharide (CPS) or exopolysaccharides (EPS), which in turn alter colony morphology, in particular to opaque, wrinkled or rugose colonies (Lee et al., 2013). *V. cholerae* contains a locus, *vps*, which encodes for proteins involved in EPS production, mainly glucose and galactose, and is associated with the formation of rugose colonies (Yildiz et al., 2014). On the other hand, locus *cps* in *V. parahaemolyticus* encodes for the production of a CPS containing galactose, glucose, and *N*-acetylglucosamine, resulting in opaque colony morphology (Bian et al., 2020). The production of exopolysaccharides is regulated by many mechanisms, including two-component signal transduction system (TCS) and *quorum sensing* (Yildiz & Visick, 2009). VpsR and VpsT are transcriptional regulators that promote the transcription of *vps* genes in *V. cholerae*, while homologues CpsR and CpsS positively regulate *cps* in *V. parahaemolyticus* (Rodrigues et al., 2018). These regulators are activated by phosphorylation by a sensor histidine kinase (HK), the primary mechanism with which bacteria respond to the extracellular environment, or by c-di-GMP, a ubiquitous second messenger that controls biofilm formation (Teschler et al., 2017). C-di-GMP binds to transcriptional regulators, stimulating transcription of exopolysaccharide production genes and prevent swarming motility (Conner et al., 2017). *Quorum sensing* (QS), as previously mentioned, controls numerous mechanisms, including biofilm formation. The main

regulator, named LuxR in *V. harveyi* (homologues OpaR, SmcR and LitR in *V. parahaemolyticus*, *V. vulnificus* and *V. fischeri*, respectively), positively regulates EPS production through c-di-GMP activation at high cell density (Yildiz & Visick, 2009). A recent study showed that a novel global regulator RobA mediates EPS biosynthesis by repressing the transcription levels of master QS regulator OpaR in *V. parahemolyticus* (Zhong et al., 2021). Mutations on the main QS regulators affect the morphology of the colonies, making them translucent (Chang et al., 2010). On the other hand, in *V. cholerae* the LuxR homolog, HapR, negatively regulates *vps* transcription (Beyhan et al., 2007). Thus in *V. cholerae*, QS responds to the high cell densities in the surrounding environment by contributing to the reduction of biofilm formation (Figure 3) (Waters et al., 2008). The biofilm will then proliferate and mature, developing multiple layers of cells and eventually creating a three-dimensional structure where, under favorable conditions, the bacterial cells detach and disperse in the environment (Sultana et al., 2018).

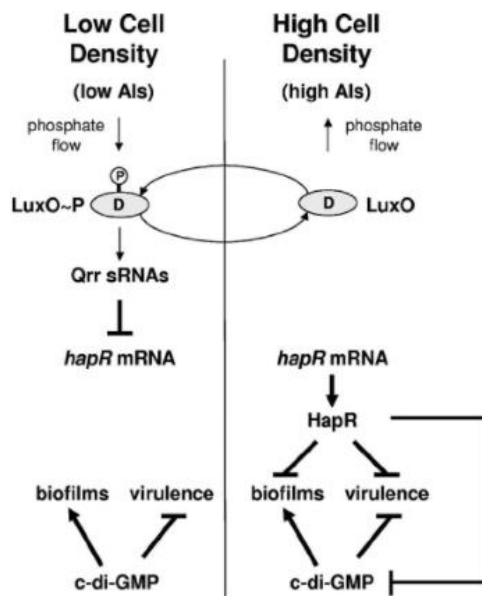


Figure 3 - Interaction between QS and c-di-GMP in the regulation of gene expression in *V. cholerae.* Adapted from Waters et al., 2008.

4. Antibiotic resistance

In 1947, Waksman defined antibiotic as *"a chemical substance, produced by microorganisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms."* (Waksman, 1947). More recent definitions describe it as an organic chemical that may be synthetic or naturally-occurring and that inhibits the growth or kills bacteria (Bentley & Bennett, 2003). Antibiotic-like substances have been used for thousands of years, with reports dating back to 1500 B.C. of the use of mushrooms, beer yeast and molds to treat infections, even

if the phenomenon couldn't be explained (Mohr, 2016). The accidental discovery of penicillin by Fleming in 1929 (Fleming, 1929) marked the beginning of mass-production of antibiotics. Since then, numerous antibacterial drugs have been developed and improved, resulting in a great decrease of mortality and morbidity associated with infection (Aminov, 2010). However, what one couldn't predict, was the rapid adaptation of the microorganisms to the changed environment by developing a wide variety of resistance mechanisms to antibiotics (Etebu & Arikekpar, 2016). Just within a year of extensive use of penicillin, there are reports of a significant number of *Staphylococcus aureus* strains that had become resistant to this drug (Schito, 2006). Many years later, the prospects have only gotten worse, with more common bacteria developing resistance to previously susceptible antibiotics. Antibiotics have been greatly used as therapeutic or prophylactic agents not only in humans, but also in agriculture and aquaculture practices (Topp et al., 2018). Nowadays, there are several antibiotics that can be grouped depending of their mechanism of action: they can inhibit the DNA synthesis, like quinolones and rifampicin or inhibit the protein synthesis, like tetracyclines, chloramphenicol and erythromycin; Others can interfere with folic acid metabolism, such as trimethoprim and sulfonamides or inhibit cell wall synthesis or function, like β-lactams (Alanis, 2005). They can also be classified based on their chemical structure into the major groups sulfonamides, tetracyclines, β-lactams, macrolides, fluoroquinolones, and aminoglycosides (Etebu & Arikekpar, 2016).

Even thought so many antibiotics have been developed over the past decades, bacteria have been able to adapt to the changing environment thanks to their plastic genome by developing genetic mutations, acquisition of antibiotic resistance genes (ARGs) due to selective pressure, or just natural resistance to certain antibiotics, a phenomenon that precedes our use of these drugs (Adedeji, 2016).

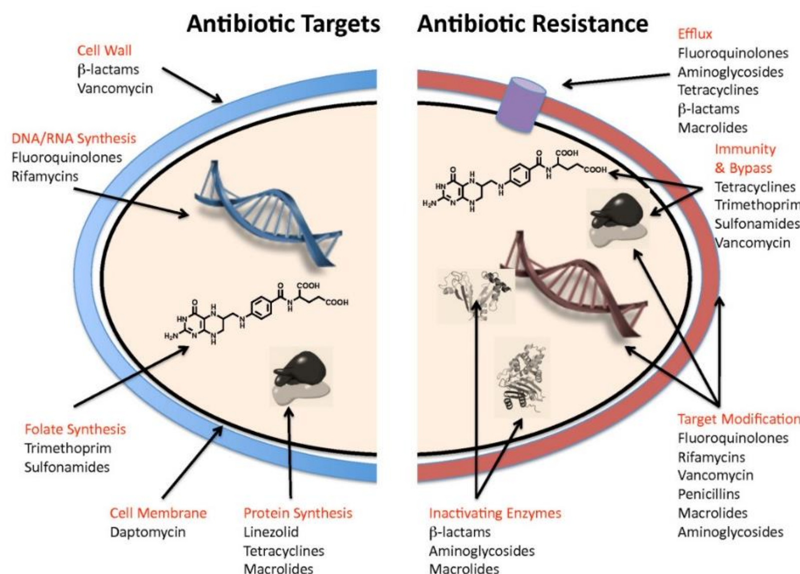


Figure 4 - Antibiotic resistance mechanisms and targets. Adapted from Wright, 2010.

Bacteria have developed an array of resistance mechanisms to overcome the effects of antibiotics such as: i) efflux pumps, ii) lower cell wall permeability, iii) enzymatic inhibition/modification of the antibiotic, iv) creation of alternative metabolic pathways to the ones inhibited by the drug, v) receptor modification (Figure 4) (Alanis, 2005). Mobile genetic elements (MGE), such as plasmids, integrative conjugative elements (ICE), transposons, and integrons, play a very important role in the dissemination of antibiotic resistance genes, allowing for the transfer and acquisition of new genetic material through horizontal gene transfer (HGT) (Frost et al., 2005). Several factors influence this transfer, such as host characteristics, selective pressure in the environment, and specific factors relating to each genetic element (Van Hoek et al., 2011).

Antibiotic resistance may be difficult to overcome since it is a natural mechanism for survival in bacteria, but it can certainly be reduced by altering behavioral factors and implement strong robust policies to control the use of antibiotics. It is estimated that every year, antimicrobial resistance (AMR) is responsible for 25,000 deaths in the EU and costs EUR1.5 billion annually (European Commission, 2017). Due to a demand of animal protein to feed the growing world population, intensive farming is instigated, which leads to excessive use of antibiotics to control the outbreaks of disease associated with animal infections. In aquaculture, one of the growing sectors with estimated profits to be US$217.5 billion every year worldwide, millions of dollars are lost due to bacterial infections (FAO, 2012). However, most antibiotics used as prophylactic or therapeutic measures, are not metabolized, and are excreted into the environment through urine and feces. Antibiotics can then accumulate unchanged in the water and soil for long periods (Husevag et al., 1991), such as fluoroquinolones and tetracyclines, where they can reach mg/kg of sediment (Roose-Amsaleg & Laverman, 2016), creating a selective pressure for antibiotic resistant bacteria (Aly & Albutti, 2014).

Excreted antibiotics by livestock and humans are disposed into the sewage system, reaching wastewater treatment plants (WWTPs) (Kummerer, 2003). If these drugs are not eliminated, they remain in the sewage system, where they create a selective pressure for the dissemination of antibiotics and resistance determinants in the environment (Bengtsson-Palme et al., 2019).

The over-prescription of antibiotics and patients not finishing their treatment also contribute to this problematic. Even though there seems to be an overall decrease in antibiotic consumption in European countries, utilization of specific antibiotics used to treat multidrug-resistant bacteria has increased in the past 10 years (ECDC, 2019). This leads to higher financial losses due to extra healthcare costs, as well as an increased morbidity and mortality. Now more than ever, it is important to reduce and limit the spread of resistance, by creating clear global guidelines regarding the use of antibiotics in human and veterinary sectors, promote awareness regarding this topic and strengthen surveillance and research (WHO, 2020).

The crisis of antibiotic resistance will not be solved solely by the development of new, improved antibiotics. Microorganisms will probably continue to adapt to their environment, creating new defense mechanisms. Therefore, new promising therapies are being studied, such as antibodies, vaccines, bacteriophages, and probiotics to be used as alternatives or in combination with antibiotics for better efficacy (Ghosh et al., 2019; Ji et al., 2020).

4.1 Antibiotic resistance associated with *Vibrio* spp.

As previously said, with the development of more antibiotics, their use became widespread. The inappropriate use of these drugs in the clinical and animal sectors has led to an increase of drug-resistant pathogens. This has contributed to a rise in infections not treatable with existing antibacterial therapies and mortality all around the world.

Vibrio species are usually susceptible to most of the antibiotics used in human and agriculture settings (Loo et al., 2020), with the antibiotics recommended for treating *Vibrio* infections being third-generation cephalosporins, quinolones, and tetracyclines (Tan et al., 2017). However, this paradigm seems to be changing with the emergence of resistant clinical and environmental strains. Gram-negative bacteria such as *Vibrio* are known to have multidrug efflux pumps that contribute to an intrinsic resistance to most narrow-spectrum agents such as some penicillins, aminoglycosides, and macrolides (Nikaido, 1998). Besides this, HGT through transduction, conjugation, and natural transformation also play an important part in the evolution and resistance of *Vibrio* spp. (Hazen et al., 2010).

The rising in resistance affects not only O1 and O139 serogroups (Dengo-Baloi et al., 2017; Iwanaga et al., 2004), but also environmental strains that find in the aquatic ecosystem a reservoir of ARGs. In 2020, a study in Kolkata, India, showed that non-O1/Non-O139 *Vibrio cholerae* are acquiring integrons containing MDR genes through newly obtained genomic islands (Morita et al., 2020). Hence, it is important to monitor the generation of these types of *V. cholerae* non-O1/non-O139 to help prevent the emergence of O1/O139 strains with MDR.

V. parahaemolyticus and *V. vulnificus* are the leading cause of seafood-associated bacterial illness worldwide. In Malaysia, a study showed that all *V. parahaemolyticus* isolated from seawater were resistant to streptomycin, while more than half showed resistance to tetracycline (Meteab Faja et al., 2019), a commonly used antibiotic to treat *V. parahaemolyticus* infections (Elmahdi et al., 2016). In Chesapeake Bay, the largest estuary in the U.S, *V. vulnificus* isolates showed a high resistance to aminoglycosides, such as streptomycin and apramycin, as well as intermediate resistance to chloramphenicol. In the same study, *V. parahaemolyticus* presented resistance to penicillins (Shaw et al., 2014).

Vibrio spp. are also known to carry quinolone-like resistance factors such as *qnrVC* genes coded in chromosomal genes, integrons, conjugative plasmids, and ICEs (Fonseca et al., 2008; Bin et al., 2010; Liu et al., 2013; Poirel et al., 2005). First reported in a class 1 integron from *V. cholerae* (Fonseca et al., 2008), these genes confer low-level resistance to quinolones and can be transferred to other gram-negative bacteria (Martínez-Martínez et al., 1998). Until now, nine alleles have been described in *Vibrionaceae*, with *qnrVC8* and *qnrVC9* reported in China in 2018 (Zhang et al., 2018a). Qnr-like genes have also been found in other gram-negative bacteria, including *Aeromonas punctata* and *Salmonella enterica* harbored in a class 1 integron (Luk-in et al., 2017; Xia et al., 2010), and in *Pseudomonas aeruginosa* in a plasmid and a class 1 integron (Lin et al., 2020; Liu et al., 2018).

Carbapenems are considered last-resort drugs to treat gram-negative and gram-positive infections (Lee et al., 2018). The overuse of β-lactams such as cephalosporins and carbapenems has led to a rise of resistant *Vibrio* in recent years, not only in the clinical setting, but also in the environment (Oyelade et al., 2018). For instance, *V. cholerae* harboring a novel carbapenemase, *bla*$_{VCC-1}$, has been isolated from coastal waters in Germany (Hammerl et al., 2017). Several authors have also reported the presence of New Delhi-metallo-beta-lactamase gene, *bla*$_{NDM-1}$, in environmental strains (Diep et al., 2015; Oyelade et al., 2018).

Quinolones, β-lactams, tetracyclines, and fluoroquinolones are the classes of antibiotics most used in aquaculture in Europe (Leal et al., 2016), while Asian countries don't have such clear guidelines regarding antibiotic use. Studies show a high prevalence of *bla*$_{TEM}$ and *bla*$_{CTX}$ in *Vibrio* spp. isolated from marketed bivalves in Korea (Dahanayake et al., 2020; Hossain et al., 2020), as well as Chinese mariculture (Zhao et al., 2018), and in rivers from Nigeria (Chikwendu et al., 2014). A report from coastal aquaculture in South Korea detected various ARG encoding for tetracycline resistance (*tetB*, *tetD*, *tetE*, *tetX*,), sulfonamide resistance (*sul1*, *sul2*), quinolone resistance (*qnrD*, *qnrS*, *aac(6')-lb-cr*), florfenicol resistance (*floR*), and the presence of the class 1 integron integrase gene (*intI1*) in *Vibrio* isolates (Hyun et al., 2018). Overall, European studies show lower prevalence of antibiotic resistant bacteria. However, due to intensive aquaculture, this prevalence is increasing. For instance, higher levels of multi-resistant bacteria have been reported in aquaculture centers compared to coastal areas in Italy (Labella et al., 2013).

MGE, including plasmids, phages, ICEs, insertion sequences, and genomic islands are frequently associated with antibiotic resistance, being major players in *V. cholerae*'s and other *Vibrio* species' antibiotic resistance (Narendrakumar et al., 2019). Plasmids belonging to the incompatibility groups A and C (IncA/C) are large conjugative plasmids associated with antibiotic resistance with a broad host range (Harmer & Hall, 2015). They were first identified in *Vibrio* spp. isolated from cultured fish, conferring multi-drug resistance phenotypes (Aoki et al., 1973; Watanabe et al., 1971). Since then, there have been multiple reports of IncA/C plasmids

coding for antibiotic resistance, mostly against β-lactams. A study found a foodborne *V. parahaemolyticus* carrying an IncA/C plasmid coding a β-lactamase gene, *bla*$_{CMY-2}$ (Li et al., 2015). *V. alginolyticus* was also found to have a *bla*$_{NDM-1}$-bearing IncA/C plasmid, which also harbored *dfrA15*, *aadA*, and *sul1* resistance genes (Zheng et al., 2018). In *V. cholerae*, IncA/C plasmids conferring high azithromycin resistance have been reported (Wang et al., 2018), as well as coding an IS*CR1* (insertion sequence common region)-mediated *bla*$_{PER-1}$ (Wu et al., 2015).

Integrons also play an important role in dissemination of antibiotic resistance. Out of the 3 classes most commonly reported, class 1 integrons seem to be the most common ones amongst *Vibrio*. Class 1 integrons lacking gene cassettes have been reported in *V. parahaemolyticus* (Jiang et al., 2014), *V. alginolyticus*, and *V. diabolicus* isolated from marine animals (Hossain et al., 2020). However, their variable regions are still able to integrate gene cassettes, especially antibiotic resistance genes, making them a possible threat to public health. Nonetheless, class 1 integrons containing *aadA2* cassette have been reported in environmental *V. alginolyticus* (Taviani et al., 2008), and harboring *bla*$_{VEB-1}$ (Lei et al., 2020), *aac6-II*, *arr-3*, and *dfrA27* reported in *V. parahaemolyticus* (Yu, 2010). Many *Vibrio* strains from different species, including *V. parahaemolyticus*, *V. mimicus*, and *V. fischeri*, possess super-integrons (SI) which, unlike resistance integrons (RI), are chromosomally-located and contain a large cassette array with a variety of functions including bacterial growth and enzymatic activity (Fluit & Schmitz, 2004; Rowe-Magnus et al., 2001).

The information detailed above highlights the importance of having clear guidelines regarding antibiotic use, not only in clinical settings, but also in aquaculture and agriculture sectors, as well as regular surveillance of antibiotic resistance in *Vibrio* spp..

5. Study area and contextualization

Ria de Aveiro is a 45 kilometers long estuarine lagoon located on the Northwest coast of Portugal where freshwater from rivers Vouga, Antuã, and Boco flow into and meet with the Atlantic salt water (Figure 5). The lagoon is an area of great importance to the region, presenting a great biodiversity and playing an important role in the reproduction and feeding of fauna, being an obligatory stop for migratory birds. It is also known for its coastal and estuarine habitats, making it favorable for fishing and hunting activities (Polis Litoral, 2019).

Figure 5 - Barra channel from Ria de Aveiro. From *"Ílhavo e a Ria de Aveiro", Município de ílhavo, n.d. (*http://www.visitilhavo.pt/dossiers-tematicos/ilhavo-e-a-ria-de-aveiro*).*

This lagoon is of high economic importance for the local population through salt production, agriculture, fishing, shellfish collection, industry, tourism, and aquaculture. The latter is a growing activity, with an increase every year in aquaculture establishments (Figure 6) (Rocha, 2017). As of 2019, there were 20 tanks and 57 nurseries (DGRM, 2019) reported. Even though this sector has a high economic importance for the region, its growth also represents a risk to the population. With more antibiotics being used, this could then lead to an increase in the prevalence of antibiotic resistant bacteria, especially in species associated with aquaculture such as *Vibrio* spp..

Despite its importance, Ria de Aveiro has been plagued by illegal discharges of pollutants from industries located on the lagoon vicinities (Lau, 2020; Quercus, 2020). A study conducted in 2007 showed fecal and urban wastewater contamination, in addition to high levels of chemical pollutants such as ammonia, phosphorus, chromium, and nickel on small lagoon channels from several municipalities of Aveiro (Almeida, 2007). Harbor facilities, sewage inputs, agriculture, and aquaculture also contribute to the high pollution levels (Henriques et al., 2006).

Figure 6 - Fish aquaculture ponds in Ria de Aveiro. From Terra Nova*, September 5th 2016 (*https://www.terranova.pt/noticia/politica/governo-quer-triplicar-producao-da-aquacultura-associacao-portuguesa-de-aquicultura*)*.

Pereira and coworkers (Pereira, 2009) reported *Vibrio* spp. in fish aquaculture in Ria de Aveiro, with a higher relative abundance between the months of October and February.

In the scope of a previous Master's project (Dias, 2020), *Vibrio* species were isolated from different sites along the estuary Ria de Aveiro (Figure 7), in three different campaigns (Spring, Summer, and Autumn). The distribution of *Vibrio* spp. was evaluated, and a low seasonal variability in the abundance of these species was noted. However, a notable variance in terms of diversity was registered, as well as an heterogeneous distribution of species throughout the estuary (Dias, 2020). Considering the clinical and environmental relevance of *Vibrio* spp., it is important to evaluate their virulence potential, as well as associated antibiotic resistance.

Figure 7 - Sampling sites where the water samples were collected marked along Ria de Aveiro in three different campaigns (Spring, Autumn, Summer). Figure adapted from Dias (2020).

AIMS OF THIS STUDY

Members of the genus *Vibrio* are disease-causing agents in humans and marine animals, being one of the primary causes of infection in the aquaculture industry, leading to millions of dollars in losses in this sector worldwide. Pathogenic strains possess an array of virulence factors, including enzymes (lipases, proteases), siderophores, and toxins, as well as the ability to form biofilms and express *quorum sensing* mechanisms. Also, there are reports of increasing levels of antibiotic resistance associated with *Vibrio* spp., rising worldwide concern.
Despite all this, little effort has been made to study and understand the virulence and antibiotic resistance of *Vibrio* spp. in estuarine water.

With this being said, the aim of this study is to contribute to assess the risk to humans and animals posed by *Vibrio* spp. isolated from the estuary Ria de Aveiro. Specific aims are:

1. To determine the antibiotic resistance phenotypes and genotypes of the strains;
2. To determine their virulence factors profiles.

MATERIALS AND METHODS

1. Bacterial Strains and Culture Conditions

As previously stated, this study was conducted using *Vibrio* isolates previously collected from the estuary Ria de Aveiro. On the scope of a PhD thesis, water samples were collected from 26 different sites along the estuary in three different campaigns: Autumn 2018, Winter 2018, Spring 2019, and Summer 2019. However, *Vibrio* isolates were not obtained from samples collected in the winter campaign. Samples of water were collected into sterile bottles (2-3 L) and passed through 0.45µm membrane filters (Pall Life Sciences, USA) in triplicate. The membranes were then placed in agar plates with TCBS (5g/L peptone from casein, 5g/L peptone from meat, 5g/L yeast extract, 10g/L sodium citrate, 10g/L sodium thiosulfate, 5g/L ox bile, 3g/L sodium cholate, 20g/L sucrose, 10g/L sodium chloride, 1g/L iron (III) citrate, 0.04g/L thymol blue, 0.04g/L bromothymol blue, and 14g/L agar-agar; Merck, Germany) supplemented with 16µg/mL of tetracycline (Sigma-Aldrich, USA), as per CLSI clinical breakpoint specific for *Vibrio* spp., and incubated at 30ºC for 24h. *Vibrio* strains were stored in 96-well plates in culture medium with 20% glycerol, at -80ºC.

Since then, on the scope of a Master's project (Dias, 2020), the *Vibrio* isolates were identified at species level through sequencing of the *pyrH* gene fragment as described previously (Dias, 2020). Hence, a collection of 77 *Vibrio* was obtained comprising 11 species, and further characterized in the present work.

2. DNA extraction and purification

For DNA extraction, the bacteria were grown in 2 mL TSB (17g/L Tryptone, 3g/L soytone, 2.5g/L glucose, 5g/L sodium chloride, and 2.5g/L dipotassium phosphate; Sigma-Aldrich, Germany) supplemented with 1.5% NaCl for 24h at 30ºC. The suspension was then centrifuged at 10.000 rpm for 10 min and the pellets were kept at -20ºC until DNA extraction. DNA was obtained using Silica Bead DNA Gel Extraction Kit (ThermoFisher Scientific, USA) according to manufacturer's instructions with some modifications. Briefly, the pellet was resuspended in 100µL of Buffer 1 (50 mM Tris-HCl pH 8.0; 50 mM EDTA pH 8.0; 0.5% Tween 20; 0.5% Trixton X-100). Afterwards, a mix of Buffer 1 and RNase A (100 mg/mL;1 mL to 2 µL ratio) was added to the cellular suspension, vortexed vigorously, and incubated at 37ºC for 30 min. Then, 70 µL of Buffer 2 (3 M Guanidine Hydrochloride; 20% Tween 20) was added and, after vigorously

vortexing, the suspension was incubated at 50ºC for 30 min. The protocol recommended by the manufacturer was then followed. The DNA was stored in 1.5 mL tubes at -20ºC until further analysis.

3. Molecular typing of *Vibrio* spp. by BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR)

To assess clonality among isolates, BOX-PCR was performed (Canellas et al., 2021a). The primer BOXA1R (5' -CTACGGCAAGGCGACGCTGACG- 3'; (Araújo et al., 2014)) was used for BOX-PCR fingerprinting. The 25µL reaction contained 6.25µL NZYTaq 2x Green Master Mix (2.5mM $MgCl_2$, 200µM dNTPs, 1.25U DNA polymerase) (NZYTech, Portugal), 15.75µL dH2O, 10 pmol primer BOXA1R, and 1µL of DNA template (50 to 100 ng/µL).
The PCR conditions included an initial denaturation at 94ºC for 7min, followed by 30 cycles of denaturation at 94ºC for 1min, annealing at 53ºC for 1min, extension at 60ºC for 1min and a final extension at 65ºC for 8min, using a MyCycler Thermal Cycler (BioRad, USA). The amplification products were resolved in a 1.5% agarose gel containing ethidium bromide (0.625 mg/mL) in TAE buffer: The molecular marker (3µL; 0.5 µg/µl) (Thermo Fischer Scientific, USA) was loaded in the first lane and 5µL of the PCR reactions in the remaining lanes. The gel was electrophoresed for 90' at 80V and photographed using a gel documentation system (BioRad Gel Doc EQ System, USA). The resulting images were processed with GelCompar II 5.10 software (Applied Maths, USA). Cluster analysis was performed using the unweighted pair-group method with average linkages (UPGMA), based on Jaccard's similarity.

4. Salt tolerance test

The isolates were streaked on TSA supplemented with 1% NaCl and incubated for 24h at room temperature. A single colony of each isolate was picked and inoculated in sterile saline to achieve a turbidity of 0.5 MacFarland. Then, 1 µL was inoculated in triplicate in TSA plates containing various concentrations of supplemented NaCl: 0, 0.5, 1, 3, 5, 7.5, and 10%, which were incubated for 24h at room temperature.

5. Antibiotic susceptibility testing

Antibiotic susceptibility testing was carried out by disk diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). A total of 8 antibiotics (ThermoFischer Scientific, USA) were assessed: doxycycline (DO, 30 µg/disk), tetracycline (TE,

30 µg/disk), ciprofloxacin (CIP, 5 µg/disk), erythromycin (E, 15 µg/disk), amoxicillin (AML, 10 µg/disk), gentamicin (CN, 10 µg/disk), ceftazidime (CAZ, 10 µg/disk), trimethoprim-sulfamethoxazole (SXT, 25 µg/disk). After incubation for 24h at 30ºC in LB medium (NZYTech, Portugal), bacterial cells were suspended in sterile saline to achieve a turbidity similar to 0.5 MacFarland standard. The inoculum was then swabbed onto Mueller Hinton agar (Oxoid, UK). The antibiotic disks were evenly placed on the plates, which were then incubated at 30ºC for 24h. The diameter of the inhibition zone was then measured and the strains were recorded as susceptible, intermediate, or resistant. Results for antibiotics tetracycline, ciprofloxacin, amoxicillin, gentamicin, and trimethoprim-sulfamethoxazole were interpreted according to the guidelines of the Clinical Laboratory Standards Institute document M45 (CLSI, 2011), while results for erythromycin were interpreted according to Bauer et al., 1966. CLSI breakpoints for *Enterobacteriaceae* were used for doxycycline (CLSI, 2018) and EUCAST 2020 breakpoints for *Aeromonas* spp. for ceftazidime (EUCAST, 2020) when breakpoints were unavailable for *Vibrio* spp.. *E. coli* ATCC 25922 was used as quality control.

The multiple antibiotic resistance (MAR) index was calculated as $\frac{a}{b}$, where “a” represents the number of antibiotics each isolate showed resistance to, and “b” represents the total number of antibiotics tested (Adenaike et al., 2016). For the purposes of this calculation, intermediate susceptibility was considered as susceptible, dose dependent (SDD) as per CLSI 2019 guidelines (Hindler et al., 2021).

6. Detection of antibiotic resistance genes

PCR assays were applied for screening of genes encoding the most reported β-lactamases TEM, SHV and CTX-M, quinolone-associated resistance determinants (*qnrVC1*, *4*, *7*, *9*, and *qnrVC3*, *5*, *6*, *10*) and tetracycline resistance genes (*tet A*, *B*, *C*, *D*, *E*, *M*) was performed. PCR conditions and primers are listed in Table 2 and Table 3. To amplify other variants of the *qnrVC* gene, namely *qnrVC3*, *5*, *6*, and *10*, these sequences were obtained from GenBank (NCBI, USA) and aligned using Clustal Omega (EMBL-EBI, UK). Two conserved regions were selected for designing forward and reverse primers (Table 2). A PCR multiplex was used for the detection of bla_{TEM} and bla_{SHV}. The PCR reactions contained 6.25 µL NZYTaq II 2x Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 1.25 U DNA polymerase) (NZYTech, Portugal), 14.75 µL dH_2O, 0.3 µM of each gene-specific primer, and 1 µL DNA in a final volume of 25 µL.

The PCR reactions for targeting $bla_{\mathrm{CTX\text{-}M}}$, *qnrVC1, 4, 7, 9*, *qnrA*, *qnrVC3, 5, 6, 10*, and *tet* genes were performed in a final volume of 25 µL, containing 6.25 µL NZYTaq II 2x Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 1.25 U DNA polymerase) (NZYTech, Portugal), 15.75 µL

dH_2O, 10 pmol of forward and reverse primers, and 1 µL of DNA previously obtained. The experiments were conducted using a MyCycler Thermal Cycler (BioRad, USA). Amplified PCR products were visualized on a 1% agarose gel with 3 µL of green safe (NZYTech, Portugal), run at 80 V for 75 min and photographed using a gel documenting system (BioRad, USA). The PCR products were purified using NZYGelpure kit (NZYTech, Portugal) according to manufacturer's instructions and sent to GATC Biotech (Germany) for sequencing to confirm the genes' presence and their sequences were compared with genes in the GenBank using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2 - Antibiotic resistance genes searched, respective primer sequence, and fragment size

Target gene	Encoded proteins	Primer Sequence (5'-3')	Amplicon size (bp)	$T_{annealing}$ (ºC)	[2]PCR *program	Reference
qnrVC1, 4, 7, 9	QnrVC1, 4, 7, 9 DNA gyrase and topoisomerase IV protection proteins	F: GGATAAAACAGACCAGTTATATGTACAAG R: AGATTTGCGCCAATCCATCTATT	650	48	A	Tacão et al., 2014
qnrVC3, 5, 6, 10	QnrVC3, 5, 6, 10 DNA gyrase and topoisomerase IV protection proteins	F: TCTCACATCAGGACTTGC R: CCCCTAATTGCTCCAGTAAC	600	62	A	This study
qnrA	QnrA DNA gyrase and topoisomerase IV protection proteins	F: TTCTCACGCCAGGATTTG R: CCATCCAGATCGGCAAA	521	53	B	Alves et al., 2014
$bla_{CTX\text{-}M}$	β-lactamase (CTX-M)	F: SCVATGTGCAGYACCAGTAA R: GCTGCCGGTYTTATCVCC	652	55	C	Alves et al., 2014
tetA	TET(A) Efflux protein	F: GCTACATCCTGCTTGCCTTC R: GCATAGATCGCCGTGAAGAG	211	53	B	Henriques et al., 2008

	Gene	Target	Primer sequence (5'–3')	Size (bp)	Annealing (°C)	Program	Reference
	tetB	TET(B) Efflux protein	F: TCATTGCCGATACCACCTCAG R: CCAACCATCATGCTATTCCATCC	391	53	B	Henriques et al., 2008
	tetC	TET(C) Efflux protein	F: CTGCTCGCTTCGCTACTTG R: GCCTACAATCCATGCCAACC	897	53	B	Henriques et al., 2008
	tetD	TET(D) Efflux protein	F: TGTGCTGTGGATGTTGTATCTC R: CAGTGCCGTGCCAATCAG	844	53	B	Henriques et al., 2008
	tetE	TET(E) Efflux protein	F: ATGAACCGCACTGTGATGATG R: ACCGACCATTACGCCATCC	744	53	B	Henriques et al., 2008
	tetM	TET(M) Ribosomal protection protein	F: GTGGACAAAGGTACAACGAG R: CGGTAAAGTTCGTCACACAC	406	55	B	Henriques et al., 2008
Multiplex I	*bla_{TEM}*	β-lactamase (TEM)	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC	800	60	D	Magoué et al., 2013
	bla_{SHV}	β-lactamase (SHV)	F: AGCCGCTTGAGCAAATTAAAC R: ATCCCGCAGATAAATCACCAC	713	60	D	Magoué et al., 2013

[2] See Table

Table 3 - PCR programs

	Temperature	A	B	C	D
1st step	94ºC	5 min	5 min	2 min	10 min
2nd step[2]	94ºC	30 s	30 s	15 s	40 s
	$T_{annealing}$	30 s	30 s	30 s	40 s
	72ºC	1min	30 s	45 s	1 min
3rd step	72ºC	7 min	7 min	10 min	1 min

[2]This step corresponds to 30 cycles (programs B, C, and D) or 35 cycles (program A)

7. Presence of virulence factors

To evaluate the presence of virulence-associated genes in *Vibrio* spp., seven genes were screened by PCR using primers listed in Table 4: *tlh, vhpA,* $toxR_{Vc}$*, flaC, hlyA, luxR,* and *chiA*. The PCR was performed in 25µL reactions, containing 6.25µL NZYTaq II 2x Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 1.25 U DNA polymerase) (NZYTech, Portugal), 15.75µL dH_2O, 10pmol of each forward and reverse primer (10µM), and 1µL of DNA template. The amplification was performed in an automatic thermal cycler (BioRad, USA) as followed: initial denaturation at 95ºC for 5min, followed by 30 cycles of 95ºC for 1min (*vhpA,* $toxR_{Vc}$*, flaC, hlyA, luxR, chiA*); 52ºC for 1min ($toxR_{Vc}$); 55ºC for 1min (*flaC*); 50ºC for 1min (*luxR, chiA, chpA*); 50ºC for 1min30s (*hlyA*); 72ºC for 1min (*flaC, luxR,* $toxR_{Vc}$*, chiA, vhpA*); 72ºC for 1min30s (*hlyA*), with a final extension of 72ºC for 10min. The amplification of the gene *tlh* was performed under the following conditions: initial denaturation at 95ºC for 3min, followed by 35 cycles of 95ºC for 30s, 60ºC for 45s, 72ºC for 30s and a final extension of 73ºC for 5min.

Amplified PCR products were visualized in a 1% agarose gel containing 2µL of ethidium bromide, run at 80V for 90', and photographed using a gel documentation system (BioRad, USA). To confirm the specificity of the amplification, about 5% of the amplified fragments for each gene were selected randomly and sent to sequence analysis. For this, the PCR products were purified using NZYGelpure kit (NZYTech, Portugal) according to manufacturer's instructions and sent to GATC Biotech (Germany) for sequencing. Sequences were compared with genes in the GenBank using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 4 - Virulence factors searched, respective primer sequence, and fragment size

Target gene	Virulence factor	Sequence (5'-3')	Amplicon size (bp)	Reference
hlyA	Hemolysin of *V. cholera*	F: GGCAAACAGCGAAACAAATACC R: CTCAGCGGGCTAATACGGTTTA	738	(Saravanan et al., 2007)
toxR$_{Vc}$	Toxin of *V. cholera*	F: ATGTTCGGATTAGGACAC R: TACTCACACACTTTGATGGC	883	(Miller et al., 1987)
tlh	Thermolabile hemolysin of *V. parahaemolyticus*	F: AAAGCGGATTATGCAGAAGCACTG R: GCTACTTTCTAGCATTTTCTCTGC	450	(Mohamad et al., 2019)
flaC	Flagella of *V. anguillarum*	F: AAATCATTCCAAATCGGTGC R: TCTTTGATTCGGCTCTTA	580	(Mohamad et al., 2019)
luxR	Quorum sensing factors	F: ATGGACTCAATTGCAAAGAG R: TTAGTGATGTTCACGGTTGT	618	(Ruwandeepika et al., 2010)
vhpA	Metalloprotease	F: CTGAACGACGCCCATTATTT R: CGCTGACACATCAAGGCTAA	201	(Ruwandeepika et al., 2010)
chiA	Chitinase	F: GGAAGATGGCGTGATTGACT R: GGCATCAATTTCCCAAGAGA	232	(Ruwandeepika et al., 2010)

8. Production of extracellular products (ECPs)

The production of extracellular products was determined through enzymatic assays on specific agar plates. The isolates were re-streaked on TSA supplemented with 1% NaCl and incubated for 24h at room temperature. A single colony of each isolate was picked and inoculated in sterile saline to achieve turbidity like 0.5 MacFarland standard. From this suspension, 2 µL were inoculated in triplicate on TSA with 1% NaCl containing 1% (v/v) Skim milk, 1% (v/v) Tween 80, 1% (v/v) Egg yolk emulsion, 0.5% (v/v) Starch, and 12% Gelatin to test for caseinase, lipase, phospholipase, amylase, and gelatinase, respectively. Hemolysin and DNase production was evaluated on blood agar (sheep blood 7%) (Liofilchem, UK) and DNase test agar (Liofilchem, UK), accordingly, using the same strategy previously described. Agar plates were then incubated at room temperature for 24h. Amylase activity was observed by adding Lugol's reagent onto the colonies grown on nutrient agar. Presence of halos around the inoculum site was considered a positive reaction for caseinase, lipase, phospholipase, hemolysin, and gelatin. DNase activity was detected by flooding the plates with 1N HCL and observing clearing zones around the colonies.

9. Detection of integrons

The presence of class 1, 2 and 3 integrons in *Vibrio* spp. was confirmed by PCR amplification of integrase genes *intI1*, *intI2* and *intI3* with specific primers as stated in Table 5. The PCR was performed in 25µL reactions, containing 6.25µL NZYTaq II 2x Green Master Mix (2.5 mM $MgCl_2$; 200 µM dNTPs; 1.25 U DNA polymerase) (NZYTech, Portugal), 15.75µL dH_2O, 10pmol of each forward and reverse primer (10µM), and 1µL of DNA template. The amplification was performed in an automatic thermal cycler (BioRad, USA) as followed: initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 59ºC (*intI1*); 55ºC (*intI2*, *intI3*) for 1 min, extension at 72ºC for 1 min and a final extension at 72ºC for 8 min. Amplified PCR products were visualized in a 1% agarose gel containing 2µL of ethidium bromide, run at 80V for 90', and photographed using a gel documentation system (BioRad, USA). The PCR products were purified using NZYGelpure kit (NZYTech, Portugal) according to manufacturer's instructions and sent to GATC Biotech (Germany) for sequencing to confirm the genes' presence and their sequences were compared with genes in the GenBank using BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 5 - Integrase primers used in this work and PCR conditions

Primer	Target gene	Sequence (5'-3')	Annealing temperature (ºC)	Amplicon size (bp)	Reference
intI1	*intI1*	F: CCTCCCGCACGATGATC R: TCCACGCATCGTCAGGC	58	280	(Araújo et al., 2017)
intI2	*intI2*	F: TTATTGCTGGGATTAGGC R: ACGGCTACCCTCTGTTATC	55	233	(Araújo et al., 2017)
intI3	*intI3*	F: AGTGGGTGGCGAATGAGTG R: TGTTCTTGTATCGGCAGGTG	55	600	(Tacão et al., 2014)

10. Plasmid extraction

A single colony from a freshly streaked selective plate was inoculated in 5 mL LB medium supplemented with 1% NaCl and incubated for 16h at 30ºC. Plasmids were purified using the NZY Miniprep Kit (NZYTech, Portugal) according to manufacturer's instructions. The extracted DNA was electrophoresed in a 0.8% agarose gel at 80V for 90 min, visualized using a gel documentation system (BioRad, USA) and stored at -20ºC.

11. Biofilm production

The biofilm production was assessed on microtiter plate by crystal violet staining with a protocol adapted from Tavares et al., 2020. Independent experiments were conducted using LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl; NZYTech, Portugal) supplemented with (1) 1% NaCl and (2) 2.5% NaCl. The experiments were performed twice. Bacteria were grown in LB broth with different NaCl concentrations to stationary phase overnight at 30ºC without agitation. The cell suspensions OD_{600nm} were adjusted to an optical density (600 nm) of approximately 0.6-0.8 and serial diluted up to a dilution corresponding to 10^{-5} (100 000 X). The experiment was conducted in a 96-well flat bottom microtiter plate, where 200 µL of each diluted culture was placed (5 replicates for each strain) and incubated for 24h at 30ºC with no agitation. The OD_{600nm} was then measured for each well and, afterwards, the inoculum was removed and the wells were washed with 200 µL of NaCl 0.9% twice and incubated at 50ºC for 1h to attach the cells. After cooling, the biofilm was stained with 200 µL of a 0.1% crystal violet solution for 30min at room temperature, re-washed with 200 µL of NaCl 0.9%, and left to dry at room temperature overnight. The biofilms were then solubilized with 200 µL of 30% acetic acid for 30min at room temperature and the OD_{590nm} at each well was measured. *E.coli* ATCC 25922 was used as quality control and sterile culture media as negative control.

The biofilm formation index (BFI) was calculated as follows: $BFI = \frac{OD_{590nm}}{OD_{600nm}}$.

The strains were classified as non-producers ($BFI \leq BFI_{negative\ control}$), weak producers ($BFI_{negative\ control} < BFI < 2 \times BFI_{negative\ control}$), producers ($BFI \geq 2 \times BFI_{negative\ control}$), and super-producers ($BFI \geq 4 \times BFI_{negative\ control}$).

RESULTS AND DISCUSSION

1. Intra-species diversity of *Vibrio* isolates in the estuary Ria de Aveiro

BOX-PCR was used to assess clonality among isolates of the *Vibrio* collection. The fingerprint patterns generated consisted of 2 – 13 amplicons with different sizes (600-6,000bp) and are shown in Figure 8. BOX-PCR revealed 69 unique patterns, with more than half of the isolates presenting more than 4 bands per pattern. Two isolates, *V. owensii* (V10E) and *V. fluvialis* (V25A) did not produce any bands. Overall, we can observe that the isolates from this study are highly heterogenous and genetically diverse.

Dendrograms were constructed for each of the most prevalent species and for the remaining isolates (Figure 9). Distinct clusters according to seasonality or sampling site were not detected. Out of the 77 *Vibrio* isolates, 6 (in pairs of 2) show high similarity (98%), suggesting clonality. However, *V. parahaemolyticus* O19C and P2E are from different locations, Estarreja and Vagos, respectively, and present a different number of virulence factors, enzymes produced, and antibiotic resistance genes. *V. parahaemolyticus* O22A and O22C and *V. campbellii* V8B and V8G are from the same campaign and location but also vary in number of virulence factors, enzymatic activity, and antibiotic resistance genes.

V. parahaemolyticus, *V. harveyi*, and *V. alginolyticus* are the most common *Vibrio* species isolated from estuaries, with *V. diabolicus* and *V. mediterranei* being less common (Canellas et al., 2021; Costa et al., 2010). However, a high diversity of *Vibrio* species (11) was collected, including a high number of *V. diabolicus* isolates, that were detected in all sampling sites (Dias, 2020). *V. campbellii* and *V. fluvialis* were only collected in the Summer campaign, while *V. jasicida*, *V. cholerae*, and *V. mytili* were only detected in the Spring campaign. Overall, the distribution of vibrio species varied according to the sampling season, while no significant correlation between environmental parameters and *Vibrio* abundance was noted (Dias, 2020). The high genetic diversity within each species, here depicted from BOX-PCR results, may result from variable environmental conditions along the estuary that promote the adaptation of different strains. In fact, pronounced variations were registered for the measured parameters along the estuary and between campaigns: water temperature values ranged from (13.5 ºC to 27.7 ºC), salinity varied between 0.2 and 34 g/kg, and pH values ranged from 6.80 and 9.42. With this being said, all 77 isolates were kept for further analysis.

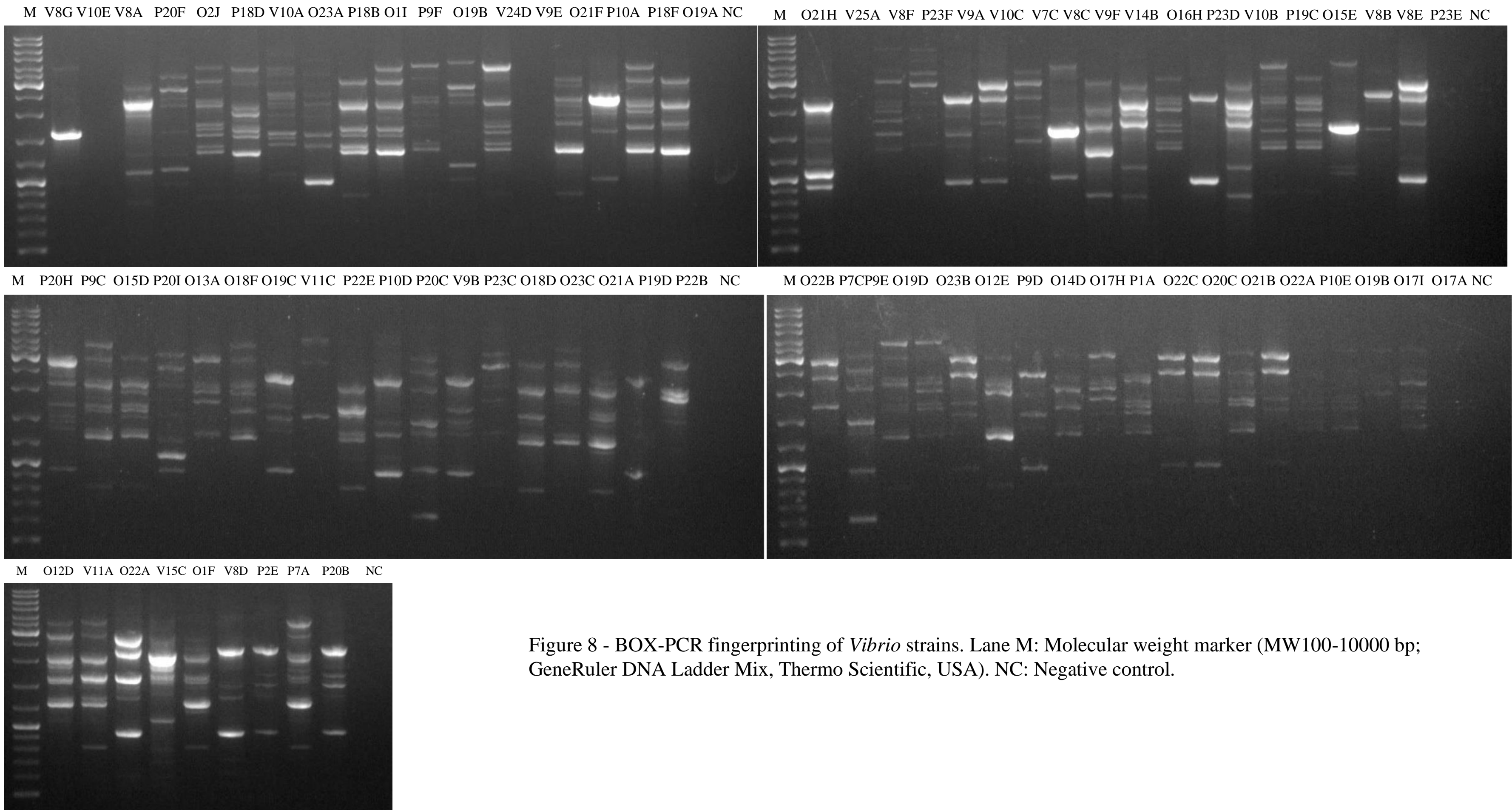
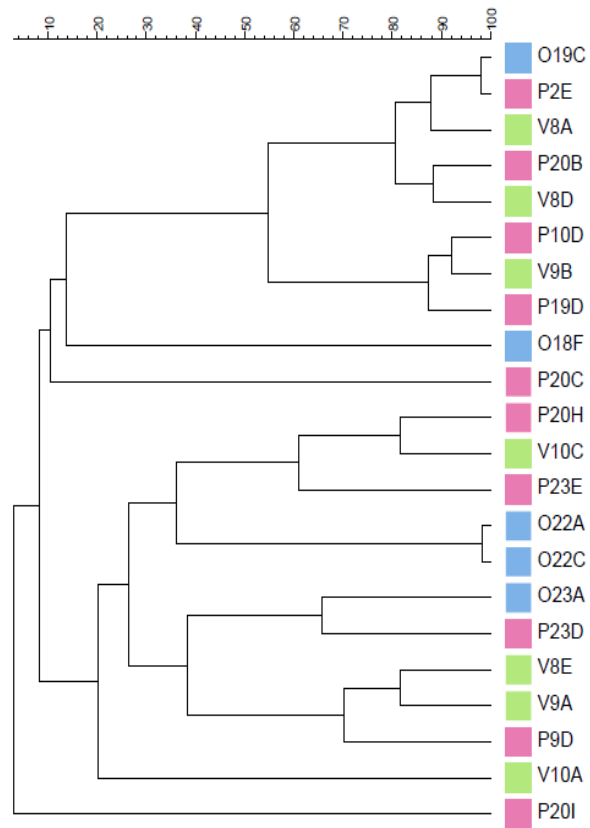
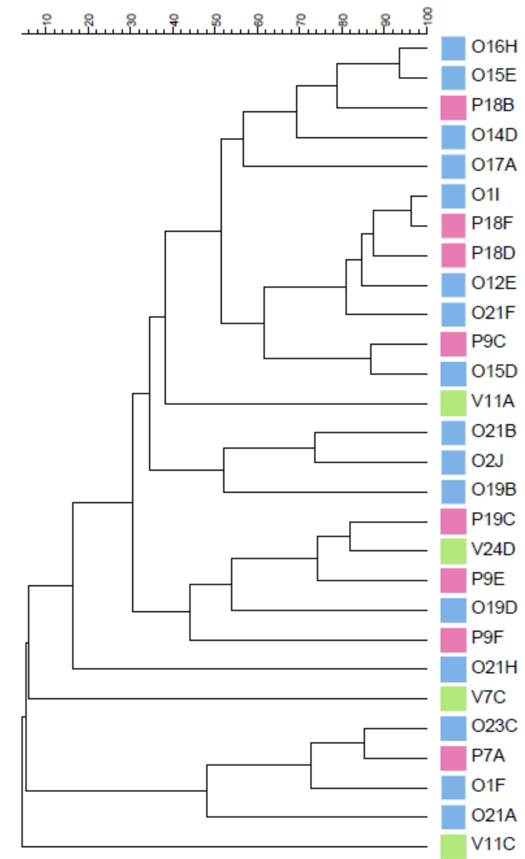


Figure 8 - BOX-PCR fingerprinting of *Vibrio* strains. Lane M: Molecular weight marker (MW100-10000 bp; GeneRuler DNA Ladder Mix, Thermo Scientific, USA). NC: Negative control.

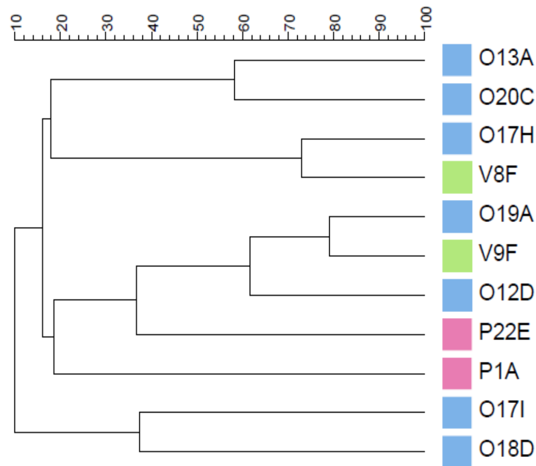
A



B



C



D

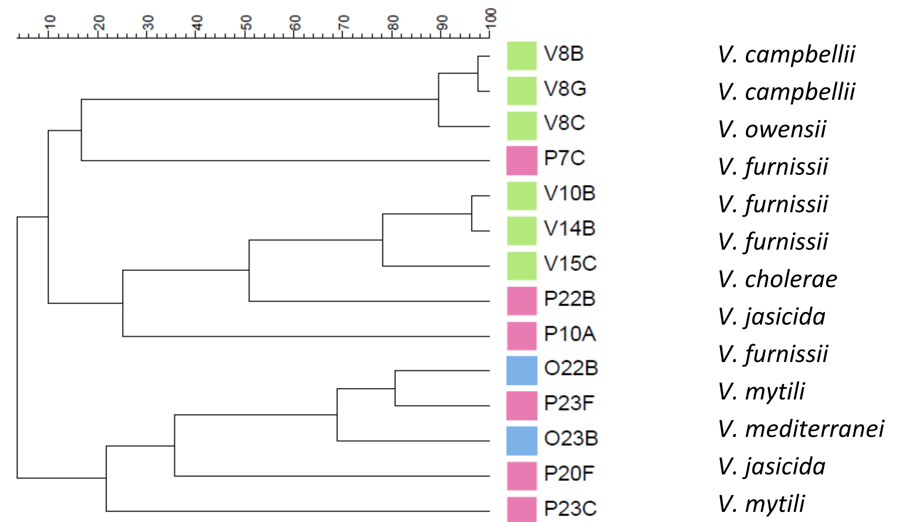


Figure 9 - UPGMA dendrogram based on the Jaccard similarity index obtained using BOX-PCR fingerprinting patterns of 77 *Vibrio* isolates from three different campaigns: Spring, Summer, and Autumn. Dendrograms A, B, C, and D correspond to *V. parahaemolyticus*, *V. diabolicus*, *V. alginolyticus*, and remaining isolates, respectively. Dendrograms were constructed using GelCompar II (Applied Maths, USA). Numbers on the horizontal axis indicate the percentage of similarity.

2. Salt tolerance of isolated *Vibrio* spp.

Vibrio species are generally halotolerant, with the exception of *V. mimicus* and *V. cholerae* (Thompson et al., 2004). To evaluate salt tolerance, the isolates were grown in TCBS supplemented with different NaCl concentrations, chosen according to the literature (Murad et al., 2010). Overall, all *Vibrio* isolates withstand salinities up to 5% (Figure 10), except *V. cholerae* isolate that stopped growing at 1% NaCl. 49% of the isolates were able to grow at 7.5% NaCl, while no growth was observed at 10% NaCl. No *V. campbellii*, *V. jasicida*, *V. mediterranei*, *V. fluvialis*, and *V. mytili* isolates grew at 7.5%, while 100% and 39% of *V. alginolyticus* and *V. diabolicus* isolates, respectively, tolerated this salinity level. Accordingly, studies suggest 4% is the most suitable salinity for the growth of *V. alginolyticus* (Dayma et al., 2015; Xu et al., 2005), but it can tolerate up to 9% salinity (Reyes-Velázquez et al., 2010). Also according to the literature, *V. mediterranei* and *V. fluvialis* grow until 6% NaCl (Kushmaro et al., 2001; Mahdi, 2007), and *V. campbellii* and *V. jasicida* seem to tolerate 5% salinity (Urbanczyk et al., 2015).
Only 2 *V. parahaemolyticus* isolates grew at 7.5% (9%), harboring 3 and 1 virulence factors. None of the strains with high pathogenicity potential, i.e. containing a higher number of virulence factors, was able to tolerate 7.5% NaCl. This is in accordance with Bauer et al., (2021) that observed higher numbers of potential pathogenic *V. parahaemolyticus* species harboring various pathogenicity factors at reduced salinity (1.5% NaCl).

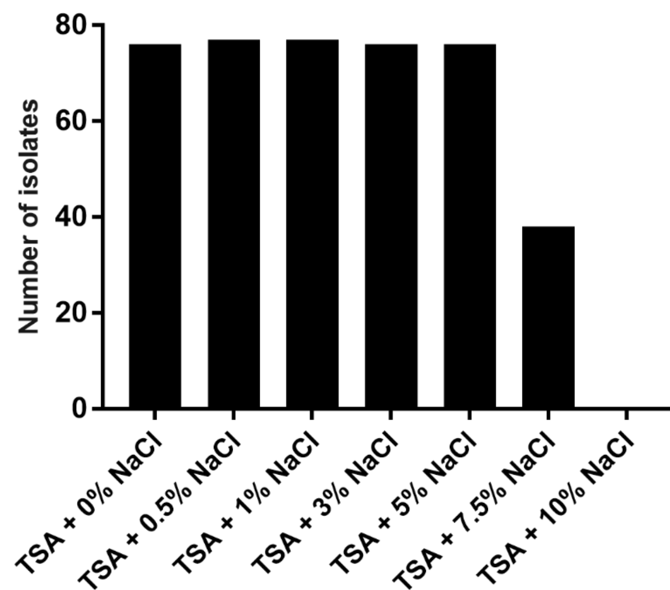


Figure 10 - Salt tolerance of *Vibrio* spp. isolates.

No association was detected between salt tolerance and sampling site or sampling season. In fact, the isolates with higher NaCl tolerance (7.5%) are mainly from the Autumn campaign (only 8 out

of 30 isolates did not show growth at this salinity) where the samples average salinity was 17.5 g/kg, while salinity values in most sampling sites were higher in the Summer, which showed an average salinity of 18.4 g/kg (Dias, 2020).
Half of the isolates tolerated salinities twice as high as the maximum measured in sampling sites, indicating that these isolates are halotolerant, which allows them to survive in the environment even at high salinity values. This may potentiate the dispersion of these isolates along the estuary and even to other coastal areas.

3. Antibiotic susceptibility profiles of *Vibrio* isolates

The 77 *Vibrio* isolates were tested against 8 different antibiotics from the main antibiotic classes: sulphonamides, fluoroquinolones, tetracyclines, macrolides, aminoglycosides, and β-lactams. The control strain *E. coli* (ATCC 25922) presented good bacterial growth and inhibition zones equal to the standards for each antibiotic tested.
As shown in Figure 11, there isn't a significant variance in antibiotic resistance levels per season. Overall, 78% of the isolates were resistant to amoxicillin and 34% presented intermediate susceptibility to erythromycin. More than 90% of the isolates showed susceptibility to gentamicin, ceftazidime, doxycycline, tetracycline, ciprofloxacin, and trimethoprim-sulfamethoxazole (SXT). β-lactams such as amoxicillin have been extensively used to treat bacterial infections (Ceccarelli et al., 2016). Due to that, many bacterial strains, including *Vibrio* strains, are known to produce extended-spectrum β-lactamases (ESBLs) that hydrolyze penicillins and extended-spectrum cephalosporins (Hernández-Robles et al., 2016). However, the *Vibrio* isolates in the collection are not resistant to third-generation cephalosporins. In this case, other resistance mechanisms are probably at play, including drug target modification, production of narrow-spectrum β-lactamases or cell impermeability (Livermore, 2012). Intrinsic resistance to erythromycin is expected in gram-negative bacteria due to the slow passage of these agents through the cell wall and their constant removal by multidrug efflux pumps (Nikaido, 1998).

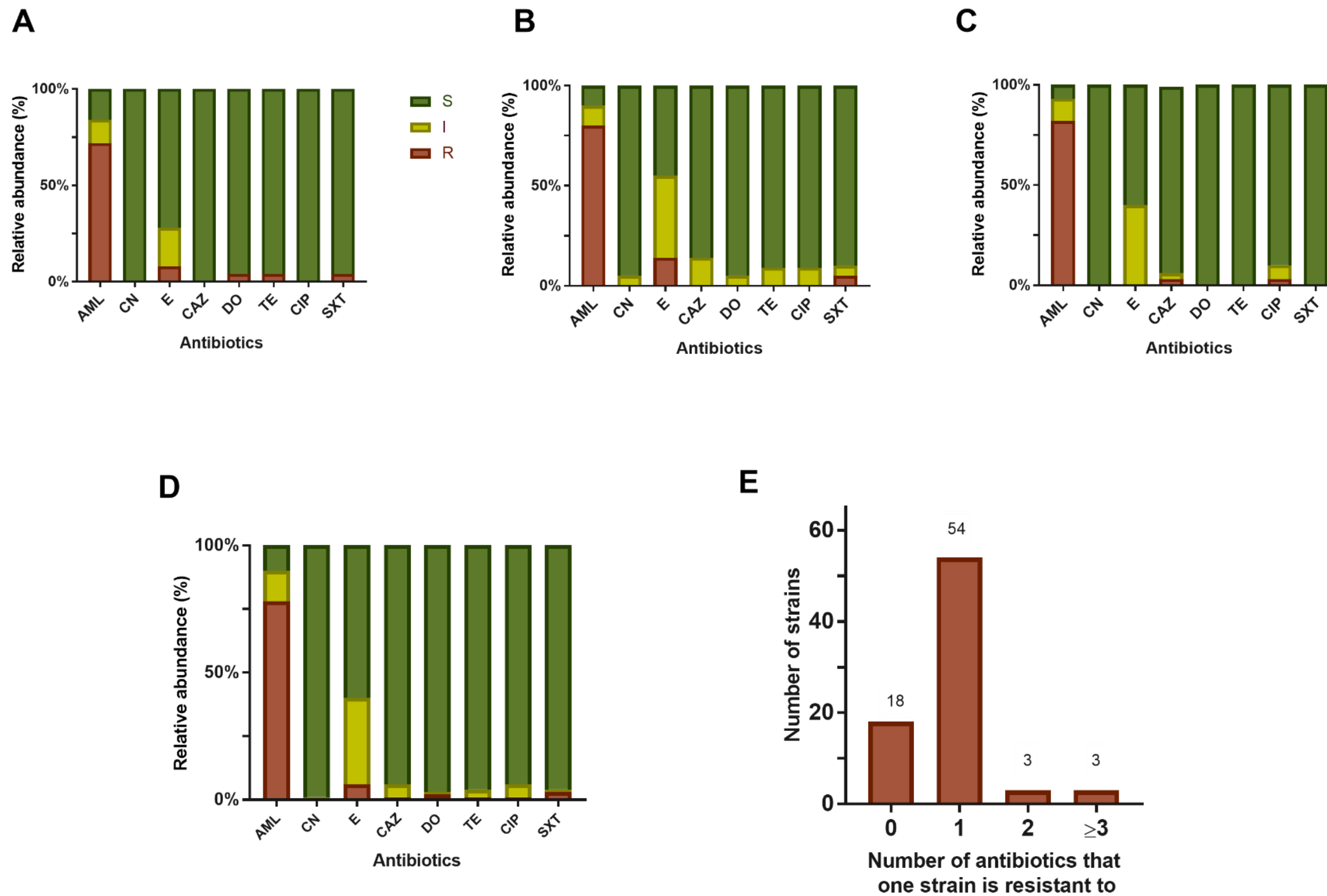


Figure 11 - Antibiotic resistance profiles of *Vibrio* isolates from different seasons, Spring (A), Summer (B), and Autumn (C). (D) Antibiotic profiles of the total number of *Vibrio* isolates included in this study. (E) Number of strains resistant to 0, 1, 2, 3 and more antibiotics.

Eighteen strains showed no resistance to the antibiotics tested, while the majority (54 out of 77 isolates) were resistant to one antibiotic, all of which were resistant to amoxicillin, except for *V. cholerae* that was resistant to trimethoprim-sulfamethoxazole. *V. cholerae* can suffer genetic mutations or acquire resistance genes through the exchange of genetic elements such as SXT element which confers resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (Iwanaga et al., 2004). Non-O1/non-O139 strains are usually susceptible to most antibiotics. However, resistance to streptomycin, carbapenems, sulfonamides, trimethoprim-sulfamethoxazole and ampicillin has been sporadically reported in environmental strains (Baron et al., 2016; Bier et al., 2015; Lepuschitz et al., 2019).

Three strains showed resistance to three or more antibiotics, all of which were resistant to amoxicillin. Besides this, *V. mytili* also showed resistance to erythromycin and doxycycline, *V. fluvialis* to erythromycin and trimethoprim-sulfamethoxazole, and *V. diabolicus* to ceftazidime and ciprofloxacin. Previous studies have found similar antibiotic resistance profiles in *V. mytili*, *V. fluvialis*, and *V. diabolicus* strains (Igbinosa et al., 2011; Scarano et al., 2014).

Aquatic environments such as estuaries and marine waters are subjected to environmental contamination from agriculture and aquaculture industry, a major factor in Ria de Aveiro, which can then lead to pollution by heavy metals and antibiotic residues, creating a selective pressure for aquatic bacteria (Siddique et al., 2021). Contrary to this assumption, the mean multi-antibiotic resistance index (MAR) of the *Vibrio* isolates was 0.11, indicating that the isolates were sampled from low antibiotic usage area (Adenaike et al., 2016). However, a higher number of antibiotics should be tested in future studies to better characterize the antibiotic resistance profiles of *Vibrio* isolates in this estuary.

The results also suggest low seasonal variability regarding antibiotic resistance, and One-way ANOVA showed no significant difference in MAR index between seasons (p = 0.850>0.05) nor between species (p =0.062>0.05). Another study showed similar results, with no significant correlation between the season of sampling and antibiotic resistance characteristics of *V. parahaemolyticus* collected from estuarine sites in Georgia and South Carolina, USA (Baker-Austin et al., 2008).

Though we registered lower levels of antibiotic resistance than those from previous studies done in estuarine environments (Kang et al., 2014; Silvester et al., 2015), they still highlight for the importance of routinely monitoring antibiotic resistance of *Vibrio*. Particularly, the fact that we have detected isolates with resistance to antibiotics that are regularly used to treat infections caused by *Vibrio* (e.g., ceftazidime, ciprofloxacin) raises concern about the possibility of dispersion of these traits among *Vibrio* present in the estuary.

4. Presence of antibiotic resistance genes in *Vibrio* spp. isolates

Taking the antibiotic resistance profiles into consideration, the presence of antibiotic resistance genes in the *Vibrio* isolates was inspected. *qnr* gene encodes a protein that binds to and protects DNA gyrase and topoisomerase IV from inhibition by quinolones. As of now, six Qnr proteins families have been described: QnrA, QnrB, QnrC, QnrD, QnrE, QnrS, and QnrVC (Zhang et al., 2018). All 77 *Vibrio* isolates were searched for *qnrVC1*, *4*, *7*, and *9* and we obtained a correct-sized band in 32 isolates. Of those, 15 were sent for sequencing, however it was only possible to confirm the identity of the fragments in 4 (5%) isolates: *V. parahaemolyticus* (V9B), *V. diabolicus* (V11C), *V. furnissii* (O22B), and *V. cholerae* (P22B) (Table 6). This could be due to the unspecific amplification of other fragments which affected the sequence quality.

Table 6 - Antibiotic resistance genes identification, gene fragment, % of identity to the closest BLAST search result, and respective accession number

Isolates	Species	Sequenced fragment	% Nucleotide Identity	Fragment size	Accession number
P22B	*V. cholerae*	*qnrVC*	99.83	587	JQ838003.1
V9B	*V. parahaemolyticus*	*qnrVC*	100	393	JQ838003.1
V11C	*V. diabolicus*	*qnrVC*	100	568	JQ838003.1
O22B	*V. furnissii*	*qnrVC*	100	570	JQ838003.1
V8A	*V. parahaemolyticus*	bla_{TEM}	100	742	MW881231.1
V8D	*V. parahaemolyticus*	bla_{TEM}	99.48	766	MW567498.1
O22A	*V. parahaemolyticus*	bla_{TEM}	99.48	762	MW567498.1
P2E	*V. parahaemolyticus*	bla_{TEM}	99.87	767	MW567498.1
V9E	*V. owensii*	bla_{TEM}	100	746	MW881231.1
V14B	*V. furnissii*	bla_{TEM}	99.74	765	MW567498.1
V8C	*V. campbellii*	bla_{TEM}	99.87	763	MW567498.1
P18F	*V. diabolicus*	bla_{TEM}	99.74	768	MW567498.1
P20F	*V. jasicida*	bla_{TEM}	99.74	766	MW567498.1
P23F	*V. mytili*	*tetB*	100	344	NG_048172.1

The about 520 nucleotide sequences were aligned in Clustal Omega (EMBL-EBI, UK) and showed 100% identity with each other and with the gene that codes for pentapeptide-repeat protein QnrVC4. To our knowledge, there are no reports of *V. diabolicus* and *V. furnissii* harboring *qnrVC* genes, possibly due to the lack of studies performed on these species. On the other hand, *V. cholerae* is a known carrier of *qnrVC* genes, many times associated with MGE (Fonseca et al., 2008; Kumar & Thomas, 2011). The presence of these genes in *V.*

parahaemolyticus has been documented as well (Zhang et al., 2018). None of the *qnrVC*-harboring isolates were resistant to ciprofloxacin. Zhao et al., (2018) stated that only 36% of *V. parahaemolyticus* were positive for *qnrVC* and none showed resistance to ciprofloxacin. This could be due to the genotype not being expressed as a result of varying growth conditions from the natural environment the isolates were collected from. For example, the antibiograms were performed in medium that did not contain NaCl and the phenotype can be influenced by the presence of this supplement. Another explanation is that these genes, as other *qnr* genes, confer low-level antibiotic resistance, not adding to the phenotypic resistance (Salah et al., 2019).

A set of primers was designed to amplify other *qnrVC* variants, namely *qnrVC3*, *5*, *6,* and *10*. Several PCR conditions were tested with different annealing temperatures: 62ºC, 55ºC, and 58ºC. Nevertheless, it was not possible to obtain an isolated band with the expected size with any of the PCR conditions. As we did not possess a positive control for these variants, we cannot positively confirm the gene was not present.

For clinical *V. parahaemolyticus*, a prevalence of 28.6% for the *qnrA* gene was previously reported (Li et al., 2017), while for environmental *V. alginolyticus* a study showed that 47% carried the *qnrA* gene, and 14% of these were not resistant to ciprofloxacin (Drais et al., 2018). In this study, the presence of *qnrA* was not detected in any of the isolates.

Furthermore, the presence of genes encoding β-lactamases, namely bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$, was evaluated. No bla_{SHV} was detected, while nine isolates from the species *V. parahaemolyticus*, *V. owensii*, *V. campbellii*, *V. diabolicus*, *V. furnissii*, and *V. jasicida* harbored the bla_{TEM} gene (Table 6). All nine nucleotide fragments were aligned using Clustal Omega (EMBL-EBI, UK) and showed 100% identity with each other and with the gene encoding for TEM-116. TEM-116 is a relevant extended-spectrum β-lactamase, widespread in clinical and environmental settings (Naidoo et al., 2020). It has been reported in many bacterial species, including *E. coli*, *Aeromonas*, and *Klebsiella* (Carminato et al., 2010; Mondal et al., 2018; Vignoli et al., 2005). This gene has been detected in 100% *V. parahaemolyticus* isolated from oysters in Brazil but in none of the other species (Rojas et al., 2011). $Bla_{\text{TEM-116}}$ has been cloned and used in pUC vectors present in some *Taq* polymerase reactions, leading to erroneous reports of TEM-116 in organisms that don't present it (Jacoby & Bush, 2016). That is not the case in this work, since all isolates were subjected to the same PCR preparations and amplification protocol and only nine were positive for TEM-116. Only one *V. diabolicus* isolate showed resistance to ceftazidime but $bla_{\text{CTX-M}}$ was not detected.

One *V. mytili* isolate showed resistance to tetracycline, while one *V. campbelli* and one *V. fluvialis* isolates presented intermediate susceptibility. The three were searched for tetracycline resistance genes, including *tetA*, *B*, *C*, *D*, *E*, and *M*. None of these genes were detected in *V. campbelli* and *V. fluvialis*, only *V. mytili* produced a band that showed 100% identity with *tetB* (Table 6), which

encodes for efflux proteins (Beheshti et al., 2020). A study conducted in China detected a prevalence of 100% of *tetB* in tetracycline-resistant *Vibrio* spp. (Kim et al., 2007).

All the genes searched for in this work, with the exception of *qnrVC* for which its intrinsic nature is still debatable (Poirel et al., 2005b), were acquired by the strains, not being a part of their intrinsic resistome. Even with low prevalence, they may be transferred to other bacteria, disseminating in the environment the resistance genotype, and contributing to the rise of antibiotic resistance in the estuary Ria de Aveiro.

5. Analysis of virulence factors of *Vibrio* spp.

A total of 7 virulence-associated genes (*chiA, vhpA, luxR, flaC, hlyA, tlh,* and $toxR_{VC}$) were detected by PCR amplification. Four virulence genes, *chiA*, *luxR*, *tlh*, and *flaC*, were frequently detected in the *Vibrio* isolates, with positive rates of 86%, 62%, 60%, and 30%, respectively. Less common in the collection were *vhpA* with a detection rate of 5%, and *hlyA* and $toxR_{VC}$, present in 1% of the isolates (Figure 12). Regarding the species' distribution, it can be observed that for *V. campbellii*, *V. mediterranei*, *V. furnissii*, and the most prevalent species (*V. parahaemolyticus*, *V. diabolicus*, and *V. alginolyticus*), 50% of the isolates contain at least 3 virulence factors, while *V. mytili* and *V. fluvialis* are the species with the least virulence factors.

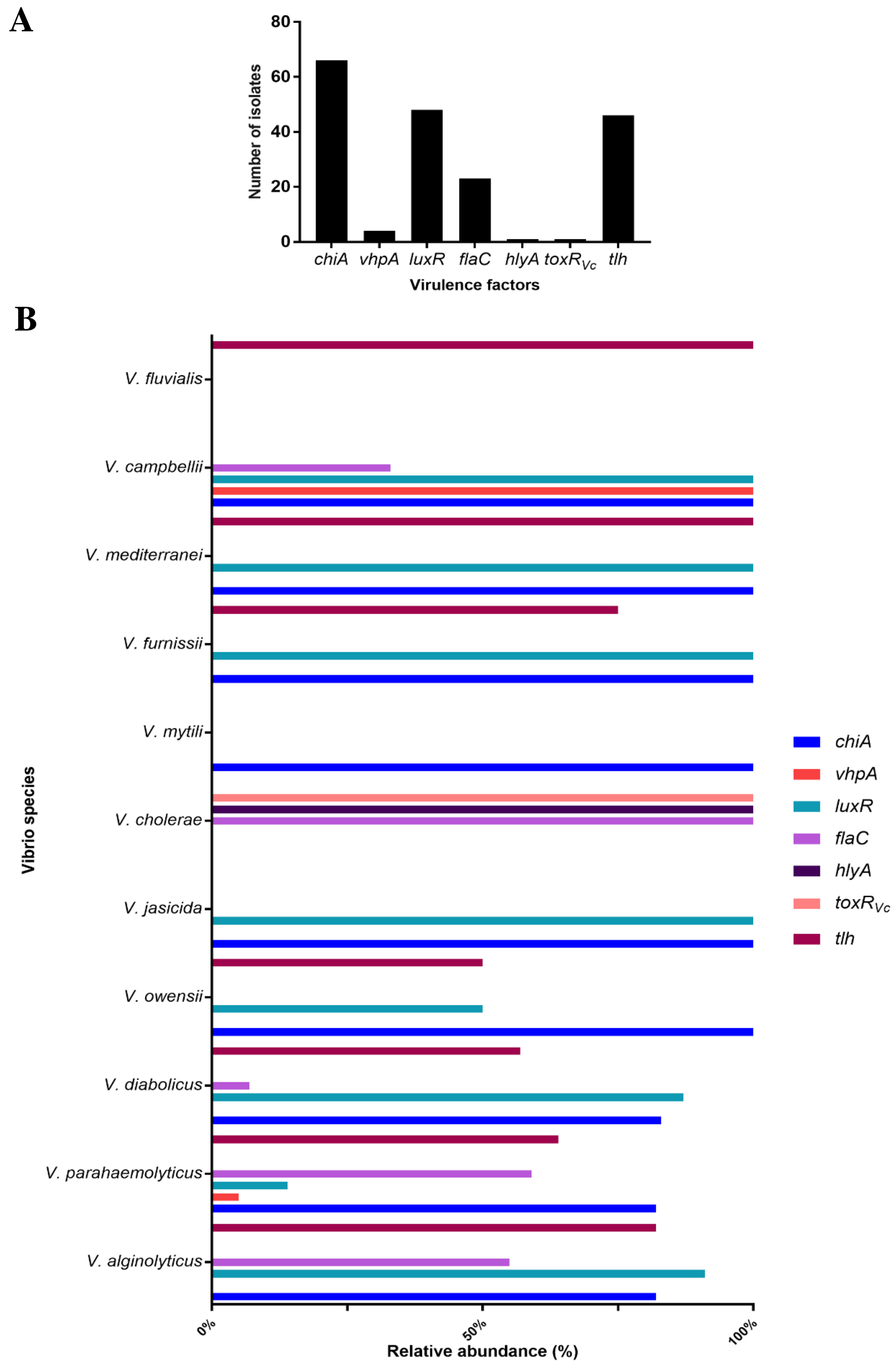


Figure 12 - (A) Relative abundance of isolates containing virulence genes. (B) Virulence genes distribution per *Vibrio* species.

The identity results of the sequenced PCR fragments are presented in Table 7. Regarding *chiA*, we obtained a band with 232 bp in 66 isolates and two were sent for sequencing, where we were able to confirm the identity of the fragments. Sequence similarity analysis using BLAST (Basic, Local Alignment Search Tool, NCBI) showed the amplified fragments to be related (>95%) to *V. parahaemolyticus chiA*. When it comes to amplification of *vhpA*, a band with 201 bp was obtained in 4 isolates, with two sent for sequencing and identity of the fragments confirmed. Both amplified fragments showed 100% identity with *V. harveyi vhpA* gene. A 580 bp band of *V. anguillarum flaC* gene was found in 23 isolates and two of them, after sequencing and alignment, presented high similarity (>99%) with *V. parahaemolyticus flaA*. *V. anguillarum* FlaC has 72% identity with FlaB of the same organism which in turn, shows 80% identity with *V. parahaemolyticus* FlaA (McGee et al., 1996). Forty-eight isolates amplified a 618 bp of *V. harveyi luxR* gene. The amplified fragments showed similarity (>93%) with *V. alginolyticus luxR*. Bands with 738 and 883 bp of *V. cholerae hlyA* and $toxR_{VC}$, respectively, were only found in the *V. cholerae* isolate. BLAST showed the amplified fragments to be highly similar (>99%) to *V. cholerae hlyA* and $toxR_{vc}$. A 450 bp band regarding *V. parahaemolyticus tlh* gene, was amplified in 46 isolates. BLAST alignment showed high similarity (>99%) with the same species' *tlh* gene. The sequences of *V. harveyi chiA*, *luxR*, and *V. anguillarum flaC* genes showed similarity to the sequences of *V. parahaemolyticus chiA*, *V. alginolyticus luxR*, and *V. parahaemolyticus flaA* genes, respectively. Most species in the collection belong to the Harveyi clade, which includes *V. harveyi*, *V. parahaemolyticus*, *V. rotiferianus*, *V. azureus*, *V. campbellii*, *V. owensii*, *V. natriegens*, *V. mytili*, *V. jasicida*, and *V. alginolyticus* (Urbanczyk et al., 2013). Therefore, a high prevalence of typical Harveyi clade genes (*chiA*, *luxR*, *vhpA*, and *tlh*) was expected. In this study, these genes were also detected in atypical hosts, such as *V. furnissii*, *V. mediterranei*, *V. fluvialis*, and *V. diabolicus*. Other works also detected the presence of these genes in atypical hosts (Deng et al., 2020; Mohamad et al., 2019; Ruwandeepika et al., 2010).

chiA, *luxR*, and *vhpA* are three typical *V. harveyi* virulence genes (Ruwandeepika et al., 2010). *chiA* was the most distributed gene amongst the *Vibrio* isolates in our collection, only absent in *V. cholerae* and *V. fluvialis* isolates. It is known that chitinases play an important part in the attack of host cells in *V. cholerae* (Frederiksen et al., 2013), so other enzymes besides the one encoded by *chiA* may be produced by *V. cholerae*. *vhpA* was present in all *V. campbellii* isolates (100%) and had a prevalence of 5% in *V. parahaemolyticus*. *V. harveyi* and *V. campbellii* are closely-related species, with 61-74% DNA-DNA similarity and over 97% 16S rDNA similarity (Ruwandeepika et al., 2012). This could explain the shared virulence genes between these species. Mohamad et al. (2019) reported the presence of *vhpA* in 100% of *V. campbellii* isolates, along with more than 50% of *V. parahaemolyticus* isolates from cultured marine fishes.

Previous studies have considered the *tlh* gene to be a specific marker for *V. parahaemolyticus* DePaola et al., 2003; Lovell, 2017). However, in this study only 64% of *V. parahaemolyticus* isolates carried the *tlh* gene, with other species such as *V. mediterranei* and *V. fluvialis* showing 100% prevalence. This indicates that detection of *V. parahaemolyticus* through *tlh* amplification is not a reliable method since it can occur in other species and may be absent from some *V. parahaemolyticus* strains. Similar findings have been reported by Yáñez et al., (2015).

Genes $toxR_{VC}$ and *hlyA* correspond to a virulence gene regulator and a gene encoding an hemolysin, respectively, and are typical of *V. cholerae* (Hasan et al., 2013). Their presence has been previously reported in *V. parahaemolyticus, V. harveyi*, *V. alginolyticus*, and *V. campbellii* (Ruwandeepika et al., 2012). However, in this study, $toxR_{VC}$ and *hlyA* were only present in the *V. cholerae* isolate.

Vibrio anguillarum's flagellum is encoded by the genes *fla*ABCDE, with *flaC* playing a role in mobility and virulence of *V. anguillarum* (Hickey & Lee, 2018). *flaC* was detected in *V. campbellii*, *V. diabolicus*, *V. cholerae*, *V. parahaemolyticus*, and *V. alginolyticus*, being more prevalent in the last 3 species. Mohamad et al., (2019) reported similar results, with *V. parahaemolyticus*, *V. alginolyticus*, and *V. campbellii* harbouring *flaC* gene.

Table 7 - Percentage of similarity of *chiA*, *vhpA*, *tlh*, *flaC*, *luxR*, *hlyA*, and $toxR_{VC}$ sequenced PCR fragments to the corresponding genes in the GenBank database

Isolate	Species	Sequenced fragment	% Nucleotide Identity	Fragment size	Accession number
O17I	*V. alginolyticus*	*chiA*	95.00	194	MW168988.1
O21F	*V. diabolicus*	*chiA*	96.20	190	AF323471.1
V8C	*V. campbellii*	*vhpA*	100	156	AY630354.1
V8B	*V. campbellii*	*vhpA*	100	154	AY630354.1
P23D	*V. parahaemolyticus*	*flaC*	99.26	540	GQ433373.1
O22C	*V. parahaemolyticus*	*flaC*	99.07	542	GQ433373.1
P19C	*V. diabolicus*	*luxR*	93.85	569	EF596781.1
O16H	*V. diabolicus*	*luxR*	93.93	569	EF596781.1
O22A	*V. parahaemolyticus*	*tlh*	100	391	JX262989.1
O19C	*V. parahaemolyticus*	*tlh*	99.21	380	JX262977.1
P22B	*V. cholerae*	*hlyA*	99.13	690	MF099997.1
P22B	*V. cholerae*	$toxR_{VC}$	99.39	820	MF099924.1

6. Presence of mobile genetic elements in the *Vibrio* collection

Integrons are important to the rapid dissemination and evolution of antimicrobial resistance, with classes 1, 2, and 3 integrons being the main integrons related to antibiotic resistance present in pathogens (Gillings, 2014). The integrase region of 280, 233, and 600 bp of class 1, 2, and 3 integrons was not found in any of the *Vibrio* isolates. Sá et al., (2010) observed an incidence of class 1 integrons of 5% in environmental non-O1/O139 *V. cholerae* strains. In this study we did not find a relationship between antibiotic resistance profiles and the occurrence of integrons. Other studies have shown similar results, with *Vibrio* spp. isolated from estuarine and marine environments exhibiting antibiotic resistance and carrying antibiotic resistance genes but not harboring integrons (Jeamsripong et al., 2020; Sulca et al., 2018). Different mobile genetic elements, such as plasmids and transposons may be involved in the transmission of resistance.

The *Vibrio* collection was also searched for the presence of plasmids. Twenty-three strains (30%) belonging to *V. alginolyticus* (6), *V. campbellii* (3), *V. cholerae* (1), *V. diabolicus* (5), *V. owensii* (2) and *V. parahaemolyticus* (6) were found to contain plasmids, whereas we couldn't extract plasmids from 54 (70%) isolates (Figure 13). Amalina et al., (2019) also detected a low prevalence of plasmid-harboring strains (39%) in *Vibrio* spp. isolated from aquaculture fish.

Out of all the strains containing plasmids, 4 harbor bla_{TEM} gene (*V. parahaemolyticus* (2), *V. campbellii* (1), and *V. owensii* (1)) and 2 *qnrVC* gene (*V. cholerae* (1) and *V. diabolicus* (1)), whilst in 8 isolates in which antibiotic resistance genes were found no plasmid was detected. A study conducted in Malaysia determined that the majority of *V. parahaemolyticus* strains isolated from seawater harbored antibiotic resistance genes but lacked plasmids (Faja et al., 2019). The strains containing plasmids also showed variable number of virulence factors, ranging from 1 to 5 genes. In the present study, we couldn't find any correlation between antibiotic resistance genes and virulence factors and presence of plasmids. However, we cannot exclude the presence of plasmids that were not purified using the method here applied. For instance, the kit here used is optimized for clinical strains containing small plasmids and the extraction of larger plasmids (>10.000 bp) may be inefficient.

Even so, integrons and plasmids don't seem to be the main mobile genetic elements responsible for the transmission of ARGs and virulence factors among *Vibrio* in the estuary Ria de Aveiro. Other MGEs should be searched in the *Vibrio* collection to better characterize the potential for horizontal gene transfer of these strains.

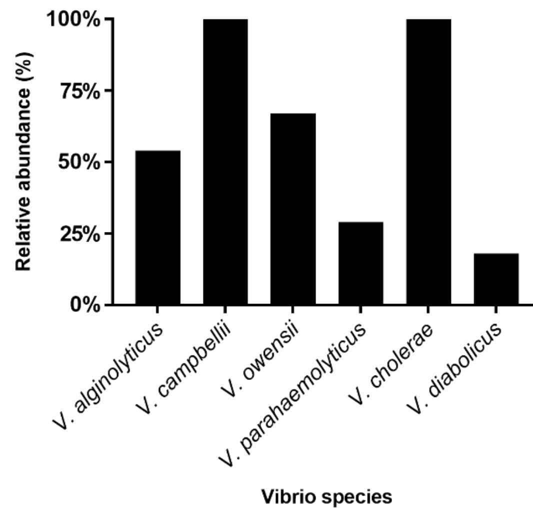


Figure 13 - Plasmid distribution per *Vibrio* species.

7. Production of extracellular enzymes and hemolysins by *Vibrio* spp. isolates

Virulence-related activities were searched among the *Vibrio* isolates, including amylase, caseinase, lipase, phospholipase, DNase, hemolysin, and gelatinase activities. Amylase, DNase, lipase, and caseinase were the enzymes most frequently detected, while gelatinase, hemolysin, and phospholipase were rarely detected. *V. mytili* and *V. mediterranei* isolates showed no extracellular activity. The most prevalent species (*V. diabolicus*, *V. parahaemolyticus* and *V. alginolyticus*) showed a higher number of extracellular activities (Table 8). One-way ANOVA showed that no seasonal variability was detected ($p<0.05$), nor between species ($p<0.05$).

Table 8 - Extracellular activity of the *Vibrio* isolates. Values presented are the number of isolates per *Vibrio* species. The color code is as follow: the number of isolates of each species is represented in a white – green color gradient and increase from white (low) to dark green (high). +++, ++, +, and – represent high, medium, low, and no extracellular activity, respectively

		Species											
		V. parahaemolyticus	*V. diabolicus*	*V. alginolyticus*	*V. owensii*	*V. jasicida*	*V. cholerae*	*V. mytili*	*V. furnissii*	*V. mediterranei*	*V. campbellii*	*V. fluvialis*	Total
	Total number of isolates	22	30	11	3	2	1	2	4	1	3	1	77
Amylase	+++	0	2	3	0	0	0	0	0	0	0	0	5
	++	1	4	2	0	0	0	0	0	0	1	0	8
	+	8	9	4	0	0	1	0	1	0	0	0	23
	-	13	14	2	3	2	0	2	3	1	2	1	43
Caseinase	+++	2	1	0	0	0	0	0	0	0	0	0	3
	++	1	0	0	0	0	0	0	1	0	0	1	3
	+	5	3	0	0	0	0	0	2	0	0	0	10
	-	14	26	11	3	2	1	2	1	1	3	0	64
Lipase	+++	1	2	3	0	0	0	0	0	0	0	0	6
	++	0	6	3	0	0	0	0	0	0	0	0	9
	+	3	2	1	0	0	0	0	0	0	1	0	7
	-	18	20	4	3	2	1	2	4	1	2	1	58
Phospholipase	+++	0	0	0	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0	0	0	0
	+	1	0	0	0	0	0	0	2	0	0	0	3
	-	21	30	11	3	2	1	2	2	1	3	1	67
Dnase	+++	0	0	0	0	0	0	0	0	0	0	0	0
	++	12	0	1	1	1	0	0	1	0	0	1	17
	+	8	3	1	0	1	1	0	3	0	0	0	17
	-	2	27	9	2	0	0	2	0	1	3	0	46
Hemolysin	β-hemolysin	1	1	0	0	0	1	0	3	0	0	1	7
	-	21	29	11	3	2	0	2	1	1	3	0	73
Gelatinase	+++	0	1	0	0	0	0	0	0	0	0	0	1
	++	1	1	0	0	0	0	0	0	0	0	0	2
	+	1	4	2	0	0	1	0	0	0	0	0	8
	-	20	24	9	3	2	0	2	4	1	3	1	69

Not many reports evaluate amylase activity from *Vibrio* spp., but some *Vibrio* amylases have been reported by Najafi & Kembhavi (2005), Hörmansdorfer et al. (2000) and Kim et al. (1999). In this work, 36 isolates (47% of the total number of isolates) from *V. parahaemolyticus* (n=9; 40% of the total number of isolates of this species), *V. diabolicus* (n=15; 50%), *V. alginolyticus* (n=9; 82%), *V. cholerae* (n=1; 100%), *V. furnissii* (n=1; 25%), and *V. campbellii* (n=1; 33%) species showed amylase activity. Caseinase and gelatinase are both proteases. However, the *Vibrio* isolates showed a variation in the ability to hydrolyze casein and gelatin. Chi et al., (2018) obtained similar results with *V. parahaemolyticus* collected from shrimp. In this work 16 isolates (20% of the total number of isolates) from *V. parahaemolyticus* (n=8; 36% of the total number of isolates of this species), *V. diabolicus* (n=4; 13%), *V. furnissii* (n=3; 75%), and *V. fluvialis* (n=1; 100%) species showed caseinolytic activity. At the same time, 11 (14% of the total number of isolates) isolates from *V. parahaemolyticus* (n=2; 9%), *V. diabolicus* (n=6; 20%), *V. alginolyticus* (n=2; 18%), *V. cholerae* (n=1; 100%) species presented gelatinase activity. Only 3 *V. diabolicus* isolates from the same sampling area, Aveiro, showed both caseinase and gelatinase activities.
Regarding lipolytic activity, 22 (28% of the total number of isolates) from *V. parahaemolyticus* (n=4; 18% of the total number of isolates of this species), *V. diabolicus* (n=10; 33%), *V. alginolyticus* (n=7; 63%), and *V. campbellii* (n=1; 33%) species showed positive results. This prevalence is lower than previously reported in studies where lipase activities were detected in more than 80 % of the environmental *Vibrio* isolates (Baffone et al., 2001; Masini et al., 2007). Phospholipase showed the lowest activity, with only *V. parahaemolyticus* (n=1; 4% of the total number of isolates of this species) and *V. furnissii* (n=2; 50%) presenting enzymatic activity.
DNase was the second most frequently detected activity. All *V. jasicida*, *V. cholerae*, *V. furnissii*, and *V. fluvialis* isolates showed DNase activity. From the remaining species, 86% (n=20) *V. parahaemolyticus*, 10% (n=3) *V. diabolicus*, 18% (n=2) *V. alginolyticus*, 33% (n=1) *V. owensii* isolates presented DNase activity. Previous reports have stated similar results, with DNase being one of the exoenzymes with higher activity in *Vibrio* (Costa et al., 2013; Silva et al., 2018). About 4% (n=1) and 3% (n=1) of *V. parahaemolyticus* and *V. diabolicus* isolates, respectively, showed hemolytic activity. As previously mentioned, *V. fluvialis* and *V. flurnissii* are closely-related species (Schirmeister et al., 2014) and belong to the Cholerae clade, along with *V. cholerae* (Sawabe et al., 2013). *V. cholerae*, *V. furnissii*, and *V. fluvialis* showed the highest hemolytic activity, with 100% (n=1), 75% (n=3), and 100% (n=1) isolates, respectively. Given the low hemolytic activity, hemolysins don't seem to be the main extracellular enzymes produced by the *Vibrio* isolates.
The results suggest that several estuarine *Vibrio* strains possess the ability to express exoenzymes and other activities associated with virulence and pathogenicity.

8. Biofilm formation of estuarine *Vibrio* spp.

Out of the *Vibrio* collection, 14 strains were selected, based on the presence of virulence factors and on their antibiotic resistance profiles, to evaluate their capacity for biofilm formation. Two assays were performed in duplicate at different salinities: 1% NaCl and 2.5% NaCl. *V. cholerae* isolate was classified as a biofilm producer in all the experiments, which is in agreement with the fact that this bacterium is a known biofilm producer (He et al., 2012). In both assays conducted in medium supplemented with 1% NaCl, *V. campbellii* V8B was classified as weak biofilm-producer. Other studies classified *V. campbellii* strains as strong biofilm producers (Bopitiya et al., 2021; Kavita et al., 2013). For the remaining isolates, there was a lack of reproducibility between experiments (Table 9). Even so, other 5 isolates were classified as biofilm producers in at least two experiments, namely *V. mytili* P23F, *V. diabolicus* O21H and V7C, *V. alginolyticus* O19A and V8F.

The inconsistency between the independent experiments may be due to the strains being in different growth stages or variations in the inoculum (Azeredo et al., 2017; Kragh et al., 2019). More experiments should be performed in order to accurately determine biofilm producers.

Table 9 - Biofilm formation of selected *Vibrio* isolates. Values are presented as mean +/- standard deviation. The color code (red, green, and white) is as follow: (a) red - strong-producer; (b) green - weak-producer; (c) white - non-producer. Data for biofilm production are average values from the five replicates, in which BFI was determined as ration O.D.590nm/O.D.600nm

Strains	Species	Biofilm production (BFI)			
		1% NaCl (1st assay)	1% NaCl (2nd assay)	2.5% NaCl (1st assay)	2.5% NaCl (2nd assay)
P23F	*V. mytili*	0.207 ± 0.049	0.067 ± 0.011	0.111 ± 0.010	0.219 ± 0.032
V8A	*V. parahaemolyticus*	0.065 ± 0.006	0.056 ± 0.004	0.112 ± 0.011	0.059 ± 0.005
P22B	*V. cholerae*	0.250 ± 0.086	0.102 ± 0.027	0.694 ± 0.076	0.198 ± 0.011
V8B	*V. campbellii*	0.111 ± 0.012	0.095 ± 0.016	0.163 ± 0.031	0.060 ± 0.004
V14B	*V. furnissii*	0.079 ± 0.009	0.074 ± 0.008	0.151 ± 0.018	0.053 ± 0.004
O21H	*V. diabolicus*	0.118 ± 0.014	0.046 ± 0.004	0.112 ± 0.019	0.096 ± 0.025
V15C	*V. furnissii*	0.084 ± 0.011	0.038 ± 0.004	0.115 ± 0.018	0.055 ± 0.006
O19A	*V. alginolyticus*	0.071 ± 0.006	0.075 ± 0.012	0.172 ± 0.029	0.082 ± 0.014
V25A	*V. fluvialis*	0.071 ± 0.010	0.058 ± 0.007	0.153 ± 0.017	0.105 ± 0.021
V8F	*V. alginolyticus*	0.072 ± 0.009	0.088 ± 0.012	0.158 ± 0.013	0.079 ± 0.011
P20B	*V. parahaemolyticus*	0.051 ± 0.006	0.047 ± 0.004	0.147 ± 0.023	0.076 ± 0.007
P18B	*V. diabolicus*	0.078 ± 0.008	0.063 ± 0.009	0.137 ± 0.017	0.053 ± 0.004
V7C	*V. diabolicus*	0.127 ± 0.012	0.061 ± 0.009	0.179 ± 0.047	0.094 ± 0.008
O12D	*V. alginolyticus*	0.060 ± 0.006	0.058 ± 0.009	0.130 ± 0.022	0.067 ± 0.010

FINAL CONCLUSIONS

This study aimed to investigate the possible risks to human and animal health represented by *Vibrio* isolates previously obtained from the estuary Ria de Aveiro in three different campaigns (Autumn, Spring, Summer). The collection is genetically diverse, with no seasonal cluster observed. This heterogenous distribution is probably related to the environmental strong gradients throughout the estuary. Notably, the *Vibrio* isolates tolerated high salinity levels, which can lead to their persistence in the estuary and even dispersion to other coastal areas.

Many of the species collected along the estuary are considered to be pathogenic to humans and/or animals, being major aquaculture pathogens. The results obtained in this study show a high prevalence and wide distribution of virulence-associated genes, with many of them detected in atypical hosts. Many species also showed extracellular activities associated with pathogenicity. Besides this, several isolates seem to be biofilm producers at different salinities, as for example those belonging to *V. cholerae* and *V. campbellii*, thus promoting survival in the estuary by retaining nutrients and conferring protection to substances like antibiotics. Additionally, this study shows high antibiotic resistance to amoxicillin, as well as the presence of antibiotic resistance genes in several isolates that are not part of their intrinsic resistome. Resistance to other antibiotics, although rare, is worth to mention since some of these antibiotics are used to treat *Vibrio* infections. Several *Vibrio* strains also harbored plasmids, which may contribute to the dissemination of antibiotic resistance and virulence-associated genes in the estuarine environment.

In summary, our results raise concerns, with possible animal and/or human pathogens harboring several virulence factors persisting in the estuary where many anthropogenic activities take place, including fish and seafood production and recreational activities, resulting in the exposure of marine animals and human populations to pathogens. Furthermore, the use of antibiotics in aquaculture and hospital settings leads to the contamination of the estuary and exerts a selective pressure for antibiotic-resistant *Vibrio*, promoting the transfer of antibiotic resistance genes between species and incrementing the estuarine resistome. This work also highlights the importance of developing alternative and more effective methods for treating infections caused by pathogenic *Vibrio*, as well as the continuous monitoring of *Vibrio* strains in the estuary Ria de Aveiro in order to prevent infections and combat antibiotic resistance.

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