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COIMBRA

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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

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Palavras-chave Resistência a antibióticos, *Vibrio* spp., ambientes estuarinos, virulência, elementos genéticos móveis.

Resumo

As bactérias do género *Vibrio* encontram-se abundantemente distribuídas por diversos ambientes marinhos, ecossistemas estuarinos e sistemas de aquacultura, podendo ser também isoladas de amostras clínicas e alimentares. Em sistemas de aquacultura, infeções bacterianas causadas por espécies de *Vibrio* (e.g. vibriose) são responsáveis por elevadas taxas de mortalidade e por quebras de produtividade. O uso intensivo de antibióticos, como agentes profiláticos ou terapêuticos, tem levado ao aumento da prevalência de estirpes de *Vibrio* multirresistentes. Antibióticos da classe das tetraciclinas são usados frequentemente para tratamento de infeções causadas por *Vibrio*, nomeadamente em animais.

Este estudo teve como objetivo investigar a distribuição de espécies de *Vibrio* resistentes a tetraciclina no estuário Ria de Aveiro. Foram analisados 376 isolados obtidos previamente em três campanhas distintas (Outono, Primavera e Verão). Pela identificação molecular através da sequenciação de um fragmento do gene *pyr*H, 78 isolados potencialmente resistentes a tetraciclina foram afiliados a 11 espécies distintas: *V. diabolicus* (n=29), *V. alginolyticus* (n=11), *V. parahaemolyticus* (n=22), *V. owensii* (n=3), *V. jasicida* (n=2), *V. campbellii* (n=3), *V. mytilli* (n=2), *V. cholerae* (n=1), *V. fluvialis* (n=1), *V. furnissii* (n=3) e *V. mediterranei* (n=1). Os resultados obtidos neste estudo sugerem uma baixa variação sazonal na abundância de *Vibrio*, mas indicam uma variação considerável em termos de diversidade (número e abundância de cada espécie). Por outro lado, verificou-se uma distribuição heterogénea ao longo do estuário, provavelmente relacionada com os gradientes de salinidade, temperatura, oxigénio dissolvido e pontualmente com valores de pH. Os resultados indicam também uma possível associação entre a abundância de bactérias resistentes e a prática de aquacultura e produção animal na região. Das espécies identificadas, *V.diabolicus* foi a mais abundante. Dado o conhecimento limitado sobre a virulência e resistência a antibióticos nesta espécie, um dos isolados obtidos (P7A) foi selecionado para sequenciação do seu genoma e análise comparativa com genomas de *V. diabolicus* depositados na base de dados PATRIC (n=11). A identificação ao nível da espécie foi confirmada através do cálculo das métricas ANIb e dDHH. A análise filogenética de múltiplos loci permitiu determinar que se trata de um ST (*sequence type*) nunca antes descrito, tendo-lhe sido atribuída a designação ST179. A análise do genoma permitiu a identificação de inúmeros genes de virulência que codificam para funções como sistemas de secreção, quimiotaxia e motilidade, entre outras. Detetaram-se também genes de resistência a antibióticos β-lactâmicos, fluoroquinolonas, fosfomicina e tetraciclinas, bem como bombas de efluxo associadas a resistência a antibióticos e a outros contaminantes (e.g metais). A elevada flexibilidade do genoma desta espécie foi confirmada pela análise do seu pangenoma, bem como pela deteção de diversos elementos que contribuem para a mobilidade de genes (i.e. ilhas genómicas, recombinases e super integrões). Desta forma, este trabalho possibilitou um avanço no conhecimento da diversidade de *Vibrio* na Ria de Aveiro, com ênfase em isolados resistentes a tetraciclina. Por outro lado, a análise do genoma de *V. diabolicus* contribuiu para esclarecer o resistoma e potencial de virulência desta espécie, até agora pouco estudada. Os nossos resultados reforçam a necessidade de estudar isolados ambientais, que podem conter determinantes genéticos de resistência com relevância clínica. Apesar de ser uma espécie geralmente considerada não patogénica, a presença de inúmeros genes de virulência associados a elementos móveis sugere a necessidade de novos estudos para melhor estabelecer a virulência desta espécie.

Keywords Antibiotic resistance, *Vibrio* spp., estuarine enviroments, virulence, mobile genetic elements.

Abstract

The genus *Vibrio* is abundantly distributed in several marine environments, estuarine ecosystems and aquaculture systems, and can also be isolated from clinical and food samples. In aquaculture systems, bacterial infections caused by *Vibrio* species (e.g. vibriosis) are responsible for high mortality rates and decrease in productivity. The intensive use of antibiotics, as prophylactic or therapeutic agents, has led to an increase of the prevalence of multidrug-resistant *Vibrio* strains. Antibiotics of the tetracycline class are often used to treat infections caused by *Vibrio*, particularly in animals.

This study aimed to investigate the distribution of *Vibrio* species resistant to tetracycline in the Ria de Aveiro estuary. A total of 376 isolates previously obtained in three different campaigns (Autumn, Spring and Summer) were analyzed. By molecular identification through partial sequence analysis of the *pyrH* gene, 78 isolates potentially resistant to tetracycline were affiliated to 11 different species: *V. diabolicus* (n=29), *V. alginolyticus* (n=11), *V. parahaemolyticus* (n=22), *V. owensii* (n=3), *V. jasicida* (n=2), *V. campbellii* (3), *V. mytilli* (n=2), *V. cholerae* (n=1), *V. fluvialis* (n=1), *V. furnissii* (n=3) and *V. mediterranei* (n=1). Results obtained in this study suggest a low seasonal variation in the abundance of *Vibrio* in the estuary,y but indicate a considerable variation in terms of diversity (number and abundance of each species). On the other hand, there was a heterogeneous distribution throughout the estuary, probably related to the gradients of salinity, temperature and dissolved oxygen and occasionally with pH values. The results also indicate a possible association between the abundance of resistant bacteria and the practice of aquaculture and animal production in the region. Of the identified species, *V. diabolicus* was the most abundant. Given the limited knowledge about virulence and resistance to antibiotics in this species, one of the isolates obtained (P7A) was selected for genome sequencing and comparative analysis with *V. diabolicus* genomes deposited in the PATRIC database (n = 11). The identification at the species level was confirmed by calculating the ANIb and dDHH metrics. The phylogenetic analysis of multiple loci allowed to determine that it represents a novel ST (sequence type) which was designated ST179. The genome analysis allowed the identification of several virulence genes that code for functions such as secretion systems, chemotaxis and motility, among others. Genes of resistance to β-lactam antibiotics, fluoroquinolones, fosfomycin and tetracyclines were also detected, as well as efflux pumps associated with resistance to antibiotics and other contaminants (e.g. metals). The high flexibility of the genome of this species was confirmed by the analysis of its pangenome, as well as by the detection of several elements that contribute to the mobility of genes (i.e. genomic islands, recombinases and superintegrons).

Thus, this work enabled an advance in the knowledge of the diversity of *Vibrio* in the Ria de Aveiro, with emphasis on isolates resistant to tetracycline. On the other hand, the analysis of the *V. diabolicus* genome has contributed to clarify the resistome and virulence potential of this species, which until now has been poorly studied. Our results reinforce the need to study environmental isolates, that may contain genetic determinants of resistance with clinical relevance. Despite being a species generally considered non-pathogenic, the presence of numerous virulence genes associated with mobile elements in the genome of *V. diabolicus* suggests the need for further studies to better establish the virulence of this species.

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ABBREVIATIONS

ABC ATP-binding cassette

ARB Antibiotic-resistant bacteria

ARG(s) Antibiotic resistance gene(s)

ANI Average nucleotide identity

BLAST Basic Local Alignment Search Tool

bp base pairs

BRIG BLAST Ring Image Generator

CARD Comprehensive Antibiotic Resistance Database

CAT Chloramphenicol Acetyltransferase

CDS Coding Sequences

CPS Capsular Polysaccharide

CRISPRs Clustered regularly interspaced palindromic repeats

CT Cholera toxin

dDDH Digital DNA-DNA Hybridization

DNA Deoxyribonucleic acid

dNTP's Deoxyribonucleotide triphosphates

DO Dissolved oxygen

EDGAR Efficient Database Framework for comparative Genome Analysis using BLAST score Ratios

EEA European Economic Area

EF-G Elongation factor G

EF-Tu Elongation factor Tu

EU European Union

FAO Food and Agriculture Organization of the United Nations

GC Guanosine and Cytosine

GEI(s) Genomic Island(s)

GIPSy Genomic island prediction software

HGT Horizontal gene transfer

ICE(s) Integrative conjugation element(s)

iTOL Interactive Tree Of Life

ISCR Insertion sequence common region

KEGG Kyoto Encyclopedia of Genes and Genomes

MATE Multidrug and toxic compound extrusion

MBL(s) Metallo-β-lactamase(s)

Mbp Mega base pairs

MDR Multiple-drug resistance

MEGA Molecular Evolutionary Genetics Analysis

MFS Major facilitator superfamily

MGE(s) Mobile genetic element(s)

MLSA Multi-locus sequence analysis

MLST Multilocus Sequence Typing

MSs Member States

NCBI National Center for Biotechnology Information

nt nucleotide

ORF Open Reading Frame

OTC Oxytetracycline

PAI(s) Pathogenicity island(s)

PATRIC Pathosystems Resource Integration Center

PCR Polymerase chain reaction

RAST Rapid Annotation using Subsystems Technology

RGI Resistance Gene Identifier

RPPs Ribosomal protection proteins

RNA Ribonucleic acid

SI Super-integron

SRVs Score ratio values

ST Sequence Type

SXT Sulfamethoxazole/Trimethroprim

TCs Tetracyclines

TCBS Thiosulfate-citrate-bile salt-sucrose agar

TCP Toxin co-regulated pilus

TDH Thermal stable direct hemolysin

TRH TDH-related hemolysin

tRNA Transfer RNA

TSA Tryptone Soya Agar

TSB Tryptone Soya Broth

T3SS Type III secretion systems

T3SS1 Type III secretion system 1

T3SS2 Type III secretion system 2

VFs Virulence factors

VFDB Virulence Factors of Pathogenic Bacteria

WGS Whole Genome Sequencing

WWTPs Wastewater treatment plants

1. INTRODUCTION

1.1. The genus *Vibrio*

1.1.1. General characteristics

In the year of 1854, the first *Vibrio* species, *Vibrio cholerae* (the causative agent of cholera) was discovered by Filippo Pacini, an Italian physician[1]. Today the genus *Vibrio* includes more than 70 species, all motile exhibiting one or more flagella[1].

Vibrio are gram-negative bacteria, straight or curved rod-shaped form, typically short and ranging from 0.5 to 0.8 μm wide and 1.5 and 3.0 μm long[2] (Fig.1). All known *Vibrio* have two chromosomes, first reported in *V. cholerae* in 1998. Chromosome 2 is usually smaller than chromosome 1, known to contain a highly variable super-integron, that harbors hundreds of gene cassettes[3].

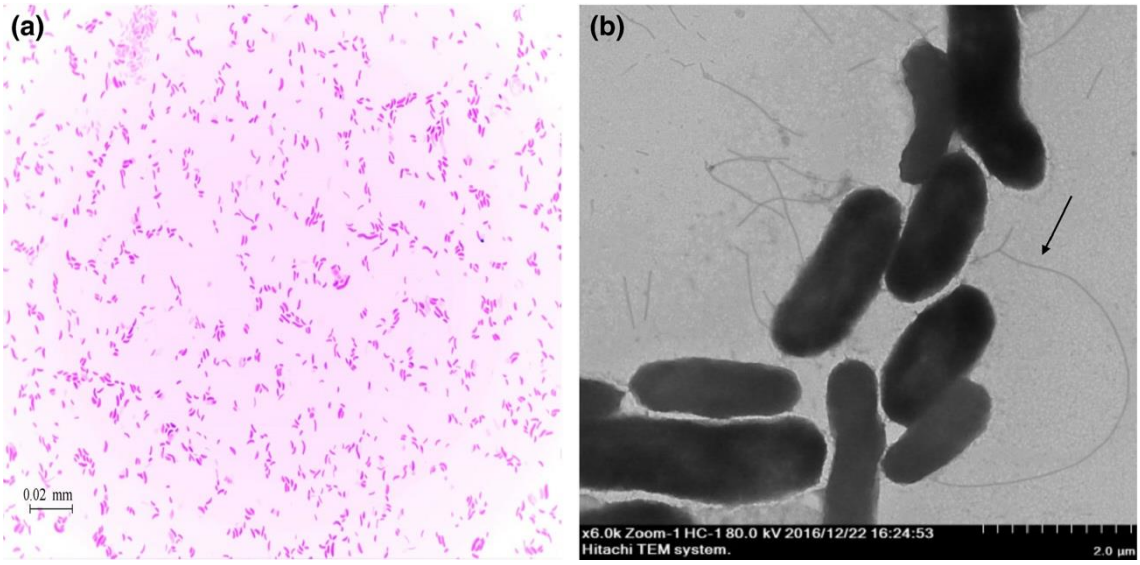

Fig. 1 - Morphology and structure of a *Vibrio alginolyticus* strain. (a) Light micrograph (1,000 ×). (b) Transmission electron micrograph (6,000 ×). Arrow shows flagellum[4].

These bacteria, which are able to grow over a wide temperature range (20 ºC to 40 ºC), have a low tolerance to acid and prefer alkaline conditions, being most species able to grow between pH 6.5 and 9.0[5]. The genus can be divided into halophilic species, as *V. parahaemolyticus and V. vulnificus* or non-halophilic, including *V. cholerae*, according to their need for sodium chloride for growth[2]. Furthermore, these bacteria are facultative anaerobes capable of both fermentative and respiratory metabolism, being most species oxidase-positive able to reduce nitrates to nitrites[2].

In aquatic habitats, *Vibrio* are essential for nutrient cycling by taking up dissolved organic matter, which leads to the regeneration of nutrients essential for sustaining primary production and oxidation of complex organic compounds in the overlying water column[6,7]. *Vibrio* are also able to produce polyunsaturated fatty acids to the aquatic food web, (which many aquatic

organisms cannot produce *de novo*) and to degrade chitin (present in exoskeletons of crustaceans)[8].

When cultured on agar media, *Vibrio* usually form two very distinct colony types, namely rugose and smooth. These colony variants formation is associated with population diversity and adaptation to environmental conditions[9]. Generally, this bacterium is able to grow on marine agar and on the selective medium thiosulfate-citrate-bile salt-sucrose agar (TCBS)[8]. In TCBS, strains will form yellow colonies if they are able to use sucrose, while the others are green (Fig. 2). This was one of the first selective media used for the isolation and purification of *Vibrio*, and is still largely employed[10].

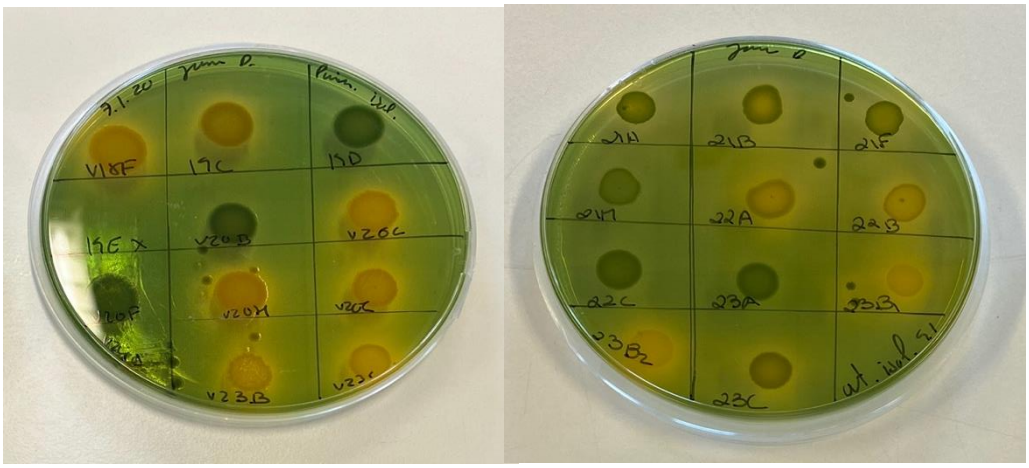

Fig. 2 - TCBS Agar plates containing *Vibrio* colonies exhibiting yellow or green colors according to Vibrio typical morphology (this study).

1.1.2. *Vibrio* environmental reservoirs

Vibrio species are ubiquitous and abundant in aquatic environments, including estuarine ecosystems, marine and coastal waters, and aquaculture systems worldwide, comprising one of the main microbiota members of these ecosystems[8].

A broad range of environmental parameters influence the persistence and density of *Vibrio* in the aquatic environment, including temperature, salinity and pH as well as biotic factors such as the presence of both phyto- and zoo-plankton[11,12,13]. These organisms have the ability to adhere and colonize the surface of marine plankton, and thus the nutrient-rich surfaces of plankton have long been known to selectively enrich for *Vibrio*, resulting in higher densities of these bacteria than the surrounding water column[11,14].

Besides plankton, *Vibrio* appear at particularly high densities in the water column, sediments, on tissues or organs of various marine animals, -e.g. corals, fish and mollusks and associated with biofilm formation[8,15]. On the other hand, *Vibrio* are less abundantly found in

aquatic vegetation[16].

The production of biofilm has been shown to be one of the bacteria's strategies to survive during starvation and/or under other environmental stresses[15], such as a decrease in temperature and salinity. Likewise, the non-culturable state also represents a response to low nutrient levels or low temperatures[17]. However, these physiological states, acting as survival tactics, may lead to difficulties when trying to isolate *Vibrio* from aquatic environments.

1.1.3. Virulence-related characteristics

The majority of *Vibrio* species are harmless to humans, and within a given species, not all strains are equally pathogenic[8,18].

As mentioned previously, the genus *Vibrio* includes more than 70 species ,and at least 12, including *V. parahaemolyticus, V. vulnificus* and *V. cholerae,* are recognized as pathogenic to humans and/or have been associated with foodborne diseases, being important to public health[14,18].

By far the best-known pathogenic *Vibrio* is *V. cholerae,* and specially the serogroups O1 and O139 (among the 200 known *V. cholerae* serotypes) are considered the most important, once they have been associated with epidemic and pandemic diarrhea outbreaks in many parts of the world[19]. In fact, cholera is a severe disease mainly in developing countries as a result of poor water supplies and sanitation, since the main routes of contamination are water and food[8,19].

Numerous virulence factors have been identified in pathogenic *Vibrio* spp.. However, infectious cycle of this bacterium is similar to other pathogenic bacteria including entry of the pathogen, establishment and multiplication, causing damage to host tissues and cells, and exit[20].

A necessary step for a successful infection of a host is the ability to colonize and adhere to host surfaces, where flagellar motility is thought to enhance the initial interaction of the bacterium with a surface[21]. However other factors, such as pili, chitin-binding proteins and chitinases, have also been associated with adhesion[20,22]. A second important phenotype in colonization of the host is biofilm formation[23]. Several factors including flagella, pili and production of extracellular exopolysaccharide are involved in this process[23]. Exopolysaccharides form a loose slime outside the cell that forms an intercellular matrix in biofilms, which enhances the growth and survival of microorganisms by providing access to nutrients and protection from antimicrobials and predators[15]. Other important virulence factors include lytic enzymes, produced by many pathogenic bacteria and that often play a central role in pathogenesis[24]. These enzymes produced by pathogenic *Vibrio* spp. include toxic extracellular proteins such as proteases, phospholipases, hemolysins[24,25], which cause damage to host tissues, thereby allowing the pathogen to obtain nutrients and to spread through tissues.

More specifically, in *V. cholerae,* the severe diarrhea manifestations result from the

presence of strains that have acquired the genes encoding the toxin co-regulated pilus (TCP), a type IV pilus, and the cholera toxin (CT), which consists of one A subunit and five B subunits, all coded for in the *ctxAB* operon of two subunits[26]. CT, is the predominant virulence factor in this species and is transferred among strains via the filamentous phage CTXϕ, in which TCP is the receptor on *V. cholerae* cells for the CTXϕ, and in addition, required for intestinal colonization[14].

Non-O1 and non-O139 *Vibrio cholerae* serotypes (also called non-agglutinable) have also been implicated as etiologic agents of mild to moderate cases of diarrhea in humans, usually associated with the consumption of raw or undercooked shellfish, or exposure of open wounds to water, indicating that there are other factors that contribute to the virulence of cholera-causing strains[14,]. However, among the many members of the *Vibrio* genus responsible for seafood-associated illnesses, two species, *V. parahaemolyticus* and *V. vulnificus*, account for the majority of cases[27,28].

V. parahaemolyticus is the leading cause of bacterial seafood-borne illness worldwide, especially in the United States[28], while infections caused by *V. vulnificus*, although not as common, are usually more severe and often lead to death[27].

Clinical strains of *V. parahaemolyticus* cause three major types of illness: gastroenteritis, wound infections, and septicemia[2,29]. The virulence of *V. parahaemolyticus* is predominantly associated with the production of thermal stable direct hemolysin (TDH) and/or the genetically similar TDH-related hemolysin (TRH)[14]. There are other factors involved in the virulence of *V. parahaemolyticus*. The association of *V. parahaemolyticus* strains to disease has also been linked to two sets of type III secretion system T3SS1 and T3SS2, responsible for both enterotoxicity and cytotoxicity activities[30].

V. vulnificus causes primary septicemia and wound infections in humans, and is characterized by the possession of several virulence factors, including the ability to evade destruction by stomach acid, the production of lipopolysaccharide and cytotoxins, and the possession of pili and flagellum[31]. Furthermore, it has a protective capsular polysaccharide, which is one of the main virulence factors associated with *V. vulnificus* infection, that facilitates host immune evasion, being encapsulated cells highly virulent[32]. Another important virulence factor is the exotoxin – hemolysin VvhA – a protein that facilitates the release of iron from hemoglobin, that may contribute to the bacterium's virulence not only through hemolytic activity but also through other cytotoxic effects[33].

Other potential human pathogenic *Vibrio* spp., along with the ones referred previously are illustrated in Table 1[2,29].

Table 1 - Summary of *Vibrio* spp. causing human infections[2,29].

	Human infection		
Species	**Gastroenteritis/diarrhea**	**Wound/ear**	**Septicemia**
V. cholerae O1/0139	YES	YES	NO
V. cholerae NON O1/0139	YES	YES	RARE
V. parahaemolyticus	YES	YES	RARE
V. vulnificus	YES	YES	YES
V. mimicus	YES	RARE	RARE
V. hollisae	YES	RARE	RARE
V. fluvialis	YES	RARE	RARE
V. alginolyticus	YES	YES	YES
V. harveyi	NO	RARE	NO
V. furnissii	RARE	NO	NO

Besides the human pathogens already described, the *Vibrio* genus also includes pathogens of aquatic animals such as *V. harveyi*, *V. campbellii* and *V. alginolyticus*, as well as *V. parahaemolyticus*[8]. These species are all members of the *Harveyi* clade, a subgroup containing major aquaculture-associated pathogens, but also non-pathogenic bacteria[34].

Vibriosis is one of the most prevalent diseases, caused by these and other *Vibrio* bacteria, in fish and other organisms (shrimps, larvae, finfish and mollusk), responsible for a high mortality rate in aquacultures worldwide, and the cause of multibillion-dollar losses in this industry[35,36]. Vibriosis is generally referred to as a systemic bacterial infection caused by members of the family of *Vibrionaceae*. It is frequently associated with both wild and farmed marine fishes, and usually fluctuation of physico-chemical properties of water and the over-population are among the factors leading to outbreaks of vibriosis in cultured fish[37]. Additionally, climate changes have become a rising concern in the recent years, since these changes may have a significative role in incrementing numerous fish pathogens, like *Vibrio*[38]. The illness caused by *Vibrio* spp. is generally diversified, and varies from species to species, but common manifestations can be observed, such as lethargic movement of affected fish, the presence of skin or mouth ulcerations and pigmentation on the body, or gastroenteritis, usually leading to the death of the organism infected[37].

The transmission of *Vibrio* spp. in farmed fish still remains unclear, however vibriosis is a water-borne infection. This means that water column works as natural transmission medium used by the etiological agent. Therefore, can be transmitted horizontally, either from open lesions or as secretion in the feces of infected fish[37].

1.2. Antibiotics and Antibiotic resistance

Antimicrobials are compounds that can kill or inhibit the growth of microorganisms including bacteria, archaea, viruses, protozoa, microalgae and fungi[39]. The term antibiotic, currently in use as natural (produced by bacteria and fungi), synthetic and semi-synthetic molecules, can be defined as a type of antimicrobial and are specifically intended to kill (bactericidal) or inhibit (bacteriostatic) the growth of bacteria, which distinguishes them from disinfectants or other microbiocides[39].

Their emergence occurred in 1928, when Alexander Fleming's never thought that by observing a piece of mould in a contaminated petri dish, he had just discovered that the mould produced penicillin, an antibacterial compound that killed the bacteria he was analyzing[40]. Along with penicillin, other antibiotics such as sulphonamides and streptomycin were discovered, and their introduction into clinical practice, was seen as an extremely efficient method against pathogenic bacteria leading many to believe that infectious diseases would become a problem of the past. In fact, antibiotics were (and are) considered one of the wonder discoveries of the 20th century [41,42].

Over the last 75 years, several new antibiotics have been developed through constant research, revolutionizing human medicine and had become also widely used as veterinary drugs, and in agriculture and aquaculture fields, emphasizing the great importance of antibiotics in today's society [40].

Currently, these complex molecules can be divided into different categories based on their mechanism of action: the inhibition of cell wall synthesis, alteration of cell membranes, protein synthesis inhibition, synthesis of nucleic acids inhibition and inhibition of metabolic pathways, as shown in Fig.3[43]. However, they can also be described by their chemical structure, based on which the major groups of antibiotics correspond to β-lactams, fluoroquinolones, tetracyclines, sulphonamides, aminoglycosides and macrolides[43].

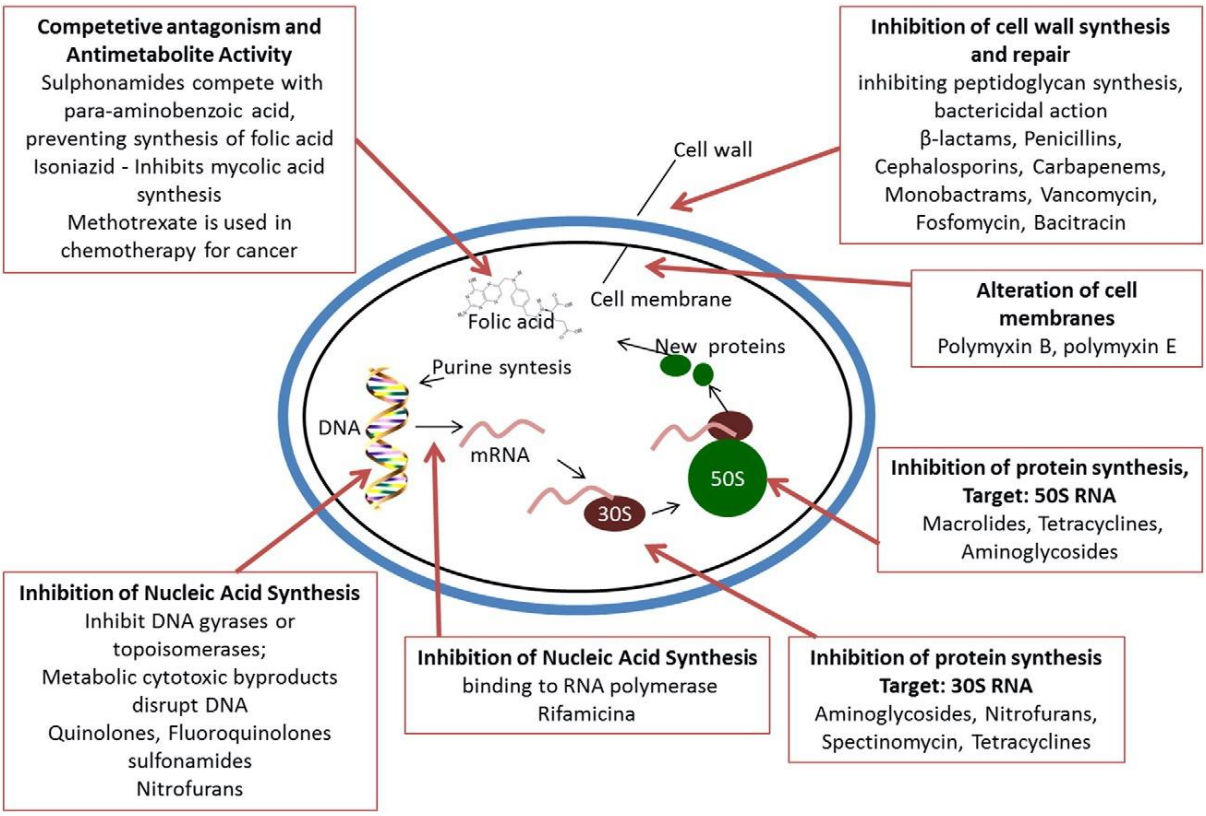

Fig. 3 - Main action mechanisms of antibiotics[39].

Most antibiotics used nowadays are synthetic or semi-synthetic molecules, however their structure is based on natural compounds produced by microorganisms present in the environment for millions of years[44]. These modifications allowed to increase the efficiency of antibiotics, to wide their spectrum of activity and to increase their capacity to escape bacterial antibiotic resistance mechanisms[44]. However, due to bacterial amazing genomic plasticity and their capacity to exchange genetic information among different species, the persistence of bacteria who had strategies to survive to natural antibiotics was allowed[45]. As such, the existence as well as the evolution of antibiotic resistance genes (ARGs) is a natural phenomenon with ancient origin and can be found in bacteria from pre-antibiotic era, e.g. viable multidrug-resistant bacteria have been cultured from the Lechuguilla Cave in New Mexico, totally isolated for >4 million years[46].

However, real concern about the emergence and diversification of antibiotic resistance mechanisms is a relatively modern crisis and is associated with the extensive use of antibiotics for clinical practices and in veterinary, agriculture and aquaculture fields[47]. The quantity of antibiotics present in aquaculture effluents varies with the type of substance, dosage, species and animal age, among other factors[48]. Since in aquaculture, antibiotics are commonly administered orally with feed, up to 30 to 90% get excreted in feces or urine[49]. Therefore, fish feces, waste food

containing antibiotics, their metabolites and degradation products (even when the molecule is largely metabolized, some of the degraded products excreted may remain bioactive)[48] end up in the surrounding environment[48]. Consequently, the accumulation of antibiotic residues in sediments, in the aquatic environment, may lead to changes in microbial communities through selection for antibiotic-resistant species[50]. Also, the persistence of ARGs in the environment for several years after actual use of the drugs, represent an even greater concern, due to the inherent connections between aquaculture systems with open water bodies, such as rivers, lakes and oceans[51]. Therefore, antibiotic-resistant bacteria (ARB) have been reported to have side effects in open water systems[51].

Furthermore, wastewater treatment plants (WWTPs) have also been recognized as a relevant source of both ARB and ARGs, since these plants receive numerous contaminants, such as metals and antibiotics, which can exert a selective pressure in the wastewater bacterial community[52].

Annually, antibiotic resistance is responsible for hundreds of thousands of deaths all over the world, currently claiming at least 50,000 deaths in Europe and United States[40] (data from 2014). However it is expected that by the year 2050, antibiotic-resistant infections will be the major cause of death, comparing to diseases like cancer and diabetes[40]. The World Health Organization has already recognized antibiotic resistance as a threat to global health, connected with multiple factors[53]. Raising even a bigger problem, because while antibiotic use is rising, the pace of the progress that has been made in the discovery of new antibiotics, has slowed drastically[40]. At this point is important to describe that antibiotic drug resistance is defined as the ability of a microorganism to resist the effects of an antibiotic agent to which it is normally susceptible. This ability can be carried out by a multitude of mechanisms, being that the nature and efficiency of these resistance tools depend on the species and the particular antibiotic[54]. Thus, the main mechanisms of resistance described so far, used by bacteria for protection against the various antibiotic effects, are enzymatic modification or degradation by hydrolysis (e.g. β - lactamases); reduced cell permeability, trough efflux pumps (that reduce intracellular antibiotic concentrations) or alterations in the cell wall (e.g. Gram-negative outer membrane); target modification (e.g. *parE* and *gyrA* point mutations); and development of alternative metabolic pathways[54,55] (Fig. 4).

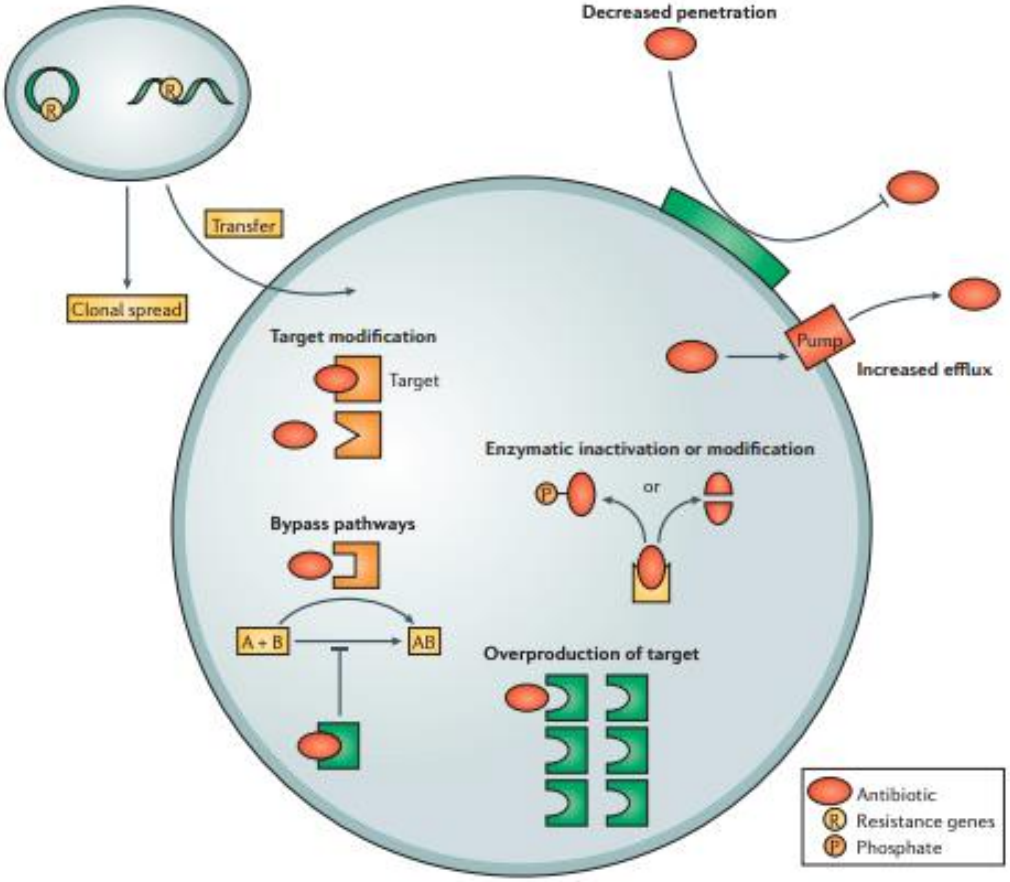

Fig. 4 – Representation of the main mechanisms of resistance available for bacteria for protection against various antibiotic effects[55].

Mobile genetic elements (MGEs) are often responsible for the capture, accumulation and dissemination of ARGs. These are genetic platforms that promote intracellular and/or intercellular DNA mobility, such as integrative conjugation elements (ICEs) and/or plasmids[56], e.g. ICE called SXT element that carries multiple resistance genes against sulfamethoxazole, trimethoprim and streptomycin[57], which can be transferred horizontally between cells, that may belong to a different species from the donor[42]. Since these elements are frequently present in multiple copies in the genome, the homologue recombination ends up to be facilitated[56]. Hence, the rapid evolution of several multipledrug-resistant (MDR) pathogenic organisms in the face of antibiotic treatment is supported by the interactions between the various types of MGEs[56,58]. Conjugation (mediated by plasmids and ICEs), transduction (mediated by bacteriophages) and transformation (extracellular DNA uptake) are the major processes of intercellular mechanisms of genetic exchange or HGT (horizontal gene transfer)[58].

The ability of these MGEs containing ARGs to spread depends on a range of factors including, selective pressures in the environment, host factors and properties of the genetic elements themselves[58].

1.3. Tetracyclines

Benjamin Duggar identified the first member of the tetracycline family in 1948, named as aureomycin (chlortetracycline), a natural fermentation product of the *Streptomyces aureofaciens* bacterium that is naturally present in the soil[59].

Currently, this group comprises the natural products obtained by the fermentation of *Streptomyces* bacteria, the first generation, including tetracycline, chlortetracycline and oxytetracycline, and the second and third generation drugs, such as metacycline, doxycycline, minocycline, and more recently the glycylcycline tigecycline[60,61]. The second and third generation drugs are semisynthetic derivatives, exhibiting improved antibacterial potency, spectrum, resistance coverage, solubility, and/ or oral bioavailability[60,61].

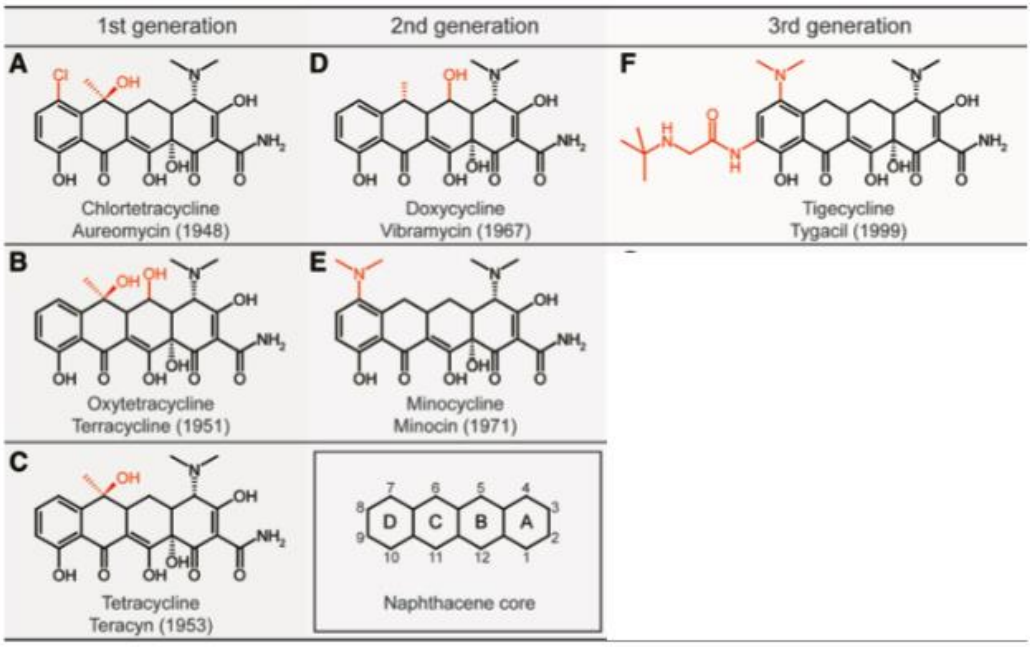

Fig. 5 - Chemical structures of tetracyclines.
Chemical structures of (A–C) first generation tetracyclines. (A) chlortetracycline, (B) oxytetracycline and (C) tetracycline, (D–E) examples of second generation tetracyclines; (D) doxycycline and (E) minocycline and (F) third generation tetracyclines, the glycylcycline tigecycline. The numbers in parentheses indicate the year the antibiotic was discovered/reported. The inset of the DCBA naphthacene core provides the carbon atom assignments for rings A–D. Adapted from[61].

In all tetracyclines (TCs) the basic structure consists of a DCBA hydronaphthacene core with four fused rings (Fig.5), therefore this family of antibiotics was named 'tetracyclines'[62]. The chemical structure of TCs varies depending on the substituents at positions 5, 6 and 7, except for the tigecycline, which has a substituent present at the C9 position (Fig.5)[61].

Tetracyclines were the first major group of antimicrobial agents for which the term 'broad-spectrum' was used, being well known for their antimicrobial activity against Gram-

positive and Gram-negative bacteria, as bacteriostatic agents[63]. Their mechanism of action, similar to aminoglycosides, works by interfering with the ability of bacteria to synthesize proteins by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site, in the mRNA-ribosome complex, by binding to the 30S ribosomal subunit[63]. More specifically in gram-negative bacteria, this access to the bacterial ribosome is possible via a two-steps process, by passive diffusion trough hydrophilic channels formed by OmpF and OmpC porin proteins in the outer membrane, and then by an energy-dependent active transport through the inner cytoplasmic membrane[64].

1.3.1. Applications of tetracyclines

These antibiotics are widely used in many countries, being chlortetracycline, oxytetracycline and tetracycline the most often used throughout the world[63]. In most countries, the amount of TCs consumed in food-producing animals markedly outweighed that consumed in humans[65]. Therefore, resistance to tetracyclines is relatively common in many bacteria from animals in most of the reporting countries[65]. In humans, TCs are usually used to treat infections such as gonorrhea, uncomplicated respiratory infections, trachoma, urinary tract infections, cholera, and other rare and serious infections[43,66].

Beyond human therapy, tetracycline antibiotics are one of the primary antibiotic groups used in veterinary medicine, for prophylaxis and therapy of several infectious diseases in animal farms[67], and in some countries including the United States, in livestock and aquaculture sectors as feed additives to promote the growth of animals[63]. In fact, oxytetracycline (OTC) and chlortetracycline are two of the ten antibiotics licensed as growth promoters in the United States[68], being also widely used in Asian aquacultures[69], and also OTC is authorized for aquaculture use in Europe as therapeutic agents[69]. Indeed, 2005 FAO (Food and Agriculture Organization of the United Nations) data reported 92% use of oxytetracycline and 23% chlortetracycline by the thirteen countries with the highest aquaculture production[70].

The practice of aquaculture continues to grow in several parts of the world due to an increase in the demand for what is now recognized as a choice of healthy protein, advances in the production of seafood, reduction of stocks of wild fish, among other factors[70]. Therefore, the amount of antibiotics (primarily TCs) added to this medium for the purpose of preventing or treating microbial infections also increases[70]. In addition, the overuse of antibiotics in aquaculture not only increases the selection of ARB and dissemination of the ARGs but also results in the presence of antibiotic residues in aquatic organisms, such as fish[71]. The Fig.6 shows a comparison regarding the consumption of antibiotic per classes in humans and animal production, including aquaculture, aggregated for the 28 European Union (EU)/European Economic Area (EEA)

Member States (MSs) in 2014, where it is possible to conclude that the tetracycline antibiotics are the class of antibiotics used in higher amounts for food-producing animals, when consumption is expressed in milligrams per kilogram of estimated biomass[65].

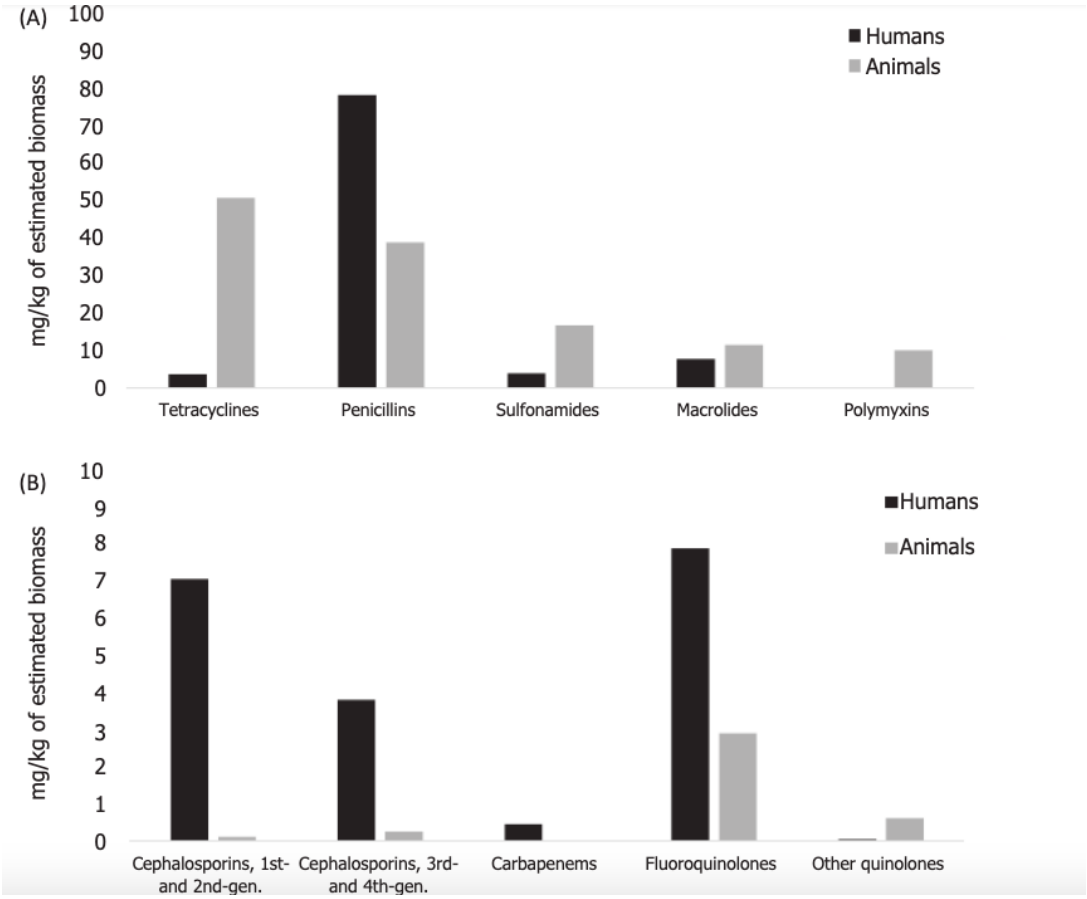

Fig. 6 - Comparison of consumption of selected antibiotic classes in humans and food-producing animals, EU/EEA MSs, 2014 (Notes: The y-axis scale differs between the graphs A and B). Adapted from [65].

In Portugal the four classes of antibiotics most consumed are penicillins (β-lactams), tetracyclines, macrolides and quinolones[72]. The total consumption of antibiotics from all classes for human health in Portugal was 86 tons in 2011, while the total consumption for veterinary purposes was 162 tons (Fig.7), corresponding to twice the human consumption[72]. For TCs, only 1% of total consumption is linked to human medicine; in 2011, OTC accounted for 30% (13.33 tons) of total TCs but in 2010 it represented 60% (46.54 tons) of total TCs administered[72].

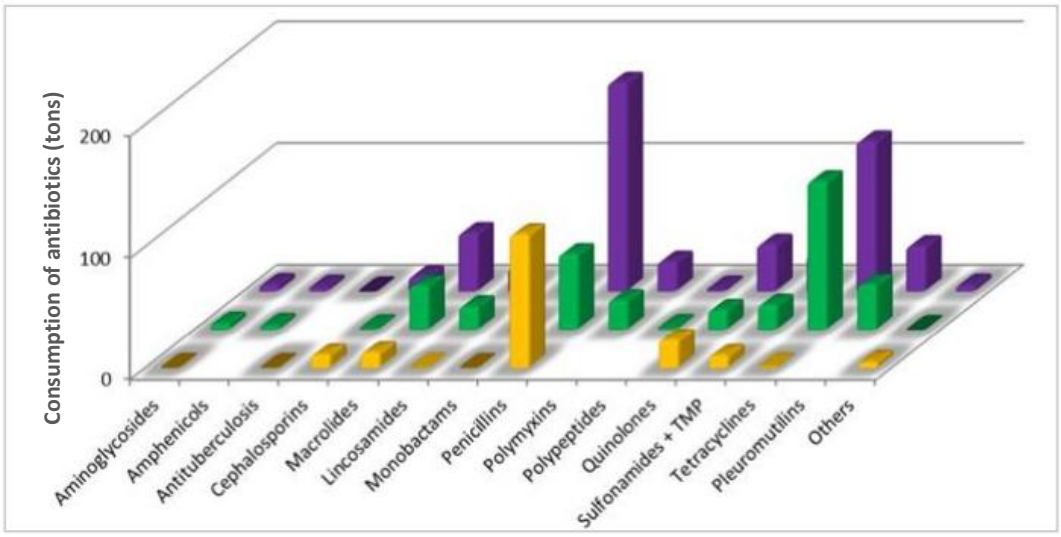

Fig. 7 - Total consumption of antibiotics in Portugal (tons) between 2010-2011. Legend: Human consumption - yellow; veterinary consumption - green; total consumption - purple[72].

Due to the extensive use of TCs, they have been frequently detected in sewage, domestic wastewater, surface and groundwater resources, and sludge[73]. Besides aquaculture, these residues are discharged into the environment (water resources in particular), through wastewater effluent of drug manufacturing companies, and also from animal and agricultural wastes[74].

Tetracyclines class exhibits an ever greater concern in this regard as they have a low rate of metabolism (<20% of the administered dose)[48], in which an estimated 70% to 80% of TCs are intact in feces, contributing to their persistence in the aquatic environment, especially in sediments and aquatic water[75] (Fig.8).

In aquatic environments, in addition to diffusing into sediments, TCs can be carried and dragged in water currents for long distances. Several studies, including in Portugal[76] documented elevated levels of bacterial antibiotic resistance in the surrounding environment of aquaculture production systems, alerting for a global health problem[70,77]. This permanence in the environment leads to a continuous selection of bacteria resistant to TCs[77].

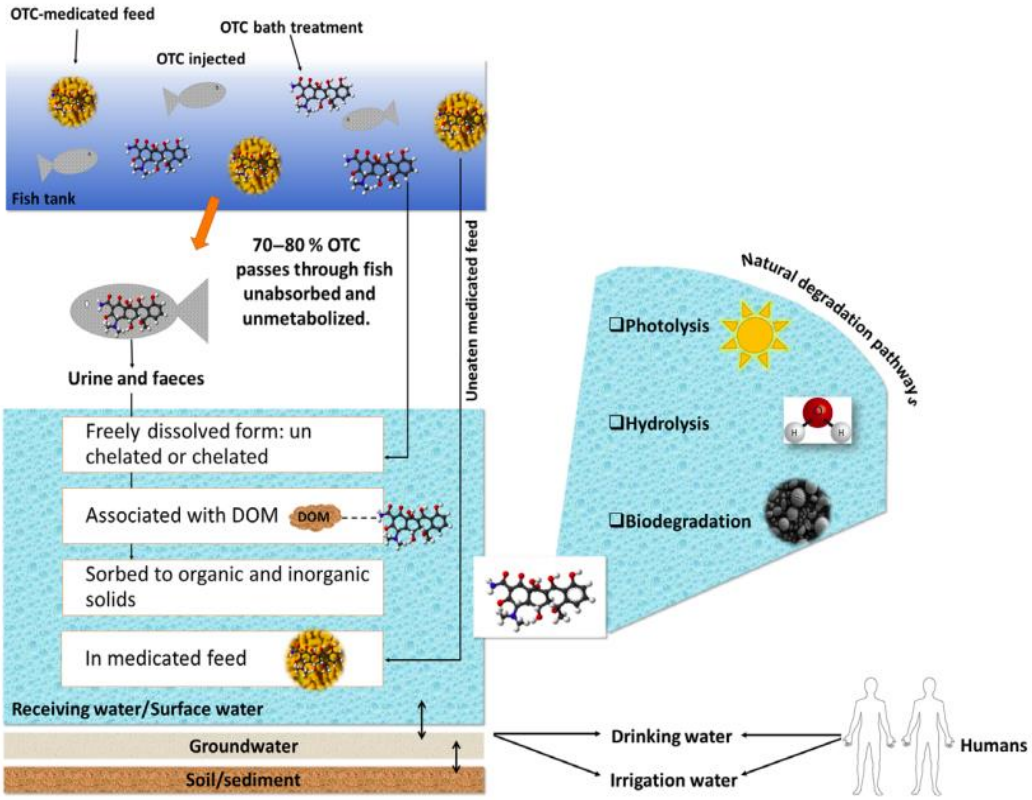

Fig. 8 - Environmental fate of oxytetracycline (similar to what happens with other tetracyclines), from its administration to its release into the aquatic environment, including the main natural degradation pathways[75]. .

1.3.2. Resistance to tetracyclines

Although TCs retain important roles in both human and veterinary medicine, such as the ones referred previously, the emergence of antibiotic resistance has limited their effectiveness.

Undoubtedly, the use of TCs in clinical practice has been responsible for the selection and increase of resistant organisms[78], and the widespread nonclinical use of these drugs and other antibiotics as animal growth promoters is becoming increasingly controversial because of concerns that this practice may be contributing as an important factor for the spread of plasmid-mediated resistance[79]. Practise that long gone has been forbidden in Europe but still allowed in Asia and in United States of America[49,68].

Prior to the mid-1950s, the majority of commensal and pathogenic bacteria were susceptible to tetracyclines. In fact, among 433 different *Enterobacteriaceae* strains (almost all from human infections, and from widely separated areas, including Europe, India, Russia and others) collected between 1917 and 1954, only 2% were resistant to tetracycline[80]. These data, along with other studies on environmental bacteria, seem to support the hypothesis that the

emergence of tetracycline-resistant bacteria followed the introduction of this antibiotic in clinical practice[81].

In 1955, the first multi-resistant *Shigella dysenteriae* was isolated and was resistant to tetracycline, streptomycin, and chloramphenicol, leading to the discovery of conjugal transfer of plasmids in bacteria, when seen that these three resistance phenotypes could be transferred together[82].

Recent studies have determined that most acquired resistance to tetracyclines, in both Gram-positive and Gram-negative bacteria, is associated with either conjugative or mobilizable elements, which may partially explain their wide distribution among bacterial species[63]. This resistance has emerged in many commensal and pathogenic bacteria due to genetic acquisition of *tet* genes, which are found in a variety of bacteria isolated from humans, animals, and the environment[66]. Other important factors associated with tetracycline resistance are related to mutations in ribosome binding sites that lead to an increase expression of intrinsic resistance mechanisms[61].

A decrease in accumulation of the antibiotic in the cell, as a result of either a decreased transport of the drug through the cell wall and membrane or acquisition of an energy-dependent efflux pathway, a decrease in the access of the drug to the ribosome as a result of the expression of ribosome protection proteins, and enzymatic inactivation of the antibiotic correspond to the three main mechanisms of resistance to tetracyclines[79].

A total of 62 different tetracycline resistance genes, including both *tet* and oxytetracycline (*otr*) genes were characterized (faculty.washington.edu/marilynr; updated Feb 20, 2020; Table 2). The presence of both *tet* and *otr* genes with the same mechanisms of resistance, such as the efflux system, is consistent with the hypothesis of lateral gene transfer from the tetracycline-producing streptomycetes to other bacteria[83].

Efflux proteins: The efflux proteins, can be found on the chromosome or plasmids in a broad array of both Gram-negative and Gram-positive bacteria and are the best studied Tet proteins[84]. There are 35 distinct tetracycline-specific efflux pumps reported in bacteria, predominantly belonging to the major facilitator superfamily (MFS) group of integral membrane transporters[85]. However, there have been rare reports of non-MFS pumps[86].

All the *tet* efflux genes code for membrane-associated proteins and are found across several microbial genera being integral membrane proteins that span the lipid bilayer of the inner cell membrane 12–14 times[87].

These efflux pumps extrude tetracycline antibiotics from the inside of cells, at the expense of a proton, and this way are able to reduce the intracellular drug concentration and thus protect the ribosomes within the cell[88].

Ribosomal Protection: In *Campylobacter jejuni* and *Streptococcus* spp., tetracycline ribosomal protection proteins (RPPs) were first described as GTPases with significant sequence and structural similarity to elongation factors EF-G and EF-Tu[89].

Currently, there are 12 RPPs described in Gram-negative and Gram-positive bacteria, from which Tet(O) and Tet(M) are the most common and better characterized[66].

These are cytoplasmic proteins that protect the ribosomes from the action of tetracycline, by binding to the ribosome and preventing the binding of tetracycline, without modifying or interrupting protein synthesis, but by inducing an alteration on ribosomal conformation[79].

Some RPP genes are conveyed by genetic mobile elements such as plasmids and conjugative transposons. Therefore, these genes can be transferred to taxonomically divergent bacteria[83].

Enzymatic inactivation: Unlike other antibiotic classes, such as the β-lactams and aminoglycosides where drug degradation and modification predominate, there are relatively few reported enzymes able to hydrolyze or modify tetracyclines.

The *tet*(X) gene encodes the better characterized example of tetracycline resistance due to enzymatic alteration, which acts by chemically modifying this drug in the presence of oxygen and NADPH. The *tet*(X) was the first tetracycline inactivation gene identified early in 1988 from *Bacteroides fragilis*[90]. Interestingly, it is an oxygen-dependent flavoprotein whereas the organism in which the gene was identified is an obligate anaerobe. This suggests that the gene must have migrated from some other organism[90].

After the discovery of *tet*(X), *tet*(34)[91] and *tet*(37)[92] identified from *Vibrio* spp., have been suggested to encode tetracycline inactivation products. Strikingly, *tet*(X) was the only gene encoding a tetracycline inactivation enzyme reported in human pathogens to date[93]. Nowadays, a family of novel tetracycline resistance genes, able to inactivate tetracycline has been proposed [94] (Table 2).

Table 2 - Mechanism of resistance for characterized *tet* and *otr* genes. Modified Feb. 20, 2020.
Originally modified Chopra & Roberts published in Microbiol. Mol. Bio. Rev. 65:232-260.[66] with permission from ASM Journals [n=62 genes + 11 mosaic genes].
(http://faculty.washington.edu/marilynr/)

Efflux (35)	Ribosomal Protection (13)	Enzymatic (13)	Unknown[a]
tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(59)[i]	*tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32),	*tet*(X)[c]	*tet*(U)
tet(G), *tet*(H), *tet*(J), *tet*(V), *tet*(Y)	*tet*(Q), *tet*(T), *tet*(36), *tet*(61)[n]	*tet*(37)[c]	
tet(Z), *tet*(30), *tet*(31), *tet*(33), *tet*(57)[g]	*otr*(A), *tetB*(P)[b], *tet*	*tet*(34)	
tet(35)[d]	*tet*(44)	*tet*(47)[j], *tet*(48)[j], *tet*(49)[j], *tet*(50)[j]	
tet(39), *tet*(41)		*tet*(51)[j],*tet*(52)[j], *tet*(53)[j], *tet*(54)[j]	
tet(K), *tet*(L), *tet*(38), tet(45)[e], *tet*(58)[l] *tet*(63)		*tet*(55)[j], *tet*(56)[j]	
tetA(P), *tet*(40)			
otr(B), *otr*(C)			
tcr3			
tet(42)	Mosiac Ribosomal Protection (11)		
tet(43)	*tet*(O/32/O), *tet*(O/W/32/O), *tet*(O/32/O		
*tet*AB(46)[f]	*tet*(O/W/32/O/W/O), *tet*(W/32/O), *tet*(O/W)		
*tet*AB(60)[k]	*tet*(W/32/O/W/O), *tet*(O/W/O), *tet*(O/W/32/O)[o]		
tet(62)[p]	*tet*(S/M), *tet*(W/N/W)		

1.4. Antibiotic resistance in *Vibrio*

As mentioned in the previous chapters, antibiotic-resistant infections have become more challenging to treat with existing antibiotics, leading to infections triggering higher mortality, which consequently imposes costs on our society at several levels[40,47]. This increasing resistance involves many human pathogens, including *Vibrio* species.

Vibrio spp. were thought to be usually susceptible to most antibiotics of veterinary and human significance[95]. The recommended antibiotics for the treatment of *Vibrio* infections are usually quinolones, cephalosporins, tetracyclines, and penicillins[96]. However, in the past few decades, antibiotic resistance has emerged and evolved in this genus due to the excessive use of antibiotics in human medicine, agriculture and aquaculture systems[50]. Consequently antibiotic-resistant *Vibrio* spp. have been increasingly reported, namely in aquaculture systems.

In *V. cholerae*, drug resistance is usually mediated by efflux pumps, by chromosomal mutations and by acquisition of conjugative plasmids, transposons, ICEs, such as SXT elements carrying ARG[57]. Aquatic ecosystems provide a suitable milieu for spreading antibiotic resistance traits through HGT among bacterial populations[97], and in fact multidrug resistant *V. cholerae* have been increasingly reported worldwide, mainly in clinical O1 and O139 strains[98], but also in environmental non-O1/non-O139 isolates in cholera epidemic areas[99].

Furthermore, several investigations reported that both *V. parahaemolyticus* and *V. vulnificus* (two foodborne bacterial pathogens) were resistant towards many clinically used antibiotics[100,101]. Increased concerns in various countries, are related with MDR exhibited in these species, and in other species from the *Harveyi* clade, most frequently against ampicillin, penicillin, tetracycline, and streptomycin[102,101]. A problem usually due to misuse of antibiotics to control infections in aquaculture production, which may raise a serious public health threat and economic concerns, as severe economic setbacks to the aquaculture industry[35,36].

With special attention to tetracycline, in 2010 a study was conducted in India, specifically in tribal areas, related to cholera epidemy (which caused high morbidity), where antibiotic susceptibility was tested, and it was concluded that all studied *V. cholerae* strains were resistant to tetracycline[103]. Likewise, a study in Malaysia revealed that most of the *V. parahaemolyticus* pathogenic strains analyzed, were resistant to ampicillin and/or tetracycline[100]. Hence, these studies highlighted the emergence and spread of tetracycline resistance, which needs to be closely monitored. In addition, *Vibrio* genome is known to have high flexibility, therefore, horizontal transfer of resistance and virulence genes between pathogenic and environmental *Vibrio* is facilitated and likely to happen[102].

Plasmids, as previously described, are one of the important mediators that facilitate the transfer of ARGs, and this correlation between the occurrence of plasmids and antibiotic resistance among *Vibrio* spp., has already been reported[104,105].

In recent years, the prevalence of tetracycline resistance genes *tetA, tetB, tetC, tetD, tetE, tetG, tetH, tetY* and *tetZ* in *Vibrio* has increased[106,107], and currently today, we know that these *tet* genes are usually associated with conjugative plasmids and/or transposons[108]. Referring to this, a study conducted in *V. parahaemolyticus* strains from penaeid shrimp, showed a high level of resistance to tetracycline. This resistance was associated with the *tetB* gene, which in particular was carried in a single copy plasmid (named as pTetB-VA1)[109]. More recently, ISCR (insertion sequence common region) elements, have received some attention for their links to multiple resistance genes. Specifically ISCR2 transposase, involved in rolling circle replication, was found in SXT of *Vibrio cholerae*[110]. Furthermore, IS*CR*2, in strains isolated from multiple animal sources, has been found adjacent to *tetA* and *tetR* in plasmids of *Escherichia coli*[111]. A study conducted from a farm fish demonstrated that IS*CR*2 is associated with tetracycline and other ARGs among *Vibrio* spp., where the resistance rates of isolates carrying IS*CR*2 were much higher than those of isolates without it. These results suggest that in fish farms, IS*CR*2 plays an important function in the dissemination of ARGs[112].

In addition to tetracycline resistance, antibiotic resistance to β-lactams, streptomycin, sulfamethoazole, etc., and associated genes, have been reported in recent studies. A study that aimed to determine antibiotic resistance patterns and genes of *V. parahaemolyticus* strains from Malaysian seawaters and fish[113] revealed that 100% of the strains collected from seawater harbored the streptomycin resistance B gene (*strB*), which confers resistance to streptomycin. Also, among the fish isolates the highest rate of resistance was found in isolates carrying the *blaP1* resistance gene, which confers resistance to β-lactams. Identical studies, in earlier years[114], had revealed lower percentage of *Vibrio* isolates with *blaP1*, therefore increasing the concerns related to antibiotic resistance emergence.

Likewise, additional studies have reported the presence of *aadA16, blaTEM1B, arr3, sul1, dfrA27, Zeo, qacEA* resistance genes and resistance nodulation division (RND) efflux pumps in *Vibrio* genus[115].

The gathering of this information allows the conclusion that the emergence of antibiotic resistance, and consequently MDR, in foodborne bacteria, like *Vibrio*, is a growing concern worldwide and its vigilance is essential to understand the implications resulting from the use of antibiotics, once they function as human foodborne pathogens, constituting a potential health concern associated with their consumption.

1.5. Study area

An estuary is a partly enclosed coastal body of water in which river water meets the sea. This ecosystem is defined by salinity boundaries rather than by geographic boundaries[116]. Tides, waves and the influx of saline water are marine influences that affect estuaries as well as the flow of freshwater and sediment from rivers[117]. Consequently, the mixing of seawater and freshwater provides high levels of nutrients both in the water column and in sediment, making estuaries among the most productive natural habitats in the world[118]. However, estuarine environments have also been reported as major repositories of anthropogenic waste for decades. Urban wastewater discharges from coastal communities, discharges from ships, riverine input and atmospheric deposition are some of the specific key pathways responsible for contaminants to enter in estuarine water. The most common anthropogenic wastes disposed in coastal areas are industrial and municipal wastes, and sewage sludge[119]. So, this extensive range of human impacts can compromise the ecological integrity of estuaries. Ria de Aveiro (study area) is an estuary of the Atlantic coast of Portugal and a protected area, located in the district of Aveiro and extending inland, parallel to the ocean, known due to the diversity of species that it hosts[120].

With a seacoast abundant in fish, fishing became a common practice, which consequently acted as a factor of population fixation in this area[121]. Today, Ria de Aveiro became a highly humanized space, where for instance aquaculture became a common practice. Aquaculture helped, and helps introducing new communities and species. However, consequences and implications related with rise of this practice are becoming more evident each day. Over time, and besides aquaculture, Ria de Aveiro has also been the target of several pollutant discharges, being the main sources of contamination the wastewater from Aveiro's city and the diffuse pollution associated to Aveiro's seaport activities, industrial activities and farming fields nearby[122].

Portugal, has currently more than 1500 aquaculture establishments, many located in Ria de Aveiro[123] (data from 2016), and strategic plans for the development and expansion of this industry have been established, with great emphasis in this estuarine area[124]. Further expansion may lead to a greater administration of antibiotics, (affecting both aquaculture facilities and surrounding areas), which in turn may lead to an increase in resistance among environmental and food-born pathogenic bacteria, like *Vibrio* (fish and seafood pathogen), which are expected to be abundant in estuarine ecosystems.

Although, studies have already reported the presence of *Vibrio* in fish aquaculture in Ria de Aveiro[125], none have determined the diversity of this genus along this coast or related their presence with antibiotic resistance.

2. AIMS OF THIS STUDY

The *Vibrio* genus includes important human and animal pathogens. In estuaries, where aquaculture production is a common practice, animal infections caused by these bacteria may result in important economic loss. On the other hand, the use of antibiotics in aquaculture and consequent contamination of the estuary, imposes a selective pressure that may alter the diversity of *Vibrio* species and select for antibiotic-resistant *Vibrio*. The emergence of antibiotic resistance in food-borne bacteria has become a growing concern worldwide and its vigilance is essential for understanding the implications resulting from the use of antibiotics in aquaculture and other settings.

Hence, this work aims are:

1) to investigate the distribution of antibiotic-resistant *Vibrio* spp. along the estuary Ria de Aveiro and in different seasons (spring, summer and autumn);

2) to perform whole-genome sequence analysis of a tetracycline-resistant *Vibrio diabolicus* isolate from Ria de Aveiro;

3) to estimate *V. diabolicus* virulence, pathogenicity and antibiotic resistance profiles based on WGS analysis;

3. MATERIALS AND METHODS

3.1. Sample collection and colony forming units counting

As referred in the previous chapter, this study was conducted along Ria de Aveiro. This area encloses sites where aquaculture, livestock and agriculture are common practices, along with industrial activity, which resulted for example in metal contamination still detected nowadays in some sites[126,123]. Samples of water were previously collected in the scope of a PhD project from 26 sites (Table 3; Fig.9) in three different sampling campaigns (Autumn 2018, Spring 2019, and Summer 2019) into sterile bottles (2-3 L, at that time immediately transported to the laboratory). Temperature, pH, conductivity, salinity and dissolved oxygen were measured on site using three distinct portable environmental meters, according to manufacturer's instructions. (WTW ProfiLine pH 3110, WTW ProfiLine Cond 3110 and WTW Oxi 330i, Germany).

Different volumes of water (5 -100 mL) were passed in triplicate through 0.45-µm membrane filters (Pall Life Sciences, USA) to concentrate bacteria. These membranes were placed in plates with TCBS agar (5 g/L Peptone from Casein, 5 g/L Peptone from Meat, 5 g/L Yeast Extract, 10 g/L Sodium Citrate, 10 g/L $Na_2S_2O_3$, 5 g/L Ox Bile, 3 g/L Sodium Cholate, 20 g/L Sucrose, 10 g/L NaCl, 1 g/L Iron(III) Citrate, 0.04 g/L Thymol Blue, 0.04 g/L Bromothymol Blue, 14 g/L Agar-Agar; Merck, Germany), a specific medium for the selective isolation and purification of *Vibrio* species, supplemented with 16 µg/mL (clinical breakpoint specific for *Vibrio* spp. described in CLSI document M45-A2[127]) of tetracycline (Sigma-Aldrich, USA), incubated at 30°C for 24 h. To determine the proportion of tetracycline-resistant bacteria, 1 mL to 5 mL of water was filtered in triplicate and membranes placed in TCBS agar without antibiotic. After incubation, presumptive *Vibrio* colonies (exhibiting yellow or green coloration, according to *Vibrio* typical morphology) were counted and the percentage of resistant colonies was determined for each sampling site.

All isolates were kept in 96-well plates in culture medium with 20% glycerol and stored at − 80 °C for further analysis.

Table 3 – Latitude (X) and Longitude (Y) coordinates of the sampling sites represented on the map.

Site	X	Y
1 Gafanha da Encarnação	-8,74033906	40,61429732
2 Vagueira	-8,75782997	40,56038595
3 Praia do Areiro	-8,77597762	40,52084478
4 Poço da Cruz	-8,78593472	40,48920371
5 Vala Real	-8,7523045	40,41548036
6 Foz do Rio Vouga	-8,6248523	40,69499827
7 Ribeira de Esgueira	-8,63681366	40,65720356
8 Ponte dos Carcavelos	-8,65452476	40,64525959
9 Lota	-8,66324337	40,65009433
10 Canal Central	-8,64798392	40,64096103
11 Coutada	-8,66715646	40,62412076
12 São Jacinto	-8,72727022	40,66105331
13 Torreira	-8,69953962	40,76144233
14 Cais do Bico	-8,64789908	40,72853515
15 Cais da Cambeia	-8,62689007	40,73175015
16 Foz do Rio Antuã	-8,5989913	40,72150641
17 Ponte da Varela	-8,67181023	40,78869819
18 Pardilhó	-8,63743313	40,80195917
19 Saltadouro	-8,62617459	40,81349784
20 Cais da Tijosa	-8,64889924	40,8208044
21 Cais da Pedra	-8,65830795	40,85836081
22 Folsa dos Couquins	-8,68564025	40,60463553
23 Vista Alegre	-8,68609247	40,58819408
24 Ponte de Vagos	-8,67201849	40,55043756
25 Porto de Aveiro	-8,72992882	40,65043743
26 Foz da Ria	-8,74873034	40,64406051

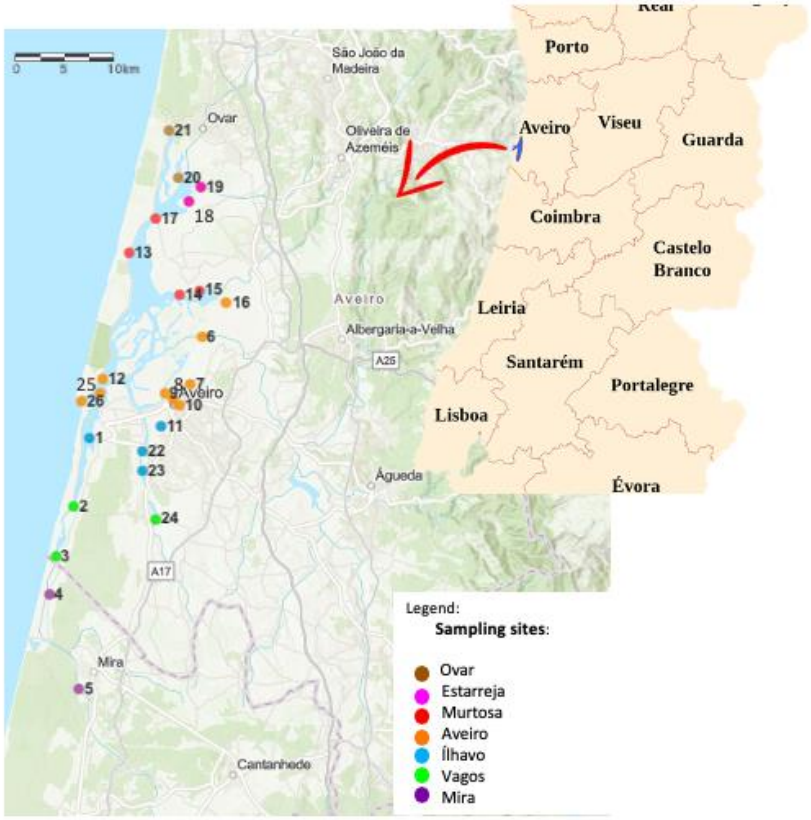

Fig. 9 - Sampling sites. Water samples were collected from the 26 sites marked on the map along Ria de Aveiro in three different campaigns (Spring, Autumn and Summer). The municipalities of Aveiro corresponding to the location of the sampling sites are outline on the map: Ovar (Brown color); Estarreja (Pink color); Murtosa (Red color); Aveiro (Orange color); lhavo (Blue color); Vagos (Green color); Mira (Purple color).

3.2. *Vibrio* cultivation

From 96-well plates, glycerol suspensions previously frozen were recovered. Their purity was confirmed in TCBS medium. Tryptone Soya Broth (TSB; 17g/L casein peptone (pancreatic), 2.5 g/L dipotassium hydrogen phosphate, 2.5 g/L glucose, 5 g/L sodium chloride, 3 g/L soya peptone (papain digest.); Merck, Germany) or Tryptone Soya Agar (TSA; 15 g/L agar, 15 g/L casein peptone (pancreatic), 5 g/L sodium chloride, 5 g/L soya peptone (papainic); Merck, Germany) supplemented with 1.5 % NaCl (1.5 g/100mL) were used as alternatives to cultivate isolates when growth was not observed in TCBS. Isolates were incubated at 30 ºC for 24-48 h to obtain pure isolates.

3.3. Molecular identification of *Vibrio* spp.

The isolates were identified based on the sequencing of a *pyrH* gene fragment (500 nt; Thermo Fisher Scientific, USA) coding for uridylate kinase, as described in previous reports[128,129]. The PCR reactions were performed in a final volume of 25 µL, containing 12.5 µL NZYTaq II 2× Green Master Mix (2.5 mM MgCl2; 200 µM dNTPs; 1.25 U DNA polymerase; NZYTech, Portugal), 9.5 µL dH_2O, 10 pmol of forward primer (*pyrH_F* - 5' GATCGTATGGCTCAAGAAG 3'), 10 pmol of reverse primer (*pyrH_R* - 5' TAGGCATTTTGTGGTCACG 3'), and 1 µL of DNA. DNA was obtained with two different procedures, using Silica Bead DNA Gel Extraction Kit (Thermo Fisher Scientific, USA) according to manufacturer's instructions, and from a cell suspension prepared by mixing 20 µL of distilled water and cells from one-two isolated colonies and boiled for 5 min at 100 ºC. The amplification was performed under the following conditions: initial denaturation at 95°C for 5 s, followed by 33 cycles of 95°C for 1 min; 59°C for 2 min 15 s and 72°C for 1 min 15 s, and a final extension of 72°C for 10s using a MyCycler Thermal Cycler (BioRad, USA). Amplified PCR products were visualized on 1.5 % agarose gel stained with ethidium bromide, run at 90 V for 60 min, and photographed using a gel documenting system (Biorad Gel Doc EQ System, USA).

The PCR products were purified using NZYGelpure purification kit (NZYtech, Portugal) according to the manufacturer's instructions and sent to GATC Biotech (Germany) for sequencing. Results were compared with published sequences in the GenBank database (www.ncbi.nlm.nih.gov/genbank/) using Basic Local Alignment Search Tool (BLAST) search[130].

3.4. Phylogenetic analysis of the isolated *Vibrio* spp.

Based on the sequences of *pyrH* gene, the phylogenetic analysis was conducted in the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0[131], initially aligned using the ClustalW[132] algorithm, integrated in the MEGA7 software. Subsequently, a phylogenetic tree was constructed using the neighbor-joining method, which included the sequences obtained in this study along with NCBI *pyrH* sequences of the closest *Vibrio* type strains. Tamura-Nei gamma distributed with invariant sites[133] was applied, which was the evolutionary model that best described the sequence data. Simultaneously, bootstrap values were applied for accessing reliability of the branches that were calculated by resampling 1000 times. The tree was posteriorly visualized using iTOL[134].

3.5. Whole Genome Sequencing, assembly and annotation

Vibrio P7A genomic DNA was purified using the Wizard Genomic DNA Purification kit (Promega, USA). The quality and quantity of the purified DNA was assessed, and DNA was sequenced using Illumina HiSeq 2500 platform (Illumina, USA) following manufacturer's instructions. Resulting raw reads were then subjected to a quality control using FastQC software (v.0.11.9), and in order to remove those with low quality and possible adaptor sequences, the merged reads were trimmered by applying Trimmomatic 0.36[135]. SPAdes (version 3.14.0) was then used to assemble the genome sequence data using the sequencing reads of phred quality score $\geq$ 20[136].

The assemble genome was annotated using the SEED and the Rapid Annotation using Subsystems Technology (RAST), a fully automated service for annotating microbial genomes[137,138]. The tRNA genes were predicted using the tRNAscan-SE[139], while rRNA genes were predicted using RNAmmer 1.2[140].

3.6. *Vibrio* diabolicus P7A species identification

In order to confirm species identification, the genome of the P7A isolate was compared with 11 type strains from *Vibrio* genus, specifically belonging to the *Harveyi* clade (obtained from PATRIC Database[141]) with an all-versus-all alignment using JSpeciesWS[142]. This web server calculates the average nucleotide identity (ANIb) between a query genome and a reference genome, representing a reliable method of genetic and evolutionary relatedness, where organisms belonging to the same species typically show 95–96% ANI among themselves[143,144]. Digital DNA-DNA hybridization (dDDH) values using the Genome-to-Genome Distance Calculator 2.1[145] were also determined, which a value of 70 % DDH is the recommended cut-off point for

species delineation[143]. G+C % divergence between P7A genome and the 11 type strains from *Vibrio* genus were also considered for the overall genome relatedness analysis[146].

3.7. Multi-Locus Sequencing Analysis

Additionally, in order to further decipher the evolutionary relationship between the P7A isolate and the closest *Vibrio* species, multi-locus sequence analysis (MLSA)[147] was used. Full length gene sequences and partial nucleotide sequences of four housekeeping genes, including *atpA, gyrB, pyrH* and *recA*, were obtained from PATRIC Database and PubMLST[148] (public database for molecular typing and microbial genome diversity), respectively. The concatenated nucleotide sequences of the four housekeeping genes (in the following order: *atpA, gyrB, pyrH* and *recA*) were imported into the software MEGA software version 7.0[131] for alignment using ClustalW, implemented in MEGA7 software. The phylogenetic neighbor joining trees (one including full length gene sequences and the other using partial nucleotide sequences) were constructed along with 11 type strains included in *Vibrio* genus and the published 11 *Vibrio diabolicus* strains by applying kimura 2-parameters gamma distributed with invariant sites. Bootstrap confidence analysis was carried out with 1,000 replicates for evaluating the robustness of trees topologies.

3.8. Comparative genomic analysis of *Vibrio diabolicus*

Comparative genome analysis was performed using the EDGAR 2.0 (Efficient Database framework for comparative Genome Analysis using BLAST score Ratios) software[149]. For orthology estimation EDGAR employs a bit score related cut-off value, which is generated based on BLAST score ratio values (SRVs). Thus, if the predicted SRVs exceeds the calculated cutoff, coding sequences (CDS) from different genomes are considered orthologous. In contrast, only CDS without any hit against any of the other genomes are considered as true singletons. This approach allows the identification of real orthologues as representatives of the calculated core genomes[149].

A private project was constructed comprising publicly available genome data of 11 *V. diabolicus* strains and *V. diabolicus* P7A isolate. EDGAR 2.0 was used for the calculation of core and pan genomes across the *V. diabolicus* strains and true singleton prediction of P7A genome.

3.9. Analysis of potential virulence, antibiotic and metal resistance genes

The occurrence and diversity of genes related to virulence, disease and defense was investigated using RAST subsystem feature counts implemented in the web-based SEED Viewer. For this purpose, the genome of P7A isolate was inspected for proteins related to five subsystems: (1) adhesion, (2) toxins and superantigens, (3) bacteriocins and ribosomally synthesized antibacterial peptides, (4) resistance to antibiotics and toxic compounds and (5) invasion and intracellular resistance. Likewise, VFDB, which is a reference database collectively presenting the virulence factors of various medical significant bacterial pathogens[150], was also used.

In addition, three web-based tools were accessed to predict genes related with antibiotic and metal resistance: Resfinder 3.2[151], which uses BLAST to determine the presence of acquired ARGs in whole-genome data, CARD[152] (Comprehensive Antibiotic Resistance Database) that uses the Resistance Gene Identifier (RGI) software for resistome analysis and prediction, and Bacmet[153] in order to identify metal resistance genes.

All the genetic determinants presenting similarity of 40% or more were noted and analyzed. Furthermore, genomic islands and putative recombinases were predicted and analyzed using the Genomic Island Prediction Software (GIPSy) v.1.1.3[154], where the *V. diabolicus* type strain CNCM I-1629^T genome was used as reference.

Ultimately, the program BRIG v.0.95 (BLAST Ring Image Generator)[155] was used to visualize the genomic similarity between *V. diabolicus* P7A and the available published *V. diabolicus* genomes, highlighting antibiotic resistance determinants, pathogenicity islands, and prophage sequences identified in *V. diabolicus* P7A's genome.

4. RESULTS AND DISCUSSION

4.1. Isolation and identification of *Vibrio* spp.

A total of 376 presumptive *Vibrio* isolates were obtained from Ria de Aveiro, collected in three different sampling campaigns (Spring, Autumn and Summer), in the scope of a previous study. During the present study, based on the partial sequencing of *pyrH* (500 nt) gene, 28 (23.7%), 30 (22.2%) and 20 (16.2%) isolates from a total of 118, 135 and 123 isolates collected in Spring, Autumn and Summer campaigns, respectively, were affiliated to *Vibrio* species. Meaning, that only 21% (78) of the total 376 presumptive *Vibrio*, were actually members of this genus. Several studies have already described that isolation/enumeration of *Vibrio* may be hampered by the variable adaptability of different taxa to TCBS medium[10,156]. Likewise, TCBS agar NaCl composition is very different from the NaCl content that is found in sea water and estuarine water and likely poor suitable to ensure the maximum recovery of *Vibrio*, allowing other bacteria to grow. Hence, it has recently been proposed a modified TCBS, adjusted for marine *Vibrio* requirements, which seems to improve their recovery in dilution plate counts[10]. In future studies, the use of this novel formulation is advisable. Additionally, the fact that TCBS medium used for isolation of *Vibrio* spp. was supplemented with tetracycline may also have affected *Vibrio* recovery, since this bacterium was thought to be usually susceptible to most antibiotics of veterinary and human significance, which includes tetracycline[95].

The evolutionary history of the *Vibrionaceae* family, which includes the genus *Vibrio* was defined and updated by Sawabe et al[147] based on the concatenated sequences of 8 housekeeping genes (*ftsZ, gapA, gyrB, mreB, pyrH, recA, rpoA* and *topA).* The *Vibrionaceae* family comprises 22 distinct clades: *Anguillarum, Cholerae, Coralliilyticus, Diazotrophicus, Gazogenes, Fischeri, Halioticoli, Harveyi, Nereis, Nigripulchritudo, Orientalis, Scophthalmi, Splendidus, Vulnificus, Damselae, Mediterranei, Pectenicida, Phosphoreum, Profundum, Porteresiae, Rosenbergii,* and *Rumoiensis*[147].

In the present study, the 78 isolates obtained (Table 4) were categorized into eleven species and three clades[147] by the construction of a phylogenetic tree using the *pyrH* gene fragment of each isolate (Fig.10): the *Harveyi, Cholerae* and *Mediterranei* clades. The *Harveyi* clade (which comprised 96.0% of the isolates) included species *V. diabolicus* (n=29 isolates), *V. alginolyticus* (n=11 isolates), *V. parahaemolyticus* (n=22 isolates), *V. owensii* (n=3 isolates), *V. jasicida* (n=2 isolates), *V. campbellii* (n=3 isolates) and *V. mytilli* (n=2 isolates). The *Cholerae* clade (6.7%) consisted of *V. cholerae* (n=1 isolates), *V. fluvialis* (n=1 isolates) and *V. furnissii* (n=3 isolates), while 1.3% of the isolates belonged to the *Mediterranei* clade, which only included *V. mediterranei* (n=1 isolates). However, from the total eleven species identified, the most frequently identified were *Vibrio diabolicus* (37.2% of the total) *Vibrio parahaemolyticus* (28.2% of the total) *and Vibrio alginolyticus* (14.1% of the total).

Table 4 - Isolates identified from each sampling campaign (the number in the Isolate indicates the number of the sampling site on the map shown below (Fig.16), size of the *pyrH* fragment from each isolate used for phylogenetic affiliation, % identity to the closest species from BLAST search and the respective accession number from the Genbank database.

SPRING					AUTUMN					SUMMER				
Isolates	**Species**	**% Nucleotide Identity**	**Fragment size**	**Accession number**	**Isolates**	**Species**	**% Nucleotide Identity**	**Fragment size**	**Accession number**	**Isolates**	**Species**	**% Nucleotide Identity**	**Fragment size**	**Accession number**
P1A	*V. alginolyticus*	99.37	476	CP051109.1	O1I	*V. diabolicus*	99.79	472	KC871681.1	V7C	*V. diabolicus*	100	475	CP014094.1
P2E	*V. parahaemolyticus*	100	477	CP014046.2	O1F	*V. diabolicus*	100	490	KC871681.1	V8A	*V. parahaemolyticus*	100	478	CP014046.2
P7A	*V. diabolicus*	100	484	KC871681.1	O2J	*V. diabolicus*	99.79	476	KC871681.1	V8B	*Vibrio campbellii*	100	466	CP033134.1
P7C	*V owensii*	99.57	463	MK690269.1	O12D	*V. alginolyticus*	100	476	JX401640.1	V8C	*V. campbellii*	100	471	CP033134.1
P9C	*V. diabolicus*	99.77	438	CP014094.1	O12E	*V. diabolicus*	100	478	AP022865.1	V8D	*V. parahaemolyticus*	100	475	CP023472.1
P9D	*V. parahaemolyticus*	100	466	CP051100.1	O13A	*V. alginolyticus*	99.79	475	CP042449.1	8VF	*V. alginolyticus*	100	468	CP051109.1
P9E	*V. diabolicus*	99.75	401	CP014094.1	O14D	*V. diabolicus*	100	471	CP014134.1	V8E	*V. parahaemolyticus*	99.58	475	CP014046.2
P9F	*V. diabolicus*	99.79	472	CP014094.1	O15D	*V. diabolicus*	99.79	475	CP014094.1	V8G	*Vibrio campbellii*	100	472	CP033134.1
P10A	*V. jasicida*	99.79	476	MK690254.1	O16E	*V. diabolicus*	99.79	472	CP014094.1	V9A	*V. parahaemolyticus*	99.58	472	CP014046.2
P10D	*V. parahaemolyticus*	100	471	CP051100.1	O16H	*V. diabolicus*	99.79	472	CP014094.1	V9B	*V. parahaemolyticus*	99.37	477	CP014046.2
P10E	*V diabolicus*	100	481	CP014094.1	O17A	*V. diabolicus*	99.79	478	CP014094.1	V9E	*V. owensii*	99.58	475	CP045859.1
P18B	*V. diabolicus*	99.79	467	CP014094.1	O17H	*V. alginolyticus*	100	475	CP042449.1	V9F	*V. alginolyticus*	100	475	JX401640.1
P18D	*V. diabolicus*	99.79	469	CP014094.1	O17I	*V. alginolyticus*	100	477	CP042449.1	V10A	*V. parahaemolyticus*	100	471	CP014046.2
P18F	*V. diabolicus*	100	440	CP014094.1	O18D	*V. alginolyticus*	99.79	477	AP022865.1	V10B	*V. owensii*	99.78	465	LC370187.1
P19C	*V. diabolicus*	100	473	CP014094.1	O18F	*V. parahaemolyticus*	99.79	477	CP051111.1	V10C	*V. parahaemolyticus*	99.79	473	CP044062.1
P19D	*V. parahaemolyticus*	100	483	CP023472.1	O19A	*V. alginolyticus*	99.58	480	AP022865.1	V10E	*V. owensii*	99.78	464	CP045859.1
P19E	*V. diabolicus*	99.79	467	CP014094.1	O19B	*V. diabolicus*	100	478	KC871681.1	V11A	*V. diabolicus*	100	475	CP014094.1
P20B	*V. parahaemolyticus*	100	482	CP051100.1	O19C	*V. parahaemolyticus*	100	477	CP051100.1	V11C	*V. diabolicus*	100	471	CP014094.1
P20C	*V. parahaemolyticus*	99.75	471	CP051111.1	O19D	*V. diabolicus*	100	478	AP022865.1	V14B	*V. furnissi*	99.79	474	CP046797.1
P20	*V. jasicida*	100	471	MK690254.1	O20C	*V. alginolyticus*	100	475	JX401640.1	V15C	*V. furnissi*	100	473	CP046797.1
P20I	*V. parahaemolyticus*	99.79	476	CP051111.1	O21A	*V. diabolicus*	100	478	KC871681.1	V24D	*V. diabolicus*	100	478	CP014094.1
P20H	*V. parahaemolyticus*	99.58	473	CP014046.2	O21B	*V. diabolicus*	100	480	CP014094.1	V25A	*V. fluvialis*	100	470	CP051108.1
P22B	*V. cholerae*	100	480	CP046737.1	O21F	*V. diabolicus*	100	475	CP014094.1					
P22E	*V. alginolyticus*	99.38	480	CP051109.1	O21H	*V. diabolicus*	100	476	CP014094.1					
P23C	*V. mytili*	100	472	GU266287.1	O22A	*V. parahaemolyticus*	100	477	CP051111.1					
P23D	*V. parahaemolyticus*	99.79	472	CP007004.1	O22B	*V. furnissii*	99.58	477	CP046795.1					
P23E	*V. parahaemolyticus*	99.79	476	CP051111.1	O22C	*V. parahaemolyticus*	99.79	478	CP051113.1					
P23F	*V. mytili*	99.78	506	GU266287.1	O23A	*V. parahaemolyticus*	99.79	476	CP034298.1					
					O23B	*V. mediterranei*	99.78	453	CP018308.1					
					O23C	*V. diabolicus*	100	479	AP022859.1					

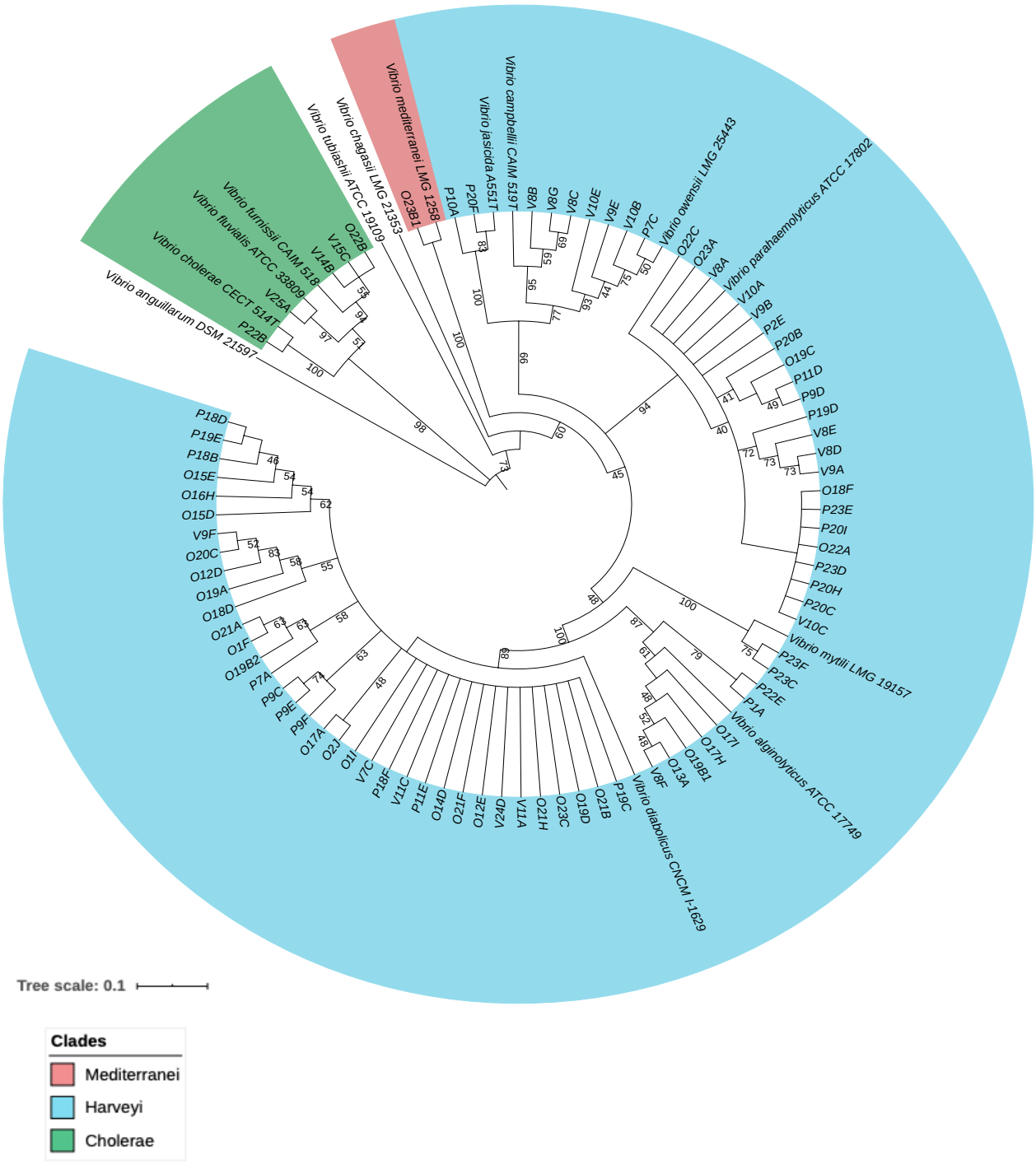

Fig. 10 - Neighbor joining tree constructed from Tamura Nei distance calculated from *pyrH* sequences, of the strains isolated from water samples and reference strains sequences acquired from the NCBI Genbank. The numbers at nodes represent levels (%) of bootstrap support from 1000 resampled datasets. Red, blue and green ranges correspond to *Mediterranei*, *Harveyi*, *Cholerae* clades, respectively.

The *Harveyi* clade corresponded to the dominant *Vibrio* group, including 72 isolates and 7 of the total 11 species identified. Members of this clade are commonly found in estuarine surface waters and sediments, as commensals on the surface or within the intestinal flora of marine animals, as opportunistic pathogens, or as primary pathogens of many commercially farmed marine invertebrate and vertebrate species[8]. *V. campbellii, V. owensii, V. alginolyticus* and *V. parahaemolyticus* are known as pathogens of commercial and medical importance, some being major aquaculture-associated pathogens[8,157], while *V. mytilli* is not known to be pathogenic to humans or animals[34].

Of these, *V. alginolyticus* and *V. parahaemolyticus* are frequently the most abundant species detected in estuarine environments[158,159]. However and interestingly, the frequent recovery of *V. diabolicus* strains, taking into account the total number of isolates obtained from Ria de Aveiro estuary, suggests that this species is more broadly distributed than previously thought, few being the reports that describe a significative presence of this species in estuarine environments[160,161]. This bacterium is in fact, originating from deep-sea hydrothermal vents[162]. The registered numbers could be associated with the fact that this species isolates were resistant to tetracycline (since this antibiotic was used in the TCBS culture medium for isolation), which could justify its prevalence in the collection. However, in several studies where *V. diabolicus* was assessed for their antibiotic susceptibility to tetracycline, this species revealed to be susceptible[163,164,165], and only trough studies of genomic analysis, resistance to tetracycline has been infered[166]. Therefore, this study may have not just revealed a more significative abundance of *V. diabolicus* in estuarine environments, as well as a possible high tetracycline resistance never experimentally registered before.

As referred for *V. diabolicus*, all the isolates identified and described in this study, are potentially resistant to tetracycline. A very interesting questionnaire study, headed to several professionals from the aquaculture sector in 25 countries, revealed that *Vibrio* spp. were among the most frequently reported ARB, most often reported for tetracycline. Also, 20% or more of respondents reported observing *Vibrio* resistance 'Frequently' for three or more antibiotics[167]. For instance, *V. parahaemolyticus* has been showing MDR due to misuse of antibiotics to control infections in aquaculture production, where most frequently observed antibiotic resistance profiles involved ampicillin, penicillin and tetracycline regardless of the countries[101,168,], similar to what is observed in *V. alginolyticus*[169]. Despite this, tetracycline remains one of the antibiotics of choice for treating infections caused by pathogenic *Vibrio*[170]. Our results raise serious concerns about its efficacy.

Furthermore, *V. cholerae, V. fluvialis* and *V. furnissii*, all members of *Cholerae* clade, are commonly isolated from estuarine water and are considered pathogenic to humans and have been associated with foodborne diseases[159]. Also, all three species have been associated with tetracycline resistance, related to aquaculture farms[171,172].

The isolation of potentially pathogenic *Vibrio* species for humans from Ria de Aveiro raises concerns, mainly because this estuary is used intensively for seafood harvesting and production, especially bivalves, usually eaten undercooked. Furthermore, sports and recreational activities are common practices in this estuary, resulting in human exposure to estuarine bacteria. Likewise, over the years, there has been a dramatic increase of touristic activity (such as, boat trips and use of water and mud for therapeutic and cosmetic purposes) in the estuary once again facilitating contact with contaminated water.

Lastly, *V. mediterranei* has been isolated from seawater, plankton, various marine organisms, such as coral and shellfish, usually in Mediterranean coast and sea[128,173]. This species has been considered an aquaculture-related pathogen, recently being related to mortality outbreaks worldwide[174]. Additionally, few are the reports concerning antibiotic resistance of these microorganisms, and the ones that reported it, shown susceptibility to tetracylcline[175]. Meaning, that the isolation of this species in tetracycline-supplemented culture medium is a further evidence of the growing emergence of antibiotic resistance.

4.2. Seasonal and spatial dynamics of *Vibrio* and effects of environmental parameters

Results obtained in this study suggested a low seasonal variability of the abundance of *Vibrio* within Ria de Aveiro. Previous studies regarding the seasonal dynamics of the *Vibrio* populations, from a marine fish aquaculture in this region, also showed lower seasonal variation in terms of abundance, although revealing new dominating populations appearing mainly in Spring[176]. However studies in other estuarine regions, revealed higher seasonal variability of the abundance of *Vibrio*, with increased abundance in warmer months[159]. In the present study, seasonal dynamics was noted in terms of diversity, with a higher number of species in Spring and Summer seasons, as shown in Fig.11.

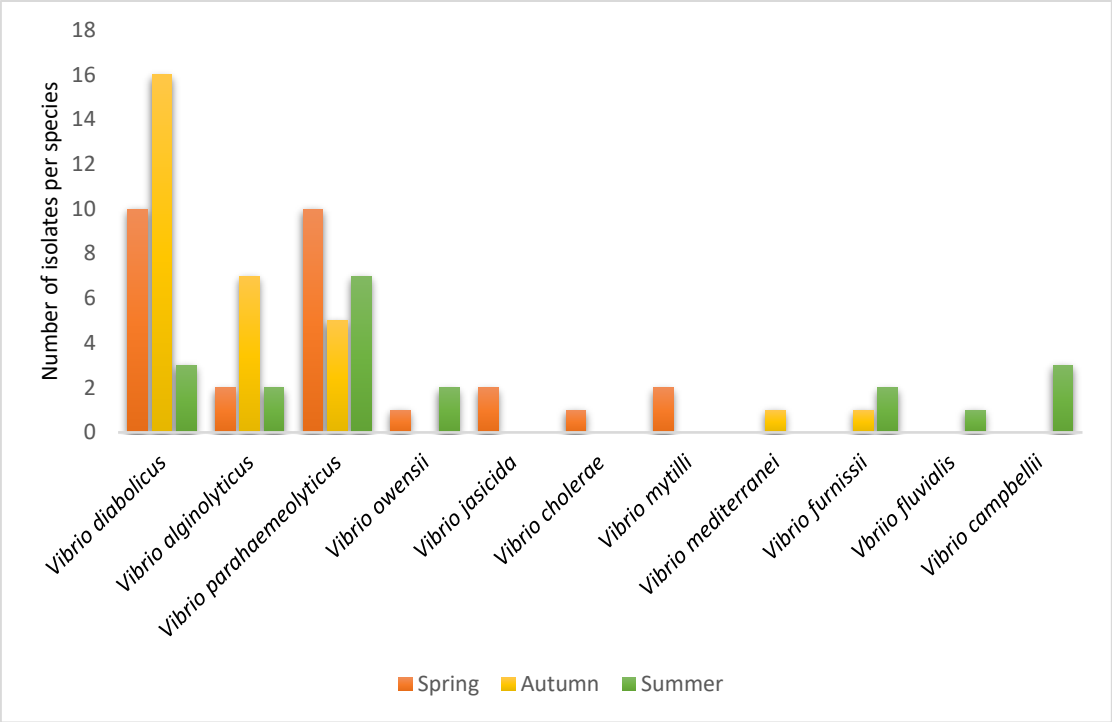

Fig. 11 - *Vibrio* species identification and number of isolates per species obtained in Spring, Autumn and Summer campaigns.

V. diabolicus, *V. alginolyticus* and *V. parahaemolyticus* were the only three species found simultaneously in all three campaigns. Although, *V. diabolicus* was in overall the most abundant species, it was only clearly dominant in the Autumn season. Along with *V. parahaemolyticus*, *V. diabolicus* was one of the two dominant species in the Spring campaign, while in Summer *V. parahaemolyticus* was the most abundant species. According to other studies, *V. diabolicus* and *V. alginolyticus* were also isolated predominantly during colder seasons[177], and in the case of the *V. diabolicus* was mostly found in the water column[178]. Contrariwise, high *V. parahaemolyticus* densities have been associated with higher water temperatures during warmer months[12]. In fact, in our study a slightly positive correlation (0.3285) between *V. parahaemolyticus* abundance with Spring and Summer campaigns was obtained. Likewise, *V. parahaemolyticus*, is a halophilic bacteria related to higher salinities[159]. While some studies have identified a significant relationship between *V. parahaemolyticus* and salinity[179], others did not[180], so the relationship with salinity may be variable and complex. In our study a very weak correlation (0.1181) was verified between *V. parahaemolyticus* abundance and salinity values.

A total of seven species were identified in Summer and Spring. Several studies reported that higher temperatures are preferable for growth of the majority of *Vibrio* spp.[159,181]. However other studies also reported that temperature alone does not affect significantly *Vibrio* abundance[182]. From Autumn campaign, only 5 species were described, including *V. diabolicus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. mediterranei*, which was only found in this campaign, and *V. furnissii*. *V. jasicida*, *V. cholerae and V. mytilli* were exclusively identified in Spring

campaign, while *V. fluvialis* and *V. campbellii* were unique to the Summer campaign. This seasonal variation is probably related to the variation in water physic-chemical parameters but may also be related to fluctuations in the use of antibiotics that may result in a different distribution of resistant strains[183].

The values of the water physic-chemical parameters, measured at each collection point (in the scope of a previous study), including dissolved oxygen (DO), conductivity, salinity, pH and temperatures are presented in Table S 1.

The correlation between the number of *Vibrio* isolates across the estuary and the environmental parameters was in general weak and statistically not significant (Table 5), probably due to the relatively low number of isolates. Even so, in Spring the strongest positive correlations were with water temperature (16.5- 23.4°C), salinity (0.200 - 33.5 g/kg) and conductivity (0.470-50.1 mS/cm); in Autumn, the number of *Vibrio* isolates was also positively correlated with salinity (0.2 - 34.8 g/kg) and conductivity (0.530-52.9 mS/cm); in Summer, the strongest positive correlation was with DO (2.90-6.18 (mg/l)).

Table 5 - Correlation coefficient between environmental parameters and the total of *Vibrio* isolates obtained. All values have *P* value of >0.05.

		Salinity	**Temperature**	**DO**	**Conductivity**	**pH**
***Vibrio* Abundance**	Spring	0.2859	0.3857	0.1101	0.2955	0.3471
	Autumn	0.3754	0.2312	-0.1923	0.3820	-0.0550
	Summer	0.2781	0.1091	0.3231	0.2729	-0.2560

Overall, the water temperature values ranged from 13.5 ºC (Autumn 2018) to 27.7 ºC (Summer 2019). In our study, a positive correlation was observed between temperature and the three campaigns (Table 5). However, besides the Spring season, did not appear to be a significative influencing factor in *Vibrio* spp. distribution or incidence, disagreeing with several similar studies, where a significant positive association was observed between *Vibrio* abundance in estuaries and temperature, as referred above[184,185].

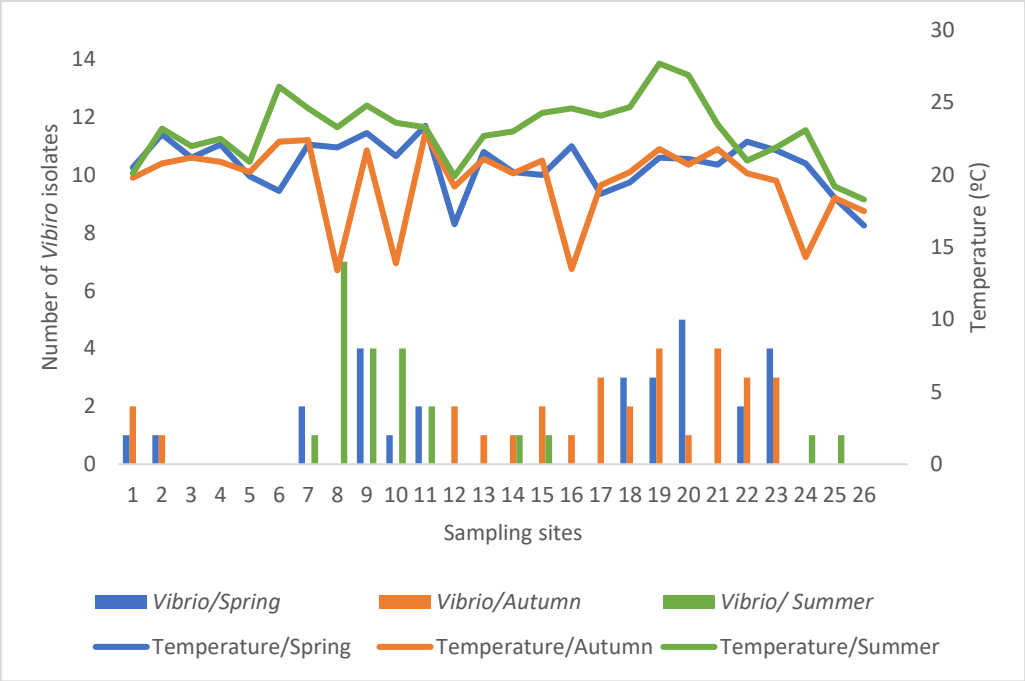

Fig. 12 - Variation of temperature levels and number of *Vibrio* isolates per sampling site from Spring, Autumn and Summer campaigns.

Additionally to temperature, the abundance and distribution of *Vibrio* have also been strongly linked to salinity, but also depending on the species and its habitat, and the geographic location[12,185,186,]. In this study, salinity oscillated between 0.2 and 34 ± 0.5 in the three campaigns. There were no *Vibrio* isolates retrieved from the sampling sites (sites 3-6; Fig. 12) where the salinity values were below 11.6, which indicated that salinity played a key role in the distribution of *Vibrio* spp.. A positive influence of water salinity on the occurrence of *Vibrio* has often been reported when the variation of salinity values was broad enough[179,186,] , as observed in our study.

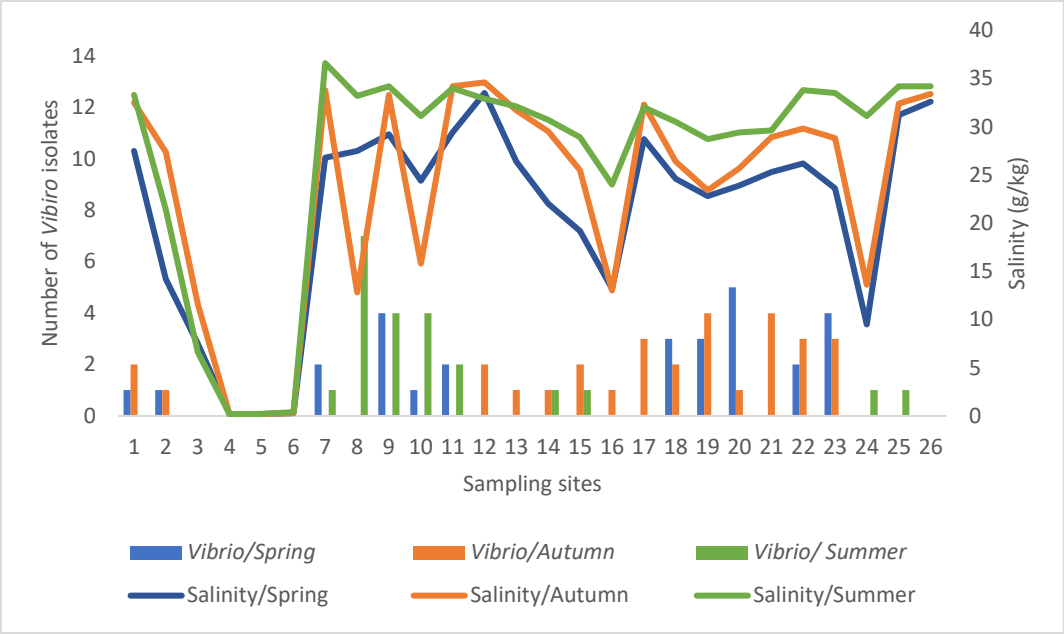

Fig. 13 - Variation of salinity levels and number of *Vibrio* isolates per sampling site from Spring, Autumn and Summer campaigns.

On the other hand, for the same sampling sites seasonal salinity variation was not pronounced. Even so, in most sampling sites salinity values during the Summer campaign were higher, possibly due to higher levels of precipitation (which brings freshwater into the estuary) during the sampling periods of Spring and Autumn. However, the abundance of *Vibrio* in the Summer campaign (20 isolates) remained almost similar to the other campaigns (28 and 30 isolates, respectively from Spring and Autumn campaigns), being in fact lower. A study conducted on the French Atlantic Coast[187] found a link between salinity and presence of *V. parahaemolyticus* in sediments regarding the influence of precipitation and other water perturbations. They realized that with less freshwater inputs, i.e rain, less would be the sea water perturbed by them, and therefore high salinity will favor sedimentation of bacteria. Likewise, in Summer season they identified a larger abundance of *Vibrio*, especially *V. parahaemolyticus*, attached to sediment particles compared to the rest of the year, which can help justify the low number of *Vibrio* isolates collected in Summer in our study, since they derived from water samples.

Furthermore, the pH values in the range of 8.4-8.6 are considered ideal for *Vibrio* growth[188]. The pH of the water measured in the sampling sites considered in this study, varied between 6.80 to 9.42 taking into account the three sampling campaigns, and therefore including sites that were not within the limits settled for *Vibrio* growth, these being 7.5 to 8.6[189]. Also, was the most constant parameter monitored during the course of the study.

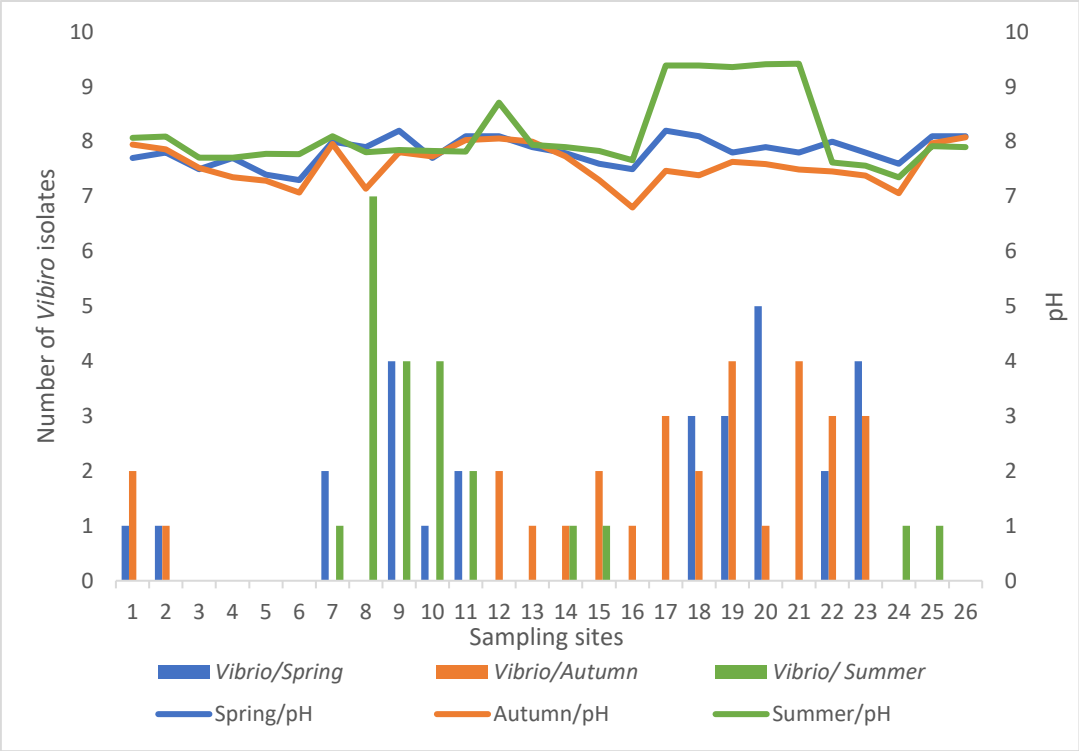

Fig. 14 - Variation of pH levels and number of *Vibrio* isolates per sampling site from Spring, Autumn and Summer campaigns.

As observed in Fig. 14, sites 18 to 23 exhibited in general a high number of *Vibrio* isolates in both Spring and Autumn campaigns, results that were not obtained in Summer campaign. Values of pH in these sites in Summer were above 9, differing significantly from values registered during the other campaigns, which may have been one of the responsible factors for the absence of *Vibrio* in these locations, in this particular campaign. As for other studies, pH has been found to be important in the ecology of *Vibrio* spp.[186].

Lastly, DO, as seen on the graphic (Fig.15) varied significantly from Autumn to Spring and Summer seasons, with lowest values appearing during Summer, where also was observed the strongest correlation between number of *vibrio* isolates and DO (0.3231). Studies have reported *V. parahaemolyticus* levels in water to be strongly correlated with DO during warmer seasons[190], however different correlations was observed in Spring and Autumn seasons.

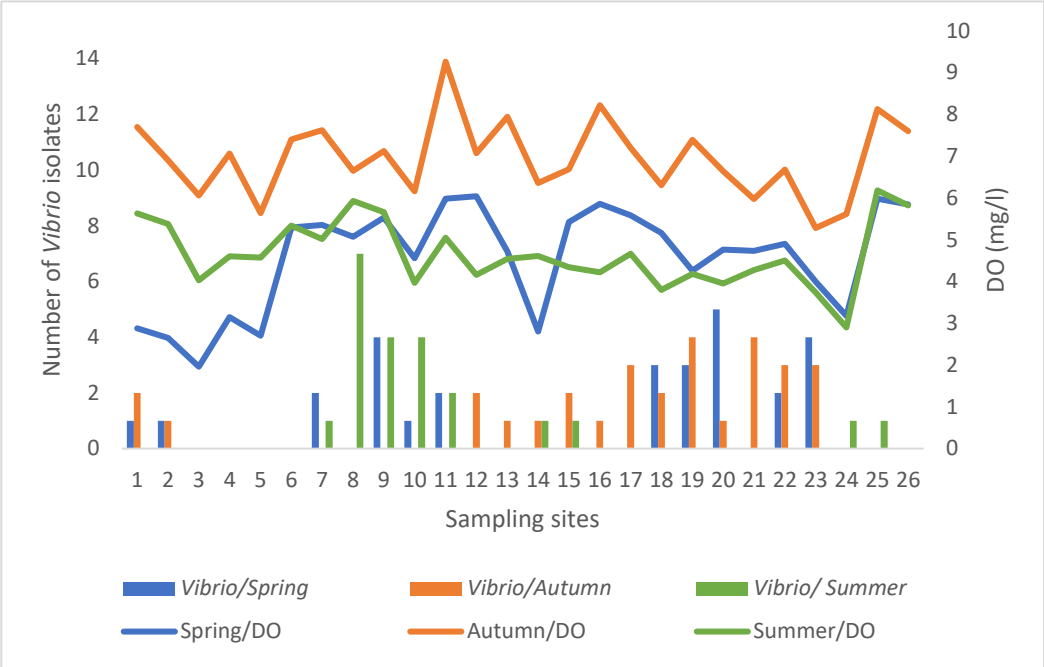

Fig. 15 - Variation of DO levels and number of *Vibrio* isolates per sampling site from Spring, Autumn and Summer campaigns.

Low DO in a water column implies deoxygenation, likely from rapid phytoplankton growth such as algal blooms. It is well known that the water temperature has a notable effect on the phytoplankton growth and metabolic processes, therefore affecting oxygen consumption and production. Correspondingly, the plankton oxygen consumption is a function of DO levels that depend on temperature[191]. Furthermore, *Vibrio* have the ability to adhere and colonize the surface of marine plankton and thus the nutrient-rich surfaces of plankton have long been known to selectively enrich for *Vibrio*, resulting in higher densities of these bacteria than the surrounding water column[11,14]. With that being said, the inclusion of phyto- and zooplankton counts, the measure of dissolved carbon in water, as well as the collection of sediments samples may further help to better understand *Vibrio* abundance and distribution.

In terms of the spatial distribution of the isolates, anthropogenic pressure may also contribute to this distribution. For instance, as referred in the previous chapters, aquaculture is a common practice in Ria de Aveiro, with currently 75 aquaculture establishments along the estuary (Table. 6; Source: DGRM), having projects in development to extend[124].

Table 6- Aquaculture establishments distributed along Ria de Aveiro estuary in the municipalities of the Aveiro district.

Aveiro municipalities	Aquaculture companies
Ovar	3
Murtosa	5
Ílhavo	**36**
Albergaria-a-velha	1
Aveiro	**22**
Vagos	2
Castelo De Paiva	1
Águeda	2
Vale De Cambra	2
Espinho	1

The majority of these aquaculture companies (77%) are located in Ílhavo and Aveiro, which corresponds to the blue and orange dots in the map (Fig. 16), respectively. From the total number of isolates obtained in this study, as seen in Fig.16, a large number (n=39; 50%) of the tetracycline-resistant isolates identified were collected from those areas, namely from sites 8, 9, 10 11, 22 and 23. Therefore, suggesting a possible association of these resistant bacteria to the aquaculture practice in this region. However, further studies are needed to confirm this relationship. For example, through the collection of water samples at these sites and characterization of the isolates obtained for their resistance to other antibiotics or by quantifying resistance genes in the surroundings of aquaculture systems and comparing them with further distant sites.

In the North of the Ria de Aveiro, Ovar and Estarreja region, namely from sites 17-21 (Fig. 16), a significative number (n=28; 36%) of *Vibrio* isolates was also identified. In that area, aquaculture production is not common. A possible reason for the incidence of *Vibrio* with tolerance/resistance to tetracycline may be the contamination from animal production farms, which are frequent in this area and where antibiotics are also administered for disease control, probably ending up affecting the estuary[192]. Also, as referred in the Introduction chapter, TCs can be carried and dragged in water currents for long distances, situation already observed in Portuguese aquaculture systems and aquatic surroundings[76]. Although the fishery sector authorities indicated excessive use of TC in aquaculture as well as their consequences[70], a lack of information on aquaculture use persists, and, concerning Portugal, very few data are available on TC residues in aquaculture systems or aquatic surroundings. In future studies, the occurrence of this and other antibiotics in the estuarine water of Ria de Aveiro should be determined in order to establish correlations with the distribution of resistant bacteria.

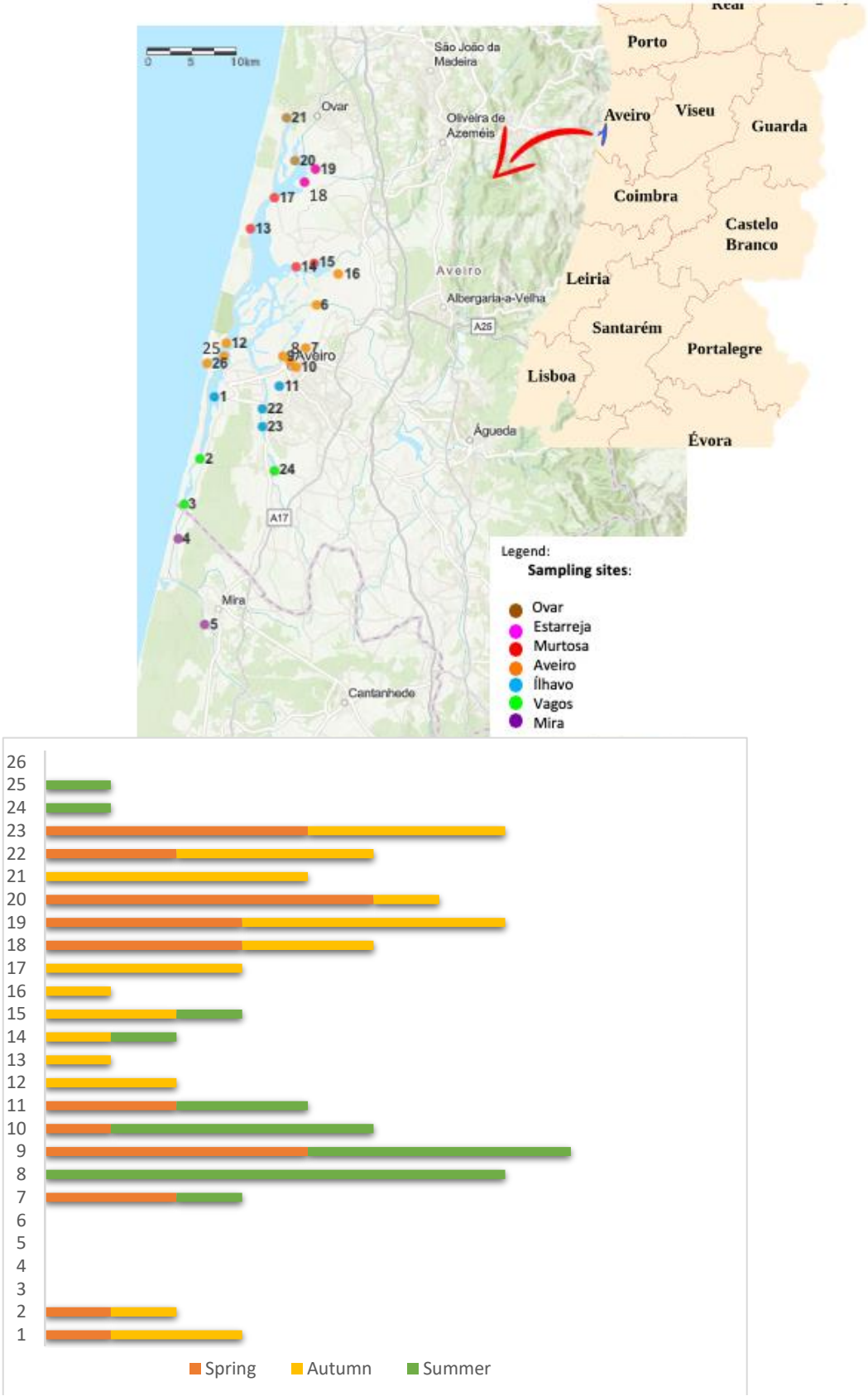

Fig. 16 -Total number of isolates per sampling site obtained from Spring, Autumn and Summer campaigns. Sampling sites. Water samples were collected from the 26 sites marked on the map along Ria de Aveiro in three different campaigns (Spring, Autumn and Summer). The municipalities of Aveiro corresponding to the location of the sampling sites are outline on the map: Ovar (Brown color); Estarreja (Pink color); Murtosa (Red color); Aveiro (Orange color); lhavo (Blue color); Vagos (Green color); Mira (Purple color).

4.3. *Vibrio diabolicus* P7A Whole Genome Sequencing, assembly and annotation

Vibrio diabolicus was one of the most prevalent species identified during this study. This species is closely related to members of the *Harveyi* clade, e.g., *V. alginolyticus* and *V. parahaemolyticus*, recognized as human and fish pathogens.

The *V. diabolicus* strains collected during this study were of particular interest, since this species has seldom been reported and studied since its description by Raguenes et al[162] in 1997, in which it was collected from a deep-sea hydrothermal field in the East Pacific Rise. Hence, the distribution, diversity and potential virulence of this *Vibrio* species has been poorly described. Thus, a detailed whole-genome analysis of a *V. diabolicus* isolate, identified as *V. diabolicus* P7A, since it was obtained during this study from the site number 7 represented on the map above (Fig.16), was conducted. More specifically, the *V. diabolicus* P7A was collected in the Spring campaign, where the values of water temperature and salinity at this site were 22.1 ºC and 26.8 g/Kg, respectively (Table S1). As well, the comparison of this genome with other *V. diabolicus* genomes (n=11) deposited in public databases was performed, in order to do a pangenome analysis and therefore to determine core and strain-specific genes. Additionally, genetic determinants encoding for virulence and antibiotic resistance in *V. diabolicus* P7A genome were also compared to those present in other in *V. diabolicus* genomes.

From the whole genome sequencing process, using Illumina HiSeq 2500 platform, a total of 1,619,724,000 bp were generated comprised in 5,399,080 reads, which were then trimmed and assembled into 70 contigs.

According to RAST, the genome had a GC content of 44.7 %, and an estimated size of 5,151,092 bp. Also based on assembly data, and taking into account the read length, the number of reads and the total genome size[193], a genome coverage value of 156X was obtained, which means that on average, each nucleotide in the genome was sequenced 156 times. This result is in accordance with the recommended, in which 35X–50X has been considered sufficient to get good coverage of small genomes such as the ones of bacteria[194]. General genome metrics of *V. diabolicus* P7A are shown in Table 7.

Table 7 - General genomic features of the whole genome sequence of *V. diabolicus* P7A.

Feature	
Genome size (bp)	5,151,092
Sequencing reads	5,399,080
Average Read lenght (bp)	150
Number of contigs	70
N50	164686
L50	11
Average GC content (%)	44.7
Number of CDS	4754
Number of tRNA genes	63
Number of rRNA gene	6
Plasmids	0

Along with genome coverage, other common metrics such as N50, number of contigs, GC content and assembly size, enable researchers to gain insight and measure success of genome assembly[195,196]. Results obtained for *V. diabolicus* P7A were within the range reported for *V. diabolicus* genomes, that is, genome length from 4,99 to 5,43 Mbp, and GC content from 44.75% to 44.89% (data retrieved from PATRIC database[141]).

This analysis is truly necessary for quality control and validation of bacterial Whole Genome Sequencing (WGS) data, in order that WGS can become part of routine diagnostics in clinical and public microbiology laboratories, since this method holds tremendous promise for pathogen identification, antibiotic resistance profiling, and outbreak detection.

The annotation process resulted in the identification of 4,754 protein-coding sequences, distributed into 553 subsystems which covered 54.0% of the genomic features, while 46.0% did not belong to any subsystem. Approximately 28% (1316/4754) of the total genes annotated were hypothetical in nature, and they account for the majority of genes that are unique to the P7A genome.

In Fig. 17, it is noticeable that the subsystems which represent a more significant part of the 553 identified, were related to a metabolic function. Of these, the most abundant were related with metabolism of amino acids and derivatives followed by metabolism of carbohydrates, which accounted for 14% (550/3795) and 12% (456/3795), respectively. These values were similar to the ones obtained from metabolomics analysis in other *Vibrio* spp. strains *(*such as *V.*

parahaemolyticus, V. vulnificus, V. harveyi, etc.*)*, where the majority of the genes were also found to be associated with these specific metabolic functions[197].

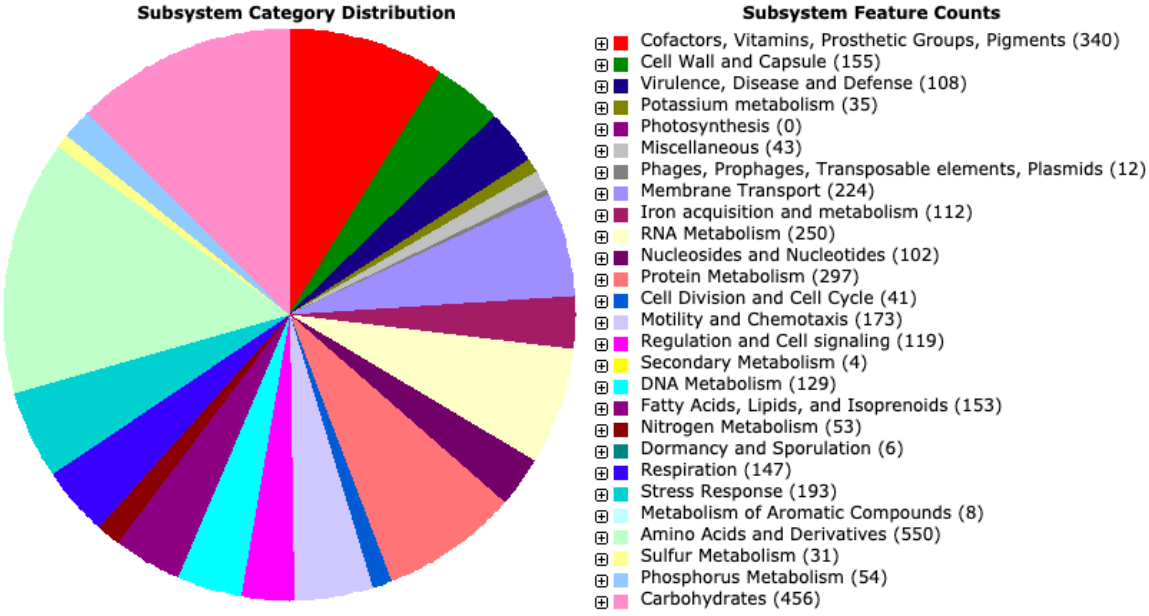

Fig. 17 - Main subsystem categories resulting from RAST annotation of the *V. diabolicus* P7A genome.

The presence and importance of amino acids and sugar metabolism genes were expected since these are largely involved in the process of survival during variable conditions, since most bacteria are exposed to a constantly changing physical and chemical environment, enabling this microorganism to adapt to multiple niches[198].

Additionally, tRNA and rRNA genes were predicted, which together detected a total of 63 tRNAs and 4 rRNAs (two relative to 5S subunit and two to the 16S subunit) in P7A genome. However, the identification of the 23S ribosomal rRNA gene was expected since bacterial 16S ribosomal rRNA, 23S ribosomal rRNA, and 5S rRNA genes are typically organized as a co-transcribed operon[199]. Therefore, although the RNAmmer 1.2 Server did not find it, a search on RAST found the 23S ribosomal RNA gene present in more than one contig. Similar numbers of tRNA and rRNA genes are present in the type strain *V. diabolicus* CNCM I-1629^T genome.

Also, no plasmids were found in the genome sequence of this bacterium. Plasmids play an important role in spreading antibiotic resistance since they frequently carry genetic determinants of antibiotic resistance. In fact, correlation between plasmid and antibiotic resistance among *Vibrio* spp. has been reported[109,200], however, in our knowledge, only one study reports this correlation in *V. diabolicus* species[163].

4.4. P7A isolate species identification

The relatedness of the P7A genome and representative genomes from its nearest phylogenetic neighbors (*Harveyi* clade members[147,201]), including the *V. diabolicus* type strain was further analyzed by calculating ANIb and dDDH values (Table 8). The ANI value of 97.72 %, above the threshold suggested for species delineation[143,144], that is 95- 96%, and the DDH value of 81.90 %, above the 70 % threshold suggested for species boundaries[143] (as referred in the previous chapter, Materials and methods) were congruent in the identification of P7A isolate as *V. diabolicus*. Additionally, the G+C content in P7A genome differed from *V. diabolicus* CNCM I-1629^T in 0.12 %. This result is endorsed by a study that showed that difference in G+C content inferred from whole-genome sequences within species is below 1 %[146].

Table 8 - The comparison of G+C content, average nucleotide identity (ANIb) and digital DNA-DNA hybridization (dDDH) values between P7A genome and representative genomes from the closest type strains.

Genome	***Vibrio* P7A**		
	ANIb %	**dDDH %**	**G+C difference %**
Vibrio jasicida CAIM 1864^T	79.70	23.50	0.29
Vibrio owensii CAIM 1854^T	80.07	24.00	0.36
Vibrio rotiferianus CAIM 577^T	79.19	23.60	0.02
Vibrio campbellii CAIM 519^T	79.95	24.10	0.40
Vibrio alginolyticus NBRC 15630^T	91.45	45.20	0.09
Vibrio azureus NBRC 104587^T	75.19	22.00	2.39
Vibrio harveyi NBRC 15634^T	79.80	23.80	0.23
Vibrio natriegens NBRC 15636^T	79.71	23.10	0.33
Vibrio sagamiensis NBRC 104589^T	74.91	21.30	3.94
Vibrio parahaemolyticus ATCC 17802^T	83.93	27.80	0.57
Vibrio diabolicus CNCM I-1629^T	**97.72**	**81.90**	0.12

4.5. Multi-locus Sequencing Analysis

To construct MLSA phylogenetic trees, a search for full length gene sequences and partial nucleotide sequences of four housekeeping genes, namely *atpA*, *gyrB*, *pyrH* and *recA* was performed. These genes are the ones used for MLST analysis of *Vibrio* spp. according to the scheme developed at the University of Padova, Italy (https://pubmlst.org/vibrio/). The partial nucleotide fragments used to construct the MLSA phylogenetic tree in Fig. 19 correspond to the region used in this MLST scheme to define sequence types. However, both trees were constructed

in order to verify if significative differences were observed. If on the one hand, analyzing full length gene sequences has the advantage to possess more information, the majority of MLSA studies performed and published only account for the use of partial nucleotide sequences[202,203]. Therefore, analyzing full length gene sequences could hamper comparison to other published MLSA analysis.

The features of the four housekeeping genes are displayed in Table 9.

Table 9 - Characteristics of single housekeeping genes and the concatenated genes from all strains

Gene	**Full length gene sequences (bp)**	**Partial nucleotide sequence (bp)**
atpA	1542	489
gyrB	2418	570
pyrH	726	501
recA	1044	462
Concatenated sequences	5030	2022
	Parsimony informative sites	
	985 (20%)	320 (16 %)
	Conserved sites	
	4351 (87 %)	1563 (77 %)

By analyzing the resulting MLSA phylogenetic trees (Fig. 18 and Fig.19) we can observe that the isolate P7A clustered with the other 12 *V. diabolicus* included in the study with a bootstrap value of 100 %, which means the node relationship is consistent and supports the inclusion of the isolate in the *Vibrio* genus and the *V. diabolicus* species.

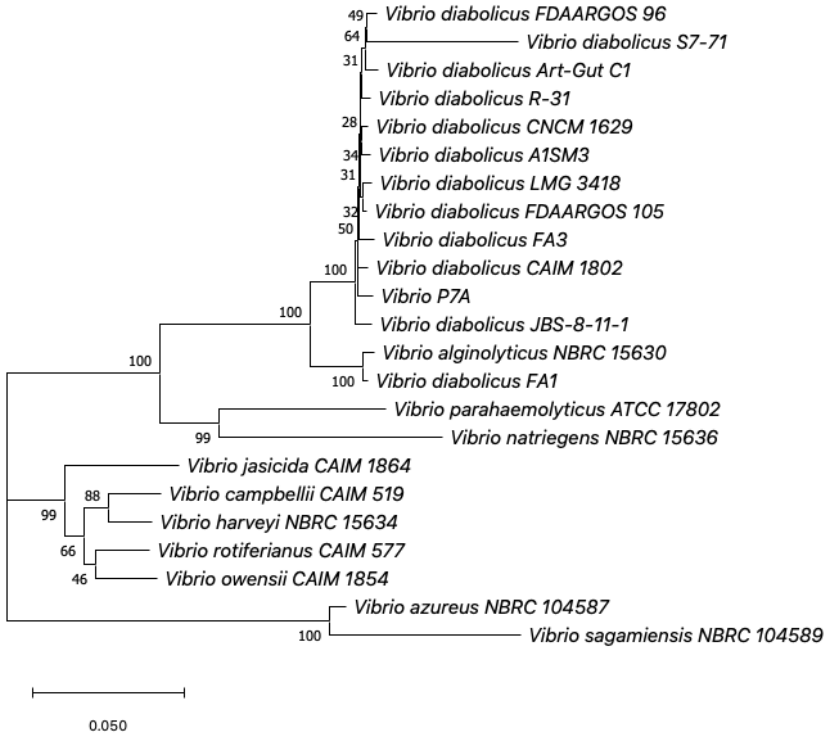

Fig. 18 – MLSA phylogenetic tree based on full lenght *atpA, gyrB, pyrH* and *recA* gene sequences of 22 strains of *Vibrio* species and the P7A isolate, generated by the neighbor-joining method. The numbers at nodes represent levels (%) of bootstrap support from 1000 resampled datasets.

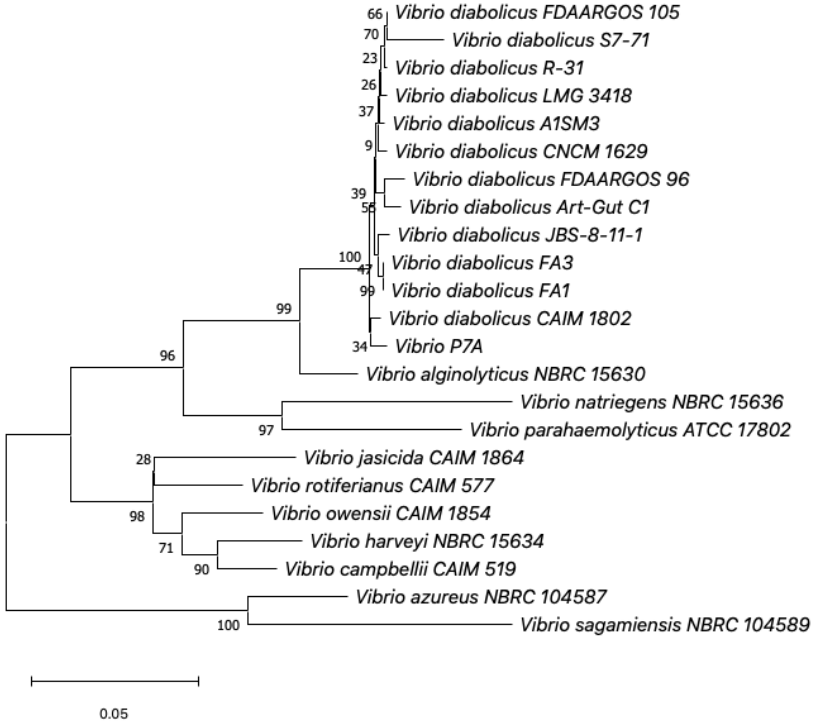

Fig. 19 – MLSA phylogenetic tree based on partial nucleotide length *atpA, gyrB, pyrH* and *recA* gene sequences of 22 strains of *Vibrio* species and the P7A isolate generated by the neighbor-joining method. The numbers at nodes represent levels (%) of bootstrap support from 1000 resampled datasets.

The phylogenetic trees showed congruence in the clustering of the large majority of strains with trees previously described[147]. However, in full length gene sequence MLSA analyze, a subclade comprised of *V. diabolicus* FA1 and *V. alginolyticus* NBRC 15630 with a bootstrap value of 100 % was observed, which may indicate that this *V. diabolicus* strain may have been incorrectly identified.

Based on these results, ANIb and dDDH values were calculated between *V. diabolicus* FA1 and several *V. diabolicus* and *V. alginolyticus* genomes. The ANIb and dDHH values of > 95 % and > 70%, respectively (Table 10), were congruent in the identification of *Vibrio diabolicus* FA1 as *V. alginolyticus* and not as *V. diabolicus.* Therefore, *V. diabolicus* FA1 was excluded of further comparative analysis.

Table 10 - Comparison of average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) values between *Vibrio diabolicus* FA1 and *V. diabolicus* and *V. alginolyticus* genomes.

Query genome	Reference genomes	dDDH %	ANIb %
***Vibrio diabolicus* FA1**	*Vibrio alginolyticus* UCD 30C	**85.50**	**97.90**
	V*ibrio alginolyticus* QD-5	**86.10**	**98.33**
	Vibrio alginolyticus NBRC 15630^{T}	**85.90**	**98.24**
	Vibrio diabolicus LMG 3818	45.90	91.57
	Vibrio diabolicus CNCM I-1639^{T}	45.30	91.42

Based on the MLST analysis it was found that *V. diabolicus* P7A belongs to a new sequence type (ST). Therefore, a new ST was requested to the PubMLST (https://pubmlst.org/vibrio/) database[204], in which the combination of the different alleles at each of the loci resulted in ST179 assigned to *V. diabolicus* P7A, now available on that database.

4.6. Comparative genomic analysis of *Vibrio diabolicus*

The pangenome calculated with the genomes of the 11 *V. diabolicus* strains (CNCM I–1629^{T}, S7-71, CAIM 1802, R-31, FA3, Art-Gut C1, A1SM3, JBS-8-11-1, FDAARGOS 96, FDAARGOS 105, LMG 3418) and *V. diabolicus* P7A consisted of 8385 genes, which included 3529 (42.1%) genes of the core genome (shared by all strains), 1931 (23%) accessory genes (present in 2 to 10 genomes), and 2925 (34.9%) strain-specific genes. A variable content (accessory and unique genes) of 57.9% reflects a high genetic diversity between the different *V. diabolicus* strains. A core/pangenome proportion of 42.1 % is one of the lowest reported for bacterial species[205]. However, it is higher, for example, than that predicted for *Clostridium botulinum* (11%) and *V. cholerae* (22%), but lower than the one estimated for *Campylobacter jejuni* (76%) or *Yersinia pestis* (89%)[205,206].

Based on the number of core, accessory, and unique genes the core-pan profile was plotted (Fig. 20). The core-pan profile plot exhibited the open nature of the pan-genome, i.e., with the sequential addition of genomes, the new genes count tends to increase significantly which again establishes the diverseness of the *V. diabolicus* genomes. In general, open pan-genomes are predominantly observed in bacteria that are prone to horizontal gene transfer[207]. However, we cannot exclude the possibility of this result being related to the low number of *V. diabolicus* genomes so far sequenced and thus available to include in this analysis.

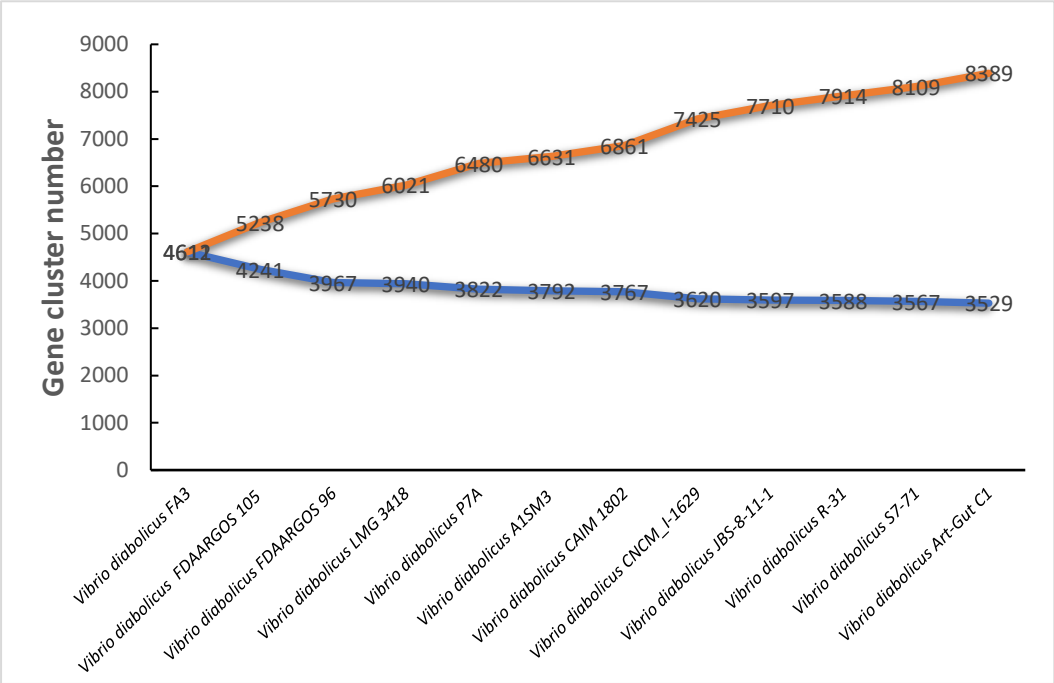

Fig. 20 - Core-pan profile of the *V. diabolicus* genomes. The orange line represents the number of new genes for each genome and the blue line represents the core gene count.

Additionally, we visualized the distribution of genes within *V. diabolicus* genomes using the Upset[208] technique (Fig. 21), implemented in the statistical computing language R (https://www.r-project.org/) available in EDGAR 2.0 database. However, values of unique orthogroup clusters for each genome (represented with a single dot in Fig. 21) are not equal to the singleton values predicted by EDGAR database. EDGAR predicted for *V. diabolicus* P7A, 248 singletons using the dedicated singleton feature (Table S2), while the Upset plot predicted 344 (shown with an orange bar in Fig. 21). The Upset plot shows the numbers of reciprocal best hits between subsets of genomes, but a gene without reciprocal best hit to another genome does not necessarily have to be a singleton. A singleton shown in the dedicated feature is a gene without any hit against any other genome. Hence, the criterion to be reported as singleton is more strict, while in other features genes ending up in the one-genome-only-groups are just genes to which no clear orthologs could be assigned. Therefore, the assigned singletons in the dedicated singleton feature in EDGAR was used for further analysis.

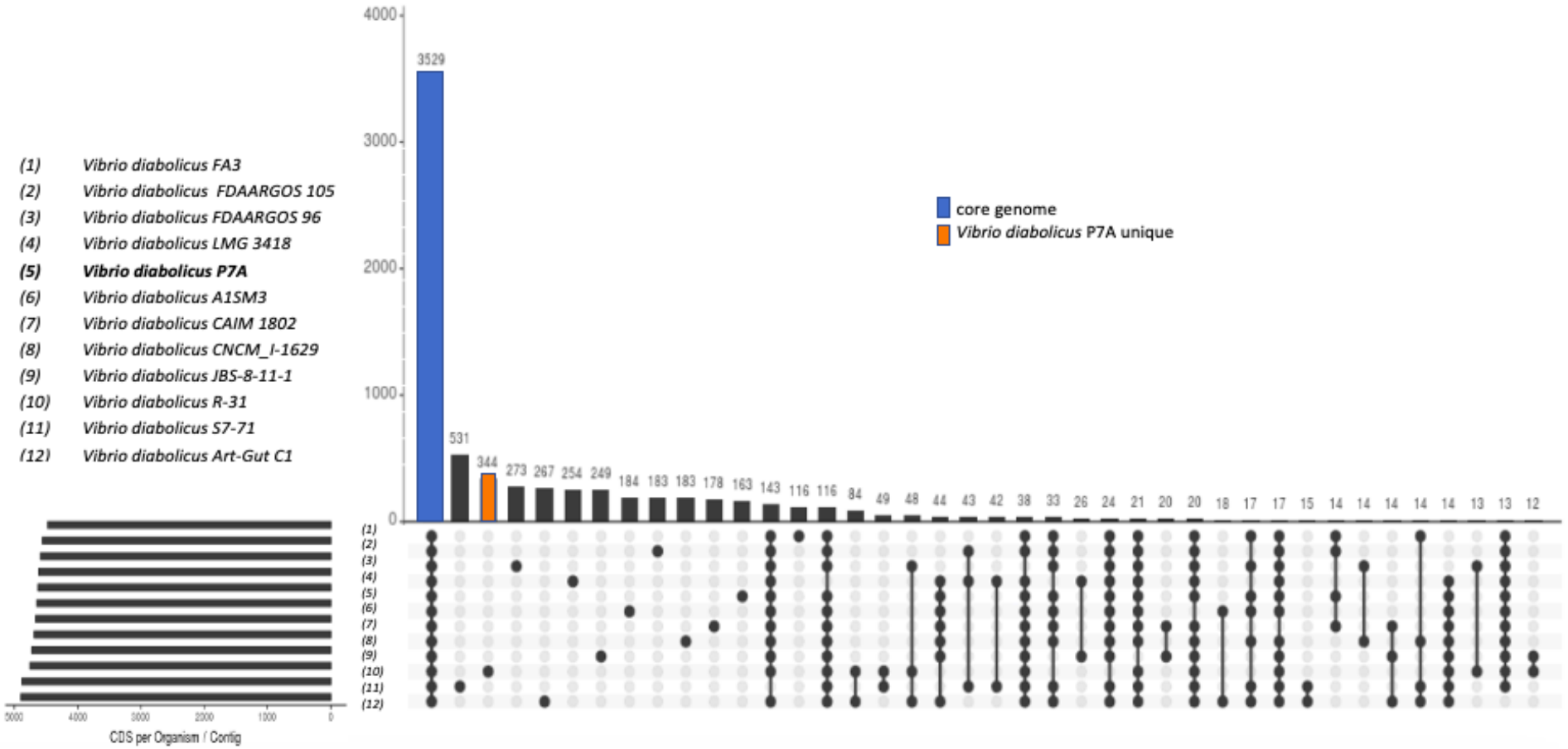

Fig. 21 - UpSet plot showing unique and overlapping gene ortholog clusters across the twelve *V. diabolicus* genomes of strains CNCM I – 1629^{T}, S7-71, CAIM 1802, R-31, FA3, Art-Gut C1, A1SM3, JBS-8-11-1, FDAARGOS_96, FDAARGOS_105, LMG 318 and P7A. The total size of each set is represented on the left barplot. The intersection matrix is sorted in descending order. Connected dots represent intersections of overlapping orthogroups while vertical bars show the size of each intersection. The core orthogroup and the *V. diabolicus P7A* unique orthogroup cluster are shown with the blue and the orange bars respectively.

The majority of *V. diabolicus* specific proteins, 200 (80.6% of the 248 singletons), had neither significant match (blastp, e-value ≤ 10^{-10}) in the NCBI-nr database, nor predicted functional domain. These were assigned as hypothetical proteins.

The functional and pathway analysis were carried out using KEGG annotation for the core and the 248 strain-specific genes of *V. diabolicus* P7A genome. KEGG annotations as represented in Fig. 22 revealed that on a broader classification, genes were found to be mainly involved in metabolism, genetic information storage, signaling and cellular processes.

By analyzing the results, we observed that core genes are mostly involved in metabolism and the strain-specific genes of *V. diabolicus* P7A genome were majorly involved in genetic information storage and signaling and cellular processes. From these results, we can infer that the genome is built in such a way that in some cases the genes belonging to the core gene set and in some cases belonging to the variable gene set are largely involved in the process and mechanism of survival during variable conditions and thus contribute to the organism's virulence and pathogenicity.

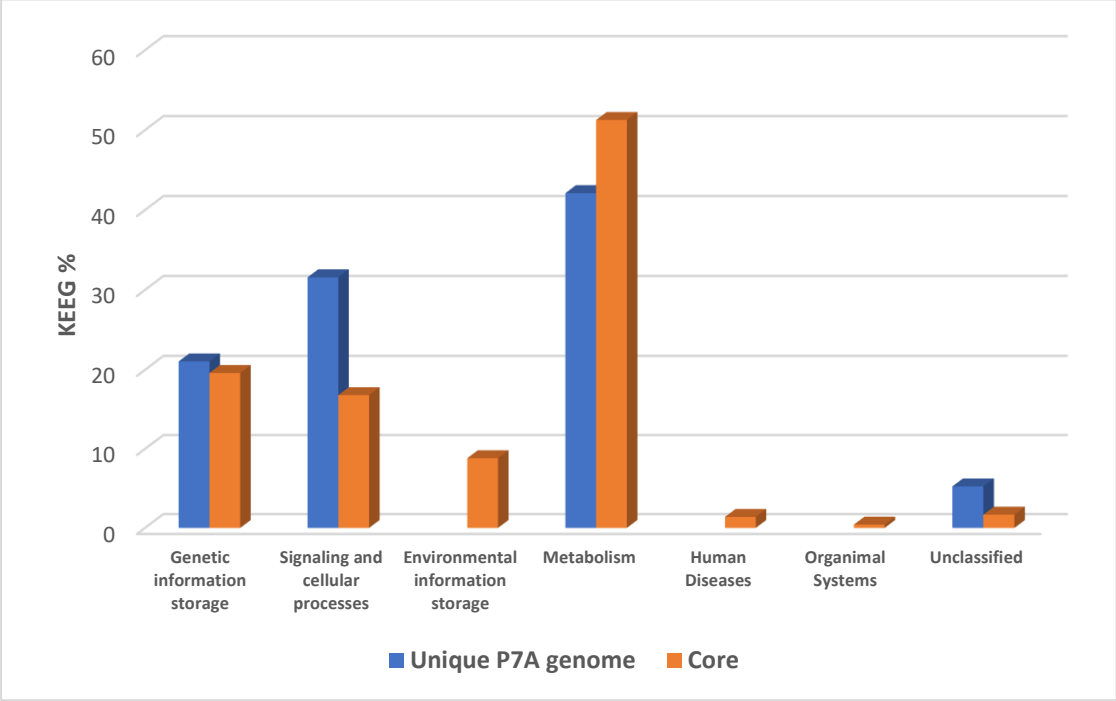

Fig. 22 - Percentage of genes involved in information processing, cellular processes, and metabolism in *V. diabolicus* core genes and unique *V. diabolicus* P7A genes, based on KEGG analysis.

4.7. *V. diabolicus* P7A genome plasticity and virulence genes

One of the important components of a pathogenic organism's genome are the genes coding for virulence factors. Hence, the prediction of putative virulence genes is necessary. In particular, it has been noted that many virulence genes are associated with genomic islands (GEIs; clusters of genes of probable horizontal origin)[209].

The GIPSy analysis allowed the identification of GEIs related to pathogenicity in *V. diabolicus* P7A genome. The software predicted 4 putative pathogenicity islands (PAIs; defined as PAI1, PAI2, PAI3 and PAI4; Table S3) in *V. diabolicus* P7A's genome. The size of GEIs varied from 9454 bp to 70569 bp, with G+C content values of 13%, 20% and two simultaneasly with 55% which are lower and higher than the overall chromosomal G+C of 44.7%. Distinct content of G+C is a common property displayed in the sequence of GEIs, along with the presence of mobility genes, such as integrases and transposases, as well flanking direct repeat sequences, that mark them as being atypical compared to the overall genome in which they are inserted[210].

The circular alignment of the *V. diabolicus* P7A with the other *V. diabolicus* genomes (n=11) (Fig. 23) highlights that the structures PAI1 and PAI4 are only present in P7A's genome, while PAI2 and PAI3 were also identified in *V. diabolicus* FDAARGOS 105 genome. However, other pathogenic islands have also been found in other *V. diabolicus* strains[211].

Based on the RAST Seed Viewer and VFDB database, we were able to determine that PAIs encoded proteins related to capsular polysaccharide (CPS) biosynthesis, which in human pathogens such as *V. parahaemolyticus* and *V. vulnificus* plays an important role in the adherence to its target cells[212,213]; phage structural proteins; ureases, increasing the ability of the pathogenic organisms to colonize the gastric mucosa and protect themselves from the acid environment of the stomach[214]; several genes encoding hemolysins, one of the major pathogenic factors among *Vibrio* species, that show hemolytic activity against erythrocytes[30]; and genes encoding for bacterial type III secretion systems (T3SS), also an important determinant of virulence among *Vibrio* pathogenic species, as well as in many other Gram-negative pathogens, contributing for their survival and colonization of the host cells[215].

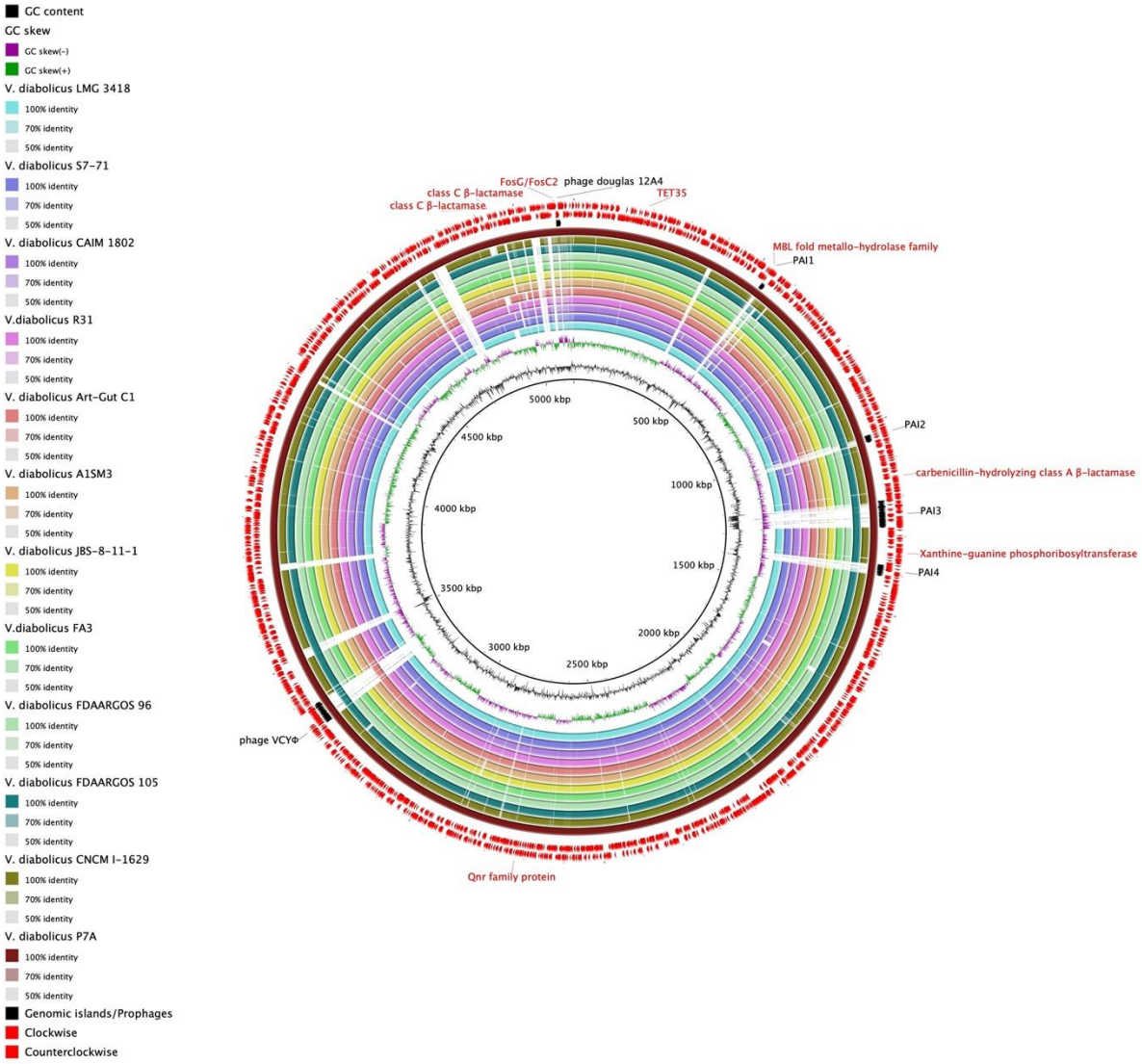

Fig. 23 - Circular genome comparison of *V. diabolicus* genomes (P7A, as the reference strain and CNCM-1629^{T}, S7-71, CAIM 1802, R-31, FA3, Art-Gut C1, A1SM3, JBS-8-11-1, FDAARGOS 96, FDAARGOS 105, LMG 3418) displaying the locations of ARGs, , putative pathogenicity islands and prophage sequences. BRIG performed the alignment using a local BLAST+ with the standard parameters (50% lower – 70% upper cut-off for identity and E-value of 10). The ring color gradients correspond to varying degrees of identity of BLAST matches. Circular genomic maps also include information on GC Skew and GC content.

As seen in P7A genome, virulence factors over-represented in GEIs are usually T3SS (including their corresponding effector proteins), as well as toxins and adherence factors, in which such proteins comprise some of the most offensive weapons available to pathogens[216,217]. GEIs, along with phages and repetitive elements have driven bacterial horizontal gene transfer and evolution[218], besides mutations and rearrangements. Therefore, bacteria possess high genomic plasticity, which makes them highly diverse organisms that are able to adapt to a broad range of environments and hosts.

In detail, PAI3 from *V. diabolicus* P7A encoded the thermostable direct related-hemolysin (*trh*) gene with 100 % nucleotide similarity to that of *V. parahaemolyticus*. *trh* and/ or thermostable direct hemolysin (*tdh*; also considered a major virulence factor in *V. parahaemolyticus*) genes are strongly associated with the pathogenicity of this organism[219], which is the leading cause of seafood-associated gastroenteritis in the United States and the world[220], as previously referred. The occurrence of these genes among environmental *V. parahaemolyticus* isolates is typically 1–10%, but depends on several factors such as, location, sample source and detection method, however other studies have already reported a higher frequency of these genes from environmental strains[161,219]. Type III secretion system 2 (T3SS2), involved as well in the virulence[30,221] of *V. parahaemolyticus* strains was also present in *V. diabolicus* P7A genome, specifically genes encoding apparatus proteins VscC2, VcrD2 and VscN2 with 96.27 %, 99.21% and 98.23%, respectively, nucleotide similarity to that of *V. parahaemolyticus.*

The high similarity of the *V. diabolicus* P7A and *V. parahaemolyticus* hemolysin gene and T3SS2 gene sequences may suggest that these genes are readily transferred among these and other *Vibrionaceae* species. Similar results were shown in other environmental *Vibrio* strains, generally considered nonpathogenic to humans[222]. The data obtained support the view of nonpathogenic *Vibrio* strains as a reservoir of virulence genes, therefore increasing their fitness in the environment, which perhaps could lead to the emerge of additional virulent *Vibrio* species with different environmental preferable conditions and different host ranges.

Furthermore, by exerting anthropogenic pressures in aquatic environments where *Vibrio* species cohabit, we may be generating selective conditions which promote the spread of this putatively self-transferable and responsive PAIs[223].

The prediction of putative recombinases was also performed by GIPSy, in which the database contains transposase, tyrosine recombinase and serine recombinase genes from bacteria. The *V. diabolicus* P7A genome had a total of 26 genes encoding for putative recombinases, in which, 18 genes encoded for phage integrases, including tyrosine recombinases or serine recombinases and 8 genes encoded for transposases. Additionally, 4 of these transposases belonged to the IS3 family, 2 belonged to IS5 family, 1 to IS4 family and 1 to IS481 family transposases. The average number of transposases described in bacterial genomes is approximately 38[224]. Therefore, the number of transposases encoding genes in *V. diabolicus* P7A is below average. However, studies that report the total number of transposases in *V. diabolicus* strains or in other *Vibrio* strains have not yet been conducted. For that reason, the prediction of putative recombinases for the 11 published *V. diabolicus* genomes available was also performed with GIPSy. The number of putative recombinases ranged from 13 to 56, which corresponds to an overall average of 23, similar to that observed in *V. diabolicus* P7A genome, which is also below the average 38 transposases described in bacterial genomes[224].

Lastly, by using PHAST for the prediction of prophage sequences, we identified 2 prophage regions on the genome of *V. diabolicus P7A*, of which 1 region was intact (*Vibrio* phage VCYΦ [GenBank: NC_016162.1] and 1 region was considered questionable (*Vibrio* phage douglas 12A4 [GenBank: NC_021068]), according to PHAST classification (Fig. 23). The presence of prophage regions in bacterial genomes is associated with an improved capacity to develop antibiotic resistance, adapt to new niches in the environment and become pathogenic[225].

The filamentous phage VCYØ of 9.4 Kb and a G+C content of 42.85 % identified in *V. diabolicus* P7A has been found with high frequency within environmental *V. cholerae* strains[226]. VCYΦ encodes two virulence genes: *ace* (encoding the accessory cholera enterotoxin) and *zot* (encoding the zonula occludens toxin), homologous to those encoded on the *V. cholerae* phage CTXΦ (which encodes the cholera toxin)[226]. Zot and Ace have been shown to be responsible for severe gastro-intestinal diseases, and are particularly common among vibriophages isolated from human pathogens, such as *V. cholerae* and *V. parahaemolyticus*[227,228], but are also present in prophage-like elements of non-human pathogens such as *V. anguillarum*[229]. Therefore, suggesting again frequent HGT among different *Vibrio* species, substantially contributing to the emergence of pathogenic *Vibrio* from non-pathogenic environmental populations[230].

Additionally, no CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) were found in the genome sequence of *V. diabolicus* P7A.

Pathogen Finder[231] survey of the *V. diabolicus* P7A genome predicted a 65% chance that this organism was pathogenic. Thus, besides the presented analysis of PAIs and other HGT-related elements, we obtained a general overview of the occurrence and diversity of genes related to virulence and defense across the chromosome of *V. diabolicus* P7A, based on RAST Seed Viewer and VFDB database analysis.

We detected a total of 172 virulence factors (VFs; Table S4). These were divided into 10 virulence-related categories (Fig. 24), adapted from the VFDB classification scheme, including adherence (19 VFs), antiphagocytosis (14 VFs), chemotaxis and motility (55 VFs), iron uptake (8 VFs), quorum sensing (2 VFs), secretion system (50 VFs), toxin (9 VFs), acid resistance (2 VFs), immune evasion (5 VFs) and colicin V and bacteriocin production cluster (8 VFs).

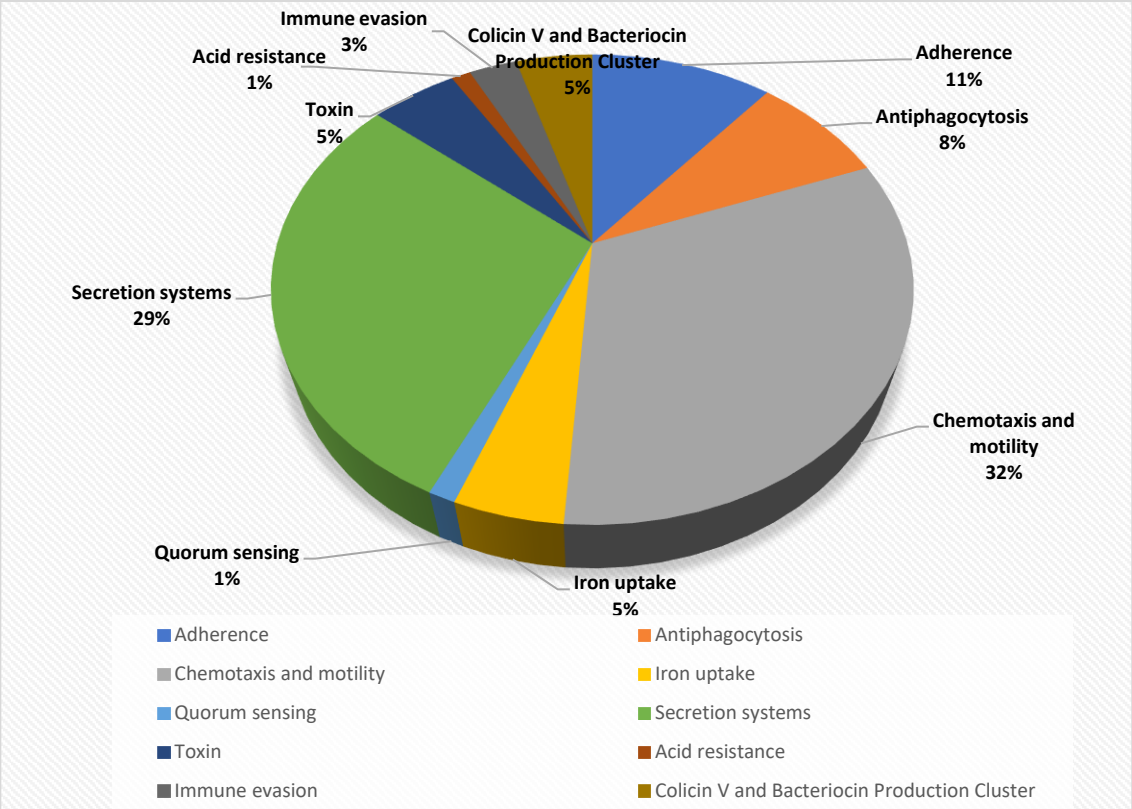

Fig. 24 - *V. diabolicus* P7A virulence factors categories resulting from RAST Seed Viewer and VFDB database.

As observed, the two major virulence-related functions represented in *V. diabolicus* P7A genome were chemotaxis and motility and secretion systems. Secretion systems, consisted almost in its totality, of genes encoding for T3SS, that as referred previously is possessed by many Gram-negative bacteria, especially those occurring as animal and plant pathogens[215]. This system is responsible for enabling injection of effector proteins directly into the cytosol of host eukaryotic cells, thus contributing to bacterial virulence against the host[232]. Regarding the other major category of virulence factors from P7A genome, flagellar-mediated motility is intimately connected to processes such as chemotaxis, biofilm formation, colonization, and virulence among this genus[21 ,23, 233,]. Thus, motility plays a significant role in the lifestyle of *Vibrio*, both in the aquatic environment and during host colonization.

V. diabolicus P7A also encoded for homologs of ToxR and ToxS, which are two integral membrane proteins that activate the transcription of virulence genes in *V. cholerae*[234]. ToxRS is widely distributed and highly homogeneous among *Vibrio* species, and it mediates environmentally induced regulation of the virulence gene expression including *tdh* and *ctxAB* expression in different *Vibrio* species[235]. Other genes involved in pathogenicity and present in P7A genome were the genes encoding RTX toxin and the type IV pilus, mannose-sensitive hemagglutinin, an important mediator of *Vibrio* interactions with different substrates in the aquatic environment, including attachment to zooplankton[236], biofilm formation[23] and adherence

to living cells[237].

Lastly, *V. diabolicus* P7A encoded for an Integron Integrase Intl4 (Fig. 25), included in a superintegron (SI), which consisted in a region with multiple repeats and unusual GC content (40.8%). The SI of *Vibrio* typically includes an integrase (*intI*), a proximal primary recombination site (*attI*), multiple target-specific recombination sites (*attC*), and associated gene cassettes[3]. Alignment of the *V. diabolicus* P7A integrase with others from closely related *Vibrio* species, such as *V. alginolyticus* revealed >99% of similarity.

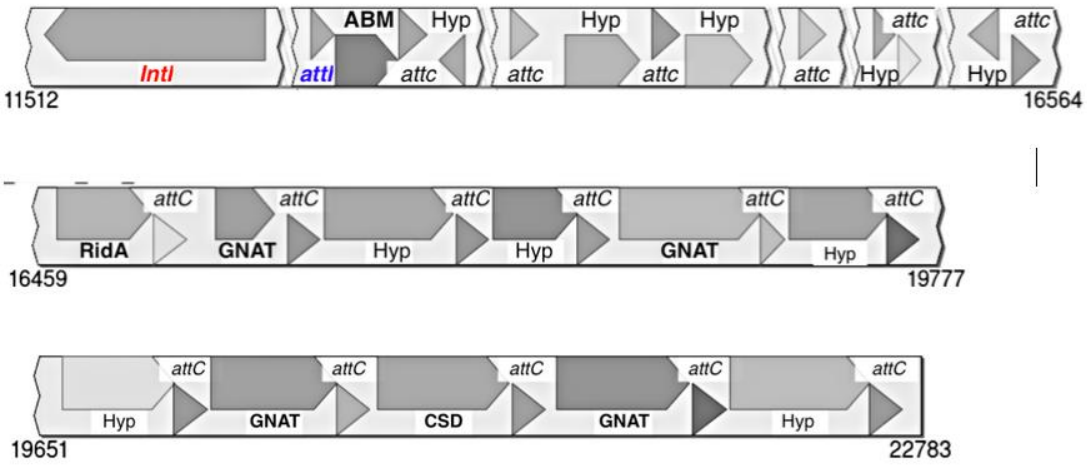

Fig. 25 - Organization of the *V. diabolicus* P7A super-integron (partial sequence). The integrase gene, *intI*, is shown in red letters and the neighboring primary integration site (attI) in purple letters next to the *intI*. The 17 putative *attC* sites possible to identify are also represented. The ORFs are indicated by the proteins they respectively encode. Hyp stands for hypothetical protein, ABM for antibiotic biosynthesis monooxygenase, CSD for cold shock domain-containing protein and GNAT for GNAT family N-acetyltransferase. The numbers correspond to the nucleotide numbering in the contig.

However, it was not possible to describe the structure of the complete SI. From the identified region, with a size of 11,2 kpb, a total of 17 *attC* sites were found, in contrast to the total 175 and 118 *attC* sites, and lengths of 126 and 138 kpb described in *V. cholerae* El Tor N16961[238] and *V. vulnificus* YJ016[239] genomes, respectively. Within the SI region of *V. diabolicus* P7A, 16 ORFs were identified, the majority of which (56%) encoding hypothetical proteins. The remaining ones codified for antibiotic biosynthesis monooxygenase, GNAT family N-acetyltransferase, RidA family and Cold shock domain-containing proteins. SI's are found in several *Vibrio* species, in which the array of gene cassettes is influenced by the niche and species[240].

4.8. Antibiotic and metal resistance profile

By analyzing the RAST data regarding the virulence, disease and defense subsystem, the CARD, Resfinder, BacMet and PATRIC databases, it was possible to identify the isolates resistance arsenal and identify specific genes related to antibiotic resistance and metal tolerance in *V. diabolicus* P7A genome.

Considering that some *V. diabolicus* strains may be virulent, as suggested by our results, intrinsic or acquired resistance to antibiotics could complicate treatment as the analysis of the species resistome revealed the presence of genes associated with resistance to fluoroquinolones, fosfomycin, tetracycline, and penicillin, common antimicrobials used for the treatment of *Vibrio* infections[96,170].

For the analysis of the antibiotic resistome of *V. diabolicus* P7A, all the genetic determinants presenting similarity of 40% or more were noted and analyzed. Taking into account this criterion, a total of 72 putative resistance genes were identified. The ARG homologs of clinical relevance identified in *V. diabolicus* P7A genome are described in Table 11 and more detailed information regarding its resistance arsenal is presented in Table S5.

Genetic determinants encoding resistance to β-lactams were identified as genes encoding class A and class C β-lactamases, which hydrolytic activity depends on having a serine residue at their active site, and a gene encoding a class B enzyme, that in contrast is a metallo-β-lactamase (MBL), and uses in their active-site zinc ions to activate a nucleophilic water molecule, which opens the β-lactam ring (through hydrolysis)[241]. More specifically, the complete genetic determinants encoding resistance to β-lactams included a gene encoding a carbenicillin-hydrolyzing class A β-lactamase; two genes encoding class C β-lactamases, both associated with resistance to cephalosporins and one gene encoding a class B β-lactamase, a member of MBL metallo-hydrolase superfamily. Class B metallo-β-lactamases, the most clinically prevalent of the classes, may confer resistance to several β-lactam antibiotics, including the last resort antibiotics carbapenems, and have been considered a threat to global public health[242].

As we see in Table 11, the percentage of similarity identified for the genes encoding for class B and C β-lactamases are low ~ 50%. After comparing these nucleotide sequences from the genome of *V. diabolicus* P7A with published sequences in the GenBank database (www.ncbi.nlm.nih.gov/genbank/) using BLAST search, the obtained results revealed that in fact, they encoded for putative class B and C β-lactamase proteins with % identity > 98%, abundantly present in different species of the genus *Vibrio*. However, to our knowledge, these proteins do not appear to have been experimentally expressed *in vivo*.

Table 11 - ARGs homologs with clinical relevance, i.e. conferring resistance to antibiotics commonly used for the treatment of *Vibrio* infections.

Antibiotic class	Putative gene (CARD)/product	Substrates/function	Contig	Length (bp)	% Nucleotide identity
β-lactam	*carB18*/class A β-lactamase	Penicillins, cephalosporins, carbenicillin[243]	4	822	87.91
	bla$_{PEDO-2}$/class B metallo β-lactamase	Broad spectrum, including carbapenems[244]	2	1350	44.9
	bla$_{OCH-2}$/class C β-lactamase	Cephalosporins, Penicillins Monobactams, Penems [243,245,246,247]	41	774	52.19
	bla$_{LRA-18}$/class C β-lactamase	Cephalosporins[245]	41	372	50.85
Tetracycline	*tet(34)*	Oxytetracylcine mechanisms[248]	5	635	92.78
	tet(35)	Efflux of tetracycline[249]	1	1602	96.47
Fosfomycin	*fosG/fosC2*	Fosfomycin inativation[250]	51	399	90.48
Fluoroquinolones	*qnrVC5*	Quinolones[251]	12	651	60.65

The environment is currently seen as a vast reservoir of resistant organisms and their associated genes, and more specifically β-lactamase producers of great clinical importance have been described namely in aquatic systems[252]. In Portugal, some studies have already reported this exact problem[253,254]. In fact, in the estuary of Ria de Aveiro, bacteria resistant to β-lactams and a great diversity of β-lactamase genes have already been found[255,256].

More specifically, the resistance to β-lactams in environmental isolates of *Vibrio* spp. is well known[257,258]. It is likely that the excessive use of β-lactam antibiotics has contributed to increasing numbers of β-lactam resistance among *Vibrio* spp. For instance, Zanetti *et al.* reported that 88.9 % of the *Vibrio* isolates from marine or coastal environments showed β-lactam resistance[258]. However, induced dynamic production and mutation of β-lactamases among the bacterial population, due to the exposure of bacterial isolates to β-lactam antimicrobials, has expand the action of these enzymes even to the newly developed β-lactam antibiotics[259].

Additionally, resistance determinants *tet34* and *tet35* genes were also identified as conferring resistance to tetracyclines. Tet35 is a tetracycline efflux pump, member of tetracycline efflux Na^+/H^+ antiporter family, found in the Gram-negative *Vibrio* and *Stenotrophomonas* and it is unrelated to other *tet* resistance genes[249]. Tet34 is a Xanthine-guanine phosphoribosyltransferase with 154 amino acids conferring resistance both to tetracycline and oxytetracycline. Oxytetracycline is one of the most widely used antibiotics in aquaculture and for that OTC-resistant bacteria are found in aquaculture sites around the world[66,260,]. OTC-

resistant bacteria have been identified not only in humans and animals but also in marine environments[261].

The detection of genes associated with resistance to tetracycline was expected since this antibiotic was used in the TCBS culture medium for isolation. The fact that this strain has these resistance determinants and was obtained from an estuary where aquaculture is an important economical activity, may be related and may be a concern given the frequent economic losses in aquacultures due to infectious diseases that are increasingly difficult to treat due to increased resistance levels.

V. diabolicus P7A genome also encoded for chloramphenicol acetyltransferase (CAT), related to resistance to chloramphenicol, an antibiotic also extensively used in aquaculture[262].

Genetic determinants related with resistance to fluoroquinolones were detected, namely the *qnrV5* gene. *Vibrio* spp. were thought to be a possible source of *qnr*-like quinolone resistance determinants[263], and the emergence of transferable *qnrVC* alleles in *Vibrionaceae* family and other bacterial species aggravated the problematic on quinolone resistance[264]. More recently, a study, reported a high prevalence of *qnrVC* genes among ciprofloxacin-resistant *Vibrio* spp. isolates[265].

These results indicate that the isolate P7A is potentially a MDR[266] isolate, since it carries genes encoding resistance to more than three classes of antibiotics. However, antibiotic susceptibility tests should be conducted to confirm this phenotype.

Additionally, genes probably related to antibiotic resistance mediated by multidrug efflux pumps were also identified, which occupied a significant part of the isolate resistance arsenal, corresponding to 46 genes in *V. diabolicus* P7A genome. Specifically, components of the MATE (Multidrug and toxic compound extrusion), MFS, RND and ABC (ATP-binding cassette) super families, known to play a predominant role in the resistance to certain antibiotics (e.g. tetracyclines, as *tet35* gene, fluoroquinolones and macrolides, among others) and other unrelated compounds such as organic solvents[267,268]. In fact, three ABC transporters were identified as conferring resistance to specific antibiotics, two exhibiting 51.13 % and 44.39 % similarity with components of a macrolide efflux system and one with 66.32 % similarity to a nitroimidazole efflux component.

Furthermore, genes encoding for the outer membrane channel TolC, were identified. TolC has been shown to be required for the functioning of many efflux systems. Several were identified on *V. diabolicus* P7A genome, as for instance homologs genes encoding for the RND AcrAB system, well characterized to be related with resistance to cephalosporins in *E. coli*[269]. *V. diabolicus* P7A harbored genes encoding VmeA and VmeB protein homologs, which were 49.86% and 63.20% identical to AcrA and AcrB in *Escherichia coli*, others have characterized VmeAB as an orthologue of AcrAB in *E. coli*[270], providing support for the hypothesis of RND involvement in cephalosporin resistance, to which resistance is emerging globally, specifically to

third-generation cephalosporins[271]. RND efflux pumps accounted for the majority of multidrug efflux pumps found on *Vibrio* P7A genome, and the obtained results suggests that RND efflux pumps in aquatic environments, may contribute to the emergence of resistance to third-generation cephalosporin drugs. Likewise, other RND efflux pumps, such as AcrD in *E. coli* are known to be responsible to counteract aminoglycosides, which are an important class of antibiotics[269]. *V. diabolicus* P7A contained genes encoding proteins with some homology to AcrD (59% identity), which could potentially mediate efflux.

Overall, these results demonstrated a connection between several RND efflux pumps and antibiotic resistance, which ends up having implications for antibiotic usage, for example, in aquaculture. However, RND efflux pumps contribute not only to antibiotic resistance but also to increased virulence and pathogenicity[272].

Overall, we found that *V. diabolicus* P7A resistome encompassed a significant collection of resistance determinants, and we cannot exclude the possibility of these genes to be transferred to pathogenic bacteria through HGT, due to the several mobile genetic elements coded in this isolate genome, such as transposases, previously described. These phenomena can also be potentiated by the presence of metals and other pollutants derived from anthropogenic activities in aquatic environments, and more specifically in estuarine environments, from where *V. diabolicus* P7A was isolated.

Estuaries are zones of complex interaction between fluvial and marine process that act as a geochemical trap for heavy metals[273,274]. Therefore, a search for metal tolerance determinants was performed. Genome analysis revealed that the genome of *V. diabolicus* P7A presented several gene clusters related to metals tolerance, namely to copper, zinc, arsenic, chromium, cobalt and cadmium (Table S6).

The polluted areas in Ria de Aveiro correspond to regions where the residence time is high and cohesive sediments are deposited. This condition is observed in Aveiro canals (close to where *V. diabolicus* P7A was collected) and Murtosa channel where sediments with the highest concentrations of Zn, Pb, Cu, and Cr are found[126]. The enrichment of heavy metals in Aveiro canals is mostly related to the past industrial production at their margins (ceramic and metallurgic), whereas in Murtosa channel effluent discharges of the chemical complex of Estarreja are the main responsible[126]. More importantly, water contaminated with industrial pollutants, as heavy metals has been well established to enhance the selection for antibiotic resistance[275,276].

The arsenic resistance mechanism conferred by *ars* operon, which is located on plasmid or chromosome, has been extensively studied in more than 50 genera. The *ars* genes appear systematically and the core genes of this system include arsenate reductase (*arsC, acr2),* membrane efflux pump (*arsB, acr3)*, and transcriptional repressor (*arsR, acr1)*[277], however despite all these determinants of resistance to arsenic being present in the genome of *V. diabolicus*

P7A, they were not found close together, and for that were not considered to be part of the same operon.

Additionally, copper resistance mechanisms such as *copA*, member of cation transport ATPase family and *cueR* were detected, already described as the responsible determinants for copper tolerance in *V. cholerae*[278], homologs to genes *copA* and *cueR* found in *E. coli*[279]. Likewise, a homolog to CopG was found, a putative cupredoxin, closely related to CopA, necessary for full copper tolerance of *V. cholerae*[278]. In addition, the *scsABCD* genes have been shown to increase copper tolerance in CutF deletion mutants[280]. *V. diabolicus* P7A encoded for *scsABCD* gene cluster, present in many but not all enteric bacteria. These genes code for proteins with homology to oxidorectusases of the thioredoxin family. However, gene *scsC* was not found close to the *scsA*, *scsB* and *scsD* genes (Fig. 26A).

Lastly, other efflux system was also characterized, the efflux system *czcCBA* (Fig.26B) probably the best-characterized members of the heavy metal efflux RND family. The *czcCBA* system functions in the detoxification of cadmium, zinc, and cobalt[281]. This structural gene region encodes outer membrane factors CzcC, membrane fusion protein CzcB, and CzcA RND protein. This operon is usually controlled by the CzcRS operon, a two-component system[282]. However, unlike described, CzcRS was not close to the CzcCBA operon it regulates in P7A isolate. Strangely, only the CzcR response regulator, of the two-component system, was present. CzcS was indeed absent, though, leaving CzcR as the only confirmed regulatory gene present. Furthermore, as a second efflux system of this determinant the protein CzcD involved in detoxification of the same metals and in regulation of expression of the CzcCBA pump, was also identified in the P7A genome[283].

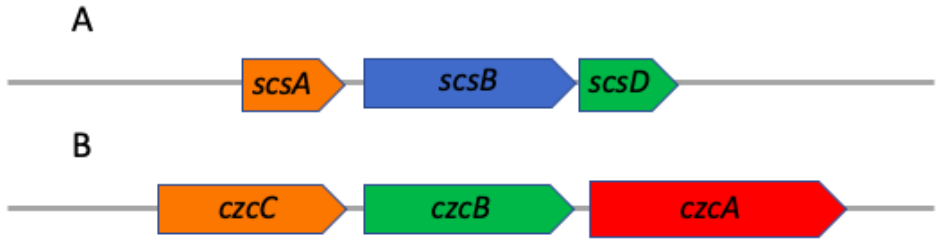

Fig. 26 – a) Diagram of part of *scsABCD* operon, with the absence of *scsC* gene; **b)** *czcCBA* efflux system schematic representation.

In fact, genome sequences suggest that efflux pumps may contribute to cross-resistance, which is when the same system confers resistance to both antibiotics and metals[275]. *The V. diabolicus* P7A genome harbored many efflux pump gene homologs, especially RND efflux pumps, that have been implicated in Gram-negative MDR[284]. This result is consistent with a recent report on the dominance of efflux pump-based cross-resistance in the whole genome sequence of

an environmental *Pseudomonas* strain from a metal-contaminated estuary, the Ria de Aveiro estuary[285].

The abundant antibiotic and heavy metal-resistant bacteria could be a serious concern due to the potential health impacts of consuming contaminated products[286].

5. FINAL CONSIDERATIONS

The first part of the study here presented aimed to investigate the distribution of tetracycline-resistant *Vibrio* spp. along the estuary Ria de Aveiro, previously obtained in three different campaigns (Autumn, Spring and Summer).

The collection of water samples from 26 sampling sites along this estuary allowed to identify eleven different species of this genus, included in three main clades. Several of the species identified have been considered pathogenic to humans and/or have been associated with foodborne diseases, some being major aquaculture-associated pathogens. Tetracycline still remains one of the antibiotics of choice for treating infections caused by pathogenic *Vibrio*. Our results raise serious concerns about its effectiveness. Furthermore, in our knowledge, species such as *V. mediterranei* and *V. diabolicus*, have not been described in previous studies as resistant to tetracycline. Meaning, that the isolation of these species in tetracycline-supplemented culture medium is a further evidence of the growing emergence of antibiotic resistance. Additionally, the results obtained in this study suggest a low seasonal variation in the abundance of *Vibrio* but indicate a considerable variation in terms of diversity (number and abundance of each species). On the other hand, there was a heterogeneous distribution throughout the estuary, probably related to the gradients of salinity, temperature, dissolved oxygen and occasionally with pH values.

Lastly, the results also indicated a possible association between the abundance of resistant bacteria anthropogenic activities.

Overall, the isolation of *Vibrio* species potentially pathogenic for humans from Ria de Aveiro raises concerns, mainly because this estuary is commonly used for practices such as seafood production, sports, recreational and touristic activities, resulting in human populations exposure to estuarine bacteria and contaminated water. On the other hand, the use of antibiotics in aquaculture and consequent contamination of the estuary, imposes a selective pressure that may alter the diversity of *Vibrio* species and select for antibiotic-resistant *Vibrio*.

The second part of the study aimed to perform whole-genome sequence analysis of a tetracycline-resistant *V. diabolicus* isolate from Ria de Aveiro and to estimate *V. diabolicus* virulence and antibiotic resistance based on WGS analysis;

Regarding *V. diabolicus P7A*, this study led to the following conclusions:

- The virulence factors over-represented in PAIs and prophages identified, support the view of *Vibrio* species previously considered nonpathogenic, such as *V. diabolicus*, as a reservoirs of virulence genes.
- The resistome of P7A is consistent with an MDR phenotype.
- Multidrug resistance efflux pumps represent the majority of the isolate resistance arsenal and may be responsible for resistance to several metals and antibiotics.

- Isolate P7A carried genes potentially conferring resistance to β-lactam antibiotics with great clinical relevance, including to carbapenems which are last-resort antibiotics.

Globally, the current study contributes to an expansion in the comprehension of the resistome owned by *V. diabolicus.* By adopting a WGS approach, we contributed with a genome sequence to public databases, which can be used to perform other comparative genomic analysis. There are still few data regarding this species since only 11 genomes have been sequenced and are currently available. These microorganisms may carry genetic determinants of antibiotic resistance mechanisms with clinical relevance, which can be transferred to pathogenic bacteria. Thus, our results highlight the interest in analyzing environmental isolates.

WGS has proven to be an excellent method to better understand virulence and antibiotic-resistance dynamics in environmental compartments. In the future, as this technology becomes integrated into the routine diagnostics in clinical and public microbiology laboratories, in conjunction with established susceptibility tests, antibiotic treatment may benefit from this technology and become more timely and effective.

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7. APPENDICE

Table S 1 - Values of water physic-chemical parameters (dissolved oxygen, conductivity, salinity, pH and temperature) in the sampling area, from Spring (orange columns), Autumn (yellow columns), and Summer (green columns) Campaigns.

	Dissolved oxygen (mg/l)			Conductivity (mS/cm)			Salinity (g/kg)			pH			Temperature (°C)		
Site	**Spring**	**Autumn**	**Summer**	**Spring**	**Autumn**	**Summer**	**Spring**	**Autumn**	**Summer**	**Spring**	**Autumn**	**Summer**	**Spring**	**Autumn**	**Summer**
1	2.88	7.70	5.63	43.0	49.9	51.1	27.5	32.5	33.3	7.70	7.95	8.07	20.5	19.8	20.1
2	2.65	6.90	5.38	23.4	42.8	34.1	14.2	27.4	21.4	7.80	7.86	8.09	22.8	20.8	23.2
3	1.96	6.06	4.03	5.00	19.5	11.5	7.5	11.6	6.60	7.50	7.52	7.71	21.2	21.2	22.0
4	3.15	7.06	4.60	0.480	0.570	0.550	0.200	0.200	0.200	7.70	7.35	7.71	22.1	20.9	22.5
5	2.70	5.64	4.57	0.470	0.530	0.450	0.200	0.200	0.200	7.40	7.29	7.78	19.9	20.2	20.9
6	5.29	7.40	5.34	0.740	0.790	1.01	0.300	0.300	0.400	7.30	7.07	7.77	18.9	22.3	26.1
7	5.35	7.62	5.02	41.8	51.6	51.0	26.8	33.8	36.6	8.00	7.96	8.10	22.1	22.4	24.6
8	5.07	6.65	5.93	42.9	21.7	50.6	27.5	12.8	33.2	7.90	7.14	7.81	21.9	13.4	23.3
9	5.53	7.12	5.66	45.0	51.0	51.7	29.2	33.3	34.2	8.20	7.81	7.85	22.9	21.7	24.8
10	4.56	6.16	3.97	38.4	26.2	47.7	24.4	15.8	31.1	7.70	7.73	7.83	21.3	13.9	23.6
11	5.98	9.26	5.05	45.3	52.0	51.7	29.4	34.2	34.0	8.10	8.03	7.82	23.4	22.9	23.3
12	6.04	7.07	4.16	51.5	52.9	50.4	33.5	34.6	32.9	8.10	8.06	8.71	16.6	19.2	19.9
13	4.72	7.94	4.54	41.1	48.6	48.9	26.4	31.7	32.1	7.90	8.00	7.94	21.6	21.1	22.7
14	2.80	6.36	4.61	35.0	45.8	47.0	22.0	29.5	30.7	7.79	7.73	7.90	20.2	20.1	23.0
15	5.42	6.69	4.34	31.0	40.3	44.5	19.2	25.5	28.9	7.60	7.30	7.83	20.0	21.0	24.3
16	5.86	8.22	4.22	22.0	21.9	37.6	13.2	13.0	24.0	7.50	6.80	7.66	22.0	13.5	24.6
17	5.58	7.20	4.66	44.7	49.7	48.8	28.7	32.3	32.0	8.20	7.47	9.39	18.7	19.3	24.1
18	5.16	6.30	3.80	38.8	41.5	46.7	24.6	26.4	30.5	8.10	7.39	9.39	19.5	20.2	24.7
19	4.26	7.39	4.18	36.1	37.0	44.4	22.8	23.4	28.7	7.80	7.63	9.36	21.2	21.8	27.7
20	4.76	6.64	3.95	37.9	40.3	45.2	23.9	25.7	29.4	7.90	7.59	9.41	21.1	20.7	26.9
21	4.73	5.97	4.27	39.7	44.9	45.6	25.3	28.9	29.6	7.80	7.49	9.42	20.7	21.8	23.5
22	4.90	6.68	4.50	40.9	46.1	51.6	26.2	29.8	33.8	8.00	7.46	7.62	22.3	20.1	21.0
23	4.00	5.28	3.74	37.3	44.7	51.1	23.6	28.8	33.5	7.80	7.38	7.56	21.7	19.6	21.9
24	3.18	5.61	2.90	16.3	22.9	47.8	9.50	13.6	31.1	7.60	7.06	7.35	20.8	14.3	23.1
25	5.98	8.13	6.18	48.0	49.6	52.3	31.2	32.4	34.2	8.10	7.97	7.92	18.4	18.4	19.2
26	5.84	7.60	5.82	50.1	51.1	52.3	32.6	33.4	34.2	8.10	8.08	7.90	16.5	17.5	18.3

Table S 2 - Encoding Proteins from the singletons identified by EDGAR database on *V. diabolicus* P7A genome relative to other 11 *V. diabolicus* genomes used for comparison. Number of singletons elements identified is also represented.

Protein	Number of elements
Hypothetical protein	204
Protein serine/threonine phosphatase PrpC, regulation of stationary phase	1
serine/threonine protein kinase	1
Retron-type RNA-directed DNA polymerase (EC 2.7.7.49)	1
prophage ps3 protein 01	1
Putative translation initiation inhibitor, yjgF family	1
YgfB and YecA	1
D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	1
RNA helicase	1
Pathogenesis-related protein	1
possible DNA helicase	1
Tryptophan synthase beta chain like (EC 4.2.1.20)	1
Putative glycosyltransferase	1
UDP-N-acetylglucosamine 2-epimerase (EC 5.1.3.14)	1
FnlB	1
UDP-N-acetylglucosamine 4,6-dehydratase (EC 4.2.1.-)	1
mannosyltransferase B	1
putative glycosyltransferase	1
Chromosome partition protein smc	1
Error-prone, lesion bypass DNA polymerase V (UmuC)	1
Exodeoxyribonuclease X (EC 3.1.11.-)	1
Heat shock protein C - stress conditions	1
Integrase/recombinase (XerC/CodV family)	1
Transposase and inactivated derivatives	1
ATPase involved in DNA repair	1
Putative DNA-binding protein in cluster with Type I restriction-modification system	1
Type I restriction-modification system, restriction subunit R (EC 3.1.21.3)	2
Type I restriction-modification system, specificity subunit S (EC 3.1.21.3)	2
Type I restriction-modification system, DNA-methyltransferase subunit M (EC 2.1.1.72)	2
Mobile element protein	6
Cytochrome c551 peroxidase (EC 1.11.1.5)	2
Plasma membrane protein involved in salt tolerance	1
Putative integrase phage protein	3
unknown	1

Table S 3 - Predicted genomic islands in the genome of *V. diabolicus* P7A

P7A genomic islands annotation					
Predicted Island	Start Position	End position	Size (bp)	GC content (%)	Annotation (The proteins more related with virulence in *Vibrio* spp.)
PAI1	532251	541705	9454	55	Capsular polysaccharide biosynthesis/export periplasmic protein;
PAI2	1028653	1048304	19651	13	
PAI3	1206937	1277506	70569	55	RTX toxins; Urease accessory protein; Urease alpha subunit; Urease beta subunit; Urease; TDH; Accessory colonization factor; transcriptional regulator ToxR, type III secretion apparatus proteins; Flagellum-specific ATP synthase,
PAI4	1376263	1405394	29131	20	

Table S 4 - Virulence factors from *V. diabolicus* P7A identified by VFDB and RAST Seed Viewer databases.

	Virulence factors	Gene name	Contig	Length (bp)
Adherence	Accessory colonization factor	*acfA*	15	648
		acfD	4	4440
	Mannose-sensitive hemagglutinin (MSHA type IV pilus)	*mshA*	28	465
		mshC	28	450
		mshD	28	567
		mshE	28	1725
		mshF	28	462
		mshG	28	1224
		mshH	28	1836
		mshI	28	1446
		mshJ	28	651
		mshK	28	333
		mshL	28	1593
		mshM	28	786
		mshN	28	1107
	Type IV pilus	*pilB*	10	1686
		pilC	10	1224
		pilD	10	870
	LPS O-antigen (*Pseudomonas*)	---	2	1038
Antiphagocytosis	Capsular polysaccharide	*cpsA*	19	1401
		cpsB	19	1206
		cpsC	19	534
		cpsD	19	2151
		cpsE	19	810
		cpsF	19	1053
		cpsG	19	1254
		cpsH	19	834
		cpsI	19	858
		cpsJ	19	1308
		wbfV/wcvB	2	1095
		wecA	2	1074
		wza	13	1122
		wzc	13	2151

Chemotaxis and motility	Flagella	*cheA*	9	2235
		cheB	9	1113
		cheR	6	828
		cheV	6	846
		cheW	18	495
		cheY	9	393
		cheZ	9	741
		filM	9	1047
		flaA	6	1122
		flaB	9	1131
		flaD	6	1134
			9	1134
		flaE	9	1134
		flaG	9	435
		flaI	9	252
		flgA	6	789
		flgB	6	351
		flgC	6	414
		flgD	6	711
		flgE	6	1290
		flgF	6	750
		flgG	6	789
		flgH	6	780
		flgI	6	1068
		flgJ	6	924
		flgK	6	1797
		flgL	6	1155
		flgM	6	282
		flgN	6	426
		flhA	9	2133
		flhB	9	993
		flhF	9	1467
		flhG	9	1971
		fliA	9	735
		fliD	9	1971
		fliE	9	312
		fliF	9	1743
		fliG	9	1056
		fliH	9	801
		fliI	9	1320
		fliJ	9	444
		fliK	9	1827
		fliL	9	504
		fliN	9	411
		fliO	9	414
		fliP	9	813
		fliQ	9	270
		fliR	9	711
		fliS	9	411
		flrA	9	1467
		flrB	9	1032
		flrC	9	1410
		motA	5	762
		motB	5	873
		motX	20	621
		motY	18	882
Iron uptake	Enterobactin receptors	*irgA*	24	1893
		vctA	15	2007
	Heme receptors	*hutA*	3	2139
		hutR	30	2139
	Periplasmic binding protein-dependent ABC transport systems	*vctC*	15	711
		vctD	15	879
		vctG	15	951
		vctP	15	909
Quorum sensing	Autoinducer-2	*luxS*	10	480
	Cholerae autoinducer-1	*cqsA*	15	1224
Secretion system	EPS type II secretion system	*epsC*	2	747
		epsE	2	1503
		epsF	2	1218
		epsG	2	444
		epsH	2	612
		epsI	2	336

		epsJ	2	678
		epsK	2	1011
		epsL	2	1146
		epsM	2	495
		epsN	2	762
		gspD	2	2019
	T3SS1 secreted effectors	*vopQ*	23	987
		vopR	23	738
		vopS	23	1185
	T3SS1	*sycN*	23	294
		tyeA	23	285
		vcrD	23	2118
		vcrG	23	285
		vcrH	23	486
		vcrR	23	414
		virF	23	861
		vopB	23	1200
		vopD	23	1005
		vopN	23	894
		vscA	23	831
		vscB	23	429
		vscC	23	1815
		vscD	23	1302
		vscF	23	249
		vscG	23	360
		vscH	23	534
		vscI	23	318
		vscJ	23	744
		vscK	23	666
		vscL	23	639
		vscN	23	1323
		vscO	23	462
		vscQ	23	969
		vscR	23	651
		vscS	23	267
		vscT	23	726
		vscU	23	1056
		vscX	23	171
		vscY	23	342
		vxsC	23	186
	T3SS2 secreted effectors	*vopA*	19	873
	T3SS2	*vcrD2*	19	1887
		vscC2	4	1422
		vscN2	4	900
Toxin	Thermolabile hemolysin	*tlh*	7	1257
	Thermostable direct related hemolysin	*trh*	4	570
	Alpha-hemolysin(Escherichia)	*hlyA*	4	2961
		hlyB	4	2124
		hlyD	4	1437
	cholerae toxin	*ace*	53	333
		zot	53	1428
		ToxR	4	885
		toxS	6	516
Acid resistance	Urease(Helicobacter)	*ureB*	6	1644
		ureG	4	639
Immune evasion	Capsule(Acinetobacter)	--	2	1131
		--	2	1050
		--	2	1110
		--	2	387
	LOS (Campylobacter)	--	2	471
	Colicin V and Bacteriocin Production Cluster	*truA*	18	795
		dedA	15	681
		---	18	927
		---	18	864
		---	18	1263
		dedD	18	594
		dedE	18	492
		purf	18	1515

Table S 5 - Antibiotic resistance determinants found in *V. diabolicus* P7A using CARD, Resfinder, PATRIC and RAST Seed Viewer

Family	Gene / homolog	Contig	Length (bp)	Annotation	Drug class
Drug transporter and efflux pumps					
MATE					
	hmrM	8	1370	MATE family efflux transporter	fluoroquinolone antibiotic; acridine dye
	------	10	1347	MATE family efflux transporter	------
	ydhE/norM	12	1341	MATE family efflux transporter	fluoroquinolone antibiotic; acridine dye
	------	22	1272	Na+-driven multidrug efflux pump	------
	------	29	1287	Na+-driven multidrug efflux pump	------
MFS					
	------	1	407	H-NS histone family protein	macrolide antibiotic; fluoroquinolone antibiotic; cephalosporin; cephamycin; penam; tetracycline antibiotic
	------	4	404	H-NS histone family protein	macrolide antibiotic; fluoroquinolone antibiotic; cephalosporin; cephamycin; penam; tetracycline antibiotic
SMR					
	qacE	2	404	family quaternary ammonium compound efflux SMR transporter	macrolide antibiotic; aminocoumarin antibiotic
	qacE	26	356	family quaternary ammonium compound efflux SMR transporter	macrolide antibiotic; aminocoumarin antibiotic
ABC					
	msbA	1	1748	macrolide ABC transporter ATP-binding protein	Nitroimidazole antibiotic
	macB	4	717	macrolide ABC transporter ATP-binding protein	macrolide antibiotic
	macB	14	699	macrolide ABC transporter ATP-binding protein	macrolide antibiotic
RND					
	smeD	1	1139	Efflux RND transporter periplasmic adaptor subunit	macrolide antibiotic; fluoroquinolone antibiotic; tetracycline antibiotic; phenicol antibiotic
	acrA	1	1130	multidrug efflux RND transporter permease subunit	fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan
	acrB	1	3161	multidrug efflux RND transporter permease subunit	fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan
	dfrA3	2	479	type 3 dihydrofolate reductase	diaminopyrimidine antibiotic
	acrB	3	3309	multidrug efflux RND transporter permease subunit	fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan
	acrB	3	3060	multidrug efflux RND transporter permease subunit	fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan
	mexF	4	2169	efflux RND transporter permease subunit	fluoroquinolone antibiotic; diaminopyrimidine antibiotic; phenicol antibiotic
	mexF	4	921	efflux RND transporter permease subunit	fluoroquinolone antibiotic; diaminopyrimidine antibiotic; phenicol antibiotic

mtrF	9	1587	Multidrug efflux pump component	fluoroquinolone antibiotic; diaminopyrimidine antibiotic; phenicol antibiotic
-----	10	3105	RND multidrug efflux transporter	-----
tolC	11	1329	Outer membrane channel protein	macrolide antibiotic; fluoroquinolone antibiotic; aminoglycoside antibiotic; carbapenem; cephalosporin; glycylcycline; cephamycin; penam; tetracycline antibiotic; peptide antibiotic; aminocoumarin antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan; penem
acrB	16	3063	multidrug efflux RND transporter permease subunit	fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan
-----	19	1158	Membrane fusion protein of RND family multidrug efflux pump	------
acrB	19	3153	multidrug efflux RND transporter permease subunit	fluoroquinolone antibiotic; cephalosporin; glycylcycline; penam; tetracycline antibiotic; rifamycin antibiotic; phenicol antibiotic; triclosan
crp	20	633	cAMP-activated global transcriptional regulator	macrolide antibiotic; fluoroquinolone antibiotic; penam
adeF	22	1143	multidrug efflux RND transporter permease subunit	fluoroquinolone antibiotic; tetracycline antibiotic
cpxA	29	1395	two-component system sensor histidine kinase	aminoglycoside antibiotic; aminocoumarin antibiotic
mexW	48	3123	multidrug efflux RND transporter permease subunit	macrolide antibiotic; fluoroquinolone antibiotic; tetracycline antibiotic; acridine dye; phenicol antibiotic
Antibiotic target alteration				
ugd	2	1167	UDP-glucose dehydrogenase (EC 1.1.1.22)	peptide antibiotic
parC	11	2286	Topoisomerase IV subunit A (EC 5.99.1.-)	fluoroquinolone antibiotic
parE	11	1881	Topoisomerase IV subunit B (EC 5.99.1.-)	fluoroquinolone antibiotic
mcr-9.1	16	1656	MCR phosphoethanolamine transferase	peptide antibiotic
gyrA	17	2721	DNA gyrase subunit A (EC 5.99.1.3)	fluoroquinolone antibiotic
gyrB	21	2418	DNA gyrase subunit B (EC 5.99.1.3)	fluoroquinolone antibiotic
tuf	37	1185	elfamycin resistant EF-Tu	elfamycin resistant
Antibiotic inactivation				
sat-2	3	435	streptothricin acetyltransferase (SAT)	nucleoside antibiotic
vatB	7	555	streptogramin vat acetyltransferase	streptogramin antibiotic
catB8	12	660	chloramphenicol acetyltransferase (CAT)	phenicol antibiotic
catB9	41	645	chloramphenicol acetyltransferase (CAT)	phenicol antibiotic

Table S 6 - Proteins Involved in Metal Resistance Encoded in Genome of *V. diabolicus* P7A identified by Rast Seed Viewer and BacMet.

	Role	Contig and length (bp)
Copper homeostasis	type cbb3 cytochrome oxidase biogenesis protein CcoI	8_2697
	multicopper oxidase	38_1080
	c-type cytochrome biogenesis protein CcmI	18_1215
	Cytochrome c heme lyase subunit CcmF	18_1977
	Copper resistance protein G	6_450
	Copper-translocating P-type ATPase CopA	6_2697
	HTH-type transcriptional regulator cueR	15_387
	copper-sensing two-component system response regulator CusR	3_663
Cobalt-zinc-cadmium resistance	cobalt-zinc-cadmium resistance protein CzcR	29_909
	cobalt-zinc-cadmium resistance protein CzcD	4_897
	CusA/CzcA family heavy metal efflux RND transporter	4_3141
	heavy metal RND efflux CzcC family	4_1422
	Co/Zn/Cd efflux system membrane fusion protein	22_1128
	Co/Zn/Cd efflux system membrane fusion protein	48_1104
	DNA-binding heavy metal response regulator	2_693
	cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB family	4_1716
	heavy metal sensor histidine kinase	2_1386
	MerR family transcriptional regulator	3_375
	MerR family transcriptional regulator	4_357
Arsenic resistance	metalloregulator ArsR family transcription factor	4_342
	arsenate reductase	11_426
	arsenate reductase arsC	9_351
	ACR3 family arsenite efflux transporter	4_1056
Copper tolerance	membrane protein suppressor for copper-sensitivity ScsA	13_303
	membrane protein suppressor for copper-sensitivity ScsA	14_276
	suppressor for copper-sensitivity ScsB	14_2112
	membrane protein, suppressor for copper-sensitivity ScsD	14_516
	secreted protein, suppressor for copper-sensitivity ScsC	13_717
	Periplasmic divalent cation tolerance protein CutA	3_309
	copper homeostasis protein CutC	5_744
	apolipoprotein N-acyltransferase/copper homeostasis protein CutE	5_1479
	CNNM family magnesium/cobalt transport protein CorC	5_900
	HlyC/CorC family transporter	5_1305
chromium tolerance	chromate efflux transporter	10_1140