Science of the Total Environment 905 (2023) 166351

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Snapshot of resistome, virulome and mobilome in aquaculture

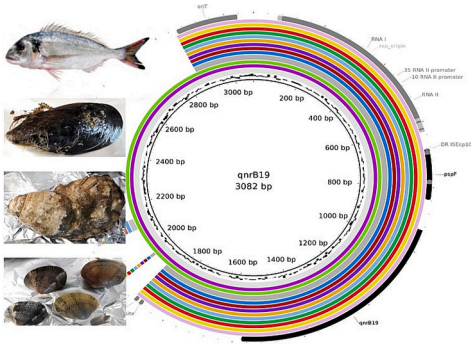
Vanessa Salgueiro [a,b,c], Vera Manageiro [a,b,c], Tânia Rosado [d], Narcisa M. Bandarra [e,f], Maria João Botelho [f,g], Elsa Dias [d], Manuela Caniça [a,b,c,h,*]

[a] National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections, Department of Infectious Diseases, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal
[b] Centre for the Studies of Animal Science, Institute of Agrarian and Agri-Food Sciences and Technologies, University of Porto, Porto, Portugal
[c] AL4AnimalS, Associate Laboratory for Animal and Veterinary Sciences, Portugal
[d] Laboratory of Biology and Ecotoxicology, Department of Environmental Health, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal
[e] Division of Aquaculture, Upgrading and Bioprospecting, Portuguese Institute for the Sea and Atmosphere, IPMA, Lisbon, Portugal
[f] CIIMAR, Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto, Matosinhos, Portugal
[g] Division of Oceanography and Marine Environment, Portuguese Institute for the Sea and Atmosphere, Lisbon, Portugal
[h] CIISA, Center for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal

HIGHLIGHTS

- New STs (17) and possible evolutionary relationships with other STs were identified.
- Over 140 resistance genes provided a snapshot of the aquaculture resistome.
- Many resistance genes found are common to those of clinical isolates (e.g. *qnrB19*).
- Many ARGs detected (e.g. *sul*) are associated to antibiotics used in aquaculture.
- Several (74.2 %) strains studied were classified as pathogenic to human.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damia Barcelo

Keywords:
Seabream
Bivalve molluscs
Gram-negative bacteria
Resistome
Virulome
Mobilome

ABSTRACT

Aquaculture environments can be hotspots for resistance genes through the surrounding environment. Our objective was to study the resistome, virulome and mobilome of Gram-negative bacteria isolated in seabream and bivalve molluscs, using a WGS approach. Sixty-six Gram-negative strains (*Aeromonadaceae*, *Enterobacteriaceae*, *Hafniaceae*, *Morganellaceae*, *Pseudomonadaceae*, *Shewanellaceae*, *Vibrionaceae*, and *Yersiniaceae* families) were selected for genomic characterization. The species and MLST were determined, and antibiotic/disinfectants/heavy metals resistance genes, virulence determinants, MGE, and pathogenicity to humans were investigated. Our study revealed new sequence-types (e.g. *Aeromonas* spp. ST879, ST880, ST881, ST882, ST883, ST887, ST888; *Shewanella* spp. ST40, ST57, ST58, ST60, ST61, ST62; *Vibrio* spp. ST206, ST205). >140 different genes were identified in the resistome of seabream and bivalve molluscs, encompassing genes associated with β-lactams, tetracyclines, aminoglycosides, quinolones, sulfonamides, trimethoprim, phenicols, macrolides and

* Corresponding author at: National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections, Department of Infectious Diseases, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal.
E-mail address: manuela.canica@insa.min-saude.pt (M. Caniça).

https://doi.org/10.1016/j.scitotenv.2023.166351
Received 10 March 2023; Received in revised form 28 July 2023; Accepted 15 August 2023
Available online 19 August 2023

fosfomycin resistance. Disinfectant resistance genes *qacE*-type, *sitABCD*-type and *formA*-type were found. Heavy metals resistance genes *mdt*, *acr* and *sil* stood out as the most frequent. Most resistance genes were associated with antibiotics/disinfectants/heavy metals commonly used in aquaculture settings. We also identified 25 different genes related with increased virulence, namely associated with adherence, colonization, toxins production, red blood cell lysis, iron metabolism, escape from the immune system of the host. Furthermore, 74.2 % of the strains analysed were considered pathogenic to humans. We investigated the genetic environment of several antibiotic resistance genes, including $bla_{\text{TEM-1B}}$, $bla_{\text{FOX-18}}$, *aph(3″)-Ib*, *dfrA*-type, *aadA1*, *catA1*-type, *tet(A)/(E)*, *qnrB19* and *sul1/2*. Our analysis also focused on identifying MGE in proximity to these genes (e.g. IntI1, plasmids and Tn*As*), which could potentially facilitate the spread of resistance among bacteria across different environments. This study provides a comprehensive examination of the diversity of resistance genes that can be transferred to both humans and the environment, with the recognition that aquaculture and the broader environment play crucial roles as intermediaries within this complex transmission network.

1. Introduction

Water is a rich environment encompassing several organisms (such as fish, bivalve molluscs, and algae) along with their respective microbiomes. Additionally, water serves as a convergence point for bacteria originated from other environments (human and animal) (Baquero et al., 2008). Aquaculture farms are examples of such diverse environments, where the use of antibiotics can increment selective pressure among commensals as well as pathogenic bacteria (Done et al., 2015; Gastalho et al., 2014).

In aquaculture settings, antibiotics are usually administrated mixed in food, which exposes both diseased and healthy animals to these substances (Ottinger et al., 2016; Topp et al., 2018). Uneaten food containing antibiotics, as well as unabsorbed antibiotics or their by-products that are excreted in urine and faeces, can be carried by water currents to other sites, thereby exposing other animals to these substances. Aquaculture, particularly in coastal environments, can be influenced by sewage and land run-off (mainly due to high rainfall in certain periods), as well as marine birds or mammals, and may concomitantly contain various bacteria, as well as various heavy metals, antibiotics and organic substances. This confluence of factors can promote selective pressure on bacteria normally present in the aquatic environment. For that reason, marine bivalve molluscs can serve as potential indicators of multidrug-resistant *Escherichia coli* and other species of the *Enterobacteriaceae* family (Grevskott et al., 2017). In addition, human activities, like maritime traffic, industry, agriculture, hospitals, tourism, and water from treatment plants can also contribute to the contamination of aquatic environments, including aquaculture farms (Done et al., 2015; Gastalho et al., 2014). The statistically significant decrease in susceptibility to oxytetracycline found in Japanese oysters during the summer, which could be directly influenced by the factors described above, such as bird migration, was previously demonstrated by us (Salgueiro et al., 2021). Additionally, Salgueiro et al. (2023) illustrated that *Staphylococcus aureus* ST398, isolated in a gilthead seabream from aquaculture, could had an environmental origin due to the tank's exposure to seawater and, consequently, to antibiotics, bacteria and resistance determinants from different locations (Salgueiro et al., 2023).

It is known that antibiotics, even in sublethal concentrations, interfere in the regulation of gene expression and interaction between bacteria, promoting genetic exchanges. In addition, these exchanges can be facilitated by the presence of bacteria at high densities in small areas, as observed in bivalve molluscs due to their filter-feeding habit, as well as in biofilms, which play a crucial role in bacterial persistence in water environments. The presence of heavy metals or disinfectants can also contribute to the selection of antibiotic resistance through cross-resistance events, as these elements serve as substrates for efflux pumps. Additionally, genes responsible for tolerance to heavy metals and disinfectants can be located in the same genetic elements as antibiotic resistance genes (ARG) (Finley et al., 2013; Lupo et al., 2012). Several theories support that some of the ARG frequently found in clinical isolates were originated in aquatic bacteria, such as *mcr* (conferring colistin resistance) and $bla_{\text{OXA-48}}$ (conferring carbapenem resistance) genes that probably derived from the chromosome of *Shewanella* spp., a bacterium typical from freshwater and marine environments (Poirel et al., 2012, 2005; Cabello and Godfrey, 2018). Likewise, plasmid-mediated bla_{AmpC} genes may have originated from other aquatic bacteria, such as *Hafnia alvei* and *Aeromonas hydrophila*, or from bacteria that circulate between various environments (like water and gut), such as *Enterobacter asburiae* and *Citrobacter freundii* (Lupo et al., 2012). The hypothesis is that these genes jumped from the chromosomes of these aquatic species under the pressure of antibiotic application, facilitated by mobile genetic elements (MGE) such as insertion sequences (IS) and plasmids. Subsequently, they disseminated into bacteria commonly found in clinical settings (Lupo et al., 2012; Poirel et al., 2012, 2005; Cabello and Godfrey, 2018). Acquired antibiotic resistance can result from mutations in genes already present in the bacterial chromosome or through the acquisition of genetic material via horizontal gene transfer (HGT) using three mechanisms: conjugation, transformation, and transduction (Nadeem et al., 2020). Due to the high abundance of phages in water environments, transduction represents an important vehicle for ARG acquisition (Breitbart, 2012). Thus, aquaculture environments can serve as reservoirs of antibiotic resistance (Nadeem et al., 2020).

Whole Genome Sequencing (WGS) represents one of the most comprehensive technologies nowadays. Therefore, this study used WGS on bacteria isolated in fish and bivalve molluscs from aquaculture production. Samples were collected directly from aquaculture farms and markets to identify which determinants of resistance and virulence, as well as MGE, are circulating in this environment in order to understand the contribution of aquaculture sources in the emergence and spread of antibiotic resistance. Thus, this study aims to comprehensively search and analyse not only the genes conferring resistance (resistome), but also the MGE that could be associated with their dissemination (mobilome), as well as genes related to virulence (virulome), whose presence unquestionably depends on the natural environment or anthropogenic factors, rather than solely relying on the metabolism of fish or bivalve molluscs (Wanyan et al., 2023). In fact, this is the first study in Portugal that deepens into the diversity of resistance genes that can be transmitted to humans and to the environment. It recognizes both the environment and aquaculture as intermediaries in a complex chain.

2. Materials and methods

2.1. Characterization of the bacterial collection

In 2018 and 2019, samples of *Sparus aurata* (muscle, gills, skin, intestine) were collected from aquaculture farm 7 in the southern region of Portugal, as well as from a market. On the other hand, in 2019, bivalve molluscs' samples were collected in 6 Portuguese aquaculture farms: *Crassostrea gigas* from aquaculture farms 1 (southern region), 5 and 6 (central region); *Mytilus* sp. from aquaculture farms 2, 3 and 4 (southern region); and *Ruditapes decussatus* from aquaculture farm 1. *Mytilus* sp. were purchased from a market (central region), imported from Spain but

with the depuration process performed in Portugal. All analyses were performed at the National Institute of Health Dr. Ricardo Jorge. Sample preparation and bacterial isolation procedures have been previously described elsewhere (Salgueiro et al., 2021, 2020). These procedures involved the use of selective media with different standard antibiotic concentrations (100 mg/L of amoxicillin, 2 mg/L of cefotaxime, 20 mg/L of chloramphenicol, 0.5 mg/L of colistin, 50 mg/L of nalidixic acid and 8 mg/L of oxytetracycline) that allowed an initial screening of decreased susceptibilities. Only individual colonies with distinct morphology were selected to ensure no duplications were included.

A total of 66 Gram-negative strains were selected for genomic characterization. Among these, 53 fulfilled the criteria of non-susceptibility to at least one group of the following antibiotics: β-lactams, aminoglycosides, folate pathway antagonists, phenicols, quinolones, polymyxins or tetracyclines. The other 13 bacterial strains were randomly selected from a susceptible group of 56 Gram-negative strains, with the purpose of understanding whether there were any ARG related to antibiotics that were not tested or ARG whose resistance phenotype was not detected by the methods used in this study. The distribution of strains among bacterial families, aquaculture species and origin (aquaculture farm and market) can be found in Table S1.

2.2. Identification of bacterial strains and antibiotic susceptibility testing

At the time of strains isolation, bacterial species identification was performed by VITEK MS (BioMérieux, Marcy-l'Étoile, France) or amplification of the 16S rRNA gene, as previously described (Jones-Dias et al., 2016b). Posteriorly, antibiotic susceptibility testing was performed by disk diffusion (Bio-Rad, Marnes-la-Coquette, France) and minimum inhibitory concentration (MIC) by an in-house broth microdilution. The antibiotics tested and their respective breakpoints are described in Table S2. Strains were considered multidrug resistant if they presented a resistant phenotype to three or more structurally unrelated antibiotics.

2.3. Genomic DNA extraction and preparation

Genomic DNA of the 66 Gram-negative strains was extracted using the MagNA Pure 96 Instrument (Roche, Manheim, Germany) and quantified using the Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions.

2.4. Whole genome sequencing

Libraries were prepared from 1 ng of genomic DNA using the dual-indexed Nextera XT Illumina library preparation kit. Subsequently, cluster generation and paired-end sequencing (2 × 150 bp for half of the strains and 2 × 250 bp for the other half; more detail in Table S3) were performed on a MiSeq Illumina platform (Illumina Inc., San Diego, CA), according to manufacturer's instructions.

2.5. Data analysis and molecular characterization

Sequence reads were subjected to trimming and filtering based on quality criteria, and de novo assembly was performed using CLC Genomics Workbench version 21.0.3 (QIAGEN, Aarhus, Denmark). Online tools and databases available at the Center for Genomic Epidemiology (CGE) (https://www.genomicepidemiology.org/; accessed on 25 July 2022) were used to confirm bacterial species identification (KmerFinder 3.2), predict multilocus sequence type (MLST), whenever available (MLST 2.0), investigate the presence of antibiotic and disinfectant resistance genes (ResFinder 4.1), virulence genes (VirulenceFinder 2.0), plasmids (PlasmidFinder 2.1), and other MGE, as well as their relation to ARG and virulence factors (MobileElementFinder version 1.0.3), and to estimate bacteria's pathogenicity towards human hosts (PathogenFinder 1.1) (Bortolaia et al., 2020; Carattoli et al., 2014; Cosentino et al., 2013; Johansson et al., 2021; Larsen et al., 2014, 2012; Tetzschner et al., 2020). New sequence types (ST) were submitted to the respective database in PubMLST (https://pubmlst.org/; accessed on 25 July 2022). A Minimum Spanning Tree (MST) was constructed using CLC Genomics Workbench version 21.0.3 based on MLST, for *Aeromonas* spp., *Citrobacter* spp., *Enterobacter cloacae* complex, *Shewanella* spp. and *Vibrio* spp. strains that exhibited new ST. The purpose was to investigate the possible evolutionary relationships between these strains, using schemes from PubMLST database, single linkage, and Manhattan distance.

Single nucleotide polymorphisms (SNPs) analysis was performed using CSI Phylogeny 1.4 web tool with default parameters and *E. coli* SCU-104 (CP053284) as reference, to construct a phylogenetic tree and a SNPs matrix with all *E. coli* detected in this study (Kaas et al., 2014). The phylogenetic tree image was edited with CLC Genomics Workbench version 21.0.3. The network of *E. coli* isolates was created with the pairwise SNP distances matrix, using the GraphSNP viewer (Permana et al., 2023). Each node represents an isolate while the edge represents the SNPs distance between two isolates. A threshold of ≤15 SNPs difference was used to filter the edges in the network. The Comprehensive Antibiotic Resistance Database (CARD) was also used to investigate the presence of ARG and PHASTER search web tool allowed the identification and annotation of prophage sequences (Alcock et al., 2020; Arndt et al., 2016). The online tool RFPlasmid was used to predict chromosomal or plasmid location of the previously identified genes (van der Graaf-Van Bloois et al., 2021). For visualizing and comparing the contigs harboring *qnrB19* genes that were determined to have a plasmid location, the BLAST Ring Image Generator (BRIG, v. 0.95) was used (Alikhan et al., 2011). This tool generated a circular image facilitating the comparison of all relevant contigs. As reference, the closest plasmid sequences obtained through the NCBI Microbial genomes BLAST analysis were used. ISsaga was used to complement the search of MGE, such as insertion sequences (Varani et al., 2011). All analysis were performed using default parameters. CLC Genomics Workbench version 21.0.3 was also used to search for integrons, heavy metals resistance genes, using Antibacterial biocide and metal resistance genes (BacMet) database version 2.0 (Pal et al., 2014), as well as to study the genetic environment of ARG.

2.6. Nucleotide sequence accession numbers

The genomes of the 66 bacterial strains included in this study were deposited in GenBank under BioProject number PRJNA762299. More information about accession numbers, contigs, consensus length and average coverage is available in Table S3. New alleles numbering for β-lactamases genes were requested at NCBI (https://www.ncbi.nlm.nih.gov/pathogens/submit-beta-lactamase/; accessed on 25 July 2022) and are the following: MZ092825 (bla_{FOX-18} from INSAq178), MZ359745 (bla_{TER-3} from INSAq228), MZ092824 ($bla_{CTX-M-246}$ from INSAq229), MZ359742 (bla_{PLA-7} from INSAq240), MZ092826 (bla_{FOX-19} from INSAq241), MZ092827 ($bla_{OXA-960}$ from INSAq243), MZ092828 ($bla_{OXA-961}$ from INSAq334), MZ092822 ($bla_{CMY-175}$ from INSAq424), MZ092830 ($bla_{OXA-963}$ from INSAq494) and MZ092832 ($bla_{OXA-965}$ from INSAq497).

3. Results and discussion

3.1. Identification of bacterial strains

The online tool KmerFinder 3.2 was utilized to confirm the species of all 66 Gram-negative strains selected for this study, previously identified by either MALDI-TOF or amplification of the 16S rRNA gene. The results revealed that 18 strains belonging to the families *Aeromonadaceae*, *Enterobacteriaceae*, *Hafniaceae*, *Morganellaceae*, and *Shewanellaceae* were misidentified. Additionally, the use of KmerFinder 3.2 allowed the identification of 7 strains at the species level, within the genera *Aeromonas*, *Enterobacter*, *Hafnia*, *Serratia*, and *Vibrio* (Table S4). However,

subsequent analysis using Average Nucleotide Identity (ANI) on GenBank revealed corrections in the identification of 6 strains. Specifically, one *Aeromonas veronii* strain was changed to *Aeromonas allosaccharophila*, one *Proteus vulgaris* strain was changed to *Proteus terrae* and three *C. freundii/C. freundii* complex strains were changed to *Citrobacter portucalensis* (Table S4). This confirms the difficulties already described in other works to identify closely related members from these genera to the species level (Fernández-Bravo and Figueras, 2020; Richter et al., 2013). Only identifications provided by KmerFinder 3.2 and confirmed by ANI will be used throughout this study.

Therefore, all strains included in this study belong to one of the following species: *A. allosaccharophila* ($n = 2$), *Aeromonas caviae* ($n = 1$), *Aeromonas media* ($n = 1$), *Aeromonas rivipollensis* ($n = 2$), *Aeromonas salmonicida* ($n = 2$), *C. freundii* ($n = 2$), *C. portucalensis* ($n = 3$), *E. asburiae* ($n = 1$), *Enterobacter hormaechei* ($n = 4$), *Escherichia coli* ($n = 11$), *Hafnia alvei* ($n = 1$), *Hafnia paralvei* ($n = 6$), *Klebsiella michiganensis* ($n = 1$), *Klebsiella pneumoniae* ($n = 2$), *Kluyvera intermedia* ($n = 1$), *Leclercia adecarboxylata* ($n = 2$), *Lelliottia amnigena* ($n = 1$), *Obesumbacterium proteus* ($n = 4$), *P. terrae* ($n = 1$), *Pseudomonas stutzeri* ($n = 2$), *Raoultella ornithinolytica* ($n = 3$), *Raoultella planticola* ($n = 1$), *Raoultella terrigena* ($n = 1$), *Serratia liquefaciens* ($n = 1$), *Shewanella algae* ($n = 4$), *Shewanella chilikensis* ($n = 2$), *Shewanella indica* ($n = 1$), *Vibrio alginolyticus* ($n = 1$), *Vibrio antiquarius* ($n = 1$), and *Vibrio diabolicus* ($n = 1$). All species, except for *P. terrae*, have been previously associated with water environments and/or aquatic animals (Harmon et al., 2019; Salgueiro et al., 2021, 2020). Nevertheless, to the best of our knowledge, this is the first report of *C. portucalensis*, *K. intermedia*, *R. terrigena*, and *S. liquefaciens* in *S. aurata* (acquired in market), as well as *P. terrae* and *S. chilikensis* in *Mytilus* sp. (acquired in market and farm 2) and *C. portucalensis* in *C. gigas* and *R. decussatus* (acquired in farm 1) from aquaculture.

3.2. Genetic diversity

MLST was performed for 40 strains (Tables 1 and 2), representing various species including *Aeromonas* spp., *C. freundii* complex, *E. cloacae* complex, *E. coli*, *K. pneumoniae*, *Shewanella* spp. and *Vibrio* spp.

3.2.1. Aeromonas spp.

Within the genus *Aeromonas*, a single strain of *A. caviae* (isolated from mussels acquired in the market) was assigned to ST368. In contrast, the other 7 strains (from gilthead seabream's muscle and mussels acquired in the market) presented new STs: ST879, ST880, ST881, ST882, ST883, ST887, and ST888. In the MST depicted in Fig. 1 A.1 (and Fig. S1 A), we can observe that the new ST879, ST880, ST882, ST883 and ST888 share a putative founder, namely ST1251, which belongs to *A. caviae* and was isolated from drinking water in Bangladesh, in 2013 (PubMLST database id 1182). These new STs show differences in 6 loci compared to ST1251. Among them, ST883 is particularly closely related to ST193 (isolated in vegetables from Italy, in 2011; PubMLST database id 213), differing by only 5 loci. On the other hand, ST113 (from *A. allosaccharophila* isolated in *Cyprinus carpio* from China, in 2011; PubMLST database id 125) and ST483 (from *A. hydrophila* recovered from meat samples from China, in 2014; PubMLST database id 547) are the closest to ST888, from which they differ in 5 loci. In Fig. 1 A.2 (and Fig. S1 A), we can see the new ST887, that differ in 5 loci compared to its putative founder, ST477, originating from *A. hydrophila* isolated in seafood from China, in 2014 (PubMLST database id 541). Finally, the remaining newly identified ST in this family, ST881, differs in 5 loci from ST459 (Fig. 1 A.3 and Fig. S1 A) previously found in *A. veronii* isolated from meat samples in China, in 2014 (PubMLST database id 524). Additionally, *A. caviae* ST368 has been previously isolated from human blood in Spain, in 2014 (PubMLST database id 424).

3.2.2. Citrobacter freundii complex

The *C. freundii* isolates in our study belonged to 2 different STs: ST64 and ST169. ST64 was recovered in this study from the muscle of a gilthead seabream (collected in farm 7) and previously reported by Villa and colleagues in association with a *C. freundii* strain harboring $bla_{\text{VIM-1}}$ and $bla_{\text{KPC-2}}$ genes, collected from a hospitalized patient (Villa et al., 2017). On the other hand, ST169, which has been linked to carbapenemase production (OXA-48 and VIM-1), was previously isolated in the digestive tract of patients with acute leukaemia (Lalaoui et al., 2019) and, in our study, ST169 was found in mussels acquired in a market.

The three strains of *C. portucalensis* identified in the present study, had different STs: ST85 isolated in gilthead seabream's gills acquired in the market, ST416 found in Japanese oysters from farm 1 and a new ST (ST971) isolated in clams also from farm 1. According to PubMLST database, ST85 and ST416 were already associated with *Citrobacter* sp. isolated in humans from China and Ireland, respectively. In MST shown in Fig. S2 A, the new ST971 clusters together with ST343 (identified in *Citrobacter* sp. isolated in food samples from China, in 2017; PubMLST database id 315) and ST834 (of unknown origin), differing by 5 loci.

3.2.3. Enterobacter cloacae complex

All strains belonging to the *E. cloacae* complex (four *E. hormaechei* and one *E. asburiae*) found in this study, were isolated in gilthead seabream from farm 7. Among these strains, only two shared the same ST, while one is new due to the presence of new alleles (ST1994). In MST shown in Fig. 1 B.1 and Fig. S1 B, the putative founder of ST1994 is ST483, which has been previously identified in *Citrobacter* species isolated from humans in the United States of America in 2018 (PubMLST database id 415). ST1994 differs from its putative founder, ST483, by 5 loci. Regarding the distribution of the strains *E. hormaechei* ST170, one *E. hormaechei* ST190 and *E. asburiae* ST1994 were found in different samples from the same fish (muscle and skin). The other *E. hormaechei* ST190 and *E. hormaechei* ST664 were detected in muscle samples from distinct fish. To the best of our knowledge, this is the first description of ST170, ST190 and ST664 in aquaculture reservoirs. However, ST190 has been previously reported in other studies involving human clinical samples, namely by Lin et al. (2020), that identified this ST in an *E. cloacae* with 19 resistance genes (such as *mcr-9*, *catA1* and *dfrA1*). Similarly Liu et al. (2021), linked this ST with non-susceptible carbapenemase isolates of *E. cloacae* complex producers of KPC-2 and IMP carbapenemases, among others (Liu et al., 2021).

3.2.4. Escherichia coli

The 11 strains of *E. coli* studied belonged to three different ST based on their origin: ST10 ($n = 8$), isolated from mussels in farm 4; ST58 ($n = 1$), found in the intestine of a gilthead seabream acquired from the market; and ST2607 ($n = 2$), isolated from the gills of a gilthead seabream in farm 7.

ST10 is a well-known ST among *E. coli* strains responsible for human extraintestinal infections worldwide (Manges et al., 2019). It has also been described in Venus clam (*Ruditapes philippinarum*) acquired from the market, where it was associated with $bla_{\text{VIM-1}}$ (Roschanski et al., 2017). Furthermore, ST10 has been associated with poultry, vegetables and water sources harboring CTX-M extended-spectrum β-lactamases (ESBL) (Müller et al., 2016). Although belonging to the same ST, phylogenetic and network analysis showed that these eight *E. coli* ST10 are not indistinguishable, with a pairwise nucleotide distance between 6 and 15 SNPs (Fig. S3A–B, and Table S5). These *E. coli* ST10 also present some differences regarding ARG, virulence factors, heavy metal resistance genes, MGE, and others (discussed in the next sections). There is no agreement between the authors regarding the SNPs cut-off to considerer whether strains are related or not. However, according to the relatedness criteria for *E. coli* SNP typing scheme of Schürch et al. (2018) ($\leq$10SNPs), INSAq316 is closely related to INSAq317 and INSAq319; additionally, INSAq317 is closely related to INSAq324; lastly, INSAq321 is closely related to INSAq354 (Fig. S3B).

ST58 is widely distributed worldwide and has been collected from various sources, including clam *Chamelea gallina*, humans, cattle, swine, poultry, birds, companion animals, water, and store-bought produce

Table 1
Summary of the results obtained by antibiotic susceptibility testing and the investigation of Multilocus sequence type (MLST), antibiotic/disinfectant resistance genes, virulence factors, mobile genetic elements (MGE), phage regions and pathogenicity towards human hosts for fish samples acquired in farm 7 (A) and market (B).

Strain	Organ	MLST	NS profile	Antibiotic resistance genes	Disinfectant resistance genes	Virulence factors	Plasmids	Others mobile genetic elements	Nr. of phage regions	Human pathogen probability (%)
A) Aquaculture farm 7										
Citrobacter freundii ($n = 1$)										
INSAq43	Muscle	ST64	AMC, FLO, FOX	bla_{CMY-41} (C)	*formA*-type (P)	–	Col (pHAD28)-type, pKPC-CAV1321, RepA	IS*30*-type, IS*102*-type, IS*Eam1*-type, IS*Ecl1*-type, IS*Kpn26*-type, IS*Sen4*	2	86.1
Enterobacter asburiae ($n = 1$)										
INSAq146	Skin	ST1994[a]	–	*fosA*-type (C)	–	–	Col440I-type, Col (pHAD28)-type	cn_4160_IS*102*-type, IS*102*-type, IS*Eam1*-type, IS*Ecl1*-type, IS*Ehe3*-type IS*Kpn26*-type, IS*Sen4*-type, Tn*5403*-type	1	83.1
Enterobacter hormaechei ($n = 4$)										
INSAq21	Muscle	ST170	AMC, CHL, FLO, FOX	bla_{ACT-17} (C)	–	*iroN*-type	Col(pHAD28), IncFIB(K)-type, IncFII (Yp)-type	IS*26*, IS*102*-type, IS*5075*-type, IS*Eam1*-type, IS*Ehe3*-type IS*Kox1*-type, IS*Kpn21*, IS*Kpn43*-type, IS*Sen4*-type	2	79.6
INSAq99	Muscle	ST190	AMC, AZT, CAZ, CTX, FEP, FLO, FOX,	bla_{ACT-17} (C), *fosA*-type (C)	–		Col(IRGK)-type, Col (pHAD28)-type, IncFIB (K)-type, IncFII(Yp)-type	IS*26*, IS*102*-type, IS*903*-type, IS*5075*-type, IS*Ehe3*-type, IS*Kox1*-type, IS*Kpn8*, IS*Kpn43*-type	2	80.2
INSAq107	Muscle	ST664	PTZ	bla_{ACT-89} (C), *fosA*-type (C)	–		Col440I-type, Col (pHAD28)-type, IncFIB (K)-type, IncFII(Yp)-type	cn_1462_IS*26*, IS*26*, IS*5075*-type, IS*Cfr26*, IS*Ehe3*-type, IS*Kpn20/38*-type	4	80.5
INSAq140	Skin	ST190	AMC, AZT, CAZ, CHL, CTX, FEP, FLO, FOX, PTZ	bla_{ACT-17} (C), *fosA*-type (C)	–		Col(IRGK)-type, Col (pHAD28)-type, IncFIB (K)-type, IncFII(Yp)-type	IS*26*, IS*5075*-type, IS*Kox1*-type, IS*Kpn8*, IS*Kpn43*-type	2	81.0
Escherichia coli ($n = 2$)										
INSAq159	Gills	ST2607	AMC, CHL, FLO	*acrA/B/E* (C), *ampH* (C), *baeS* (C), *cpxA* (C), *emrB/R* (C), *eptA* (C), *evgA* (C), *hns* (C), *marA* (C), *mdf (A)*-type (C), *mdtE/G/H* (C), *msbA* (C), *tolC* (C)	–	*astA*, *gad*-type, *hra*-type, *iss*-type, *lpfA*, *ompT*, *ompT*-type, *papC*-type, *terC*, *terC*-type	–	MITEEc1-type	4	94.5
INSAq163	Gills	ST2607	AMC, FLO	*acrA/B/E* (C), *ampH* (C), *baeS* (C), *cpxA* (C), *emrB/R* (C), *eptA* (C), *evgA* (C), *hns* (C), *marA* (C), *mdf (A)*-type (C),	–		–	IS*3*-type MITEEc1-type	3	94.4

(*continued on next page*)

Table 1 (*continued*)

Strain	Organ	MLST	NS profile	Antibiotic resistance genes	Disinfectant resistance genes	Virulence factors	Plasmids	Others mobile genetic elements	Nr. of phage regions	Human pathogen probability (%)
				mdtE/G/H (C), *msbA* (C)						
Klebsiella michiganensis ($n = 1$)										
INSAq73	Muscle	NA	–	$bla_{OXY-1-3}$ (C)	–	*fyuA*-type	Col440I-type, Col (pHAD28)-type, IncFIB (K)-type, IncN-type	IS*26*, IS*102*-type, IS*5075*-type, IS*Ecl1*-type, IS*Esa1*-type, IS*Kpn8*, IS*Kpn28/38*-type, IS*Ppu12*-type, IS*Sen4*, IS*Sm3*-type, Tn*5403*-type	1	83.3
Klebsiella pneumoniae ($n = 2$)										
INSAq40	Muscle	ST134	FLO	bla_{SHV-60} (C), *fosA*-type (C), *kpnF* (C), *lptD* (C), *oqxA*-type (C), *oqxB19* (C)	–	*iutA*-type, *traT*-type	Col440I-type, Col440II-type, IncFIA (HI1)-type, IncFIB(K)-type	cn_1587_IS*Ec36*-type, IS*26*-type, IS*102*-type, IS*903*-type, IS*5075*-type, IS*Ec36*-type, IS*Kpn1*-type, IS*Plge2*-type	4	89.0
INSAq121	Intestine									89.1
Leclercia adecarboxylata ($n = 2$)										
INSAq143	Skin	NA	FLO	*qnrB19* (P)	–	–	Col(pHAD28), Col (pHAD28)-type	IS*903*-type, IS*5075*-type	2	82.8
INSAq160	Gills	NA	–	–	–	–	Col (pHAD28)-type	IS*26*, IS*30*-type, IS*Eam1*-type, IS*Ec33*-type, IS*Ppu12*-type, IS*Raq1*-type, IS*Sen4*-type, Tn*5403*-type	1	82.3
Pseudomonas stutzeri ($n = 2$)										
INSAq87	Muscle	NA	CHL, ERT, FLO, FMQ	–	–	–	–	IS*Cfr1*-type, IS*Pa16/86*-type, IS*Ppu23*-type, IS*Pre3*-type, IS*Pst3/4*-type	–	15.9
INSAq93	Muscle	NA		–	–	–	–	IS*Cfr1*-type, IS*Pa86*-type, IS*Pre3*-type, IS*Pst3/4*-type	–	15.7
B) Market										
Aeromonas allosacharophila ($n = 1$)										
INSAq178	Muscle	ST881[a]	ERT	bla_{FOX-18}[b] (P), $bla_{OXA-958}$ (C), *cphA*-type (C)	–	–	–	IS*Aeme3/15/23*-type, IS*Ahy3*-type, IS*As12/23/25/26*-type, IS*As27*, IS*Kpn3*-type, MITEAeme1-type	–	46.9
Aeromonas media ($n = 1$)										
INSAq193	Muscle	ST883[a]	OTC	*ampH* (C), *aph (3″)-Ib* (P), *aph (6)-Id* (P), bla_{TEM-1B} (P), *dfrA5*-type (P), *qnrB19* (P), *sul2* (P), *tet(A)* (P), *tet(E)*-type (P)	*sitABCD*-type (P)	*cia*, *cvaC*, *etsC*, *hlyF*, *iroN*, *iss*, *iucC*, *iutA*-type, *mchF*, *ompT*, *ompT*-type, *sitA*, *traT*	Col440I-type, IncFIB (AP001918)-type, IncQ1-type, IncFII	cn_3415_IS*Apu1*, *intI1/tniA*, IS*3*-type, IS*26*, IS*100*-type, IS*629*-type, IS*Aeme8/9*-type, IS*Aeme12/15*, IS*Ahy2*-type, IS*Apu1*, IS*As2/6/12/16/19/25/26/32*-type, IS*As20*, IS*Ec1/31*-type, ISEc32, IS*Ecl8*-type, MITEAeme2, MITEEc1-type, MITEKpn1-type, Tn*3*	–	89.9
Aeromonas rivipollensis ($n = 2$)										
INSAq177	Muscle	ST880[a]	–	–	–	–	–	IS*Aeme1*-type IS*As2/7/13/18/31*-type,	2	47.1

(*continued on next page*)

Table 1 (*continued*)

Strain	Organ	MLST	NS profile	Antibiotic resistance genes	Disinfectant resistance genes	Virulence factors	Plasmids	Others mobile genetic elements	Nr. of phage regions	Human pathogen probability (%)
INSAq180	Muscle	ST882[a]	–	–	–	–	–	IS*As9*/21, IS*Ec28*-type, IS*Kpn3/10*-type, MITEAeme2 cn_10729_IS*5*-type, IS*5*-type, IS*Aeme20*-type, IS*Ahy2*-type, IS*As2/13/31*-type, IS*Kpn10*-type	2	46.3
Aeromonas salmonicida (*n* = 2)										
INSAq169	Muscle	ST879[a]	–	*cphA5*-type (C)	*qacE*-type (P)	–	–	cn_3415_IS*Apu1*, *intI1/tniA/tniQ*, IS*Apu1*, IS*As3/12/13/31*-type, IS*As22/27*, IS*Ec28*-type, IS*Sba16*-type	1	29.0
INSAq195	Intestine	ST887[a]	–	*cphA5*-type (C)	–	–	–	cn_2101_IS*Apu1*, IS*Aeme5/21*-type, IS*Apu1*, IS*Apu2*-type, IS*As1/2/3/7/12/15/16/23/29/32/34*-type, IS*As13/21*, IS*Ave3*-type, IS*Kpn3/10*-type, IS*Kpn9*-type, Tn*4671*-type, *tniA/tniB/tniQ*	2	27.6
Citrobacter portucalensis (*n* = 1)										
INSAq234	Gills	ST85	AMC, FLO	$bla_{CMY-176}$ (C), *qnrB18* (C)	–	–	IncFIB(K)-type	cn_3415_IS*Apu1*, IS*102*-type, IS*5075*-type, IS*Apu1*, IS*Cfr13/26*-type, IS*Ecl1*-type, IS*Ec17*-type, IS*Ehe3*-type, IS*Esa1*-type, IS*Sm1*-type	8	84.6
Escherichia coli (*n* = 1)										
INSAq183	Intestine	ST58	CIP, FLO, FMQ, OTC, SXT	*acrA/B/E* (C), *ampH* (C), *aph(3″)-Ib* (P), *aph(6)-Id* (P), *baeR/S* (C), bla_{TEM-1B} (P), *cpxA* (C), *dfrA5*-type (P), *emrB/R* (C), *evgA* (C), *hns* (C), *marA* (C), *mdf(A)*-type (C), *mdtG/H* (C), *msbA* (C), *qnrB19* (P), *sul2* (P), *tet(A)* (P), *tolC* (C),	*sitABCD*-type (P)	*capU*-type, *cia*, *cvaC*, *etsC*, *fyuA*, *gad*-type, *hlyF*, *iroN*, *irp2*, *iss*, *iucC*, *iutA*-type, *lpfA*, *mchF*, *ompT*, *ompT*-type, *sitA*, *terC*, *terC*-type, *traT*	Col440I-type, Col(pHAD28), IncFIB (AP001918)-type, IncFII, IncQ1	IS*26*, IS*100*-type, IS*629*-type, IS*Ec31/38*-type, IS*Ec32*, MITEEc1, MITEEc1-type, Tn*3*, Tn*As1*, *intI1/tniA*	1	94.4
Hafnia paralvei (*n* = 6)										
INSAq191	Intestine	NA	AMC, CHL, FLO	bla_{ACC-1a} (C)	–	–	–	IS*Cfr26*-type, IS*Ecl1*-type MITEYpe1-type	3	64.0
INSAq204	Skin	NA	AMC, CAZ, FLO	bla_{ACC-1c} (C), *qnrD2* (P)	–	–	–	IS*Cfr26,* IS*Sen4*-type	1	58.4
INSAq217	Gills								–	57.6
INSAq225									–	55.9
INSAq215	Gills	NA	FLO	bla_{ACC-1d} (C)	–	–	Col3M-type, Col(Ye4449)	IS*26*-type, IS*5075*-type, IS*Ehe3*-type, IS*Kpn26*-type, MITEYpe1-type	–	71.7

(*continued on next page*)

Table 1 (*continued*)

Strain	Organ	MLST	NS profile	Antibiotic resistance genes	Disinfectant resistance genes	Virulence factors	Plasmids	Others mobile genetic elements	Nr. of phage regions	Human pathogen probability (%)
INSAq219	Gills	NA	AMC, CHL, FLO	*bla*$_{ACC-1d}$ (C)	–	–	Col3M-type, Col(Ye4449)-type, IncN-type, pKPC-CAV1321, RepA	IS*30*-type, IS*5075*-type, IS*Cfr26*-type, IS*Ehe3*-type, IS*Sen4*-type, MITEYpe1-type	–	69.5
Kluyvera intermedia ($n = 1$)										
INSAq229	Skin	NA	–	*bla*$_{CTX-M-246}$[b] (C)	–	–	IncFIB(K)-type, IncR	IS*5075*-type, IS*Eam1*-type, IS*Ec33*-type, IS*Ecl1*-type, IS*Sen4*-type, Tn*5403*-type	1	76.5
Lelliottia amnigena ($n = 1$)										
INSAq176	Muscle	NA	–	–	–	–	Col (pHAD28)-type	IS*26*-type, IS*5075*-type, IS*Kpn26*-type	1	75.0
Obesumbacterium proteus ($n = 4$)										
INSAq172	Muscle	NA	AMC, CHL, FLO, OTC	*bla*$_{ACC-3-type}$ (P)	–	–	–	–	1	55.0
INSAq192	Muscle	NA	AMC, CHL, FLO	*bla*$_{ACC-3-type}$ (C)	–	–	–	–	3	51.5
INSAq197	Muscle	NA	AMC, CAZ, CHL, FLO, FOX, OTC, PTZ		–	–	–	–	3	54.8
INSAq216	Gills	NA	AMC, CHL, FLO		–	–	–	–	3	54.8
Raoultella terrigena ($n = 1$)										
INSAq228	Skin	NA	–	*bla*$_{TER-3}$[b] (C), *fosA*-type (C)	–	–	IncFIB(K)-type	IS*30*-type, IS*102*-type, IS*5075*-type, IS*Ec52*-type, IS*Kpn2/26*-type	2	78.4
Serratia liquefaciens ($n = 1$)										
INSAq207	Skin	NA	FLO, OTC	–	–	–	Col (pHAD28)-type, Col (Ye4449)-type	IS*Ec31*-type, IS*Esa2*-type	5	73.0

NS profile: non-susceptibility profile. NA: Not applicable (databases for determination of MLST profiles for these organisms were not available in MLST 2.0 web tool). (C) Gene with predicted chromosomal location, determined by RFPlasmid tool. (P) Gene with predicted plasmid location, determined by RFPlasmid tool. -type: genetic element with <100 % coverage and/or identity.

[a] New MLST profiles found in this study.

[b] New alleles found in this study.

(Reid et al., 2022; Vignaroli et al., 2016). No literature was found regarding ST2607.

E. coli is a commensal of the intestines of worm-blooded animals; thus, its presence is commonly associated with faecal contamination. These bacteria can enter aquaculture farms through runoff from land (especially during periods of high precipitation), sewage, maritime traffic and birds or marine mammals (Grevskott et al., 2017). This situation may have occurred in the aquaculture farms studied, since some were located nearby agricultural farms, wastewater treatment plants, sewage discharges, bathing areas and/or ports/marinas/quays.

3.2.5. *Klebsiella pneumoniae*

The two strains of *K. pneumoniae* isolated from the muscle and intestine of fish from the same farm (7) belonged to ST134. This ST has previously been identified in *K. pneumoniae* strains recovered from patients in hospitals in China and Japan, and it has been associated with several resistance genes, including *bla*$_{KPC-2}$, *bla*$_{IMP-1}$, *qnr*A1, *qnrB6*, *sul1*, and *tet(B)* (Liu et al., 2015; Mori et al., 2020).

3.2.6. *Shewanella spp.*

Within the *Shewanella* genus, only two strains belonged to the same new ST (ST57; isolated in mussels from farm 2). In Fig. 1 C, it can be observed that ST57 differs in 6 loci from ST9, which corresponds to

Table 2
Summary of the results obtained by antibiotic susceptibility testing and the investigation of Multilocus sequence type (MLST), antibiotic/disinfectant resistance genes, virulence factors, mobile genetic elements (MGE), phage regions and pathogenicity towards human hosts for bivalve samples collected in farms 1/2/3/4/5 (A) and market (B).

Strain	Aquaculture species	MLST	NS profile	Antibiotic resistance genes	Disinfectant resistance genes	Virulence factors	Plasmids	Others MGE	Nr. of phage regions	Human pathogen probability (%)
A) Aquaculture farms										
Citrobacter portucalensis (*n* = 2)										
INSAq424 (Farm 1)	*Crassostrea gigas*	ST416	AMC, FLO, FOX, OTC	$bla_{CMY-175}$[b] (C), *qnrB7* (C)	–	–	Col440I-type	IS*26*-type, IS*30*-type, IS*Ehe3*-type, IS*Kpn24*-type, IS*Sba14*-type, IS*Sen4*-type, IS*Yps3*	4	82.4
INSAq485 (Farm 1)	*Ruditapes decussatus*	ST971[a]	AMC, AMX, CHL, FLO, FOX, OTC	$bla_{CMY-119}$ (C), *qnrB*-type (C),	–	*traT*-type	IncFIB(K)-type	IS*30*-type, IS*Kox1*-type, IS*Kpn24*-type	5	86.2
Escherichia coli (*n* = 8)										
INSAq316 (Farm 4)	*Mytilus* sp.	ST10	AMC, AMX, CHL, CIP, FLO, FMQ, OTC, SXT	*aadA1* (P), *acrA/B/F/S* (C), *ampH* (C), *aph(3″)-Ib* (P), *aph(6)-Id* (P), *bacA* (C), *baeR/S* (C), bla_{AMPC} (C), bla_{TEM-1B} (P), *catA1*-type (P), *cpxA* (C), *dfrA1*-type (P), *emrA/R* (C), *eptA* (C), *evgA* (C), *gadX* (C), *hns* (C), *kdpE* (C), *kpnH* (C), *marA* (C), *mdf(A)*-type (C), *mdtE/F/G/N/O/P* (C), *mph(B)* (P), *pmrF* (C), *qnrB19* (P), *sul1/2* (P), *tet(A)* (P), *yojI* (C)	*qacE*-type (P), *sitABCD*-type (P)	*gad*, *iha*-type, *iucC*, *iutA*, *mchB/F*-type, *mchC*, *sigA*-type, *sitA*, *terC*, *terC*-type, *traT*	Col156-type, Col (BS512), Col(MG828)-type, Col(pHAD28), IncFIB(AP001918)-type, IncFII-type, IncQ1-type	*intI1/tniA/tniB/qacdelta1/qacE1/sul1*, IS*3*-type, IS*4*-type, IS*26*, IS*30*-type, IS*421*-type, IS*682*-type, IS*1326*, IS*Ec1/31/37/38*-type, IS*Ec32*, IS*Kpn26*-type, IS*Sso4*-type, MITEEc1-type, MITEEc1, Tn*3*	1	93.1
INSAq317 (Farm 4)	*Mytilus* sp.	ST10		*aadA1* (P), *acrA/B/F/S* (C), *ampH* (C), *aph(3″)-Ib* (P), *aph(6)-Id* (P), *bacA* (C), *baeR/S* (C), bla_{AMPC1} (C), bla_{TEM-1B} (P), *catA1*-type (P), *cpxA* (C), *dfrA1*-type (P), *emrA/R* (C), *eptA* (C), *evgA* (C), *gadX* (C), *hns* (C), *kdpE* (C), *kpnH* (C), *marA* (C), *mdf(A)*-type (C), *mdtE/F/G/N/O/P* (C), *mph(B)* (P), *msbA* (C), *pmrF* (C), *qnrB19* (P), *sul1/2* (P), *tet(A)* (P), *yojI* (C)		*iucC*, *iutA*, *mchB/F*-type, *mchC*, *sigA*-type, *sitA*, *terC*, *terC*-type, *traT*	Col156-type, Col440I-type, Col(BS512), Col (MG828)-type, Col (pHAD28), IncFIB (AP001918)-type, IncFII-type, IncQ1-type		1	93.1
INSAq319 (Farm 4)	*Mytilus* sp.	ST10	AMC, CHL, CIP, FLO, FMQ, OTC, SXT	*aadA1* (P), *acrA/B/F/S* (C), *ampH* (C), *aph(3″)-Ib* (P), *aph(6)-Id* (P), *bacA* (C), *baeR/S* (C), bla_{AMPC1} (C), bla_{TEM-1B} (P), *catA1*-type (P), *cpxA* (C), *dfrA1*-type (P), *emrA/K/R* (C), *eptA* (C), *evgA* (C), *gadX* (C), *hns* (C), *kpnH* (C), *marA* (C), *mdf(A)*-type (C), *mdtE/F/G/N/O/P* (C), *mph(B)* (P), *msbA* (C), *pmrF* (C), *qnrB19* (P), *sul1/2* (P), *tet(A)* (P), *tolC* (C), *yojI* (C)		*gad*-type, *iucC*, *iutA*, *mchB/F*-type, *mchC*, *sigA*-type, *sitA*, *terC*, *terC*-type, *traT*			1	93.2
INSAq320 (Farm 4)	*Mytilus* sp.	ST10		*aadA1* (P), *acrA/B/F/S* (C), *ampH* (C), *aph(3″)-Ib* (P), *aph(6)-Id* (P),		*gad*-type, *iha*, *iucC*, *iutA*, *mchB/F*-type, *mchC*, *sigA*-type, *sitA*, *terC*, *terC*-type, *traT*			1	93.1
INSAq321 (Farm 4)	*Mytilus* sp.	ST10		*bacA* (C), *baeR/S* (C), bla_{AMPC1} (C), bla_{TEM-1B} (P), *catA1*-type (P), *cpxA* (C), *dfrA1*-type (P), *emrA/R* (C), *eptA* (C), *evgA* (C), *gadX* (C), *hns* (C), *kdpE* (C), *kpnH* (C), *marA* (C), *mdf(A)*-type (C), *mdtE/F/G/N/O/P* (C), *mph(B)*			Col156-type, Col (BS512), IncFIB (AP001918)-type, IncFII-type, IncQ1-type		1	93.1

(*continued on next page*)

Table 2 (*continued*)

Strain	Aquaculture species	MLST	NS profile	Antibiotic resistance genes	Disinfectant resistance genes	Virulence factors	Plasmids	Others MGE	Nr. of phage regions	Human pathogen probability (%)
INSAq322 (Farm 4)	*Mytilus* sp.	ST10	AMC, CAZ, CHL, CIP, FLO, FMQ, OTC, SXT	(P), *msbA* (C), *pmrF* (C), *qnrB19* (P), *sul1/2* (P), *tet(A)* (P), *tolC* (C), *yojI* (C)			Col156-type, IncFIB (AP001918)-type, IncFII-type, IncQ1-type		1	93.1
INSAq324 (Farm 4)	*Mytilus* sp.	ST10	AMC, CHL, CIP, FLO, FMQ, OTC, SXT				Col156-type, Col440I-type, Col(BS512), Col (MG828)-type, Col (pHAD28), IncFIB		1	93.1
INSAq354 (Farm 4)	*Mytilus* sp.	ST10	AMC, CHL, FLO, FMQ, OTC, SXT				(AP001918)-type, IncFII-type, IncQ1-type		1	93.2
Raoultella ornithinolytica (*n* = 3)										
INSAq311 (Farm 4)	*Mytilus* sp.	NA	FMQ, NAL, OTC	bla_{ORN-1} (C)	–	*fyuA*-type	Col440I-type, IncFIB(K)-type, IncFII(K)-type, IncR-type	IS*Ehe3*-type, IS*Kpn21/26/38/42/43*-type, IS*Ppu12*-type, IS*Sm3*-type, Tn*5403*-type	2	77.1
INSAq315 (Farm 4)	*Mytilus* sp.	NA	CIP, FMQ, NAL, OTC						2	77.3
INSAq326 (Farm 4)	*Mytilus* sp.	NA	FMQ, NAL, OTC						2	77.4
Shewanella algae (*n* = 4)										
INSAq258 (Farm 2)	*Mytilus* sp.	ST57[a]	AMX, FMQ, OTC	$bla_{OXA-SHE-type}$ (C), *qnrA3* (C)	–	–	–	IS*Kpn2*-type, IS*Slo2*-type	–	30.7
INSAq494 (Farm 2)	*Mytilus* sp.	ST61[a]	FMQ, OTC	$bla_{OXA-963}$[b] (C), *qnrA3* (P),	–	–	–	–	–	28.5
INSAq495 (Farm 2)	*Mytilus* sp.	ST57[a]	OTC	$bla_{OXA-964}$ (C), *qnrA3* (C)	–	–	–	IS*Kpn2*-type, IS*Slo2*-type	–	29.1
INSAq497 (Farm 5)	*Crassostrea gigas*	ST62[a]	COL	$bla_{OXA-965}$[b] (C), *qnrA12* (P)	–	–	–	IS*Vpa2*-type	–	39.1
Shewanella chilikensis (*n* = 1)										
INSAq334 (Farm 2)	*Mytilus* sp.	ST58[a]	FMQ, OTC	$bla_{OXA-961}$[b] (C), *qnrA11* (P)	–	–	–	–	–	30.7
Shewanella indica (*n* = 1)										
INSAq347 (Farm 3)	*Mytilus* sp.	ST60[a]	FMQ, OTC	$bla_{OXA-962}$ (C), *qnrA2* (P)	–	–	–	IS*Shes3*-type	–	35.5
B) Market										
Aeromonas allosaccharophila (*n* = 1)										
INSAq241	*Mytilus* sp.	ST888[a]	ERT, IMP, MEM	bla_{FOX-19}[b] (C), $bla_{OXA-959}$ (C), *cphA7*-type (C)	–	–	–	IS*Ahy2*-type, IS*As7/34*-type	1	42
Aeromonas caviae (*n* = 1)										

(*continued on next page*)

Table 2 (*continued*)

Strain	Aquaculture species	MLST	NS profile	Antibiotic resistance genes	Disinfectant resistance genes	Virulence factors	Plasmids	Others MGE	Nr. of phage regions	Human pathogen probability (%)
INSAq239	*Mytilus* sp.	ST368	FOX	bla_{MOX-8} (C)	–	–	–	IS*Aeme19/21*-type, IS*As1/7/12/13/16/17/18/19/21/22/25/34*-type, IS*Ecl8*-type, IS*Kpn3/10*-type, MITEKpn1-type, *tniA/tniB/tniQ*	–	42.2
Citrobacter freundii ($n = 1$)										
INSAq237	*Mytilus* sp.	ST169	AMC, FLO, FOX	bla_{CMY-65} (C), *qnrB38* (C)	–	–	Col3M-type, Col440I-type, IncFIA(HI1)-type, IncY-type	IS*26*, IS*30*-type, IS*903*-type, IS*Ec30*-type, IS*Kpn43*-type, Tn*5403*-type	–	88.2
Raoultella planticola ($n = 1$)										
INSAq240	*Mytilus* sp.	NA	–	bla_{PLA-7}[b] (C), *fosA*-type (C)	–	–	IncFIB(K)-type, IncFII (Yp)-type	IS*102*-type, IS*903*-type, IS*5075*-type, IS*Ech12*-type, IS*Ec52*-type, IS*Kpn26*-type, IS*Sm3*-type, IS*Spu2*	4	84.6
Hafnia alvei ($n = 1$)										
INSAq249	*Mytilus* sp.	NA	AMC, CHL, FLO, OTC	$bla_{ACC-3-type}$ (C)	–	–	–	IS*26*-type, IS*5075*-type, IS*Ecl1*-type, IS*Sm1*-type	3	60.6
Proteus terrae ($n = 1$)										
INSAq246	*Mytilus* sp.	NA	AMC, CHL, FLO, OTC	*hugA*-type (C), *tet(H)*-type (C)	–	–	–	IS*Shes4*-type	2	68.4
Shewanella chilikensis ($n = 1$)										
INSAq243	*Mytilus* sp.	ST40[a]	–	$bla_{OXA-960}$[b] (C), *qnrA1* (P)	–	–	–	–	–	55.9
Vibrio alginolyticus ($n = 1$)										
INSAq252	*Mytilus* sp.	ST183	AMP	$bla_{CARB-56}$ (C)	–	–	–	–	4	65.1
Vibrio antiquarius ($n = 1$)										
INSAq251	*Mytilus* sp.	ST206[a]	AMP	$bla_{CARB-57}$ (C), *fos*-type (C)	–	–	–	IS*Va1/2*-type, IS*Vch1*-type, IS*VpaI*	2	65.2
Vibrio diabolicus ($n = 1$)										
INSAq250	*Mytilus* sp.	ST205[a]	AMP	$bla_{CARB-57}$ (C)	–	–	–	–	–	66.6

NS profile: Non-susceptibility profile; NA: Not applicable (databases for determination of MLST profiles for these organisms were not available in MLST 2.0 web tool). (C) Gene with predicted chromosomal location, determined by RFPlasmid tool. (P) Gene with predicted plasmid location, determined by RFPlasmid tool. -type: genetic element with <100 % coverage and/or identity.

[a] New MLST profiles, found in this study.

[b] New alleles found in this study.

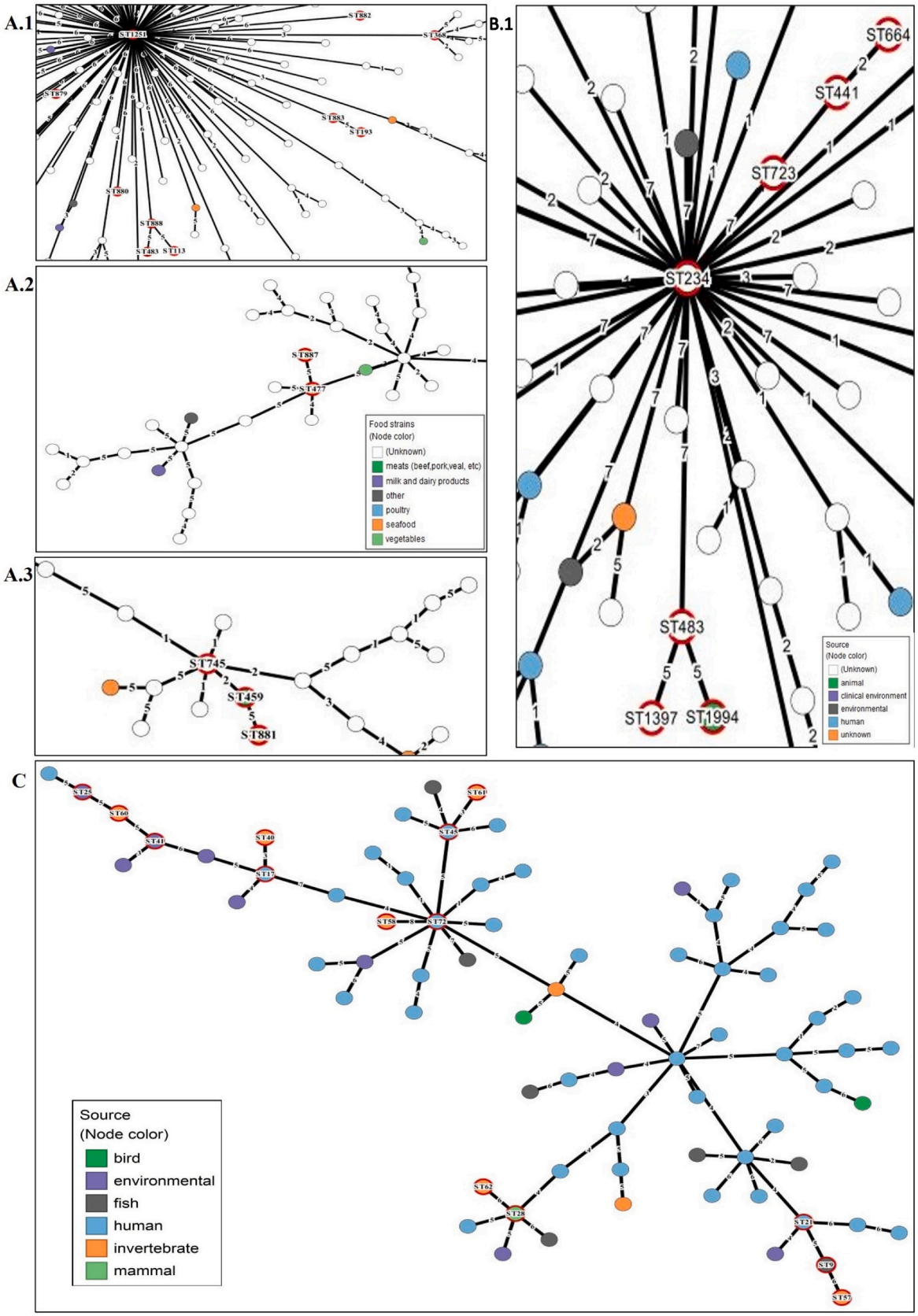


Fig. 1. Minimum spanning trees (MST) constructed with CLC Genomics Workbench version 21.0.3, using MLST schemes to understand possible evolutionary relationships among the new ST detected in: **A.** Aeromonas spp.; **A.1.** Zoom of the MST, highlighting the new ST879, ST880, ST882, ST883 and ST888; **A.2.** Zoom of the MST, highlighting the new ST887. **A.3.** Zoom of the MST highlighting the new ST881; **B.1.** *Enterobacter cloacae* complex, zoom of the MST highlighting the new ST1994; **C.** *Shewanella* spp., with the new ST found in this study (ST40, ST57, ST58, ST60, ST61 and ST62) and their closest neighbours highlighted. ST with a red circle are ST relevant to the analysis.

S. algae isolated from fish in France in 1980 (PubMLST database id 9). The other five strains, isolated from mussels in market and farms 2 and 3 (southern region of Portugal) and Japanese oysters from farm 5 (central region of Portugal), revealed ST described here for the first time (ST40, ST58, ST60, ST61, and ST62). According to MST in Fig. 1 C, ST40 differs from ST17 by only 3 loci. Interestingly, ST17 was detected in *S. chilikensis* isolated from human samples in Spain (in 2012; PubMLST database id 17). This finding is consistent with the *Shewanella* species that harbour ST40 in our study and the country of origin of the mussels acquired in the market, where *S. chilikensis* ST40 was found. ST58 is clustered with ST72 (which belongs to *S. algae* isolated from human in Taiwan, in 2016; PubMLST database id 104), although it differs from ST72 in 8 loci. In the same Fig. 1 C, it can be observed that ST60 differs from ST25 (*S. indica* isolated from sea sediments in the USA, in 1992; PubMLST database id 25) and ST41 (*S. indica* isolated also from sediments in India, in 2006; PubMLST database id 63) by 5 loci. ST61 differs from ST45 (*S. algae* isolated from human in Norway, in 1997; PubMLST database id 70) by 3 loci, and ST62 differs from ST28 (*S. algae* isolated from *Neophocaena phocaenoides* in South Korea, in 2017; PubMLST database id 28) by 6 loci.

3.2.7. *Vibrio spp.*

All *Vibrio* spp. were isolated from mussels acquired in the market. One strain of *V. alginolyticus* belonged to ST183, which has been previously identified in human clinical samples in Norway, as well as in marine animals and water in China (PubMLST database). Additionally, one strain of *V. diabolicus* and one strain of *V. antiquarius* presented new STs (ST205 and ST206, respectively). The MST in Fig. S4 A demonstrates that ST205 differs from ST63 (*V. alginolyticus/diabolicus* isolated from live marine animals in Italy, in 2007; PubMLST database id 70) by only 1 locus. ST63 and ST205 clustered together with ST32 (PubMLST database id 33) and ST57 (PubMLST database id 64), both isolated from live marine animals of the same species, country, and year as ST63. The other new ST, ST206, differs from ST574 (identified in *V. alginolyticus/diabolicus* isolated from *Penaeus vannamei* in Italy, in 2011; PubMLST database id 1188) by 2 loci, as shown in Fig. S4 B. Together with ST143 (identified in *Vibrio chagasii* from unknown origin; PubMLST database id 175) and ST567 (identified in *V. alginolyticus/diabolicus* isolated from *Hymenopenaeus muelleri* in Italy, in 2011; PubMLST database id 1181), ST206 and ST574 form a cluster, with ST574 being the putative founder.

More studies are needed to understand the specific characteristics of each ST in terms of their host range and environmental preferences. However, it is evident that these STs have the capability to thrive in diverse environments and infect various hosts, including fish and bivalve molluscs and humans.

3.3. *Resistome*

The investigation of genes associated with antibiotic, disinfectant and heavy metals resistance has identified over 140 different genes (Tables 1 and 2; Figs. 2 and S5). This comprehensive analysis provides a valuable snapshot of the resistome present in seabream and bivalve molluscs cultivated in aquaculture farms.

3.3.1. *Antibiotic resistance genes*

In the *Aeromonadaceae* family, genes usually associated with β-lactams resistance (e.g. $bla_{\text{TEM-1B}}$, $bla_{\text{FOX-18/19}}$) were found to be predominant. This was followed by genes associated with tetracyclines resistance (e.g. *tet(A)*, *tet(E)*-type), as well as genes linked to aminoglycosides resistance (e.g. *aph(3″)-Ib*, *aph(6)-Id*). Notably, we highlight $bla_{\text{FOX-18}}$ and $bla_{\text{FOX-19}}$ genes described here for the first time. The $bla_{\text{FOX-18}}$ gene displayed 23 missense mutations (G11T, C13T, G74A, A148G, G213T, G227A, C228T, T310G, T314A, G361A, G366T, C389A, A433G, A458G, A459C, C653T, A693G, C767T, C799G, T800C, A884C, A902C, and C956A) compared to $bla_{\text{FOX-3,}}$ with 95.5 % identity shared between the two genes. Similarly, $bla_{\text{FOX-19}}$ exhibit 22 missense mutations (T19C, G52A, G94A, A106G, T107C, T108C, C443T, A509T, A586G, A651G, C653T, C698T, A766G, C802G, A902T, C914T, G915A, T937C, C940A, G952A, T954C, and T958C) that differentiate it from $bla_{\text{FOX-7}}$, sharing 95.8 % identity. These genes were detected in 2 strains of *A. allosaccharophila* isolated from gilthead seabream ($bla_{\text{FOX-18}}$; INSAq178) and mussels ($bla_{\text{FOX-19}}$; INSAq241) obtained from different markets. These strains also tested positive for $bla_{\text{OXA-958/959}}$ and *cphA*-type genes and were categorized as "Susceptible, increased exposure" according to EUCAST guidelines for carbapenems (ertapenem for INSAq178; ertapenem, imipenem and meropenem for INSAq241) (Tables 1 and 2). The bla_{FOX} genes were previously described by Maravić et al. (2013) in *A. caviae* isolated from wild-growing *Mytilus galloprovincialis* in Croatia and in water and sediments collected from aquaculture ponds in China (Huang et al., 2017). To our knowledge, this is the first report of $bla_{\text{OXA-958}}$ in gilthead seabream and $bla_{\text{OXA-959}}$ in aquaculture mussels. These bla_{OXA} genes were already described in *Aeromonas* spp. isolated from faeces of dairy cattle and wastewater treatment plant effluent (accession numbers: LKKI01000011 and AP022264, respectively). Additionally, two susceptible strains harboured a *cphA5*-type gene (intrinsic in some species of *Aeromonas*) yet they remained susceptible to carbapenems. This was already described and be attributed to the low enzymatic activity of this metallo-β-lactamase (Juan et al., 2017). Most of the ARG detected in this family have been previously associated with aquaculture activities (Pham et al., 2018; Salgueiro et al., 2021).

Enterobacteriaceae represented the family with the highest diversity of ARG (Tables 1 and 2). These genes were predominantly associated with efflux systems, which are commonly found in aquatic environments, possibly due to their ability to transport a wide variety of substrates, including essential cellular components (Muziasari et al., 2016). The efflux pumps identified belonged to four different families: the small multidrug resistance (SMR) family, the ATP-binding cassette (ABC) family, the resistance-nodulation-cell division (RND) family, and the major facilitator superfamily (MFS). These pumps from our study are usually associated with the capacity of extruding several antibiotics from different classes such as quinolones (e.g. OqxAB), tetracyclines [e.g. Tet(A)] and nitroimidazole (e.g. MsbA). Further identified ARG are commonly associated with various mechanisms, including decreasing the uptake of antibiotics (e.g. *marA* for several antibiotics), modification of the antibiotic target (e.g. *dfrA1*-type for trimethoprim; *sul1/2* for sulfonamides) and inactivation of antibiotics (e.g. $bla_{\text{SHV-60}}$ and $bla_{\text{TEM-1B}}$ for β-lactams; *aadA1* and *aph(3″)-Ib* for aminoglycosides; *fosA*-type for fosfomycin; *catA1*-type for phenicols; and *mphB* for macrolides). Noteworthy, $bla_{\text{TER-3}}$, $bla_{\text{CTX-M-246}}$, $bla_{\text{PLA-7}}$ (which encode class A β-lactamases) and $bla_{\text{CMY-175}}$ (which encodes a class C β-lactamase) were found in this study for the first time. The gene $bla_{\text{TER-3}}$, found in one *R. terrigena* strain isolated from gilthead seabream's skin in the market, displayed 1 nonsense mutation (G160A) and 2 missense mutations (A350G and G351A) compared to $bla_{\text{TER-2}}$, sharing a homology of 99.3 %. This class A β-lactamase is characteristic of the chromosome of *R. terrigena* and is mainly found in water environments (Walckenaer et al., 2015). Also identified in gilthead seabream's skin from market, $bla_{\text{CTX-M-246}}$ from *K. intermedia* shows 99.1 % homology with $bla_{\text{CTX-M-10}}$. It differs from $bla_{\text{CTX-M-10}}$ due to 3 missense mutations (G367A, A483C and A636G) and belongs to the CTX-M-1 group. It is widely accepted that $bla_{\text{CTX-M}}$ genes originated from chromosomal *bla* genes found in *Kluyvera* spp. and were subsequently mobilized to other species, leading to their worldwide dissemination (Sivaraman et al., 2021). Other studies have also detected $bla_{\text{CTX-M}}$ genes in *E. coli* and *K. pneumoniae* strains isolated from aquaculture environments. For instance, Said et al. (2017) identified these genes in gilthead seabream, similar to our findings, and Sivaraman et al. (2021) reported their presence in samples of shrimp, water, and sediment, where the CTX-M-1 group predominated. In our study, chromosomal $bla_{\text{PLA-7}}$ was isolated from *R. planticola* obtained from mussels in the market, demonstrated 4 missense mutations compared with $bla_{\text{PLA-1a}}$ (G578A, A644C, A793G and G845C) and $bla_{\text{PLA-2a}}$ (G578A, A644C,

A793G and G841A) genes, sharing 99.0 % homology with them. No literature was found regarding the prevalence of bla_{PLA} genes in aquaculture. The lately newly identified variant in *Enterobacteriaceae*, $bla_{CMY-175}$ (from *C. portucalensis* isolated in Japanese oysters from farm 1), shares 99.7 % homology with bla_{CMY-34}, differing by only 1 missense mutation (C602A). Previous studies have reported the presence of bla_{CMY} genes in several fish species, including *C. freundii* from *Pagrus major* in Japan and salmon in Chile, *A. salmonicida* from salmon aquaculture facilities in Canada, *Morganella* sp. and *Klebsiella oxytoca* from retail aquaculture products and *E. coli* isolated from freshwater *Perca fluviatilis* in Switzerland (Ahmed et al., 2015; Ye et al., 2013). Among the 31 ARG-positive strains, 25 had a resistance phenotype to at least one antibiotic (amoxicillin, amoxicillin/clavulanic acid, aztreonam, cefepime, cefotaxime, cefoxitin, ceftazidime, piperacillin/tazobactam, chloramphenicol, florfenicol, ciprofloxacin, flumequine, nalidixic acid, oxytetracycline and/or trimethoprim/sulfamethoxazole) and three showed susceptibility with increased exposure to ertapenem. These last 3 strains of *E. hormaechei* (INSAq99, INSAq107 and INSAq140; Table 2) were positive for the presence of bla_{ACT-17} and bla_{ACT-89} genes, which can confer resistance to carbapenems only when associated with other mechanisms, such as the loss of outer membrane porins (Jacoby, 2009). Genes that are weakly expressed, namely $bla_{OXY-1-3}$ in *K. michiganensis,* bla_{TER-3} in *R. terrigena*, $bla_{CTX-M-246}$ in *K. intermedia*, and bla_{PLA-7} in *R. planticola*, can explain the susceptible phenotypes, similar to the findings in the studies developed by Cantón et al. (2012) for bla_{CTX-M}, González-López et al. (2009) for bla_{OXY} and Walckenaer et al. (2015, 2004) for bla_{TER} and bla_{PLA}. Nine *E. coli* were classified as multidrug resistant: one was recovered from gilthead seabream in the market and eight were isolated from mussels in farm 4. Most of the ARG found in this bacterial family were punctually described in a few previous studies related with different aquaculture reservoirs (Libisch et al., 2022; Sivaraman et al., 2021; Shah et al., 2014). Nonetheless, our study allowed a more complete approach, as it not only covers acquired genes but also investigates chromosomal genes, which are less studied, leading to the identification of several new alleles and genes that are reported for the first time in aquaculture reservoirs (e.g. bla_{ORN-1}, $bla_{OXY-1-3}$, *qnrB38*, *mdtE*, *mdtN*, *mdtO*, *mdtP*, *baeS*, *eptA*, *emrA*, *emrK*, *yojI*, and *kdpE*).

All strains from the *Hafniaceae* family analysed in this study revealed the presence of at least one ARG, specifically $bla_{ACC-1a/1c/1d/3-type}$, which is associated with a non-susceptibility phenotype to β-lactam antibiotics, such as cefoxitin and ceftazidime, and to the amoxicillin/clavulanic acid and piperacillin/tazobactam associations (with the exception of INSAq215, which showed increased susceptibility to ceftazidime). The bla_{ACC} genes are ubiquitous in the chromosome of *Hafnia* spp. and encode a class C β-lactamase that can be categorized into two groups: low-level inducible cephalosporinases (susceptible to ceftazidime) and high-level constitutive cephalosporinases (resistant to ceftazidime, as observed in seven strains in our study; Table 2) (Janda and Abbott, 2006; Jayol et al., 2017). To the best of our knowledge, there are no studies identifying a chromosomal class C β-lactamase in *O. proteus*. However considering its high homology with *Hafnia* genus (Koivula et al., 2006), it is plausible to assume that *O. proteus* also harbours a similar β-lactamase, encoded by $bla_{ACC-like}$ genes, as observed in the four strains of *O. proteus* identified in our study (Table 2). Previous studies have detected the presence of bla_{ACC} genes in various samples, namely blue mussels and *Venus* clams, acquired from a market in *H. alvei* and *E. coli*, respectively (Vu et al., 2018), although the authors did not specify whether the samples originated from aquaculture farms. In our study, three strains of *H. paralvei*, from gills and muscle samples of gilthead seabream purchased in a market, were found to harbour the plasmid-mediated quinolone resistance (PMQR) determinant *qnrD2* gene. However, these strains exhibited a susceptible phenotype to all tested quinolones, as PMQRs are associated with low-level resistance to quinolones, which may not be detectable using conventional phenotypic methods (Briales et al., 2012). The *qnrD* genes have been relatively rare, but have been found in food sources, such as lettuce and broiler samples (Jones-Dias et al., 2016a; Kraychete et al., 2019). They have also been detected in aquaculture reservoirs, such as the shrimp intestinal tract, shrimp pond waters and sediments samples (Su et al., 2017), as well as in the effluents of coastal aquaculture of *Paralichthys olivaceus* (Jang et al., 2018). However, some studies do not specify the *qnrD* variant, so it is unclear if our study represents the first description of *qnrD2* in aquaculture reservoirs. Nevertheless, to our knowledge, this is the first description of *qnrD2* in *H. paralvei*. The *qnrD2* gene was firstly described in a human isolate of *Salmonella enterica* serovar Hadar (Abgottspon et al., 2014) and, according to NCBI database, it has also been detected in *Providencia rettgeri* (NZ_ABFNOU020000056), *Proteus mirabilis* (NZ_JAAATT010000048) and *Morganella morganii* (NZ_JADNHI010000046) isolated from human samples.

The only strain of *Morganellaceae* family analysed in this study was *P. terrae* isolated from mussels obtained from the market. This strain tested positive for the presence of *tetH*-type and *hugA*-type ARG, which are associated with resistance to tetracyclines and β-lactams, respectively. Additionally, this strain was also resistant to chloramphenicol (no corresponding ARG detected), indicating multidrug resistance. The *tetH* gene has previously been identified in effluents from coastal aquaculture of *P. olivaceus* in south Korea (Jang et al., 2018) and in sediments from aquaculture fish farms (rainbow trout and common white fish) in the northern Baltic Sea (Tamminen et al., 2011). On the other hand, to our knowledge, this is the first description of *hugA* gene in mussels from aquaculture origin. The chromosomal *hugA* gene has been identified in *Plesiomonas shigelloides* in wild-caught fish (Herrera et al., 2006) and in *Proteus* spp. in cecum samples from chicken slaughterhouses (Zheng et al., 2022).

In the *Shewanellaceae* family, all 7 strains analysed were found to harbour a gene encoding a class D β-lactamase ($bla_{OXA-960/961/962/963/964/965,}$ or $bla_{OXA-SHE-type}$) and one variant of the PMQR gene *qnrA* (*qnrA1*, *qnrA2*, *qnrA3*, *qnrA11* or *qnrA12*). This study identified new genes, $bla_{OXA-960/961/963/965}$, which encode class D β-lactamases belonging to the OXA-55 family. The gene $bla_{OXA-960}$ was found in *S. chilikensis* from mussels obtained from the market and shares 93.1 % of identity with bla_{OXA-55}, with 20 missense mutations (A98C, C117A, T121G, C137T, T138C, A199T, G212T, C292A, G316C, T383C, G514A, A592C, G593A, G713A, G716A, T717A, G781A, T792G, G796A, and A857T) distinguishing the two. The gene $bla_{OXA-961}$, also identified in *S. chilikensis* but from mussels originated in farm 2, exhibits 94.9 % of identity with bla_{OXA-55} and possesses 17 missense mutations (A98C, T121G, C134T, C292A, G316C, T383C, A470C, A471G, A517G, A592C, G593A, G713A, G716A, T717A, C764T, G781A, and G796A) compared bla_{OXA-55}. The gene $bla_{OXA-963}$ was found in a *S. algae* strain isolated from the same sample as $bla_{OXA-961}$. It shares 98.7 % identity with bla_{OXA-55}, differing by only 5 missense mutations (T121A, C292A, T383C, G499C and A584T). The last new bla_{OXA} found in this family, $bla_{OXA-965}$, was identified in *S. algae* isolated from Japanese oysters originating from farm 5 and shares 97.7 % homology with bla_{OXA-55}, with 9 missense mutations (T121A, A128G, C292A, T383C, G520A, G560A, A561G, G629T and G781A) distinguishing these two. The genes $bla_{OXA-962}$ and $bla_{OXA-964}$ are described here for the first time in mussels sourced from aquaculture, *S. indica* from farm 3 and *S. algae* from farm 2, respectively. The only descriptions regarding these two genes were found in NCBI database, the gene $bla_{OXA-962}$ was previously associated with *S. chilikensis* from an unspecified origin in Japan (AP024611) while $bla_{OXA-964}$ was linked to *S. algae* isolated from *Neophocaena phocaenoides* in South Sea of South Korea (CP033575). All OXA genes detected in this family were predicted to be located on the chromosome and did not demonstrate hydrolysis activity against the tested carbapenems. Regarding the *qnrA* variants found in this family, they have been previously described in aquaculture reservoirs, such as water columns, sediments, mussels and Japanese oysters (Salgueiro et al., 2021; Tomova et al., 2015; Zago et al., 2020). Tomova et al. (2015) conducted a study in water and sediment samples from aquaculture origin, as well as urinary tract isolates from individuals residing in the aquaculture area.

They identified the presence of *qnrA1* in putative uncultured marine bacteria and in urinary tract isolate *E. coli*. These findings suggested the possibility of HGT occurring between marine bacteria and human pathogens (Tomova et al., 2015). In our study, one strain of *S. algae*, isolated from mussels collected in farm 2, was considered multidrug resistant, although only *qnrA3* and $bla_{\text{OXA-SHE-type}}$ genes were found.

The three strains belonging to the *Vibrionaceae* family analysed showed resistance to ampicillin and carried two variants of the bla_{CARB} gene, $bla_{\text{CARB-56}}$ and $bla_{\text{CARB-57}}$. Previous studies developed by Zhang et al. (2013) and Hossain et al. (2020) detected bla_{CARB} genes in *E. coli* isolated from fishponds waters and in *Vibrio parahaemolyticus* isolated from aquaculture shrimp, respectively (Hossain et al., 2020; Zhang et al., 2013). However, some studies, such as the one developed by Zhang et al. (2013), did not specify the variant of the bla_{CARB} genes, making direct comparisons with the variants found in our study difficult. Nonetheless, according to the NCBI database, $bla_{\text{CARB-57}}$ has been detected in *V. diabolicus*, which is consistent with our findings, and was isolated from *Epinephelus coioides* (AMPD01000002). One strain of *V. antiquarius* was found to carry a *fos*-type gene, which is typically associated with fosfomycin resistance.

No ARG were detected in the only strain from the *Yersiniaceae* family (*S. liquefaciens*) and in the two strains from the *Pseudomonadaceae* family (*P. stutzeri*), which were isolated from gilthead seabream obtained from market and farm 7, respectively. The *P. stutzeri* strains showed a resistance phenotype to ertapenem, chloramphenicol, florfenicol (intrinsic resistances) and flumequine. This suggests that the methods used in this study may not have detected all the resistance genes present in these strains. Both *P. stutzeri* and *S. liquefaciens* species have previously been detected in aquaculture environments, such as biofilters in a recirculating aquaculture system for seabass in France and in salmon farms in Chile (Michaud et al., 2009; Miranda et al., 2003).

Overall, the study of resistome highlights several new ARGs. Indeed, water is a rich environment that can be contaminated by different sources (e.g. maritime traffic, land run-off, marine birds or mammals), promoting selective pressure on the bacteria normally present in the aquatic environment, which possibly explains the identification of new ARGs and high diversity (Done et al., 2015; Gastalho et al., 2014).

3.3.2. Disinfectant resistance genes

In the present study, disinfectant resistance genes were detected only in the *Aeromonadaceae* and *Enterobacteriaceae* families (Table 2). Two strains of the *Aeromonas* genus (*A. media* and *A. salmonicida*) from *S. aurata* acquired in the market, tested positive for the *qacE*-type or *sitABCD*-type genes. These genes encode resistance to quaternary ammonium compounds and peroxides, respectively. This seems to be the first description of *sitABCD* operon in *Aeromonas* sp. and aquaculture environments, unlike *qacE* gene that has been previously described in bacteria isolated from a tilapia aquaculture system (Chenia and Jacobs, 2017). In addition to the *Aeromonas* strains, the study also detected *sitABCD*-type and *qacE*-type genes in nine multidrug resistant *E. coli* and *formA*-type gene (aldehydes resistance) in a *C. freundii* with resistance to β-lactams antibiotics from gilthead seabream's muscle collected in farm 7. The *formA*-type gene had already been identified in another study carried out by this team, where it was found in an *Enterobacter ludwigii* strain isolated from *S. aurata* in an aquaculture setting (Manageiro et al., 2022). These three genes are associated with resistance to disinfectants frequently used in aquaculture settings. Quaternary ammonium compounds, such as benzalkonium chloride, disrupt the cell membranes, destroy enzymes involved in energy production, and denature vital proteins (Mon-On et al., 2018). Indeed, aldehydes disinfectants, such as formaldehyde, are commonly used in aquaculture to control ectoparasites in fish (Hu et al., 2019). This may justify the detection of these genes in the present study. Peroxides, namely hydrogen peroxide, are used in aquaculture and in the food industry in general to cope with bacterial and parasitic infections. This disinfectant is known for generating free hydroxyl radicals, which cause damage to DNA, membrane lipids and other cell components crucial for bacterial survival (Jia et al., 2021; Výrostková et al., 2020). The *sitABCD* operon, which was detected in *Aeromonas* strains and multidrug-resistant *E. coli* strains in this study, are not exclusively related to disinfectants resistance. It encodes an ABC cassette-ATPase type transporter that is involved in the transport of iron and manganese, as well as to virulence in *E. coli* and *S. enterica* serovar Typhimurium (Boyer et al., 2002; Galardini et al., 2020; Sabri et al., 2006). In fact, the detoxification of free radicals and protection against oxidative damage caused by hydrogen peroxide are associated with the acquisition of iron and manganese by the bacteria (Sabri et al., 2006). This system can be particularly important in water environments where the concentrations of manganese can be low (Green et al., 2013).

3.3.3. Heavy metals resistance/tolerance genes

Heavy metals found in aquaculture settings, as well as in the farmed animals, can have different origins, since natural processes (e.g. coastal erosion) to human activities (e.g. industrial wastewater, mining, pesticide and fertilize applications in agriculture) (Lv et al., 2021; Razak et al., 2021). Moreover, heavy metals, such as copper and zinc, can be incorporated into fish feed as preservatives and/or additives or used as anti-fouling agents in fish cages (Farmaki et al., 2014). In our study, we observed a wide variety of heavy metals resistance/tolerance genes, with the *mdt* gene group being the most prevalent (16.6 %), followed by *acr* gene group (11.7 %) and the *sil* genes (11.0 %) (Fig. S5). These genes are involved in resistance to copper and zinc (*mdt* and *acr* genes), arsenic (*acr* genes) and silver (*sil* genes), mainly through efflux systems (Sinha et al., 2021; Wang and Fierke, 2013; Zagui et al., 2021). Other resistance/tolerance genes were identified, which were associated with mercury (e.g. *merA*), tungsten (e.g. *baeS*), nickel (e.g. *niKA*), iron (e.g. *fieF*), chromium (e.g. *ruvB*), manganese (e.g. *mntH*) and tellurium (e.g. *terC*) (Jie et al., 2016; Sherpa et al., 2020). Indeed, we observed a diverse range of heavy metal resistance/tolerance genes in bacteria isolated from both fish and bivalves, indicating the presence of these genes in multiple reservoirs (Fig. 2 A). The top five most frequently identified genes in both reservoirs were *sil* (15.5 %) > *mdt* (14.6 %) > *pco* (11.9 %) > *acr* (11.5 %) > *ars* (7.9 %) in fish and *mdt* (18.6 %) > *acr* (11.9 %) > *cus* (8.5 %) > *sil* (6.7 %) > *pco* (5.4 %) in bivalve molluscs. Furthermore, genes *merA*, *nirA*, *ruvB*, and *ncr* were exclusively present in fish samples (Fig. 2 A).

In the perspective of market vs. aquaculture farm, we observed a wide variety in both reservoirs (Fig. 2 B). The top five of the genes most frequently found in the market were *mdt* (15.8 %) > *ars* (14.0 %) > *sil* (11.7 %) > *acr* (11.1 %) > *pco* (8.8 %), while in aquaculture farms were *mdt* (16.9 %) > *acr* (11.8 %) > *sil* (10.8 %) > *pco* (8.5 %) > *cus* (6.5 %). Genes *ruvB* and *terC* were only present in aquaculture farm samples (Fig. 2 B). Several studies have been conducted to quantify heavy metals and investigate heavy metal resistance in aquaculture samples (Jiang et al., 2020; Ju et al., 2017). However, only a few studies have focused on the investigation of the specific genes responsible for conferring resistance, as in our study (Zago et al., 2020; Zhou et al., 2019). Zago et al. (2020) investigated the resistome, mobilome and virulome of 12 multidrug-resistant strains from the *Vibrionaceae* and *Shewanellaceae* families, isolated from aquaculture fish farms in Italy. These authors identified 26 different genes associated with the resistance to various metals, including arsenic (e.g. *arsA* and *acr3*), copper (e.g. *cusA* and *copA*), cobalt-zinc-cadmium (*czcA*, *zur* and *znuC*), chromium (e.g. *chrA* and *srpC*), molybdenum (e.g. *moeA* and *moeB*), magnesium (e.g. *corA* and *mgtE*) and nickel (e.g. *nikR*) (Zago et al., 2020). Similarly, Zhou and colleagues collected soil, water, and sediment samples from three duckfish farms in China and concluded that metal resistance genes were more abundant in sediment samples compared to soil samples. These authors found genes related to copper resistance, *pcoA* and *pcoD*, in all samples analysed. The gene *zntA*, which is associated with zinc resistance, was also detected and was the most abundant metal resistance gene in the majority of the samples analysed (Zhou et al., 2019). However, Zhou et al. (2019) focused specifically on zinc and copper resistance genes and

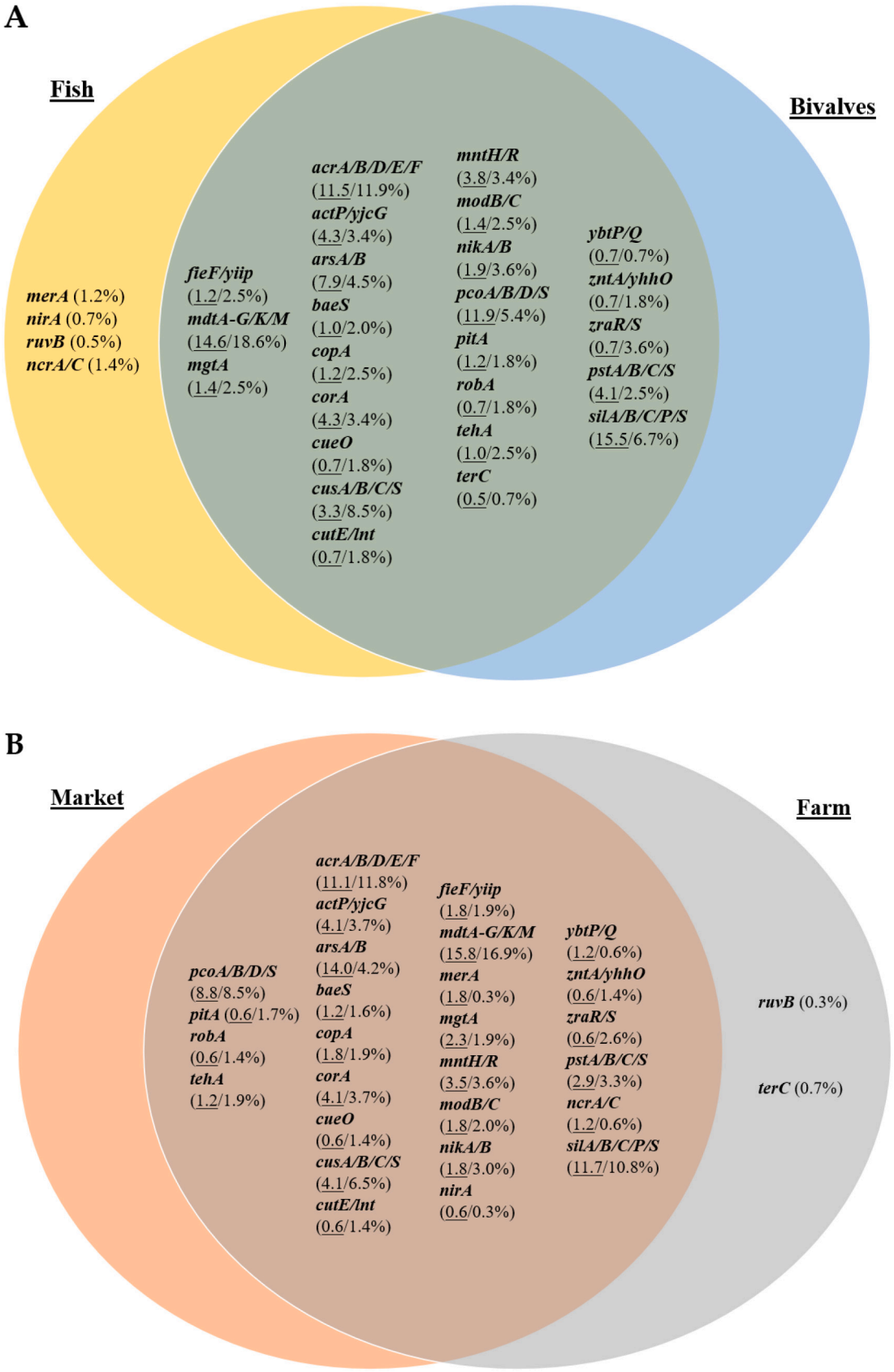


Fig. 2. Venn diagrams representing genes (and their prevalence in brackets) found in this study associated with tolerance to heavy metals in: **A.** Fish vs. bivalve samples (in the group of genes found in both reservoirs, the underlined percentages correspond to the prevalence of that gene in fish samples and the non-underlined ones correspond to bivalve samples); **B.** Market vs. farm samples (in the group of genes found in both reservoirs, the underlined percentages correspond to the prevalence of that gene in market samples and the non-underlined ones correspond to farm samples).

did not investigate a broader range of metal resistance genes.

Heavy metals can persist in the environment, which have a significant impact on bacterial communities and can drive the selection of heavy metals resistance/tolerance genes (Wang et al., 2021). Indeed, some studies indicate that the transfer of ARG through conjugation process is promoted by the presence of heavy metals (Meng et al., 2022). While some of these metals, such as iron, copper, and zinc, are vital for bacterial functions, excessive amounts can be toxic (Ju et al., 2017). This holds true not only for bacteria but also for aquatic animals and humans. Therefore, the presence and persistence of heavy metals in the environment, namely through intensive aquaculture, pose risks to both microbial communities and higher organisms, highlighting the importance of studying and understanding heavy metal resistance/tolerance genes in these contexts (Farmaki et al., 2014; Nabinger et al., 2018; Razak et al., 2021).

3.4. Virulome

The investigation of aquaculture's virulome has allowed the identification of 25 different genes in the families *Aeromonadaceae* and *Enterobacteriaceae*, which have been associated in the literature with an increase in virulence (Tables 1 and 2).

3.4.1. In Aeromonadaceae family

In the *Aeromonadaceae* family, a strain of *A. media* (INSAq193 from *S. aurata* acquired in the market) exhibited the presence of 13 genes associated with various virulence factors. These genes are related to toxin production (*cia*, *cvaC*), transportation (*etsC*, *mchF*), red blood cell lysis (*hlyF*), iron metabolism (*iroN*, *iucC*, *iutA*-type, *sitA*), increased serum survival (*iss*, *traT*), and the escape from the immune system of the host (*ompT*, *ompT*-type) (Daga et al., 2019; Messaili et al., 2019). To our knowledge, this is the first description of *cia*, *cvaC*, *etsC*, *iucC*, *mchF*, *hlyF*, *iroN* and *iss* genes in aquaculture. However, some of these genes have been found in fishery products, just like in the studies developed by Massella et al. (2020) and Zhang et al. (2017), although the origin of the samples was not specified. These seven genes have been found in various other reservoirs, such as drinking water, marine and freshwater, dog, seal, birds, and South Australian grey-headed flying fox pups (Ewbank et al., 2022; McDougall et al., 2022; Medina-Pizzali et al., 2022; Vingino et al., 2021). Genes *iutA*, *traT* and *ompT* were identified in aquaculture reservoirs, specifically in *K. pneumoniae* from shrimp aquaculture farms, *Acinetobacter johnsonii* isolated from cultured *Oreochromis niloticus* and *Vibrio ichthyoenteri* from *Paralichthys olivaceus* cultured in a marine farm (Elabd et al., 2020; Sivaraman et al., 2021; Tang et al., 2019).

Overall, in *Aeromonadaceae* family only one strain was found to possess virulence genes (Table 1), which did not correspond to the commonly studied virulence genes in this family, such as *hlyA* (hemolysin), *act* (cytotoxic heat-labile enterotoxin), *ast* (cytotonic heat-stable enterotoxin), *aerA* (aerolysin), and *alt* (cytotonic heat-labile enterotoxin) (Nhinh et al., 2021; Pattanayak et al., 2020).

The fact that all genes detected in our study, except for the *ompT* gene, were predicted to be located on plasmids suggests that these genes may have been acquired through MGE from other bacterial species, particularly from *Enterobacteriaceae* family.

3.4.2. In Enterobacteriaceae family

In *Enterobacteriaceae*, 66.7 % of the strains (22/33) tested positive for virulence genes. These genes were identified in gilthead seabream acquired from farm 7 and market, as well as in clams and mussels from farms 1 and 4, respectively. In addition to the genes previously described in *A. media* INSAq193, several other virulence genes were found in the *Enterobacteriaceae* family. These included genes associated with toxin production (*astA*, *mchB/C*, *sigA*), adherence and colonization (*lpfA*, *papC*, *terC*, *iha*, *hra*), iron metabolism (*fyuA*, *irp2*), resistance to acid environments (*gad*) and hexosyltransferase homologue (*capU*) (Ferdous et al., 2016; Rodríguez-Villodres et al., 2019). To our knowledge, among the virulence genes detected in our study, only *terC* and *papC* genes had already been described in aquaculture. The *terC* gene was found in *Pangasianodon hypophthalmus* in Vietnam while the *papC* gene was identified in *S. aurata* in Portugal (Manageiro et al., 2022; Ngo et al., 2022). The genes *astA*, *lpfA*, *iha* and *irp* were identified by Bueris et al. (2022) in wild-caught seafood, more specifically, marine bivalves, in Brazil. The genes *mchB*, *mchC* and *gad* have been associated to *E. coli* isolated from water, sediments, and seal samples (Nadya et al., 2016; Vingino et al., 2021). Foysal et al. (2019) detected *sigA* gene in *Shigella* spp. isolated from hisla fish (*Tenualosa ilisha*) acquired in the market (unknown origin). The genes *hra*, *capU* and *fyuA* were previously identified in *E. coli* strains isolated from surface water of agricultural drainage in Mexico (Magaña-Lizárraga et al., 2022) and coastal marine sediments from the Adriatic Sea (Vignaroli et al., 2012).

In general, *traT* was the most identified virulence gene (9.0 %) in this study, followed by *iutA* (8.3 %).

Most previous studies have focused on virulence genes in specific bacteria responsible for disease in animals produced in aquaculture. Deng et al. (2020) studied 70 strains belonging to nine *Vibrio* species and analysed their prevalence in marine fish with diseases from an aquaculture area in South China, as well as the presence of ARG and virulence factors. Similarly, Nhinh et al. (2021) investigated the prevalence of infection, ARG and virulence factors of *A. hydrophila* isolated from diseased fish (tilapia, channel catfish and carp) from several farms in Vietnam. Our study takes a more complete approach by encompassing several genera and species of Gram-negative bacteria, isolated from not only fish (*S. aurata*), but also three species of bivalve molluscs (*C. gigas*, *Mytilus* spp., and *R. decussatus*). Additionally, we investigated the presence of virulence genes in both the bacterial chromosome and plasmids. In contrast to other studies involving animals produced in aquaculture, all *Vibrio* spp. identified in our work were negative for the presence of virulence genes, such as *flaC* (flagella C), *tdh* (thermostable direct hemolysin), *vvh* (hemolysin), and *trh* (*tdh*-related hemolysin) (Deng et al., 2020; Ryu et al., 2019). These differences in the presence of virulence genes can be attributed to the complex nature of virulence expression, which is influenced by several factors such as environmental conditions (e.g. water temperature, salt conditions), the presence of competing bacteria either from the same species or different species, and hosts (Çam and Brinkmeyer, 2020; Ceccarelli et al., 2013; Rubio-Portillo et al., 2020). However, several of the virulence genes mentioned above are related to humans and land animals and not necessarily to aquatic animals; indeed, they may have other functions, as is the case of the *traT* gene that can also prevent the self-mating of carrier cells of identical or closely related conjugative plasmids, which may explain their presence in this study (Sukupolvi and O'Connor, 1990).

3.5. Mobilome and its importance in the spread of resistance

Our study revealed a significant number and diverse distribution of MGE across the 8 bacterial families analysed (Tables 1 and 2). These MGE included a total of 133 plasmids, 412 IS, 20 transposons, 9 composite transposons, 20 miniature inverted-repeat transposable elements (MITEs), 11 class 1 integron-integrases (*intI1*) and 109 prophage regions. Among the plasmids, Col[pHAD28] was the most predominant (13.5 %), while IS*26* was the most prevalent among the IS (5.6 %). Tn*3* accounted for the majority of the transposons (50.0 %), cn_3415_IS*Apu1* was the most common composite transposons (33.3 %), and MITEEc1 had the highest prevalence among the MITEs (60.0 %).

The MGE described below can potentially facilitate the spread of resistance genes between bacteria and the environment. Therefore, in addition to prophages (Section 3.5.1), we also investigated the genetic environment of several ARG (Section 3.5.2), where we highlighted the most significant findings of contigs that were predicted, through in silico analysis, to be located on plasmids.

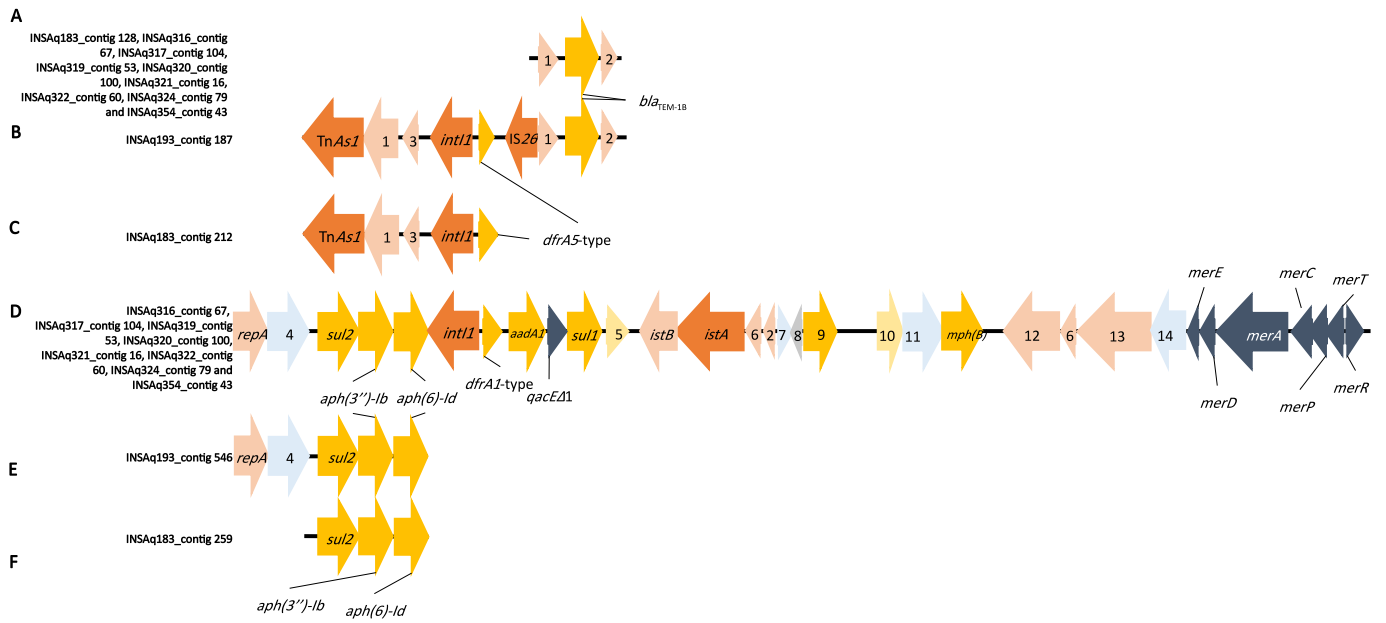


Fig. 3. A and B: alignment of all bla$_{TEM-1B}$ found in this study and their respective genetic environments. **B, C and D:** alignment of all dfrA-type found in this study and their respective genetic environments. **D, E and F:** alignment of all sul2, aph(3″)-Ib and aph(6)-Id found in this study and their respective genetic environments. INSAq183 (*Escherichia coli*) was isolated in intestine of gilthead seabream from market. INSAq193 (*Aeromonas media*) was detected in muscle of gilthead seabream from market. All strains in group D (*E. coli*) were isolated in mussels from farm 4. Arrows are drawn to scale. Elements in dark and light yellow are associated with antibiotic resistance. Elements in light and dark orange are associated with mobile genetic elements. Elements in dark blue are associated with disinfectants and heavy metals resistance. Elements in light blue are associated with normal functions of the bacterial cell. **1.** Recombinase family protein. **2.** Transposase zinc-binding domain-containing protein. **3.** DUF3330 domain-containing protein. **4.** Replication protein. **5.** GNAT family *N*-acetyltransferase. **6.** TniB family NTP-binding protein. **7.** Diguanylate cyclase. **8.** Hypothetical protein. **9.** Beta-lactamase family protein. **10.** TetR/AcrR family transcriptional regulator. **11.** Alpha/beta fold hydrolase. **12.** Transposase. **13.** DDE-type integrase/transposase/recombinase. **14.** EAL domain-containing protein.

3.5.1. Prophages

Among the 66 strains analysed, prophages were present in 72.7 % of them, with values ranging from 1 to 8 prophage regions. The most frequently identified prophages were Entero_Mu and Escher_HK639 (7.3 % each) (Fig. S6). Mu phages are known to infect several Gram-negative bacteria and play a role in the dissemination of bacterial transposable elements, while Escher_HK639 prophage was previously described in a carbapenem-resistant *C. freundii* strain isolated from a patient admitted in a Hospital in South Africa (Ramsamy et al., 2020; Ranquet et al., 2005). Interestingly, fish samples exhibited a higher diversity of prophages compared to bivalves, with Escher_HK639 as the most frequent in fish (9.9 %; Fig. S7 A). In bivalve samples, the most frequent prophage was Entero_Mu (21.1 %) (Fig. S7 A). Among the prophages identified, only Entero_mEp213, Entero_mEp235, Entero_mEp390, Escher_500465_1, Escher_HK639, Klebsi_phiKO2, Salmon_118970_sal3, Salmon_SEN34 and Vibrio_12B12 were found in both fish and bivalve samples (Fig. S7 A). Both market and farms reservoirs revealed a wide diversity of prophages. In the market samples, the most frequent prophages were Klebsi_phiKO2 and Vibrio_12B12 (7.3 % each one), while in aquaculture farms it was Entero_Mu (14.8 %) (Fig. S7 B). Prophage Klebsi_phiKO2 has previously been associated with human clinical isolates of *K. pneumoniae*, whereas Vibrio_12B12 has been isolated from a *Vibrio penaeicida* in a Japanese shrimp farm (Enany et al., 2022; Ragab et al., 2022). Among the phages studied, only Entero_c_1, Entero_mEp235, Erwini_vB_EhrS_59, Escher_500465_1, Escher_HK639, Klebsi_phiKO2, Mannhe_vB_MhM_3927AP2, Salmon_118970_sal3, Salmon_Fels_2 and Salmon_SP_004 were found in both the market and farm reservoirs (Fig. S7 B). Our investigation confirms that aquaculture harbour a great abundance and diversity of phages. These phages can play a role in the spread of ARG and virulence factors through generalized or specialized transduction. If generalized transduction is responsible for the transfer of any section of the bacterial genome, specialized transduction only affects some specific genes (Meng et al., 2022). Such elements can integrate into bacterial chromosomes, becoming known as prophages, and representing up to 20 % of the bacterial genome. Prophages carrying antibiotic resistance and/or virulence genes can confer selective advantages to bacteria under certain environmental conditions, such as exposure to sub-inhibitory concentrations of antibiotics. Notable examples of genes acquired through prophages integration in bacterial genomes include *stx* (Shiga toxin) in *E. coli* O157:H7 and *bla*$_{TEM}$ in *E. coli* (Olszak et al., 2017; Wendling et al., 2021).

3.5.2. Plasmids, transposons, integrons, IS

3.5.2.1. Genetic environment of bla$_{TEM-1B}$ and dfrA genes. The bla$_{TEM-1B}$ genes were identified in one *E. coli* strain isolated from a gilthead seabream acquired in the market, as well as in eight *E. coli* strains isolated from mussels in farm 4. In these strains, a recombinase family protein was found upstream of the bla$_{TEM-1B}$ gene, while a transposase zinc-binding domain-containing protein was found downstream. This genetic arrangement was similar to that observed in *A. media* strains obtained from gilthead seabream acquired in the market (Fig. 3 A and B). Transposons belonging to the Tn*3* family (Tn*As1*) are widespread across bacterial phyla and are frequently associated with several ARG, such as bla$_{TEM-1}$, particularly in aquatic environments (Jones-Dias et al., 2016c, 2013; Sultan et al., 2020).

As in our study (Fig. 3 B), it's not uncommon to observe the integration of other MGE such as class 1 integrons and IS (Nicolas et al., 2015; Sultan et al., 2020). Previous studies have recognized the significant role of IS*26* in the dissemination of multidrug resistance genes, especially in Gram-negative bacteria. IS*26* has been frequently associated with class 1 integrons, and its insertion at various sites can lead to rearrangements of antibiotic resistance loci (Partridge et al., 2018; Zhao et al., 2018).

The arrangement and orientation of IS*26* and the *dfrA*, bla$_{TEM-1B}$

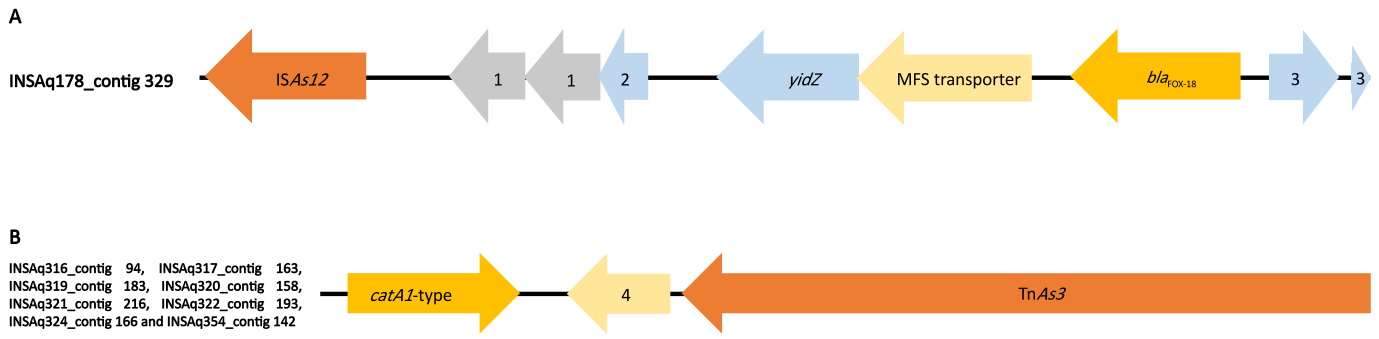


Fig. 4. A. Gene bla_{FOX-18} and its respective genetic environment detected in *Aeromonas allosaccharophila* isolated in the muscle of a gilthead seabream acquired in the market. **B.** Gene catA1-type and its respective genetic environment detected in eight *Escherichia coli* isolated in mussels from farm 4. Arrows are drawn to scale. Elements in dark and light yellow are associated with antibiotic resistance. Elements in orange are associated with mobile genetic elements. Elements in light blue are associated with normal functions of the bacterial cell. Elements in grey have unknown function. **1.** Hypothetical protein. **2.** Bor family protein. **3.** AAA family ATPase. **4.** GNAT family *N*-acetyltransferase.

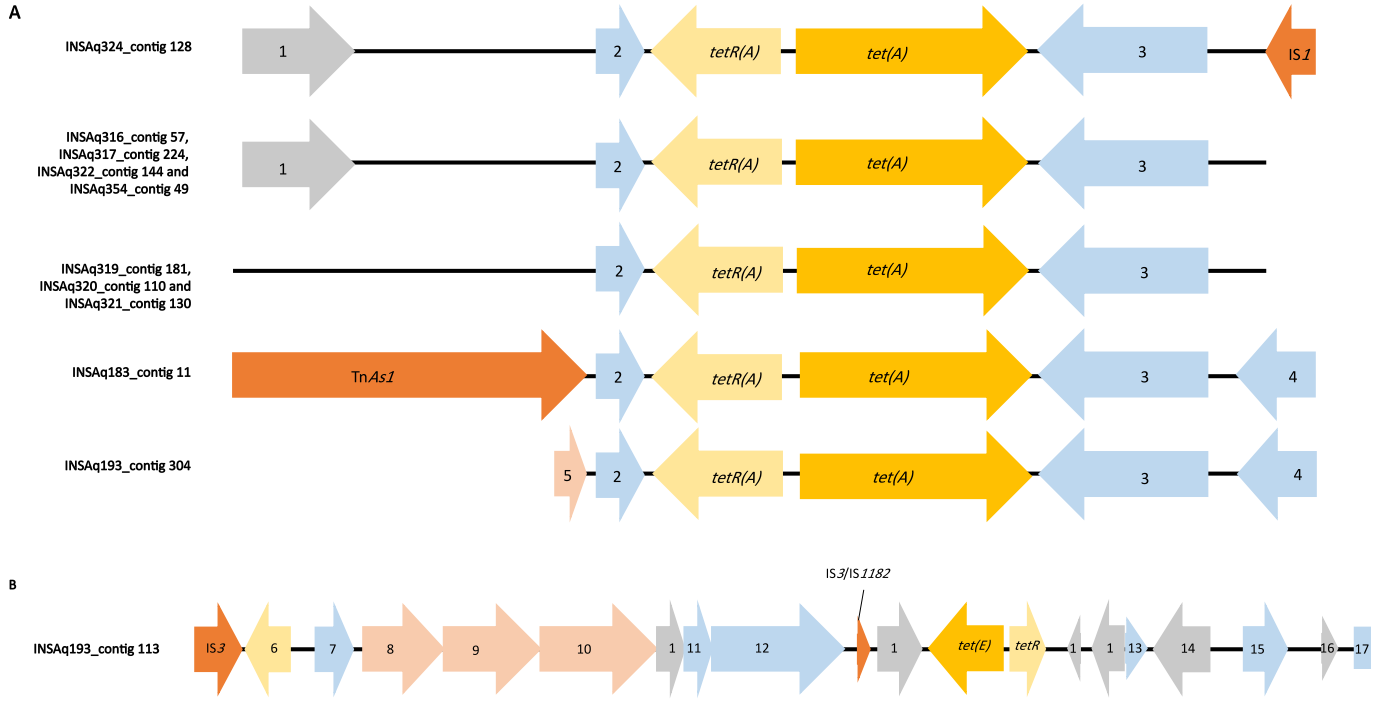


Fig. 5. A. Genes tet(A) and their respective genetic environment detected in our study. INSAq183 (*Escherichia coli*) and INSAq193 (*Aeromonas media*) were isolated in intestine and muscle of gilthead seabream from market, respectively. All other strains (*E. coli*) were isolated in mussels from farm 4. **B.** Gene tet(E) and their respective genetic environment detected in INSAq193. Arrows are drawn to scale. Elements in dark and light yellow are associated with antibiotic resistance. Elements in light and dark orange are associated with mobile genetic elements. Elements in light blue are associated with normal functions of the bacterial cell. Elements in grey have unknown function. **1.** Hypothetical protein. **2.** Relaxase. **3.** EamA family transporter. **4.** Cysteine hydrolase. **5.** Transposase. **6.** LuxR C-terminal-related transcriptional regulator. **7.** Single-stranded DNA-binding protein. **8.** Site-specific integrase. **9.** Tyrosine-type recombinase/integrase. **10.** Integrase. **11.** DivIVA domain-containing protein. **12.** ATP-dependent RecD-like DNA helicase. **13.** Helix-turn-helix domain-containing protein. **14.** DUF4113 domain-containing protein. **15.** Transporter substrate-binding domain-containing protein. **16.** DUF3811 domain-containing protein. **17.** Cold shock domain-containing protein.

genes observed in our study (Fig. 3 B) have also been reported in other studies. Kubomura et al. (2020) identified IS*26* and the *dfrA* gene in enteropathogenic and enteroaggregative *E. coli*, while Gomes-Neves et al. (2015) detected IS*26* and bla_{TEM-1B} in strains of *Salmonella enterica* isolated from slaughtered swine samples (Gomes-Neves et al., 2015; Kubomura et al., 2020). The *dfrA*-type genes identified in both *A. media* and *E. coli* strains isolated from gilthead seabream obtained from the market are located not only downstream of the integrase gene *intI1*, but also adjacent to the transposon Tn*As1* (Tn*3* family), as shown in Fig. 3 B, C and D. Moreover, in *A. media* strains, an IS*26* element is found downstream of the *dfrA5*-type gene, along with a recombinase family protein and bla_{TEM-1B}.

3.5.2.2. Genetic environment of sul2, aph(3″)-Ib and aph(6)-Id genes. Fig. 3 D, E and F, present the alignment of *sul2*, *aph(3″)-Ib*, *aph(6)-Id* genes along with their respective genetic environment. The presence of the *repA* gene, which is essential for plasmid replication, in strains belonging to groups D and E provides evidence for a possible association of these ARG with a plasmid (Betteridge et al., 2004). All *E. coli* strains from group D (isolated from mussels from farm 4) harboured additional ARG within this plasmid, such as: *dfrA1*-type, *aadA1*, *sul1* and *mph(B)*; disinfectants and heavy metals resistance genes, such as *qacEΔ1* and the *mer* operon; and additional mobile genetic elements, like *intI1*, located downstream of *sul2*, *aph(3″)-Ib* and *aph(6)-Id* genes, and upstream of *dfrA1*-type, *aadA1*, *sul1* and *qacEΔ1* genes. Class 1 integrons have been

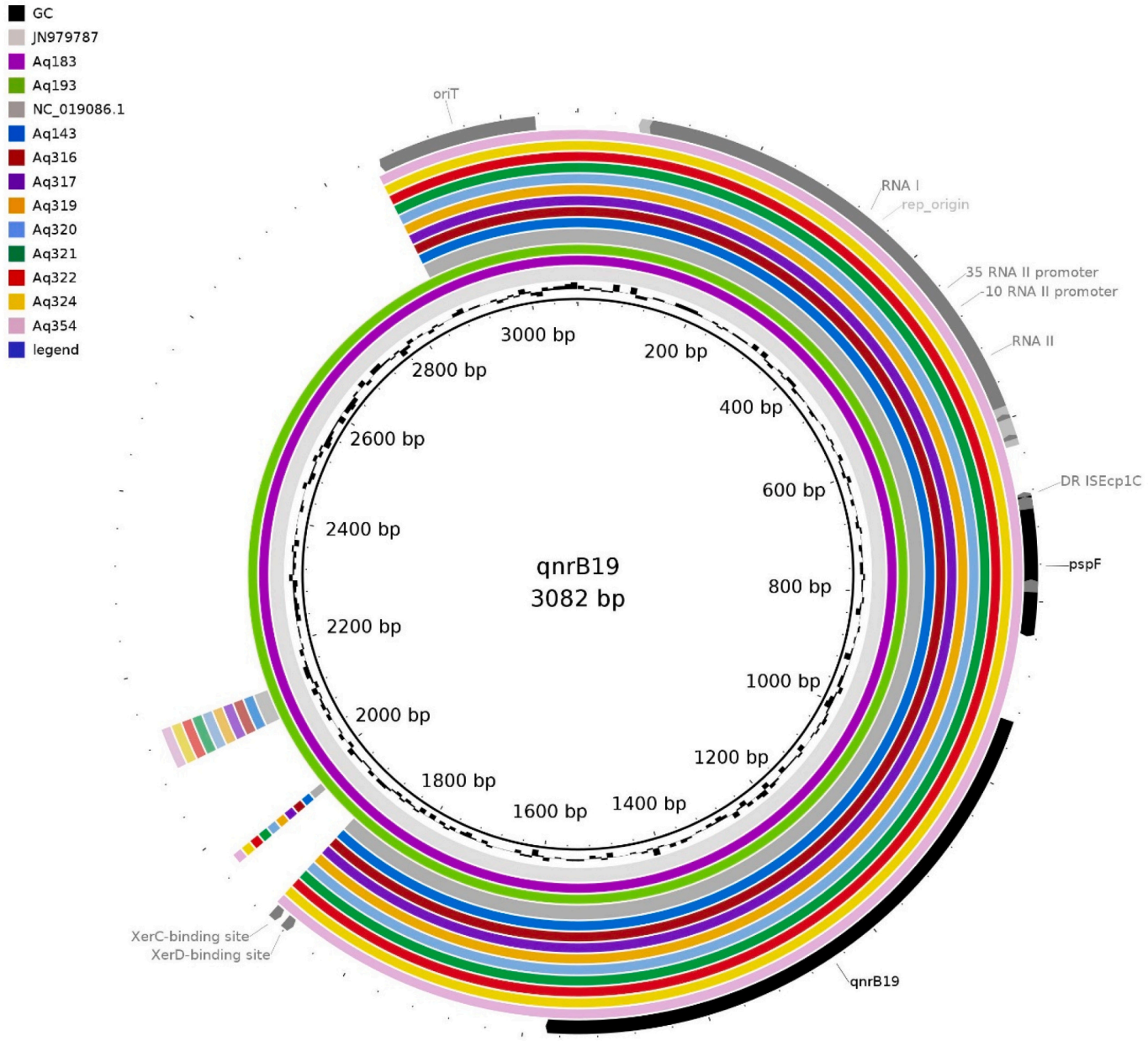


Fig. 6. Circular comparison, generated by BLAST Ring Image Generator (BRIG, v. 0.95; Alikhan et al., 2011), between all contigs harboring qnrB19 genes detected in this study, as having a plasmid location. Plasmids used as reference are the closest plasmid sequences obtained using the NCBI Microbial genomes BLAST analysis.

reported to be associated with *aadA*, *dfrA* and *mph(B)* genes (Jiang et al., 2019; Muziasari et al., 2016; Suzuki et al., 2022). The ARG *sul*, *dfrA* and *mph(B)* are involved in resistance to antibiotics used in aquaculture, especially in fish (e.g. China, South Africa, Turkey, Norway and Italy), and their presence contributes to a selective pressure that promotes the survival of bacteria carrying these genes, as well as HGT and recombination (Andersson and Hughes, 2014; Federation of Veterinarians of Europe, 2016; Ferry et al., 2022; Santos and Ramos, 2018). Additionally, other genes, like *aadA*, can thrive in these environments due to co-selection (Muziasari et al., 2016).

3.5.2.3. Genetic environment of bla$_{FOX-18}$ gene. Noteworthy, an IS*As12* element was detected 4864 bp upstream of a major facilitator superfamily (MFS) transporter and the *bla*$_{FOX-18}$ gene in an *A. allosaccharophila* from gilthead seabream acquired in the market (Fig. 4 A). Although *bla*$_{FOX}$ genes are normally encoded on the chromosome of *Aeromonas* spp. (Maravić et al., 2013), previous studies have reported their association with plasmids in this genus (as in our study), isolated from active sludge in urban wastewater treatment plant in Poland (Piotrowska et al., 2017).

All eight *E. coli* strains isolated from mussels in farm 4 had a *catA1*-type gene, sharing the same genetic environment (Fig. 4 B). This gene was located upstream of a GNAT family *N*-acetyltransferase and the transposon Tn*As3*. Tn*As3* is a transposon belonging to the Tn*3* family and has previously been associated with *cat* genes (Mbelle et al., 2019; Ross et al., 2021). Once again, our findings provide evidence that transposons from Tn*3* family are frequently linked to several ARG and may play an essential role in their mobilization and dissemination.

3.5.2.4. Genetic environment of tet genes. In Fig. 5 A, we can see the alignment of *tet(A)* genes found in this study. In all strains *tet(A)* gene was found downstream *tetR(A)* [which codes for the repressor TetR, responsible for the regulation of *tet(A)* mRNA expression (Møller et al., 2016)] and a relaxase, and upstream a EamA family transporter

(belonging to the drug-metabolite transporter superfamily). The remaining genetic environment varies according to the origin of the farmed animals (farm 4 or market). We highlight *E. coli* INSAq324 which has an IS*1* downstream of the *tet(A)* gene and the EamA family transporter, and *E. coli* INSAq183 which has Tn*As1* upstream of the relaxase and the *tetR(A)* gene. *A. media* INSAq193 proved to be an important reservoir of ARG, as this strain had another ARG associated with tetracyclines resistance, *tet(E)* (Fig. 5 B), upstream of a *tetR* gene and downstream of a hypothetical protein and the IS*3*. Tn*As1* was observed in other studies in association with the *tet(A)* gene, isolated in human samples with diarrhoea and located in a plasmid along with the *mcr-1* gene (Li et al., 2021).

For this study, we did not find any reference in the literature to the association of IS*1* and *tet(A)*, as well as IS*3* with *tet(E)* (Fig. 5 A and B). However, it is noteworthy that tetracycline resistance genes are often associated with plasmids and transposons (Hong et al., 2014).

3.5.2.5. Genetic environment of qnrB19 gene. All the *qnrB19* genes found in this study were located in small plasmids with approximately 3000 bp. In Fig. 6, a comparative analysis is presented between the *qnrB19*-harboring contigs identified in our study and the *qnrB19*-harboring reference plasmids (accession numbers: JN979787 and NC_019086.1). Our finding indicate that the *qnrB19*-harboring plasmids of INSAq183 and INSAq193, isolated from the intestine and muscle of a gilthead seabream obtained from the market, respectively, exhibit a higher similarity (99.6 %) to the *qnrB19*-harboring small ColE1-type plasmid (JN979787), which was isolated from a clinical *E. coli* isolate in Argentina (Tran et al., 2012).

Among the other *qnrB19*-harboring contigs identified in our study (INSAq143 isolated from L. *adecarboxylata* in a gilthead seabream collected from farm 7; INSAq316, INSAq317, INSAq319, INSAq320, INSAq321, INSAq322, INSAq324 and INSAq354 isolated in eight *E. coli* in mussels collected from farm 4), a higher identity (99.8 %) was observed with another small ColE-type plasmid isolated from an *E. coli* in a faecal sample, collected from a healthy child in Peru (NC_019086.1; Pallecchi et al., 2010). The genetic environment of the *qnrB19* genes is identical in all analysed strains, with the *pspF* (coding to phage shock protein transcriptional activator for *pspABCE* operon) gene disrupted by an IS*Ecp1C*-like located upstream of the *qnrB19* gene. Some authors have proposed that IS*Ecp1C*-like elements may have played a significant role in the mobilization of the *qnrB19* gene (Tran et al., 2012). The *qnrB19* gene can be found on both large and small plasmids. Small plasmids, similar to those found in our study, have been described in previous studies encompassing different bacterial species within the *Enterobacteriaceae* family, multiple host species (e.g. horse, chicken, pigs, wild birds, humans, and environmental samples) and different geographical regions (Moreno-Switt et al., 2019; Pallecchi et al., 2010; Tran et al., 2012). This suggests that this type of small plasmids could be responsible for the dissemination of the *qnrB19* gene in different reservoirs through HGT. Indeed, Moreno-Switt et al. (2019) demonstrated that the transfer of these small plasmids is possible, from *Salmonella* Heidelberg to *Salmonella Typhimurium*, through the action of a P22 bacteriophage by transduction (Moreno-Switt et al., 2019).

MGE play a fundamental role in the adaptation and evolution of bacteria in diverse environments. They range from the simple elements, such IS capable of translocating to several locations and influencing the expression of nearby genes, to more complex MGE, such as plasmids. Plasmids can incorporate in their structure numerous other MGE, like transposons and integrons, as well as resistance and virulence genes. They can be transmitted to other bacteria through HGT of the same or different species, genus, or family (Partridge et al., 2018; Ross et al., 2021).

3.6. Pathogenicity to humans

All genes described above, from antibiotic, disinfectant and heavy metals resistance genes to virulence factors (such as those related with adherence, fimbriae, toxins secretion, efflux proteins and other features essential for the survival and spread of bacteria within a human host), prophages and MGE, contribute to the pathogenicity of these strains. The analysis of the 66 strains studied, allowed the classification of 49 (74.2 %) as pathogenic to humans, with values ranging from 54.8 % to 94.5 % (Tables 1 and 2). This is a concerning fact since farmed animals will inevitably come into contact with humans, whether through handling of these animals in aquaculture settings, markets or through their consumption, thereby exposing humans to potential risk of infection.

4. Conclusions

Our study provides new information about resistance/virulence determinants that are circulating in the aquaculture environment and emphasizes the importance of MGE and the mobilome in the dissemination of these determinants to both humans and to the environment.

New ST were identified in *Aeromonas* spp., *C. portucalensis*, *E. asburiae*, *Shewanella* spp. and *Vibrio* spp., shedding light on their potentially evolutionary relationships with other studied STs. This contributes to the understanding of the bacterial diversity in aquatic farmed animals. The extensive array of antibiotic, disinfectants and heavy metals resistance genes discovered (e.g. bla_{TEM}, *sul2*, *qnrB19*, *qnrD2*, *catA1*-type, *tet*, *sitABCD*, *merA* and *copA*) confirms aquaculture environments as hotspots for resistance genes, many of which are also prevalent in clinical human isolates. MGE, such as IntI1, plasmids and Tn*As*, were found to play a crucial role in the dissemination of resistance to tetracyclines, trimethoprim, β-lactams, phenicols, sulfonamides, quinolones, aminoglycosides, macrolides, disinfectants (like quaternary ammonium compounds), and heavy metals (such as mercury). Antibiotics, disinfectants and heavy metals can persist in the environment for extended periods, subjecting bacterial communities to selective pressures and altering their abundance and composition. This can favour resistant bacteria over sulfur-reducing, aromatic-compound-oxidizing, methanotrophic and photosynthetic bacteria, with a possible impact in ecosystem functioning. Likewise, it is known that even low amounts of antibiotics can influence microbial signalling and interactions (Anand et al., 2021; Posada-Perlaza et al., 2019). Given the appropriate conditions, these genes can transfer to new reservoirs, posing a significant threat to human health (Caniça et al., 2015). This emphasizes the concerning and highly probable occurrence of bidirectional transmission of antibiotic resistant bacteria between the aquaculture and the environment, potentially impacting human health. Therefore, it is crucial to implement One Health strategies to monitor these reservoirs and the surrounding environment (Mcewen and Collignon, 2018).

Overall, these results show the need to apply Council Recommendation/2002/77/EC on the prudent use of antimicrobial agents in human medicine, as it highlights the need of establishing a relationship between the occurrence of antimicrobial resistance in certain human pathogens and its occurrence in animals and the environment (European Council, 2001).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.166351.

CRediT authorship contribution statement

Conceptualization: M.C.; methodology: V.S., N.M.B. and M.J.B.; validation: V.M. and M.C.; formal analysis: V.S. and V.M.; investigation:

V.S., V.M., T.R., E.D. and M.C.; writing original draft preparation: V.S.; writing review and editing: V.S., V.M., T.R., N.M.B., M.J.B., E.D. and M.C.; visualization: M.C.; supervision: M.C.; funding acquisition: M.C. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Online and in the PDF.

Acknowledgements

None.

Funding

V.S. has a Ph.D. fellowship granted by the FCT (Fundação para a Ciência e a Tecnologia) with the reference SFRH/BD/133100/2017 cofinanced by European Social Fund and the Operational Program for Human Capital (POCH), Portugal. This work was supported by funding from the European Union's Horizon 2020 Research and Innovation programme under grant agreement No 773830: One Health European Joint Programme (WORLDCOM project), and by FCT/MCTES [UIDB/00211/2020] through national funds.

References

Abgottspon, H., Zurfluh, K., Nüesch-Inderbinen, M., Hächler, H., Stephan, R., 2014. Quinolone resistance mechanisms in *Salmonella enterica* serovars Hadar, Kentucky, Virchow, Schwarzengrund, and 4,5,12:i:-, isolated from humans in Switzerland, and identification of a novel *qnrD* variant, *qnrD2*, in *S.* Hadar. Antimicrob. Agents Chemother. 58, 3560–3563. https://doi.org/10.1128/AAC.02404-14.

Ahmed, A.M., Maruyama, A., Khalifa, H.O., Shimamoto, Toshi, Shimamoto, Tadashi, 2015. Seafood as a reservoir of gram-negative bacteria carrying integrons and antimicrobial resistance genes in Japan. Biomed. Environ. Sci. 28, 924–926. https://doi.org/10.3967/bes2015.128.

Alcock, B.P., Raphenya, A.R., Lau, T.T.Y., Tsang, K.K., Bouchard, M., Edalatmand, A., Huynh, W., Nguyen, A.L.V., Cheng, A.A., Liu, S., Min, S.Y., Miroshnichenko, A., Tran, H.K., Werfalli, R.E., Nasir, J.A., Oloni, M., Speicher, D.J., Florescu, A., Singh, B., Faltyn, M., Hernandez-Koutoucheva, A., Sharma, A.N., Bordeleau, E., Pawlowski, A.C., Zubyk, H.L., Dooley, D., Griffiths, E., Maguire, F., Winsor, G.L., Beiko, R.G., Brinkman, F.S.L., Hsiao, W.W.L., Domselaar, G.V., McArthur, A.G., 2020. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res. 48, D517–D525. https://doi.org/10.1093/nar/gkz935.

Alikhan, N.F., Petty, N.K., Ben Zakour, N.L., Beatson, S.A., 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC Genomics 12, 402. https://doi.org/10.1186/1471-2164-12-402.

Anand, U., Reddy, B., Singh, V.K., Singh, A.K., Kesari, K.K., Tripathi, P., Kumar, P., Tripathi, V., Simal-Gandara, J., 2021. Potential environmental and human health risks caused by antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs) and emerging contaminants (ECs) from municipal solid waste (MSW) landfill. Antibiotics 10, 374. https://doi.org/10.3390/antibiotics10040374.

Andersson, D.I., Hughes, D., 2014. Microbiological effects of sublethal levels of antibiotics. Nat. Rev. Microbiol. 12, 465–478. https://doi.org/10.1038/nrmicro3270.

Arndt, D., Grant, J.R., Marcu, A., Sajed, T., Pon, A., Liang, Y., Wishart, D.S., 2016. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res. 44, W16–W21. https://doi.org/10.1093/nar/gkw387.

Baquero, F., Martínez, J.L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19, 260–265. https://doi.org/10.1016/j.copbio.2008.05.006.

Betteridge, T., Yang, J., Pittard, A.J., Praszkier, J., 2004. Role of RepA and DnaA proteins in the opening of the origin of DNA replication of an IncB plasmid. J. Bacteriol. 186, 3785–3793. https://doi.org/10.1128/JB.186.12.3785-3793.2004.

Bortolaia, V., Kaas, R.S., Ruppe, E., Roberts, M.C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R.L., Rebelo, A.R., Florensa, A.F., Fagelhauer, L., Chakraborty, T., Neumann, B., Werner, G., Bender, J.K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B. B., Malhotra-Kumar, S., Westh, H., Pinholt, M., Anjum, M.F., Duggett, N.A., Kempf, I., Nykäsenoja, S., Olkkola, S., Wieczorek, K., Amaro, A., Clemente, L., Mossong, J., Losch, S., Ragimbeau, C., Lund, O., Aarestrup, F.M., 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J. Antimicrob. Chemother. 75, 3491–3500. https://doi.org/10.1093/jac/dkaa345.

Boyer, E., Bergevin, I., Malo, D., Gros, P., Cellier, M.F.M., 2002. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica* serovar Typhimurium. Infect. Immun. 70, 6032–6042. https://doi.org/10.1128/IAI.70.11.6032.

Breitbart, M., 2012. Marine viruses: truth or dare. Annu. Rev. Mar. Sci. 4, 425–448. https://doi.org/10.1146/annurev-marine-120709-142805.

Briales, A., Rodriguez-Martinez, J.M., Velasco, C., Machuca, J., Díaz de Alba, P., Blazquez, J., Pascual, A., 2012. Exposure to diverse antimicrobials induces the expression of *qnrB1*, *qnrD* and *smaqnr* genes by SOS-dependent regulation. J. Antimicrob. Chemother. 67, 2854–2859. https://doi.org/10.1093/jac/dks326.

Bueris, V., Sellera, F.P., Fuga, B., Sano, E., Carvalho, M.P.N., Couto, S.C.F., Moura, Q., Lincopan, N., 2022. Convergence of virulence and resistance in international clones of WHO critical priority enterobacterales isolated from marine bivalves. Sci. Rep. 12, 5707. https://doi.org/10.1038/s41598-022-09598-8.

Cabello, F.C., Godfrey, H.P., 2018. Aquaculture, exaptation, and the origin of *mcr*-positive colistin resistance. Antimicrob. Agents Chemother. 62 https://doi.org/10.1128/AAC.01903-18 e01903-18.

Çam, S., Brinkmeyer, R., 2020. Differential expression of *vvhA* and *CPS operon allele 1* genes in *Vibrio vulnificus* under biofilm and planktonic conditions. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 113, 1437–1446. https://doi.org/10.1007/s10482-020-01452-z.

Caniça, M., Manageiro, V., Jones-Dias, D., Clemente, L., Gomes-Neves, E., Poeta, P., Dias, E., Ferreira, E., 2015. Current perspectives on the dynamics of antibiotic resistance in different reservoirs. Res. Microbiol. 166, 594–600. https://doi.org/10.1016/j.resmic.2015.07.009.

Cantón, R., González-Alba, J.M., Galán, J.C., 2012. CTX-M enzymes: origin and diffusion. Front. Microbiol. 3, 110. https://doi.org/10.3389/fmicb.2012.00110.

Carattoli, A., Zankari, E., Garciá-Fernández, A., Larsen, M.V., Lund, O., Villa, L., Aarestrup, F.M., Hasman, H., 2014. *In Silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob. Agents Chemother. 58, 3895–3903. https://doi.org/10.1128/AAC.02412-14.

Ceccarelli, D., Hasan, N.A., Huq, A., Colwell, R.R., 2013. Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. Front. Cell. Infect. Microbiol. 3, 97. https://doi.org/10.3389/fcimb.2013.00097.

Chenia, H.Y., Jacobs, A., 2017. Antimicrobial resistance, heavy metal resistance and integron content in bacteria isolated from a South African tilapia aquaculture system. Dis. Aquat. Org. 126, 199–209. https://doi.org/10.3354/dao03173.

Cosentino, S., Larsen, M.V., Aarestrup, F.M., Lund, O., 2013. PathogenFinder - distinguishing friend from foe using bacterial whole genome sequence data. PLoS One 8, e77302. https://doi.org/10.1371/journal.pone.0077302.

Daga, A.P., Koga, V.L., Soncini, J.G.M., de Matos, C.M., Perugini, M.R.E., Pelisson, M., Kobayashi, R.K.T., Vespero, E.C., 2019. *Escherichia coli* bloodstream infections in patients at a university hospital: virulence factors and clinical characteristics. Front. Cell. Infect. Microbiol. 9, 191. https://doi.org/10.3389/fcimb.2019.00191.

Deng, Y., Xu, L., Chen, H., Liu, S., Guo, Z., Cheng, C., Ma, H., Feng, J., 2020. Prevalence, virulence genes, and antimicrobial resistance of *Vibrio* species isolated from diseased marine fish in South China. Sci. Rep. 10, 14329. https://doi.org/10.1038/s41598-020-71288-0.

Done, H.Y., Venkatesan, A.K., Halden, R.U., 2015. Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture? AAPS J. 17, 513–524. https://doi.org/10.1208/s12248-015-9722-z.

Elabd, H., Abd El-latif, A., Shaheen, A., Matter, A., 2020. Identification of emerging *Acinetobacter johnsonii* virulence and antibiotic resistance genes associated with high mortality in cultured Oreochromis niloticus. Egypt. J. Aquac. 10, 19–33. https://doi.org/10.21608/eja.2020.29010.1021.

Enany, S., Zakeer, S., Diab, A.A., Bakry, U., Sayed, A.A., 2022. Whole genome sequencing of *Klebsiella pneumoniae* clinical isolates sequence type 627 isolated from Egyptian patients. PLoS One 17, e0265884. https://doi.org/10.1371/journal.pone.0265884.

European Council, 2001. Council Recommendation of 15.11.2001 on the Prudent Use of Antimicrobial Agents in Human Medicine (2002/77/EC). OJ L34 of 5.2.2002. 13. Brussels, Belgium. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32002H0077. (Accessed 20 June 2023).

Ewbank, A.C., Fuentes-Castillo, D., Sacristán, C., Esposito, F., Fuga, B., Cardoso, B., Godoy, S.N., Zamana, R.R., Gattamorta, M.A., Catão-Dias, J.L., Lincopan, N., 2022. World Health Organization critical priority *Escherichia coli* clone ST648 in magnificent frigatebird (*Fregata magnificens*) of an uninhabited insular environment. Front. Microbiol. 13, 940600 https://doi.org/10.3389/fmicb.2022.940600.

Farmaki, E.G., Thomaidis, N.S., Pasias, I.N., Baulard, C., Papaharisis, L., Efstathiou, C.E., 2014. Environmental impact of intensive aquaculture: investigation on the accumulation of metals and nutrients in marine sediments of Greece. Sci. Total Environ. 485–486, 554–562. https://doi.org/10.1016/j.scitotenv.2014.03.125.

Federation of Veterinarians of Europe, 2016. Antimicrobial Use in Food Producing Animals. Brussels, Belgium. Available at: https://www.ema.europa.eu/en/documents/report/annex-replies-efsa/ema-questions-use-antimicrobials-food-producing-animals-eu-possible-measures-reduce-antimicrobial_en.pdf. (Accessed 30 December 2022).

Ferdous, M., Friedrich, A.W., Grundmann, H., de Boer, R.F., Croughs, P.D., Islam, M.A., Kluytmans-van den Bergh, M.F.Q., Kooistra-Smid, A.M.D., Rossen, J.W.A., 2016. Molecular characterization and phylogeny of Shiga toxin–producing *Escherichia coli* isolates obtained from two Dutch regions using whole genome sequencing. Clin. Microbiol. Infect. 22, 642.e1–642.e9. https://doi.org/10.1016/j.cmi.2016.03.028.

Fernández-Bravo, A., Figueras, M.J., 2020. An update on the genus *Aeromonas*: taxonomy, epidemiology, and pathogenicity. Microorganisms 8, 129. https://doi.org/10.3390/microorganisms8010129.

Ferry, G., Lauteri, C., Vergara, A., 2022. Antibiotic resistance in the finfish aquaculture industry: a review. Antibiotics 11, 1574. https://doi.org/10.3390/antibiotics11111574.

Finley, R.L., Collignon, P., Larsson, D.G.J., McEwen, S.A., Li, X.-Z., Gaze, W.H., Reid-Smith, R., Timinouni, M., Graham, D.W., Topp, E., 2013. The scourge of antibiotic resistance: the important role of the environment. Clin. Infect. Dis. 57, 704–710. https://doi.org/10.1093/cid/cit355.

Foysal, M.J., Momtaz, F., Robiul Kawser, A.Q.M., Chaklader, M.R., Siddik, M.A.B., Lamichhane, B., Tay, A.C.Y., Rahman, M.M., Fotedar, R., 2019. Microbiome patterns reveal the transmission of pathogenic bacteria in hilsa fish (*Tenualosa ilisha*) marketed for human consumption in Bangladesh. J. Appl. Microbiol. 126, 1879–1890. https://doi.org/10.1111/jam.14257.

Galardini, M., Clermont, O., Baron, A., Busby, B., Dion, S., Schubert, S., Beltrao, P., Denamur, E., 2020. Major role of iron uptake systems in the intrinsic extra-intestinal virulence of the genus *Escherichia* revealed by a genome-wide association study. PLoS Genet. 16, e1009065 https://doi.org/10.1371/journal.pgen.1009065.

Gastalho, S., Silva, G.J., Ramos, F., 2014. Antibiotics in aquaculture and bacterial resistance: health care impact. Acta Farm. Port. 3, 29–45. https://doi.org/10.13140/2.1.4697.0560.

Gomes-Neves, E., Manageiro, V., Ferreira, E., Correia da Costa, J.M., Caniça, M., 2015. First description of food-borne *Salmonella enterica* resistance regions R1 and R3 associated with IS*26* elements. Res. Microbiol. 166, 570–573. https://doi.org/10.1016/j.resmic.2015.03.007.

González-López, J.J., Coelho, A., Larrosa, M.N., Lavilla, S., Bartolomé, R., Prats, G., 2009. First detection of plasmid-encoded bla_{OXY} β-lactamase. Antimicrob. Agents Chemother. 53, 3143–3146. https://doi.org/10.1128/AAC.01473-08.

Green, R.T., Todd, J.D., Johnston, A.W.B., 2013. Manganese uptake in marine bacteria; the novel MntX transporter is widespread in Roseobacters, Vibrios, Alteromonadales and the SAR11 and SAR116 clades. ISME J. 7, 581–591. https://doi.org/10.1038/ismej.2012.140.

Grevskott, D.H., Svanevik, C.S., Sunde, M., Wester, A.L., Lunestad, B.T., 2017. Marine bivalve mollusks as possible indicators of multidrug-resistant *Escherichia coli* and other species of the *Enterobacteriaceae* family. Front. Microbiol. 8, 24. https://doi.org/10.3389/fmicb.2017.00024.

Harmon, D.E., Miranda, O.A., McCarley, A., Eshaghian, M., Carlson, N., Ruiz, C., 2019. Prevalence and characterization of carbapenem-resistant bacteria in water bodies in the Los Angeles–Southern California area. Microbiologyopen 8, e00692. https://doi.org/10.1002/mbo3.692.

Herrera, F.C., Santos, J.A., Otero, A., García-López, M.L., 2006. Occurrence of *Plesiomonas shigelloides* in displayed portions of saltwater fish determined by a PCR assay based on the *hugA* gene. Int. J. Food Microbiol. 108, 233–238. https://doi.org/10.1016/j.ijfoodmicro.2005.12.008.

Hong, H., Jung, J., Park, W., 2014. Plasmid-encoded tetracycline efflux pump protein alters bacterial stress responses and ecological fitness of *Acinetobacter oleivorans*. PLoS One 9, e107716. https://doi.org/10.1371/journal.pone.0107716.

Hossain, M.M.M., Uddin, M.I., Islam, H., Fardoush, J., Rupom, M.A.H., Hossain, M.M., Farjana, N., Afroz, R., Hasan-Uj-Jaman, Roy, H.S., Shehab, M.A.S., Rahman, M.A., 2020. Diagnosis, genetic variations, virulence, and toxicity of AHPND-positive *Vibrio parahaemolyticus* in *Penaeus monodon*. Aquac. Int. 28, 2531–2546. https://doi.org/10.1007/s10499-020-00607-z.

Hu, J., Le, Q., Wang, Y., Yu, N., Cao, X., Kuang, S., Zhang, M., Gu, W., Sun, Y., Yang, Y., Xu, S., Yan, X., 2019. Effects of formaldehyde on detoxification and immune responses in silver pomfret (*Pampus argenteus*). Fish Shellfish Immunol. 88, 449–457. https://doi.org/10.1016/j.fsi.2019.03.019.

Huang, L., Xu, Y. Bin, Xu, J.X., Ling, J.Y., Chen, J.L., Le Zhou, J., Zheng, L., Du, Q.P., 2017. Antibiotic resistance genes (ARGs) in duck and fish production ponds with integrated or non-integrated mode. Chemosphere 168, 1107–1114. https://doi.org/10.1016/j.chemosphere.2016.10.096.

Jacoby, G.A., 2009. AmpC β-lactamases. Clin. Microbiol. Rev. 22, 161–182. https://doi.org/10.1128/CMR.00036-08.

Janda, J.M., Abbott, S.L., 2006. The genus *Hafnia*: from soup to nuts. Clin. Microbiol. Rev. 19, 12–28. https://doi.org/10.1128/CMR.19.1.12-28.2006.

Jang, H.M., Kim, Y.B., Choi, S., Lee, Y., Shin, S.G., Unno, T., Kim, Y.M., 2018. Prevalence of antibiotic resistance genes from effluent of coastal aquaculture, South Korea. Environ. Pollut. 233, 1049–1057. https://doi.org/10.1016/j.envpol.2017.10.006.

Jayol, A., Saly, M., Nordmann, P., Ménard, A., Poirel, L., Dubois, V., 2017. *Hafnia*, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods. J. Antimicrob. Chemother. 72, 2507–2511. https://doi.org/10.1093/jac/dkx154.

Jia, R., Du, J., Cao, L., Feng, W., He, Q., Xu, P., Yin, G., 2021. Application of transcriptome analysis to understand the adverse effects of hydrogen peroxide exposure on brain function in common carp (*Cyprinus carpio*). Environ. Pollut. 286, 117240 https://doi.org/10.1016/j.envpol.2021.117240.

Jiang, H., Cheng, H., Liang, Y., Yu, S., Yu, T., Fang, J., Zhu, C., 2019. Diverse Mobile genetic elements and conjugal transferability of sulfonamide resistance genes (*sul1*, *sul2*, and *sul3*) in *Escherichia coli* isolates from *Penaeus vannamei* and pork from large markets in Zhejiang, China. Front. Microbiol. 10, 1787. https://doi.org/10.3389/fmicb.2019.01787.

Jiang, H., Yu, T., Yang, Y., Yu, S., Wu, J., Lin, R., Li, Y., Fang, J., Zhu, C., 2020. Co-occurrence of antibiotic and heavy metal resistance and sequence type diversity of *Vibrio parahaemolyticus* isolated from *Penaeus vannamei* at freshwater farms, seawater farms, and markets in Zhejiang Province, China. Front. Microbiol. 11, 1294. https://doi.org/10.3389/fmicb.2020.01294.

Jie, S., Li, M., Gan, M., Zhu, J., Yin, H., Liu, X., 2016. Microbial functional genes enriched in the Xiangjiang River sediments with heavy metal contamination. BMC Microbiol. 16, 179. https://doi.org/10.1186/s12866-016-0800-x.

Johansson, M.H.K., Bortolaia, V., Tansirichaiya, S., Aarestrup, F.M., Roberts, A.P., Petersen, T.N., 2021. Detection of mobile genetic elements associated with antibiotic resistance in *Salmonella enterica* using a newly developed web tool: MobileElementFinder. J. Antimicrob. Chemother. 76, 101–109. https://doi.org/10.1093/jac/dkaa390.

Jones-Dias, D., Manageiro, V., Francisco, A.P., Martins, A.P., Domingues, G., Louro, D., Ferreira, E., Caniça, M., 2013. Assessing the molecular basis of transferable quinolone resistance in *Escherichia coli* and *Salmonella* spp. from food-producing animals and food products. Vet. Microbiol. 167, 523–531. https://doi.org/10.1016/j.vetmic.2013.08.010.

Jones-Dias, D., Clemente, L., Moura, I.B., Sampaio, D.A., Albuquerque, T., Vieira, L., Manageiro, V., Caniça, M., 2016a. Draft genomic analysis of an avian multidrug resistant *Morganella morganii* isolate carrying *qnrD1*. Front. Microbiol. 7, 1660. https://doi.org/10.3389/fmicb.2016.01660.

Jones-Dias, D., Manageiro, V., Caniça, M., 2016b. Influence of agricultural practice on mobile *bla* genes: IncI1-bearing CTX-M, SHV, CMY and TEM in *Escherichia coli* from intensive farming soils. Environ. Microbiol. 18, 260–272. https://doi.org/10.1111/1462-2920.13021.

Jones-Dias, D., Manageiro, V., Graça, R., Sampaio, D.A., Albuquerque, T., Themudo, P., Vieira, L., Ferreira, E., Clemente, L., Caniça, M., 2016c. QnrS1- and Aac(6′)-Ib-cr-producing *Escherichia coli* among isolates from animals of different sources: susceptibility and genomic characterization. Front. Microbiol. 7, 671. https://doi.org/10.3389/fmicb.2016.00671.

Ju, Y.R., Chen, C.W., Chen, C.F., Chuang, X.Y., Di Dong, C., 2017. Assessment of heavy metals in aquaculture fishes collected from southwest coast of Taiwan and human consumption risk. Int. Biodeterior. Biodegrad. 124, 314–325. https://doi.org/10.1016/j.ibiod.2017.04.003.

Juan, C., Torrens, G., González-Nicolau, M., Oliver, A., 2017. Diversity and regulation of intrinsic β-lactamases from non-fermenting and other Gram-negative opportunistic pathogens. FEMS Microbiol. Rev. 41, 781–815. https://doi.org/10.1093/femsre/fux043.

Kaas, R.S., Leekitcharoenphon, P., Aarestrup, F.M., Lund, O., 2014. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. PLoS One 9, e104984. https://doi.org/10.1371/journal.pone.0104984.

Koivula, T.T., Juvonen, R., Haikara, A., Suihko, M.L., 2006. Characterization of the brewery spoilage bacterium *Obesumbacterium proteus* by automated ribotyping and development of PCR methods for its biotype 1. J. Appl. Microbiol. 100, 398–406. https://doi.org/10.1111/j.1365-2672.2005.02794.x.

Kraychete, G.B., Campana, E.H., Picão, R.C., Bonelli, R.R., 2019. *qnrD*-harboring plasmids in *Providencia* spp. recovered from food and environmental Brazilian sources. Sci. Total Environ. 646, 1290–1292. https://doi.org/10.1016/j.scitotenv.2018.07.378.

Kubomura, A., Sekizuka, T., Onozuka, D., Murakami, K., Kimura, H., Sakaguchi, M., Oishi, K., Hirai, S., Kuroda, M., Okabe, N., 2020. Truncated class 1 integron gene cassette arrays contribute to antimicrobial resistance of diarrheagenic *Escherichia coli*. Biomed. Res. Int. 2020, 4908189. https://doi.org/10.1155/2020/4908189.

Lalaoui, R., Djukovic, A., Bakour, S., Hadjadj, L., Sanz, J., Salavert, M., López-Hontangas, J.L., Sanz, M.A., Ubeda, C., Rolain, J.M., 2019. Genomic characterization of *Citrobacter freundii* strains coproducing OXA-48 and VIM-1 carbapenemase enzymes isolated in leukemic patient in Spain. Antimicrob. Resist. Infect. Control 8, 167. https://doi.org/10.1186/s13756-019-0630-3.

Larsen, M.V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R.L., Jelsbak, L., Sicheritz-Pontén, T., Ussery, D.W., Aarestrup, F.M., Lund, O., 2012. Multilocus sequence typing of total-genome-sequenced bacteria. J. Clin. Microbiol. 50, 1355–1361. https://doi.org/10.1128/JCM.06094-11.

Larsen, M.V., Cosentino, S., Lukjancenko, O., Saputra, D., Rasmussen, S., Hasman, H., Sicheritz-Pontén, T., Aarestrup, F.M., Ussery, D.W., Lund, O., 2014. Benchmarking of methods for genomic taxonomy. J. Clin. Microbiol. 52, 1529–1539. https://doi.org/10.1128/JCM.02981-13.

Li, Y., Zhang, Y., Chen, M., Hu, J., Zhang, H., Xiang, Y., Yang, H., Qiu, S., Song, H., 2021. Plasmid-borne colistin resistance gene *mcr-1* in a multidrug resistant *Salmonella enterica* serovar Typhimurium isolate from an infant with acute diarrhea in China. Int. J. Infect. Dis. 103, 13–18. https://doi.org/10.1016/j.ijid.2020.11.150.

Libisch, B., Abdulkadir, S., Keresztény, T., Papp, P.P., Olasz, F., Fébel, H., Sándor, Z.J., Rasschaert, G., Lambrecht, E., Heyndrickx, M., Szabó, A., Kovács, M., Posta, K., 2022. Detection of acquired antibiotic resistance genes in domestic pig (*Sus scrofa*) and common carp (*Cyprinus carpio*) intestinal samples by metagenomics analyses in Hungary. Antibiotics 11, 1441. https://doi.org/10.3390/antibiotics11101441.

Lin, M., Yang, Y., Yang, Y., Chen, G., He, R., Wu, Y., Zhong, L.L., El-Sayed Ahmed, M.A. E., Feng, S., Shen, C., Wen, X., Huang, J., Li, H., Zheng, X., Tian, G.B., 2020. Co-occurrence of *mcr-9* and bla_{NDM-1} in *Enterobacter cloacae* isolated from a patient with bloodstream infection. Infect. Drug Resist. 13, 1397–1402. https://doi.org/10.2147/IDR.S248342.

Liu, S., Huang, N., Zhou, C., Lin, Y., Zhang, Y., Wang, L., Zheng, X., Zhou, T., Wang, Z., 2021. Molecular mechanisms and epidemiology of carbapenem-resistant *Enterobacter cloacae* complex isolated from Chinese patients during 2004–2018. Infect. Drug Resist. 14, 3647–3658. https://doi.org/10.2147/IDR.S327595.

Liu, Y., Wan, L.G., Deng, Q., Cao, X.W., Yu, Y., Xu, Q.F., 2015. First description of NDM-1-, KPC-2-, VIM-2- and IMP-4-producing *Klebsiella pneumoniae* strains in a single Chinese teaching hospital. Epidemiol. Infect. 143, 376–384. https://doi.org/10.1017/S0950268814000995.

Lupo, A., Coyne, S., Berendonk, T.U., 2012. Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. Front. Microbiol. 3, 18. https://doi.org/10.3389/fmicb.2012.00018.

Lv, J., Hu, R., Wang, N., Zhu, L., Zhang, X., Yuan, X., Liu, B., 2021. Distribution and movement of heavy metals in sediments around the coastal areas under the influence of multiple factors: a case study from the junction of the Bohai Sea and the Yellow Sea. Chemosphere 278, 130352. https://doi.org/10.1016/j.chemosphere.2021.130352.

Magaña-Lizárraga, J.A., Gómez-Gil, B., Rendón-Maldonado, J.G., Delgado-Vargas, F., Vega-López, I.F., Báez-Flores, M.E., 2022. Genomic profiling of antibiotic-resistant *Escherichia coli* isolates from surface water of agricultural drainage in North-Western Mexico: detection of the international high-risk lineages ST410 and ST617. Microorganisms 10, 662. https://doi.org/10.3390/microorganisms10030662.

Manageiro, V., Salgueiro, V., Rosado, T., Bandarra, N.M., Smith, T., Dias, E., Caniça, M., 2022. Genomic analysis of a *mcr-9.1*-harbouring IncHI2-ST1 plasmid from *Enterobacter ludwigii* isolated in fish farming. Antibiotics 11, 1232. https://doi.org/10.3390/antibiotics11091232.

Manges, A.R., Geum, H.M., Guo, A., Edens, T.J., Fibke, C.D., Pitout, J.D.D., 2019. Global extraintestinal pathogenic *Escherichia coli* (ExPEC) lineages. Clin. Microbiol. Rev. 32 https://doi.org/10.1128/CMR.00135-18 e00135-18.

Maravić, A., Skočibušić, M., Šamanić, I., Fredotović, Ž., Cvjetan, S., Jutronić, M., Puizina, J., 2013. *Aeromonas* spp. simultaneously harbouring $bla_{CTX-M-15}$, bla_{SHV-12}, bla_{PER-1} and bla_{FOX-2}, in wild-growing Mediterranean mussel (*Mytilus galloprovincialis*) from Adriatic Sea, Croatia. Int. J. Food Microbiol. 166, 301–308. https://doi.org/10.1016/j.ijfoodmicro.2013.07.010.

Massella, E., Reid, C.J., Cummins, M.L., Anantanawat, K., Zingali, T., Serraino, A., Piva, S., Giacometti, F., Djordjevic, S.P., 2020. Snapshot study of whole genome sequences of *Escherichia coli* from healthy companion animals, livestock, wildlife, humans and food in Italy. Antibiotics 9, 782. https://doi.org/10.3390/antibiotics9110782.

Mbelle, N.M., Feldman, C., Osei Sekyere, J., Maningi, N.E., Modipane, L., Essack, S.Y., 2019. The resistome, mobilome, virulome and phylogenomics of multidrug-resistant *Escherichia coli* clinical isolates from Pretoria, South Africa. Sci. Rep. 9, 16457. https://doi.org/10.1038/s41598-019-52859-2.

McDougall, F., Boardman, W., Power, M., 2022. High prevalence of beta-lactam-resistant *Escherichia coli* in South Australian Grey-headed flying fox pups (*Pteropus poliocephalus*). Microorganisms 10, 1589. https://doi.org/10.3390/microorganisms10081589.

McEwen, S.A., Collignon, P.J., 2018. Antimicrobial resistance: a one health perspective. Microbiol. Spectr. 6 https://doi.org/10.1128/microbiolspec.ARBA-0009-2017.

Medina-Pizzali, M.L., Venkatesh, A., Riveros, M., Cuicapuza, D., Salmon-Mulanovich, G., Mäusezahl, D., Hartinger, S.M., 2022. Whole-genome characterisation of ESBL-producing *E. coli* isolated from drinking water and dog faeces from rural Andean households in Peru. Antibiotics 11, 692. https://doi.org/10.3390/antibiotics11050692.

Meng, M., Li, Y., Yao, H., 2022. Plasmid-mediated transfer of antibiotic resistance genes in soil. Antibiotics 11, 525. https://doi.org/10.3390/antibiotics11040525.

Messaili, C., Messai, Y., Bakour, R., 2019. Virulence gene profiles, antimicrobial resistance and phylogenetic groups of fecal *Escherichia coli* strains isolated from broiler chickens in Algeria. Vet. Ital. 55, 35–46. https://doi.org/10.12834/VetIt.799.3865.2.

Michaud, L., Lo Giudice, A., Troussellier, M., Smedile, F., Bruni, V., Blancheton, J.P., 2009. Phylogenetic characterization of the heterotrophic bacterial communities inhabiting a marine recirculating aquaculture system. J. Appl. Microbiol. 107, 1935–1946. https://doi.org/10.1111/j.1365-2672.2009.04378.x.

Miranda, C.D., Kehrenberg, C., Ulep, C., Schwarz, S., Roberts, M.C., 2003. Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. Antimicrob. Agents Chemother. 47, 883–888. https://doi.org/10.1128/AAC.47.3.883-888.2003.

Møller, T.S.B., Overgaard, M., Nielsen, S.S., Bortolaia, V., Sommer, M.O.A., Guardabassi, L., Olsen, J.E., 2016. Relation between *tetR* and *tetA* expression in tetracycline resistant *Escherichia coli*. BMC Microbiol. 16, 39. https://doi.org/10.1186/s12866-016-0649-z.

Mon-On, N., Surachetpong, W., Mongkolsuk, S., Sirikanchana, K., 2018. Roles of water quality and disinfectant application on inactivation of fish pathogenic *Streptococcus agalactiae* with povidone iodine, quaternary ammonium compounds and glutaraldehyde. J. Fish Dis. 41, 783–789. https://doi.org/10.1111/jfd.12776.

Moreno-Switt, A.I., Pezoa, D., Sepúlveda, V., González, I., Rivera, D., Retamal, P., Navarrete, P., Reyes-Jara, A., Toro, M., 2019. Transduction as a potential dissemination mechanism of a clonal *qnrB19*-carrying plasmid isolated from *Salmonella* of multiple serotypes and isolation sources. Front. Microbiol. 10, 2503. https://doi.org/10.3389/fmicb.2019.02503.

Mori, N., Kagawa, N., Aoki, K., Ishi, Y., Tateda, K., Aoki, Y., 2020. Clinical and molecular analyses of bloodstream infections caused by IMP metallo-β-lactamase-producing *Enterobacteriaceae* in a tertiary hospital in Japan. J. Infect. Chemother. 26, 144–147. https://doi.org/10.1016/j.jiac.2019.07.017.

Müller, A., Stephan, R., Nüesch-Inderbinen, M., 2016. Distribution of virulence factors in ESBL-producing *Escherichia coli* isolated from the environment, livestock, food and humans. Sci. Total Environ. 541, 667–672. https://doi.org/10.1016/j.scitotenv.2015.09.135.

Muziasari, W.I., Pärnänen, K., Johnson, T.A., Lyra, C., Karkman, A., Stedtfeld, R.D., Tamminen, M., Tiedje, J.M., Virta, M., 2016. Aquaculture changes the profile of antibiotic resistance and mobile genetic element associated genes in Baltic Sea sediments. FEMS Microbiol. Ecol. 92, fiw052. https://doi.org/10.1093/femsec/fiw052.

Nabinger, D.D., Altenhofen, S., Bitencourt, P.E.R., Nery, L.R., Leite, C.E., Vianna, M.R.M. R., Bonan, C.D., 2018. Nickel exposure alters behavioral parameters in larval and adult zebrafish. Sci. Total Environ. 624, 1623–1633. https://doi.org/10.1016/j.scitotenv.2017.10.057.

Nadeem, S.F., Gohar, U.F., Tahir, S.F., Mukhtar, H., Pornpukdeewattana, S., Nukthamna, P., Moula Ali, A.M., Bavisetty, S.C.B., Massa, S., 2020. Antimicrobial resistance: more than 70 years of war between humans and bacteria. Crit. Rev. Microbiol. 46, 578–599. https://doi.org/10.1080/1040841X.2020.1813687.

Nadya, S., Delaquis, P., Chen, J., Allen, K., Johnson, R.P., Ziebell, K., Laing, C., Gannon, V., Bach, S., Topp, E., 2016. Phenotypic and genotypic characteristics of Shiga toxin-producing *Escherichia coli* isolated from surface waters and sediments in a Canadian Urban-agricultural landscape. Front. Cell. Infect. Microbiol. 6, 36. https://doi.org/10.3389/fcimb.2016.00036.

Ngo, T.P.H., Vu, H.T.T., Le, T.T.T., Bui, H.C.N., Liles, M.R., Rodkhum, C., 2022. Comparative genomic analysis of hypervirulent *Aeromonas hydrophila* strains from striped catfish (*Pangasianodon hypophthalmus*) in Vietnam. Aquaculture 558, 738364. https://doi.org/10.1016/j.aquaculture.2022.738364.

Nhinh, D.T., Le, D.V., Van Van, K., Giang, N.T.H., Dang, L.T., Hoai, T.D., 2021. Prevalence, virulence gene distribution and alarming the multidrug resistance of *Aeromonas hydrophila* associated with disease outbreaks in freshwater aquaculture. Antibiotics 10, 532. https://doi.org/10.3390/antibiotics10050532.

Nicolas, E., Lambin, M., Dandoy, D., Galloy, C., Nguyen, N., Oger, C.A., Hallet, B., 2015. The Tn*3*-family of replicative transposons. Microbiol. Spectr. 3 https://doi.org/10.1128/microbiolspec.MDNA3-0060-2014.

Olszak, T., Latka, A., Roszniowski, B., Valvano, M.A., Drulis-Kawa, Z., 2017. Phage life cycles behind bacterial biodiversity. Curr. Med. Chem. 24, 3987–4001. https://doi.org/10.2174/0929867324666170413100136.

Ottinger, M., Clauss, K., Kuenzer, C., 2016. Aquaculture: relevance, distribution, impacts and spatial assessments - a review. Ocean Coast. Manag. 119, 244–266. https://doi.org/10.1016/j.ocecoaman.2015.10.015.

Pal, C., Bengtsson-Palme, J., Rensing, C., Kristiansson, E., Larsson, D.G.J., 2014. BacMet: antibacterial biocide and metal resistance genes database. Nucleic Acids Res. 42, D737–D743. https://doi.org/10.1093/nar/gkt1252.

Pallecchi, L., Riccobono, E., Sennati, S., Mantella, A., Bartalesi, F., Trigoso, C., Gotuzzo, E., Bartoloni, A., Rossolini, G.M., 2010. Characterization of small ColE-like plasmids mediating widespread dissemination of the *qnrB19* gene in commensal enterobacteria. Antimicrob. Agents Chemother. 54, 678–682. https://doi.org/10.1128/AAC.01160-09.

Partridge, S.R., Kwong, S.M., Firth, N., Jensen, S.O., 2018. Mobile genetic elements associated with antimicrobial resistance. Clin. Microbiol. Rev. 31 https://doi.org/10.1128/CMR.00088-17 e00088-17.

Pattanayak, S., Priyadarsini, S., Paul, A., Kumar, P.R., Sahoo, P.K., 2020. Diversity of virulence-associated genes in pathogenic *Aeromonas hydrophila* isolates and their in vivo modulation at varied water temperatures. Microb. Pathog. 147, 104424 https://doi.org/10.1016/j.micpath.2020.104424.

Permana, B., Beatson, S.A., Forde, B.M., 2023. GraphSNP: an interactive distance viewer for investigating outbreaks and transmission networks using a graph approach. BMC Bioinform. 24, 209. https://doi.org/10.1186/s12859-023-05332-x.

Pham, T.T.H., Rossi, P., Dinh, H.D.K., Pham, N.T.A., Tran, P.A., Ho, T.T.K.M., Dinh, Q.T., De Alencastro, L.F., 2018. Analysis of antibiotic multi-resistant bacteria and resistance genes in the effluent of an intensive shrimp farm (Long An, Vietnam). J. Environ. Manag. 214, 149–156. https://doi.org/10.1016/j.jenvman.2018.02.089.

Piotrowska, M., Przygodzinska, D., Matyjewicz, K., Popowska, M., 2017. Occurrence and variety of ß-lactamase genes among *Aeromonas* spp. isolated from urban wastewater treatment plant. Front. Microbiol. 8, 863. https://doi.org/10.3389/fmicb.2017.00863.

Poirel, L., Rodriguez-Martinez, J.M., Mammeri, H., Liard, A., Nordmann, P., 2005. Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob. Agents Chemother. 49, 3523–3525. https://doi.org/10.1128/AAC.49.8.3523-3525.2005.

Poirel, L., Potron, A., Nordmann, P., 2012. OXA-48-like carbapenemases: the phantom menace. J. Antimicrob. Chemother. 67, 1597–1606. https://doi.org/10.1093/jac/dks121.

Posada-Perlaza, C.E., Ramírez-Rojas, A., Porras, P., Adu-Oppong, B., Botero-Coy, A.M., Hernández, F., Anzola, J.M., Díaz, L., Dantas, G., Reyes, A., Zambrano, M.M., 2019. Bogotá River anthropogenic contamination alters microbial communities and promotes spread of antibiotic resistance genes. Sci. Rep. 9, 11764. https://doi.org/10.1038/s41598-019-48200-6.

Ragab, W., Kawato, S., Nozaki, R., Kondo, H., Hirono, I., 2022. Comparative genome analyses of five *Vibrio penaeicida* strains provide insights into their virulence-related factors. Microb. Genom. 8, 000766 https://doi.org/10.1099/mgen.0.000766.

Ramsamy, Y., Mlisana, K.P., Amoako, D.G., Allam, M., Ismail, A., Singh, R., Abia, A.L.K., Essack, S.Y., 2020. Pathogenomic analysis of a novel extensively drug-resistant *Citrobacter freundii* isolate carrying a bla_{NDM-1} carbapenemase in South Africa. Pathogens 9, 89. https://doi.org/10.3390/pathogens9020089.

Ranquet, C., Toussaint, A., De Jong, H., Maenhaut-Michel, G., Geiselmann, J., 2005. Control of bacteriophage Mu lysogenic repression. J. Mol. Biol. 353, 186–195. https://doi.org/10.1016/j.jmb.2005.08.015.

Razak, M.R., Aris, A.Z., Zakaria, N.A.C., Wee, S.Y., Ismail, N.A.H., 2021. Accumulation and risk assessment of heavy metals employing species sensitivity distributions in Linggi River, Negeri Sembilan, Malaysia. Ecotoxicol. Environ. Saf. 211, 111905 https://doi.org/10.1016/j.ecoenv.2021.111905.

Reid, C.J., Cummins, M.L., Börjesson, S., Brouwer, M.S.M., Hasman, H., Hammerum, A. M., Roer, L., Hess, S., Berendonk, T., Nešporová, K., Haenni, M., Madec, J.Y., Bethe, A., Michael, G.B., Schink, A.K., Schwarz, S., Dolejska, M., Djordjevic, S.P., 2022. A role for ColV plasmids in the evolution of pathogenic *Escherichia coli* ST58. Nat. Commun. 13, 683. https://doi.org/10.1038/s41467-022-28342-4.

Richter, S.S., Sercia, L., Branda, J.A., Burnham, C.A.D., Bythrow, M., Ferraro, M.J., Garner, O.B., Ginocchio, C.C., Jennemann, R., Lewinski, M.A., Manji, R., Mochon, A.

B., Rychert, J.A., Westblade, L.F., Procop, G.W., 2013. Identification of *Enterobacteriaceae* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using the VITEK MS system. Eur. J. Clin. Microbiol. Infect. Dis. 32, 1571–1578. https://doi.org/10.1007/s10096-013-1912-y.

Rodríguez-Villodres, Á., Bonnin, R.A., de la Rosa, J.M.O., Álvarez-Marín, R., Naas, T., Aznar, J., Pachón, J., Lepe, J.A., Smani, Y., 2019. Phylogeny, resistome, and virulome of *Escherichia coli* causing biliary tract infections. J. Clin. Med. 8, 2118. https://doi.org/10.3390/jcm8122118.

Roschanski, N., Guenther, S., Vu, T.T.T., Fischer, J., Semmler, T., Huehn, S., Alter, T., Roesler, U., 2017. VIM-1 carbapenemase-producing *Escherichia coli* isolated from retail seafood, Germany 2016. Eurosurveillance 22, 17–00032. https://doi.org/10.2807/1560-7917.ES.2017.22.43.17-00032.

Ross, K., Varani, A.M., Snesrud, E., Huang, H., Alvarenga, D.O., Zhang, J., Wu, C., McGann, P., Chandlere, M., 2021. TnCentral: a prokaryotic transposable element database and web portal for transposon analysis. MBio 12, e0206021. https://doi.org/10.1128/mBio.02060-21.

Rubio-Portillo, E., Martin-Cuadrado, A.B., Caraballo-Rodríguez, A.M., Rohwer, F., Dorrestein, P.C., Antón, J., 2020. Virulence as a side effect of interspecies interaction in *Vibrio* coral pathogens. MBio 11. https://doi.org/10.1128/mBio.00201-20 e00201-20.

Ryu, A.R., Mok, J.S., Lee, D.E., Kwon, J.Y., Park, K., 2019. Occurrence, virulence, and antimicrobial resistance of *Vibrio parahaemolyticus* isolated from bivalve shellfish farms along the southern coast of Korea. Environ. Sci. Pollut. Res. 26, 21034–21043. https://doi.org/10.1007/s11356-019-05426-1.

Sabri, M., Léveillé, S., Dozois, C.M., 2006. A SitABCD homologue from an avian pathogenic *Escherichia coli* strain mediates transport of iron and manganese and resistance to hydrogen peroxide. Microbiology 152, 745–758. https://doi.org/10.1099/mic.0.28682-0.

Said, L. Ben, Hamdaoui, M., Jouini, A., Boudabous, A., Slama, K. Ben, Torres, C., Klibi, N., 2017. First detection of CTX-M-1 in extended-spectrum β-lactamase-producing *Escherichia coli* in seafood from Tunisia. J. Food Prot. 80, 1877–1881. https://doi.org/10.4315/0362-028X.JFP-16-324.

Salgueiro, V., Manageiro, V., Bandarra, N.M., Reis, L., Caniça, M., 2020. Bacterial diversity and antibiotic susceptibility of *Sparus aurata* from aquaculture. Microorganisms 8, 1343. https://doi.org/10.3390/microorganisms8091343.

Salgueiro, V., Reis, L., Botelho, M.J., Manageiro, V., Caniça, M., 2021. Assessing the bacterial community composition of bivalve mollusks collected in aquaculture farms and respective susceptibility to antibiotics. Antibiotics 10, 1135. https://doi.org/10.3390/antibiotics10091135.

Salgueiro, V., Manageiro, V., Bandarra, N.M., Ferreira, E., Clemente, L., Caniça, M., 2023. First comparative genomic characterization of the MSSA ST398 lineage detected in aquaculture and other reservoirs. Front. Microbiol. 14, 1035547. https://doi.org/10.3389/fmicb.2023.1035547.

Santos, L., Ramos, F., 2018. Antimicrobial resistance in aquaculture: current knowledge and alternatives to tackle the problem. Int. J. Antimicrob. Agents 52, 135–143. https://doi.org/10.1016/j.ijantimicag.2018.03.010.

Schürch, A.C., Arredondo-Alonso, S., Willems, R.J.L., Goering, R.V., 2018. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. Clin. Microbiol. Infect. 24, 350–354. https://doi.org/10.1016/j.cmi.2017.12.016.

Shah, S.Q.A., Cabello, F.C., L'Abée-Lund, T.M., Tomova, A., Godfrey, H.P., Buschmann, A.H., Sørum, H., 2014. Antimicrobial resistance and antimicrobial resistance genes in marine bacteria from salmon aquaculture and non-aquaculture sites. Environ. Microbiol. 16, 1310–1320. https://doi.org/10.1111/1462-2920.12421.

Sherpa, M.T., Najar, I.N., Das, S., Thakur, N., 2020. Distribution of antibiotic and metal resistance genes in two glaciers of North Sikkim, India. Ecotoxicol. Environ. Saf. 203, 111037 https://doi.org/10.1016/j.ecoenv.2020.111037.

Sinha, R.K., Krishnan, K.P., Kurian, P.J., 2021. Complete genome sequence of *Alteromonas pelagimontana* 5.12T, a marine exopolysaccharide-producing bacterium isolated from hydrothermally influenced deep-sea sediment of eastern Southwest Indian Ridge. Mar. Genomics 55, 100804. https://doi.org/10.1016/j.margen.2020.100804.

Sivaraman, G.K., Rajan, V., Vijayan, A., Elangovan, R., Prendiville, A., Bachmann, T.T., 2021. Antibiotic resistance profiles and molecular characteristics of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from shrimp aquaculture farms in Kerala, India. Front. Microbiol. 12, 622891 https://doi.org/10.3389/fmicb.2021.622891.

Su, H., Liu, S., Hu, X., Xu, X., Xu, W., Xu, Y., Li, Z., Wen, G., Liu, Y., Cao, Y., 2017. Occurrence and temporal variation of antibiotic resistance genes (ARGs) in shrimp aquaculture: ARGs dissemination from farming source to reared organisms. Sci. Total Environ. 607–608, 357–366. https://doi.org/10.1016/j.scitotenv.2017.07.040.

Sukupolvi, S., O'Connor, C.D., 1990. TraT lipoprotein, a plasmid-specified mediator of interactions between gram-negative bacteria and their environment. Microbiol. Rev. 54, 331–341. https://doi.org/10.1128/mr.54.4.331-341.1990.

Sultan, I., Ali, A., Gogry, F.A., Rather, I.A., Sabir, J.S.M., Haq, Q.M.R., 2020. Bacterial isolates harboring antibiotics and heavy-metal resistance genes co-existing with mobile genetic elements in natural aquatic water bodies. Saudi J. Biol. Sci. 27, 2660–2668. https://doi.org/10.1016/j.sjbs.2020.06.002.

Suzuki, S., Kadoya, A., Masuda, N., Sugimoto, Y., Takada, H., Mizukawa, K., Takei, A., Chou, H.Y., Wu, J.H., 2022. Macrolide resistance genes and mobile genetic elements in waterways from pig farms to the sea in Taiwan. J. Glob. Antimicrob. Resist. 29, 360–370. https://doi.org/10.1016/j.jgar.2022.04.024.

Tamminen, M., Karkman, A., Lõhmus, A., Muziasari, W.I., Takasu, H., Wada, S., Suzuki, S., Virta, M., 2011. Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. Environ. Sci. Technol. 45, 386–391. https://doi.org/10.1021/es102725n.

Tang, X., Wang, H., Liu, F., Sheng, X., Xing, J., Zhan, W., 2019. Recombinant outer membrane protein T (OmpT) of *Vibrio ichthyoenteri*, a potential vaccine candidate for flounder (*Paralichthys olivaceus*). Microb. Pathog. 126, 185–192. https://doi.org/10.1016/j.micpath.2018.11.001.

Tetzschner, A.M.M., Johnson, J.R., Johnston, B.D., Lund, O., Scheutz, F., 2020. *In Silico* genotyping of *Escherichia coli* isolates for extraintestinal virulence genes by use of whole-genome sequencing data. J. Clin. Microbiol. 58 https://doi.org/10.1128/JCM.01269-20 e01269-20.

Tomova, A., Ivanova, L., Buschmann, A.H., Rioseco, M.L., Kalsi, R.K., Godfrey, H.P., Cabello, F.C., 2015. Antimicrobial resistance genes in marine bacteria and human uropathogenic *Escherichia coli* from a region of intensive aquaculture. Environ. Microbiol. Rep. 7, 803–809. https://doi.org/10.1111/1758-2229.12327.

Topp, E., Larsson, D.G.J., Miller, D.N., Van den Eede, C., Virta, M.P.J., 2018. Antimicrobial resistance and the environment: assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. FEMS Microbiol. Ecol. 94, fix185. https://doi.org/10.1093/femsec/fix185.

Tran, T., Andres, P., Petroni, A., Soler-Bistué, A., Albornoz, E., Zorreguieta, A., Reyes-Lamothe, R., Sherratt, D.J., Corso, A., Tolmaskya, M.E., 2012. Small plasmids harboring *qnrB19*: a model for plasmid evolution mediated by site-specific recombination at *oriT* and Xer sites. Antimicrob. Agents Chemother. 56, 1821–1827. https://doi.org/10.1128/AAC.06036-11.

van der Graaf-Van Bloois, L., Wagenaar, J.A., Zomer, A.L., 2021. RFPlasmid: predicting plasmid sequences from short-read assembly data using machine learning. Microb. Genom. 7, 000683 https://doi.org/10.1099/mgen.0.000683.

Varani, A.M., Siguier, P., Gourbeyre, E., Charneau, V., Chandler, M., 2011. ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. Genome Biol. 12, R30. https://doi.org/10.1186/gb-2011-12-3-r30.

Vignaroli, C., Luna, G.M., Rinaldi, C., Di Cesare, A., Danovaro, R., Biavasco, F., 2012. New sequence types and multidrug resistance among pathogenic *Escherichia coli* isolates from coastal marine sediments. Appl. Environ. Microbiol. 78, 3916–3922. https://doi.org/10.1128/AEM.07820-11.

Vignaroli, C., Di Sante, L., Leoni, F., Chierichetti, S., Ottaviani, D., Citterio, B., Biavasco, F., 2016. Multidrug-resistant and epidemic clones of *Escherichia coli* from natural beds of Venus clam. Food Microbiol. 59, 1–6. https://doi.org/10.1016/j.fm.2016.05.003.

Villa, J., Arana, D.M., Viedma, E., Perez-Montarelo, D., Chaves, F., 2017. Characterization of mobile genetic elements carrying VIM-1 and KPC-2 carbapenemases in *Citrobacter freundii* isolates in Madrid. Int. J. Med. Microbiol. 307, 340–345. https://doi.org/10.1016/j.ijmm.2017.07.001.

Vingino, A., Roberts, M.C., Wainstein, M., West, J., Norman, S.A., Lambourn, D., Lahti, J., Ruiz, R., D'angeli, M., Weissman, S.J., Rabinowitz, P., 2021. Surveillance for antibiotic-resistant *E. coli* in the Salish Sea Ecosystem. Antibiotics 10, 1201. https://doi.org/10.3390/antibiotics10101201.

Vu, T.T.T., Alter, T., Roesler, U., Roschanski, N., Huehn, S., 2018. Investigation of extended-spectrum and AmpC β-lactamase-producing *Enterobacteriaceae* from retail seafood in Berlin, Germany. J. Food Prot. 81, 1079–1086. https://doi.org/10.4315/0362-028X.JFP-18-029.

Výrostková, J., Pipová, M., Semjon, B., Jevinová, P., Regecová, I., Malová, J., 2020. Antibacterial effects of hydrogen peroxide and caprylic acid on selected foodborne bacteria. Pol. J. Vet. Sci. 23, 439–446. https://doi.org/10.24425/pjvs.2020.134689.

Walckenaer, E., Poirel, L., Leflon-Guibout, V., Nordmann, P., Nicolas-Chanoine, M.H., 2004. Genetic and biochemical characterization of the chromosomal class A β-lactamases of *Raoultella* (formerly *Klebsiella*) *planticola* and *Raoultella ornithinolytica*. Antimicrob. Agents Chemother. 48, 305–312. https://doi.org/10.1128/AAC.48.1.305-312.2004.

Walckenaer, E., Delmas, J., Leflon-Guibout, V., Bonnet, R., Nicolas-Chanoine, M.H., 2015. Genetic, biochemical characterization and mutagenesis of the chromosomal class A β-lactamase of *Raoultella* (formerly *Klebsiella*) *terrigena*. Pathol. Biol. 63, 158–163. https://doi.org/10.1016/j.patbio.2015.05.002.

Wang, C., Wang, Y., Liu, P., Sun, Y., Song, Z., Hu, X., 2021. Characteristics of bacterial community structure and function associated with nutrients and heavy metals in coastal aquaculture area. Environ. Pollut. 275, 116639 https://doi.org/10.1016/j.envpol.2021.116639.

Wang, D., Fierke, C.A., 2013. The BaeSR regulon is involved in defense against zinc toxicity in *E. coli*. Met. Integr. Biometal Sci. 5, 372–383. https://doi.org/10.1039/c3mt20217h.

Wanyan, R., Pan, M., Mai, Z., Xiong, X., Su, W., Yang, J., Yu, Q., Wang, X., Han, Q., Li, H., Wang, G., Wu, S., 2023. Distribution and influencing factors of antibiotic resistance genes of crayfish (*Procambarus clarkii*) intestine in main crayfish breeding provinces in China. Sci. Total Environ. 857, 159611 https://doi.org/10.1016/j.scitotenv.2022.159611.

Wendling, C.C., Refardt, D., Hall, A.R., 2021. Fitness benefits to bacteria of carrying prophages and prophage-encoded antibiotic-resistance genes peak in different environments. Evolution (N. Y) 75, 515–528. https://doi.org/10.1111/evo.14153.

Ye, L., Zhang, L., Li, X., Shi, L., Huang, Y., Wang, H.H., 2013. Antibiotic-resistant bacteria associated with retail aquaculture products from Guangzhou, China. J. Food Prot. 76, 295–301. https://doi.org/10.4315/0362-028X.JFP-12-288.

Zago, V., Veschetti, L., Patuzzo, C., Malerba, G., Lleo, M.M., 2020. Resistome, mobilome and virulome analysis of *Shewanella algae* and *Vibrio* spp. strains isolated in Italian aquaculture centers. Microorganisms 8, 572. https://doi.org/10.3390/microorganisms8040572.

Zagui, G.S., Moreira, N.C., Santos, D.V., Darini, A.L.C., Domingo, J.L., Segura-Muñoz, S.I., Andrade, L.N., 2021. High occurrence of heavy metal tolerance genes in bacteria

isolated from wastewater: a new concern? Environ. Res. 196, 110352 https://doi.org/10.1016/j.envres.2020.110352.

Zhang, R.Q., Ying, G.G., Su, H.C., Zhou, L.J., Liu, Y.S., 2013. Antibiotic resistance and genetic diversity of *Escherichia coli* isolates from traditional and integrated aquaculture in South China. J. Environ. Sci. Health - B Pestic. Food Contam. Agric. Wastes 48, 999–1013. https://doi.org/10.1080/03601234.2013.816611.

Zhang, Y., Zhao, L., Chen, W., Huang, Y., Yang, L., Sarathbabu, V., Wu, Z., Li, J., Nie, P., Lin, L., 2017. Complete genome sequence analysis of the fish pathogen *Flavobacterium columnare* provides insights into antibiotic resistance and pathogenicity related genes. Microb. Pathog. 111, 203–211. https://doi.org/10.1016/j.micpath.2017.08.035.

Zhao, H., Chen, W., Xu, X., Zhou, X., Shi, C., 2018. Transmissible ST3-IncHI2 plasmids are predominant carriers of diverse complex IS*26*-class 1 integron arrangements in multidrug-resistant *Salmonella*. Front. Microbiol. 9, 2492. https://doi.org/10.3389/fmicb.2018.02492.

Zheng, X., Ma, J., Lu, Y., Sun, D., Yang, H., Xia, F., Tang, B., 2022. Detection of *tet(X6)* variant–producing *Proteus terrae* subsp. *cibarius* from animal cecum in Zhejiang, China. J. Glob. Antimicrob. Resist. 29, 124–130. https://doi.org/10.1016/j.jgar.2022.02.011.

Zhou, Q., Wang, M., Zhong, X., Liu, P., Xie, X., Wangxiao, J., Sun, Y., 2019. Dissemination of resistance genes in duck/fish polyculture ponds in Guangdong Province: correlations between Cu and Zn and antibiotic resistance genes. Environ. Sci. Pollut. Res. 26, 8182–8193. https://doi.org/10.1007/s11356-018-04065-2.