NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTMENT OF LIFE SCIENCES

DECRYPTING THE DIVERSITY OF MICROBIOME IN AQUACULTURE

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Master in Human Biology and Environment

DOCTORATE IN BIOLOGY

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Decrypting the diversity of microbiome in aquaculture

To my parents.

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"(…) a caligrafia é uma lição de humildade. Não pode haver vaidade no alfabeto. A letra que, nesta frase, é maiúscula, na frase seguinte, já desceu do pedestal. Somos como as letras: o tamanho que temos depende dos outros que, junto connosco, costuram as frases."

(Mia Couto, Lição de caligrafia. In *Visão Nº 1519*, p.8).

Abstract

Aquaculture can play an important role in reducing the overexploitation of natural resources and feeding the world's growing population. However, the use of e.g., antibiotics in aquaculture can favor the development of resistant bacteria and jeopardize the safety of its products. Thus, this Ph.D. thesis aimed to contribute to the deciphering of aquaculture's microbiome and resistome, as well as to the understanding of the role of mobile genetic elements (MGE) in the dissemination of resistance genes in these environments. Several approaches were used, to obtain the results that most reflect the microbiome and resistome of seabream and bivalve mollusks from aquaculture. All microbiomes studied were very diverse, encompassing commensal and pathogenic bacteria from seabream and bivalve mollusks (e.g., *Aeromonas*, *Kocuria*, *Pseudomonas* and *Vibrio* genera), as well as bacteria important in human medicine (e.g., *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*). Twenty-one new sequence types were described in *Aeromonas* spp., *Citrobacter* sp., *Enterobacter* spp., *Shewanella* spp., *Staphylococcus* sp. and *Vibrio* spp. Decreased susceptibilities to phenicols, oxytetracycline, β-lactams (namely carbapenems), quinolones, glycopeptides, mupirocin, erythromycin, and colistin were found. The resistome also revealed a great diversity of genes in all samples studied associated with antibiotics (e.g., $bla_{\text{TEM-1B}}$, *mecA*, *sul2*, *mcr-9.1*), disinfectants (e.g., *formA*-type), and heavy metals (e.g., *sil*) resistance. Twenty-five different genes related with increased virulence were also detected. Thirteen new β-lactams resistance genes were identified (e.g., $bla_{\text{CTX-M-246}}$, $bla_{\text{FOX-18}}$, and $bla_{\text{OXA-958}}$) and 35 other resistance genes, namely for antibiotics (e.g., *mcr-9* and *qnrD2*), heavy metals (e.g., *emrA* and *mdtE*) and disinfectants (*sitABCD*-type), and virulence factors (e.g., *astA* and *hlyF*) were here described for the first time associated with aquaculture. Our results suggest that some of these resistance genes (e.g., *erm(T)*-type, *qnrB19*, *catA1*-type, *tet(A)*, *dfrA*-type, *aph(6)-Id*, *qacEΔ1* and *merA*) are being disseminated by MGE such as plasmids, class 1 integrons, and Tn*As1*. These findings not only expand our knowledge about aquaculture's microbiome and resistome, but also provide the necessary

information to implement the most suitable measures to control antibiotic resistance in aquaculture environments.

Keywords: microbiome, resistome, mobilome, antibiotic resistance, aquaculture, seabream, bivalve mollusks.

RESUMO

A aquacultura pode desempenhar um papel importante na redução da sobre-exploração dos recursos naturais e no fornecimento de alimentos para a crescente população mundial. No entanto, o uso de, por exemplo, antibióticos em aquacultura pode favorecer o desenvolvimento de bactérias resistentes e comprometer a segurança dos seus produtos. Assim, esta tese de Doutoramento teve como objetivo contribuir para a decifração do microbioma e resistoma em aquacultura, bem como compreender o papel dos elementos genéticos móveis na disseminação de genes de resistência nestes ambientes. Foram usadas diversas abordagens com o intuito de obter os resultados que melhor refletem o microbioma e o resistoma das douradas e moluscos bivalves de aquacultura. Os microbiomas estudados apresentaram uma grande diversidade, englobando bactérias comensais e patogénicas das douradas e moluscos bivalves (ex.: os géneros *Aeromonas*, *Kocuria*, *Pseudomonas* e *Vibrio*), assim como bactérias importantes em medicina humana (ex.: *Staphylococcus aureus*, *Escherichia coli* e *Klebsiella pneumoniae*). Foram descritos 21 novos *sequence type* em *Aeromonas* spp., *Citrobacter* sp., *Enterobacter* spp., *Shewanella* spp., *Staphylococcus* sp. e *Vibrio* spp. Foram encontradas suscetibilidades diminuídas aos fenicois, oxitetraciclina, β-lactâmicos (como carbapenemes), quinolonas, glicopéptidos, mupirocina, eritromicina e colistina. Também os resistomas revelaram uma grande diversidade de genes associados a resistências aos antibióticos (ex.: $bla_{\text{TEM-1B}}$, *mecA*, *sul2*, *mcr-9.1*), desinfetantes (ex.: *formA*-type) e metais pesados (ex.: *sil*). Foram também detetados 25 genes diferentes, associados a uma maior virulência. Encontram-se aqui descritos 13 novos genes de resistência aos β-lactâmicos (ex.: $bla_{\text{CTX-M-246}}$, $bla_{\text{FOX-18}}$, $bla_{\text{OXA-958}}$) e outros 35 genes de resistência, nomeadamente aos antibióticos (ex.: *mcr-9* e *qnrD2*), metais pesados (ex.: *emrA* e *mdtE*) e desinfetantes (*sitABCD*-type), e fatores de virulência (ex.: *astA* e *hlyF*) são aqui identificados pela primeira vez associados à aquacultura. Os nossos resultados sugerem que alguns destes genes de resistência (como *erm(T)*-type, *qnrB19*, *catA1*-type, *tet(A)*, *dfrA*-type,

aph(6)-Id, *qacEΔ1* e *merA*) estarão a ser disseminados por elementos genéticos móveis, como plasmídeos, integrões de classe 1 e Tn*As1*. Estes estudos não só ampliam o nosso conhecimento sobre o microbioma e o resistoma em aquacultura, mas também providenciam a informação necessária para a implementação das medidas mais adequadas ao controlo da resistência aos antibióticos em aquacultura.

Palavras chave: microbioma, resistoma, mobiloma, resistência aos antibióticos, aquacultura, douradas, moluscos bivalves.

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Abbreviations, Acronyms, and Initialisms

A - Autumn.

ABC - ATP-binding cassette.

ABX - Antibiotics.

Acc. – Accession.

ACCs - Aminoglycoside *N*-acetyltransferases.

***agr* -** Accessory gene regulator.

AIP - Autoinducing peptide.

AMC - Amoxicillin/clavulanic acid.

AMP - Ampicillin.

AMR – Antimicrobial resistance.

AMX - Amoxicillin.

AN - Amikacin.

ANI - Average nucleotide identity

ANTs - Aminoglycoside *O*-nucleotidyltransferases.

AP - Alignment percentage.

APHs - Aminoglycoside *O*-phosphotransferases.

AR - Antibiotic resistance.

ARG - Antibiotic resistance genes

ARO – Antibiotic resistance ontology.

ATP - Adenosine triphosphate.

Att - Phage attachment site.

***attC* -** Attachment site of the integrons cassette.

***attI* -** Attachment site of the integron.

AZT - Aztreonam.

B.C. - Before Christ.

BLAST – Basic local alignment search tool.

bp - Base pairs.

BPN – Benzylpenicillin.

BRIG - BLAST ring image generator.

C - Predicted chromosomal location.

CA – California.

CA-MRSA - Community-acquired MRSA.

CARD - Comprehensive antibiotic resistance database.

CASFM VET - Comité de l'antibiogramme de la Société Française de Microbiologie Recommandations Vétérinaires.

CATs - Chloramphenicol *O*-acetyltransferases.

CAZ - Ceftazidime.

CC(s) - Clonal Complex(es).

CDSs – Coding sequences.

CDT - Combined disk test.

CGE - Center for genomic epidemiology.

CHL - Chloramphenicol.

CI - Confidence intervals.

CIP - Ciprofloxacin.

CLSI - Clinical and Laboratory Standards Institute.

Coa - Phage coat protein.

COL - Colistin.

CSI Phylogeny - Call SNPs & infer phylogeny.

CTX - Cefotaxime.

dATP - Deoxyadenosine triphosphate.

DCT - Disc combination test.

dCTP - Deoxycytidine triphosphate.

DDST - Double disc synergy test.

dGTP - Deoxyguanosine triphosphate.

DHF - Dihydrofolate.

DHFR - Dihydrofolate reductase.

DHPS - Dihydropteroate synthetase.

DNA - Deoxyribonucleic acid.

dNTPs - Deoxyribonucleotides triphosphates.

DOR - Doripenem.

DPC - Daptomycin.

DRG - Disinfectant resistance genes.

dTTP - Deoxythymidine triphosphate.

EAEC - Enteroaggregative *Escherichia coli*.

ECCMID - European Congress of Clinical Microbiology and Infectious Diseases.

ECDC - European Centre for Disease Prevention and Control.

ECOFFs - Epidemiological cut-off values

EF-G - Elongation factor G.

EFSA - European Food Safety Authority.

e.g. - *exempli gratia* (for example).

EHEC - Enterohaemorrhagic *E. coli*.

EIEC - Enteroinvasive *E. coli*.

EJP – European Joint Programme.

EMA - European Medicines Agency.

EPEC - Enteropathogenic *E. coli*.

ERT - Ertapenem.

ERY – Erythromycin.

ESBL - Extended-spectrum β-lactamase.

et al. - *et alii* (and other people)

etc. - *et cetera*.

ETEC - Enterotoxigenic *E. coli*.

EUCAST - European Committee on Antimicrobial Susceptibility Testing.

ExPEC - Extraintestinal pathogenic *E. coli*.

FAO - Food and Agricultural Organization of the United Nations.

FCT - Fundação para a Ciência e a Tecnologia.

FEP - Cefepime.

Fib - Phage tail fiber.

FLO - Florfenicol.

FMQ - Flumequine.

FOX - Cefoxitin.

FUS - Fusidic acid.

GC - Gene cassette.

GEN - Gentamycin.

GNAT - Gcn5-related *N*-acetyltransferase.

GRD - Glycopeptide resistance detection.

GTP - Guanosine triphosphate.

GTPases - GTP hydrolases.

HC - High concentration.

hGISA - Heterogeneous glycopeptide-intermediate *Staphylococcus aureus*.

HMM - Hidden Markov models.

HMRG - Heavy metals resistance genes.

HPP - Human pathogen probability.

HGT – Horizontal gene transfer

hVISA - Heterogeneous-vancomycin-intermediate *S. aureus*.

Hyp - Hypothetical protein.

I - Intermediate/susceptible, increased exposure.

IC²AR - International Caparica Conference in Antibiotic Resistance.

i.e. - *id est* (that is).

IEC - Immune evasion cluster.

IgG - Immunoglobulin G.

iMLS$_B$ - Inducible macrolide-lincosamide-streptogramin B resistance.

IMP - Imipenem.

In - Integrons.

Int - Phage integrase.

IR - Inverted repeats.

IS - Insertion sequences.

IS*CRs* - Insertion sequence common regions.

INIAV - National Institute of Agrarian and Veterinary Research.

INSA - National Institute of Health Dr. Ricardo Jorge.

IPMA - Portuguese Institute of Sea and Atmosphere.

LA-MRSA - Livestock-associated MRSA.

LEV - Levofloxacin.

LNZ - Linezolid.

LPS - Lipopolysaccharide.

MA – Massachusetts.

MALDI-TOF - Matrix-assisted laser desorption ionization–time of flight.

MATE - Multidrug and toxic compound extrusion.

MBL - Metallo-β-lactamase.

MCTES - Ministério da Ciência, Tecnologia e Ensino Superior.

MEM - Meropenem.

MFS - Major facilitator superfamily.

MGE - Mobile genetic elements.

MIC - Minimum inhibitory concentration.

MICROBIOTEC - Congress of Microbiology and Biotechnology.

MITE(s) - Miniature inverted-repeat transposable element(s).

MLST - Multilocus sequence typing.

MO – Missouri.

MOX - Moxifloxacin.

mRNA - Messenger RNA.

MRSA - Methicillin resistant *S. aureus*.

MSSA - Methicillin susceptible *S. aureus*.

MST - Minimum spanning tree.

MUP - Mupirocin.

n.a. - Data not available.

NA - Not applicable.

NAL - Nalidixic acid.

NCBI – National center for biotechnology information.

ND – Not detected.

NET - Netilmicin.

No. - Number.

NS - Non-susceptibility.

NT - Not typable.

OMPs - Outer membrane proteins.

OR - Odds Ratio.

OTC - Oxytetracycline.

OTUs - Operational taxonomic units

P - Predicted plasmid location.

PABA - Para-aminobenzoic acid.

PBPs - Penicillin binding proteins.

Pc - Promotor of cassette.

PCR - Polymerase chain reaction.

PFGE - Pulsed-field gel electrophoresis.

Pla - Phage plate protein.

PLP - Phage-like protein.

PMCR - Plasmid mediated colistin resistance.

pMLST – plasmid MLST.

PMQR(s) - Plasmid-mediated quinolone resistance(s).

POCH - Programa Operacional do Capital Humano.

Por - Portal protein.

PTC - Peptidyltransferase center.

PTZ - Piperacillin/tazobactam.

QRDRs - Quinolone resistance determining regions.

R - Resistant.

RFLP - Restriction fragment length polymorphism.

RGI - Resistance gene identifier.

RIF - Rifampicin.

RNA - Ribonucleic acid.

RND - Resistance-nodulation-cell division.

ROS - Reactive oxygen species.

RPPs - Ribosomal protection proteins.

rRNA - Ribosomal RNA.

RRDR - Rifampicin resistance-determining region.

RTE - Ready-to-eat.

S - Susceptible.

SaPI - *S. aureus* pathogenicity islands.

SCC*mec* - Staphylococcal cassette chromosome *mec*.

SCIN - Staphylococcal complement inhibitor.

SEs - Staphylococcal enterotoxins.

Sha - Phage tail shaft protein.

SLV - Single locus variant.

SMR - Small multidrug resistance.

SNPs - Single nucleotide polymorphisms.

SP - Susceptibility profile.

sp. - A single species.

spp. - More than one species.

ST - Sequence Type.

STEC - Shiga toxin-producing *E. coli*.

STR - Streptomycin.

Su - Summer.

SXT - Trimethoprim/sulfamethoxazole.

TDH - Thermostable direct hemolysin.

Ter – Terminase.

TET - Tetracycline.

THF - Tetrahydrofolate.

TMN - Tobramycin.

Tn - Transposons.

TP - Teicoplanin.

tRNA - Transfer RNA.

UPGMA - Unweighted pair group method with arithmetic mean.

USA - United States of America.

VA - Vancomycin.

VF(s) - Virulence factor(s).

VISA - Vancomycin-intermediate *S. aureus.*

VRSA - Vancomycin-resistant *S. aureus*.

vs. - *versus*.

WHO - World Health Organization.

WGS - Whole genome sequencing.

WMS - Whole metagenome sequencing.

THESIS OUTLINE

The main body of these Ph.D. thesis is based on nine chapters, including an initial overview of aquaculture and antibiotic resistance (Chapter 1, General introduction), the objectives (Chapter 2, Aim of the thesis), six manuscripts presented as individual chapters (3 to 8) and a general discussion that connect the results obtained (Chapter 9, General discussion and conclusions). Five of the six manuscripts have already been published and the remainder is submitted for publication in an international peer reviewed journal at the time this thesis was finalized. Each manuscript-based chapter is composed by a title page, with the reference of the publication (if applicable) and the personal contributions of the author of this Ph.D. thesis, a specific abstract, introduction, material and methods, results, discussion, and conclusions according to the scope of the manuscript. Chapters 3 to 8 do not follow a chronological order but a rational order taking into account the objectives established for this Ph.D. thesis. Briefly, each chapter includes the following contents:

Chapter 1 consists of a general introduction that provides the reader with the state of the art in aquaculture and antibiotic resistance, necessary to contextualize the relevance of the studies presented in the subsequent chapters. It includes the bases for the understanding of aquaculture and its production methods, as well as an overall description of its evolution in the world and specifically in Portugal, the most relevant pathogenic bacteria that can affect the animals reared in these systems, important bacteria associated with food poisoning outbreaks in humans after the consumption of contaminated fish or bivalve mollusks, followed by a description of how and why are antibiotics used in aquaculture, how they can influence animal and human health, as well as the environment, and their mechanisms of action/resistance. At the end, the contributions of next generation sequencing methods to the search of resistance and virulence genes are underlined.

Chapter 2 comprises the main and specific objectives of this Ph.D. thesis, contextualizing them.

In **Chapter 3**, we studied the bacterial diversity in samples of *Sparus aurata* (gilthead seabream) collected in a fish farm and investigated their antibiotic susceptibility profiles, as well as the genes related with the reduced susceptibilities found. This chapter corresponds to the following published manuscript: Salgueiro, V.; Manageiro, V.; Bandarra, N. M.; Reis, L.; Ferreira, E.; Caniça, M. Bacterial diversity and antibiotic susceptibility of *Sparus aurata* from aquaculture. *Microorganisms* 2020, *8*, 1343, doi: 10.3390/microorganisms8091343.

In **Chapter 4**, we investigated the bacterial composition of three species of bivalve mollusks (*Crassostrea gigas*, *Mytilus* spp. and *Ruditapes decussatus*) collected from 6 aquaculture farms in the central and south regions of Portugal, in summer and autumn. Antibiotic susceptibility and presence of resistance genes were also explored. This chapter corresponds to the following published manuscript: Salgueiro, V.; Reis, L.; Ferreira, E.; Botelho, M.J.; Manageiro, V.; Caniça, M. Assessing the Bacterial Community Composition of Bivalve Mollusks Collected in Aquaculture Farms and Respective Susceptibility to Antibiotics. *Antibiotics* 2021, *10*, 1135, doi: 10.3390/antibiotics10091135.

Chapter 5 includes a study that compares *Staphylococcus aureus* found in aquaculture (in Chapter 3) with *S. aureus* from other reservoirs, namely humans and animals of livestock, poultry, and zoo, through an analysis of several genes that provide us genetic relatedness and diversity. Antibiotic susceptibility profiles were also determined, and resistance genes were investigated to all strains from the different reservoirs. This chapter corresponds to the following published manuscript: Salgueiro, V.; Manageiro, V.; Bandarra, N. M.; Ferreira, E.; Clemente, L.; Caniça, M. Genetic relatedness and diversity of *Staphylococcus aureus* from different reservoirs: humans and animals of livestock, poultry, zoo and aquaculture. *Microorganisms* 2020, *8*, 1345, doi: 10.3390/microorganisms8091345.

In **Chapter 6** we deepened the study of ten *S. aureus* from the lineage ST398 collected in humans, dolphin, and gilthead seabream from aquaculture, through a whole genome sequencing (WGS) approach. Phylogenetic analysis and investigation of resistome, virulome and mobilome of these strains were performed. This chapter corresponds to the following published manuscript: Salgueiro, V.; Manageiro, V.; Bandarra, N. M.; Ferreira, E.; Clemente, L.;

Caniça, M. First comparative genomic characterization of MSSA ST398 lineage from aquaculture with different reservoirs. *Frontiers in Microbiology* 2023, *14*, 1035547, doi: 10.3389/fmicb.2023.1035547

Chapter 7 represents the study of the resistome, virulome and mobilome of Gram-negative bacteria isolated in previous studies (Chapters 3 and 4) in gilthead seabream and bivalve mollusks from aquaculture farms, using WGS. This chapter corresponds to the following submitted manuscript: Salgueiro, V.; Manageiro, V.; Rosado, T.; Bandarra, N. M.; Botelho, M.J.; Dias, E.; Caniça, M. Aquaculture as a hotspot for MGE, virulence and resistance genes (antibiotics, disinfectants and heavy metals) and their contribution to the environmental resistome. Submitted to *Science of the total environment*.

Chapter 8 contains an in-depth report of the resistome, virulome and mobilome of an *mcr-9.1*-harbouring *Enterobacter ludwigii* collected in a muscle sample of gilthead seabream from an aquaculture farm. This chapter corresponds to the following published manuscript: Manageiro, V.; Salgueiro, V.; Rosado, T.; Bandarra, N. M.; Ferreira, E.; Smith, T.; Dias, E.; Caniça, M. Genomic Analysis of a *mcr-9.1*-Harbouring IncHI2-ST1 Plasmid from *Enterobacter ludwigii* Isolated in Fish Farming. *Antibiotics* 2022, *11*, 1232, doi: 10.3390/antibiotics11091232.

Chapter 9 provides a global overview and discussion of the previous manuscript-based chapters, emphasizing the main results and conclusions achieved throughout this Ph.D. thesis.

Considering the different layouts and in-text reference styles adopted by the journals in which the manuscripts were published or submitted, chapters 3 to 8 were formatted in a unique style, with all references gathered in a single "Bibliography" section. Supplementary data concerning each chapter was compiled in the last section of this Ph.D. thesis designated "Supplementary material". Numbering of tables and figures is presented according with the number of the chapter.

1

GENERAL INTRODUCTION

1.1 Aquaculture

1.1.1 Definition and production systems

According to Food and Agricultural Organization of the United Nations (FAO), aquaculture is *"the farming of aquatic organisms including fish, mollusks, crustaceans, and aquatic plants. Farming implies some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated, the planning, development and operation of aquaculture systems, sites, facilities and practices, and the production and transport."* [1] Aquaculture is a diverse sector and can be practice in distinct environments, implementing different production systems. The three environments where aquaculture can be practice are: freshwater (e.g., lakes, rivers, and groundwater), seawater/saltwater (e.g., open waters, inland seas and inshore) and brackish water (e.g., estuaries and lagoons). Aquaculture in freshwater environments represents the main production. Furthermore, according to the production system, aquaculture can be classified into extensive, semi-intensive and intensive. An extensive system is closest to the natural environment, where sometimes only competitivity species and predators are removed (e.g., mussels farming). A semi-intensive system comprises additional feed to achieve higher population densities (e.g., shrimps farming). Finally, in an intensive system all nutritional needs are provided and implies greater technological investment and maintenance (e.g., salmon farming) [2,3].

1.1.2 Evolution over the decades

Aquaculture is an ancient activity, practiced since 1100 B.C. in China and 140 B.C. in Europe [4,5]. Nevertheless, it was in the last few decades that this sector experienced an exponential growth caused by an increased in aquatic foods consumption, fluctuations in natural stocks and technological developments that allowed a higher productivity with reduction of costs and waste. An increasing world population and more awareness of the positive effects of aquatic foods in human health led to an average annual growth of 3.0% of the consumption of these products (1961-2019), outpacing other animal proteins such as eggs, meat, and milk (2.1% per year in the period 1961-2017). Worryingly, data collected from 1974 to 2019 show a decreased in the amount of fish stocks within biological sustainable levels (90.0% in 1974 to 64.6% in 2019), due to numerous factors such as overfishing and climate changes, that have a negative effect in the ecosystem and biodiversity. Therefore, aquaculture is seen as an alternative to feed the increasing world population. In 2018, aquatic animals' production reached its record with 179 million tons. A minor reduction was registered in 2020, to 178 million tons, due to restrictions caused by COVID-19 pandemic that affected capture fisheries, aquaculture, ports, and markets all over the world. Of these, 88 million tons corresponded to aquaculture production (mainly finfish, followed by mollusks, crustaceans, marine invertebrates, aquatic turtles, and frogs). Estimations from the same year confirmed China as the main fisheries and aquaculture producer, with 35.0% of the global production and 56.7% of aquaculture global production (excluding algae farming). Other important producers are Norway, Chile, Egypt, Bangladesh, Vietnam, Indonesia, and India. As already described in section 1.1.1, aquaculture can be practice in distinct environments. This feature associated to the development of new technologies allowed the cultivation of a great diversity of genera/species (more than 600 units in 2020, that include species, hybrids, and groups identified at the genus, family, or higher level). As it grew, aquaculture sector involved more and increasingly specialized human resources (approximately 20.6 million people in 2020), acquiring a greater importance at a social and economic level [6,7].

1.1.3 Aquaculture in Portugal

Portugal is the third major consumer of fish worldwide [8]. This country followed the trend of the rest of Europe, where decreased/stagnation of natural stocks led to the development of the aquaculture sector. Although aquaculture represents a small portion of aquatic foods production and despite some oscillations, this sector has generally grown in the last

decades (from 4 457 tons in 1990 to 16 999 tons in 2020). Portugal has a great diversity of habitats and weather conditions favorable to aquaculture practice, especially in estuaries, rivers, lagoons, and coastal areas (with the use of the most sheltered bays, since sea conditions can be unfavorable in the winter months, e.g., suspended cables for the cultivation of bivalves in the Algarve and floating cages for fish in Madeira). Taking into to account the species produced, Portuguese aquaculture has changed over the past few decades. Fish of the *Mugilidae* family dominated aquaculture production until the 1970s. Subsequently, in the 1980s, there was a shift to the cultivation of mainly rainbow trout and bivalves (specifically clams). Since the 1990s, there was a growth in the cultivation of marine species, namely seabream, seabass, sole and turbot. Data from 2020 revealed turbot (*Scophthalmus maximus;* 3 407 tons), gilthead seabream (*Sparus aurata;* 1 768 tons) and European seabass (*Dicentrarchus labrax;* 904 tons) as the main species produced, although mollusks continue to represent the largest portion of aquaculture production (58.0%). Of these, oysters (3 838 tons; e.g., *Crassostrea gigas*), clams (3 659 tons; e.g., *Ruditapes decussatus*), and mussels (2 007 tons; e.g., *Mytilus galloprovincialis*) represent the main species produced in 2020 [9–11].

1.1.3.1 *Sparus aurata* (gilthead seabream)

The species *Sparus aurata* (common name: gilthead seabream) belongs to *Sparidae* family and is characterized by an oval body, silvery grey color, with a golden band between the eyes and a black spot at the origin of the lateral line (Figure 1.1) [12]. This omnivorous species feeds mainly on bivalve mollusks, crustaceans, and other fish. *S. aurata* can be found along the Eastern Atlantic coasts from Great Britain to Senegal but is more common in the Mediterranean Sea. The natural habitat of this species is brackish water and marine environments between 15 to 30 m, especially in sandy and rocky bottoms with algae.

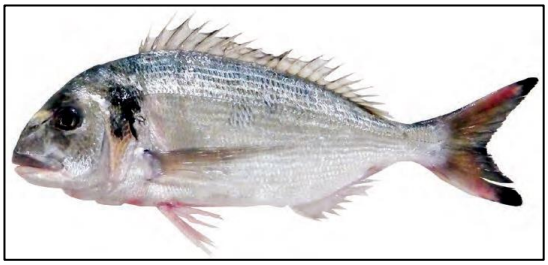

Figure 1.1 — *Sparus aurata*. In this figure we can observe specific characteristics of this species: oval body, silvery grey color, golden band between the eyes and a black spot at the origin of the lateral line (adapted from [12]).

Aquaculture production of gilthead seabream can be extensive, semi-intensive (coastal ponds and lagoons) and intensive (sea cages and land-based installations, such as tanks; Figure 1.2). The extensive system is characterized by a low density of fish and a diet based exclusively on natural resources. In the semi-intensive system, densities of fish can fluctuate and there is greater human intervention, such as supplementary feed and/or oxygen, fertilization of the farming area to improve natural resources availability and introduction of juveniles pre-fattened in an intensive system (reducing farming time and mortality). In the intensive system there is more control in all farming phases (reproduction, larval rearing, nursery, pre-fattening, and grow-out; Figure 1.2) [13,14].

Figure 1.2 — Structures used for aquaculture production of *Sparus aurata*. **(a)** Nursery tanks for the growth of juveniles, where water, salinity, temperature, and food are controlled. **(b)** Land-based tanks for grow-out phase. This system allows higher density of fish than sea cages. **(c)** Sea cages for grow-out phase. In this system, water and temperature controls are not possible (adapted from [15,16]).

1.1.3.2 *Mytilus* spp. (mussels)

Like fish, bivalve mollusks are recognized as a good source of proteins, vitamin D, long-chain omega-3 fatty acids, iodine, and selenium, contributing to a healthy diet (with visual, neural, cognitive and cardiovascular benefits) [17]. Members of *Mytilidae* family, *Mytilus* spp. (common name: mussels), are characterized by a soft body surrounded by a shell with two valves, mainly composed of calcium carbonate, triangular shape, inequilateral, equivalve, with beaks at the anterior end and a purple, blue, or dark brown coloration (Figure 1.3). This genus

can be also found in the Mediterranean coast, European west coast, and North Africa. Rocky environments in subtidal and intertidal areas of coastal shores and estuaries are natural habitats for *Mytilus* spp., although they can be found up to 40 m depth [18,19]. These areas are rich in phytoplankton and organic matter, food sources for these filter feeders [20]. The wide distribution of *Mytilus* spp. is facilitated by their rapid growth, high fertility, and resistance to environmental variations (temperature, salinity, etc.) [19]. Feeding by filtration leads to an accumulation of toxins, antibiotic residues, bacteria, viruses, and protozoa in bivalve mollusks, making them important "sentinel species" for the detection of contaminants in the environments where they are present [21].

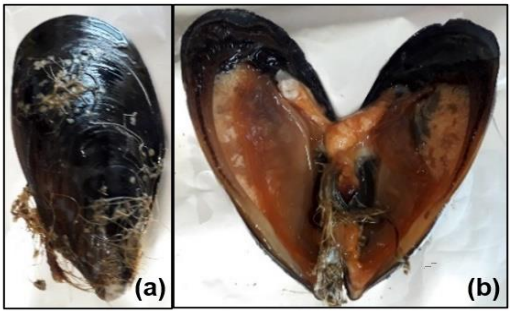

Figure 1.3 — *Mytilus galloprovincialis*. **(a)** In this figure we can observe some characteristics of *Mytilus* spp. such as the coloration, and a triangular and inequilateral shell. **(b)** Soft body of *M. galloprovincialis* surrounded by an equivalve shell (photos by the author).

The aquaculture production system used in *Mytilus* spp. is mainly extensive. Normally, mussel seeds are collected in natural beds, although there are also available seeds produced in hatcheries, where the natural maturation cycle of mussels is simulated. Seeds can be attached in ropes with a special cotton or rayon mesh or deployed in setting tanks and later transferred into a nursery, where mussels will grow until they reach 6-10 mm in size and be subsequently transferred into grow-out systems. These grow-out systems are diverse and can consist in on-bottom culture (mussels grow in the ocean floor), *bouchot* culture (mussels are reared in wooden poles located into the intertidal seabed; each pole can produce 60 kg of mussels), raft culture (mussels are reared in ropes attached to rafts supported by floats; each raft can produce 45 tons of mussels in a 18 month rearing cycle) or longline/rope culture (mussels grow in vertical ropes that are supported by horizontal lines connected to floats; this technique can produce 18-20 tons of mussels) (Figure 1.4). When mussels reach commercial size

(5-10 cm), they are harvested by hand or using a mechanical method. After harvesting, mussels can go to depurations plants, to remove possible contaminants and be sold in markets as fresh products, or to canning factories, where they go through several processes since sterilization, frying or boiling to obtain a variety of processed products [19,20].

Figure 1.4 — Types of grow-out systems in aquaculture production of mussels. **(a)** On-bottom culture. **(b)** *Bouchot* culture. **(c)** Raft culture. **(d)** Longline culture (adapted from [22–25]).

1.1.3.3 *Crassostrea gigas* (Japanese oyster)

Crassostrea gigas (common names: Japanese oyster, Pacific cupped oyster, Giant oyster) belongs to *Ostreidae* family and possesses a solid shell, inequilateral, inequivalve, with the top valve markedly concave and the bottom valve flat or slightly concave that fits inside the top valve, an outer surface extremely rough and laminated, with a white color with purple spots and streaks (Figure 1.5). This species originated in East Asia but is currently distributed all over the world (Australia, Asia, Mediterranean/European west coast, North Africa, and America) and represents the most consumed bivalve mollusk. Like *Mytilus* spp., this euryhaline species has a high growth and fertility rates, and a broad temperature tolerance [4,18,26]. In Portugal, *C. gigas* was introduced after 1974, due to high mortality rates within the populations of Portuguese-oyster (*Crassostrea angulata*). This mortality was caused by an iridovirus, whose spread was facilitated by excessive exploitation and water pollution, that weakened the immune system of Portuguese-oyster's populations [4]. The natural habitat of *C. gigas* is the intertidal until the subtidal zones in estuaries and coastal lagoons, attached to rocks/other oysters shells, or mud-sand bottoms (5 to 40 m depth) [18].

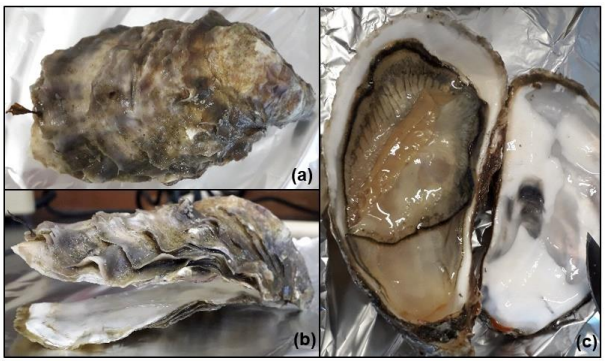

Figure 1.5 — *Crassostrea gigas*. **(a)** In this figure we can observe some phenotypic characteristics of *C. gigas*, such as inequilateral shell, coloration, and rough surface. **(b)** Here we can observe the laminated surface and the inequivalve shape, with the bottom valve smaller than the top valve that is markedly concave. **(c)** Soft body of *C. gigas* surrounded by two valves (photos by the author).

Aquaculture production of Japanese oysters is very similar to mussels' production already described. It begins with the seed collection (through wild capture or collection in hatcheries), followed by the transfer to setting tanks (for larvae growth), posterior transfer to a land-based or sea-based nursery (where they grow until 12 to 15 mm in length), and a final transfer to grow-out systems, where they are harvest when they reach commercial size (>75 mm in length and 70 to >100 g in weight). The grow-out system used depends on the environmental conditions of the region and can vary between bottom (seeds are placed in intertidal/subtidal ground pre-prepared with gravel or shells and covered by nets or fences, to avoid predation), off-bottom (Japanese oysters grow in mesh bags or plastic trays attached to a wooden/steel structure in intertidal zones; Figure 1.6) and suspended cultures (longline or raft; Figure 1.6). Both off-bottom and suspended cultures involve regular maintenance to transfer and divide the growing oysters to new bags/trays/nets/ropes, since a high density slows the growth rate. Like mussels, Japanese oysters can be sold live (sometimes after depuration), frozen, canned, or smoked [26].

Figure 1.6 — Different grow-out systems used in aquaculture production of oysters. **(a)** Off-bottom culture in mesh bags. **(b)** Off-bottom culture in trays. **(c)** Suspended culture (long-line) (adapted from [27]).

1.1.3.4 *Ruditapes decussatus* (clams)

Veneridae family includes the species *Ruditapes decussatus* (common name: clams), characterized by an oval to quadrate shell, inequilateral, equivalve, beaks at the anterior half, and very diverse in color (yellow, white, or light brown) and pattern (streaks, zigzags, rays, and spots) (Figure 1.7). This species is distributed along the Mediterranean coast, Iberian Peninsula, southern and western England and Morocco, west Africa, and Senegal. *R. decussatus* is usually found buried in mud, sand, or clay from shallow waters. Like *Mytilus* spp. and *C. gigas*, *R. decussatus* feeds by filtration of phytoplankton and organic matter from the water [18,28,29].

Aquaculture's processes and systems used for *R. decussatus* are very similar to those already described for *Mytilus* spp. and *C. gigas* (sections 1.1.3.2. and 1.1.3.3). Briefly, seed supply for aquaculture production of *R. decussatus* can also be obtain in natural populations or hatcheries. The second phase of the process is nursery (in greenhouses, meshed containers or inland tanks with controlled water and food), followed by grow-out systems where algae and predators (starfish, crabs, gastropods, and birds) are controlled, and substrate is oxygenated. Harvesting process is usually performed by hand, with shovels or rakes, and with a minimal length of 30 mm [28]. The destination of harvest products is the same as for mussels and Japanese oyster, described above.

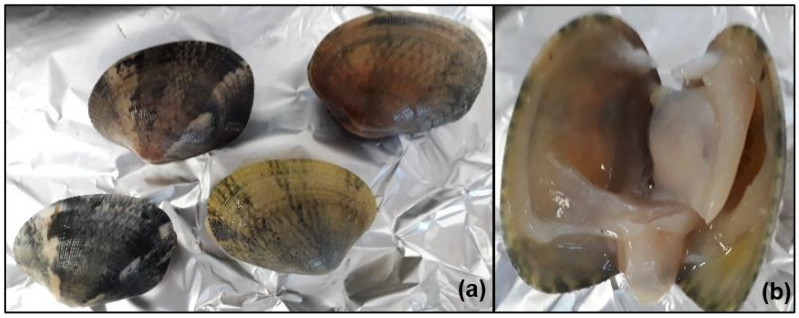

Figure 1.7 — *Ruditapes decussatus*. **(a)** Here we can observe the inequilateral shell and several colors and patterns that *R. decussatus* can exhibit. **(b)** Soft body of *R. decussatus* with a highly developed muscular foot, surrounded by an equivalve shell (photos by the author).

1.2 Most relevant pathogenic bacteria in aquaculture

Bacteria are normally present in water, sediment, and animals (as commensals) from aquatic environments. However, disturbances in bacteria-host-environment interactions can evolve to disease in aquatic animals (fish, bivalve mollusks, crustaceans, etc.) [30]. In aquaculture, these diseases can originate high morbidity and mortality rates in the animals produced, consequently, creating large economic losses [31]. Among disturbances that can cause disease in aquaculture animals are water pollution (that can affect water quality and alter the microbiome of aquatic environment), environmental changes, high density cultures, transportation between different aquaculture structures and diet. These alterations can cause stress conditions in aquatic animals, thus weakening their immune system. Potentially pathogenic bacteria can be also introduced in aquaculture settings through feed, water changes, transportation, and handling [32–35]. Many different bacterial genera are responsible for disease in animals raised in aquaculture settings. In this section, it will be discussed some of the bacteria most frequently isolated in diseased fish and other aquatic animals, responsible for major economic losses (*Vibrio* spp., *Photobacterium damselae*, *Aeromonas* spp., *Pseudomonas* spp., *Edwardsiella* spp., and *Lactococcus garvieae*).

1.2.1 *Vibrio* spp.

Bacteria belonging to the genus *Vibrio* are Gram-negative, facultative anaerobic, oxidase positive, halophilic, rod-shaped, and curved, with polar flagella that contributes to their motility. They are naturally present in water, plankton, sediments, and several aquatic animals [17]. Several *Vibrio* spp. (*Vibrio alginolyticus*, *Vibrio vulnificus*, *Vibrio anguillarum*, *Vibrio ordalii*,

Vibrio salmonicida, etc.) were already associated with disease in fish (such as *S. aurata* and *Salmo salar*), causing lethargy, anorexia, hemorrhages, skin lesions and a dark skin coloration [13,36]. These bacteria are also responsible for diseases in bivalve mollusks (such as oysters and clams), particularly in larvae and juveniles reared in hatcheries, causing bacillary necrosis, loss of velar epithelial, deciliation and abnormal swimming behavior. In *R. decussatus*, *Vibrio tapetis* can colonize the surface of periostracal lamina at the mantle edge of the shell and cause the "brown ring disease". This disease is characterized by a disorder in normal calcification of the shell, resulting in disturbances in growth, that can lead to death if bacteria penetrate in the mantle epithelium and in the soft tissues [26,28,32].

This genus is also associated with disease in humans (namely *Vibrio parahaemolyticus* and *V. vulnificus*), through ingestion of raw or undercooked fish and shellfish or through direct contact of open wounds with water or aquatic animals infected with these bacteria. Clinical manifestations can go from gastroenteritis and wound infection to septicemia. These species can have several virulence factors that contribute to their pathogenicity. Among those virulence factors are *tdh* (thermostable direct hemolysin, TDH), *trg* (TDH-related hemolysin) and *vvh* (cytotoxin-hemolysin) genes, capsule, proteins in type IV pilus and type II, zinc metalloprotease, ability to acquire iron and ToxR (a transmembrane regulatory protein) [17].

1.2.2 *Photobacterium damselae*

Photobacterium damselae are rod-shaped Gram-negative bacteria belonging to *Vibrionaceae* family [37,38]. These marine bacteria include two subspecies, *P. damselae* subsp. *piscicida* and *P. damselae* subsp. *damselae*. The first one is a well-known causative agent of photobacteriosis (or pasteurellosis, a worrying disease due to high mortality rates) in fish (e.g., *S. aurata* and *D. labrax*, among others) and the second is considered an emerging pathogen in aquaculture, capable of causing disease (mostly, ulcerative lesions and hemorrhage areas) in several aquatic animals such as mollusks, cetaceans, fish, and crustaceans [39,40].

In humans, these bacteria are responsible for wound infections (while handling fish or when expose to seawater) that can evolve into necrotizing fasciitis with multiple organ failure [40]. Bacteria belonging to this species can have hemolytic activity and be able to produce cytotoxins (such as damselysin, phobalysin P/C and phospholipase PlpV) [37].

1.2.3 *Aeromonas* spp.

Species from the genus *Aeromonas* are Gram-negative bacilli from *Aeromonadaceae* family, catalase- and oxidase-positive, capable of fermenting glucose and degrading nitrates to nitrites. Although already described in several environments, *Aeromonas* spp. are indigenous to aquatic environments. This genus is an important pathogen of fish (specially *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria*), being capable of causing lesions in gills and fins, skin ulcerations, hemorrhages, and septicemia. Mortality rates can reach 80% in infections caused by this microorganism [30,41]. Like *Vibrio* spp., *Aeromonas* spp. can infect bivalve mollusks larvae, causing bacillary necrosis [32].

Besides fish and bivalve mollusks, *Aeromonas* spp. were already described in vegetables and fruits (possible, because of irrigation with contaminated water), meats and dairy products. Ingestion of raw food contaminated with these bacteria can cause infection in humans, namely gastroenteritis. Wound infections can also occur in immunocompetent and immunocompromised people while handling aquatic animals or through direct contact of a wound with infected water. Numerous cases of septicemia and bacteremia were also registered. Human infections are usually associated with *A. caviae*, *Aeromonas dhakensis*, *A. veronii* and *A. hydrophila*. Virulence of this genus is related to genes encoding for specific structural components (such as *maf-5* and *flaA*), toxins (*alt*, *exoA*, *act*, among others), proteins linked with metals, and secretion systems (e.g., T6SS, T3SS) that together enable these bacteria to defeat the immune system of the host [41].

1.2.4 *Pseudomonas* spp.

Pseudomonas spp. belong to *Pseudomonadaceae* family and are rod shaped, motile, and aerobic Gram-negative bacteria. This genus is usually found in water and soil. Although considered as part of the normal microbiota of fish, some species (like *Pseudomonas fluorescens*, *Pseudomonas anguilliseptica*, and *Pseudomonas aeruginosa*) can be opportunistic pathogens and cause diseases, such as necrotic and hemorrhagic disease, cold water strawberry disease, and septicemia [30,36,42,43]. In bivalve mollusks, these bacteria are more problematic in larvae phase.

Several species of *Pseudomonas* are known to cause infections in humans (such as pneumonia, urinary tract infections, eye and wound infections, and bacteremia), among them *P. aeruginosa* is the most significant in hospital settings [44,45].

1.2.5 *Edwardsiella* spp.

Edwardsiella genus comprises short, rod-shaped, and facultative anaerobic Gram-negative bacteria from *Enterobacteriaceae* family, usually found in marine and freshwater environments. Bacteria from this genus were already described from infections in fish, reptiles, birds, and humans. In fish, species like *Edwardsiella piscicida*, *Edwardsiella anguillarum* and *Edwardsiella ictaluri* are important pathogens responsible for loss of skin's pigmentation, hemorrhages, septicemia, abundant ascitic fluid in internal organs, as well as nodules and abscesses [36,46,47].

Although not very often, *Edwardsiella* species can enter the food chain and cause disease in humans, such as gastroenteritis, biliary tract and wound infections, meningitis, peritonitis, liver abscess and septicemia, among others [36,46].

1.2.6 *Lactococcus garvieae*

Lactococcus garvieae is an important pathogen in aquaculture farming. These facultative anaerobic, nonhemolytic, and catalase-negative Gram-positive bacteria are responsible for lactococcosis disease (a fatal hemorrhagic septicemia, normally associated with summer months, and that can result in 50% of mortality) in several fish species. Fish affected by this disease usually exhibit erratic swimming, anorexia, melanosis, swollen abdomen, exophthalmia, and hemorrhages in various areas of the body. These bacteria are also able to affect shrimps and are the causative agent of bovine mastitis.

Already isolated in dairy and meat products, poultry, cattle, cereals, and vegetables, these bacteria demonstrate an ability to adapt to different environmental conditions, such as pH, salinity, and temperature [48–51].

L. garvieae is not a common pathogen in humans, although several infectious were already described, such as meningitis, lumbar osteomyelitis, infective endocarditis, and hepatic abscess. Some authors hypothesized that these diseases are associated with consumption of raw fish, in people with digestive disorders [48].

1.3 Most relevant bacteria associated with food poisoning outbreaks

An WHO (World Health Organization) report, estimates a total of 600 million foodborne diseases and 420 000 deaths worldwide in 2010. The highest burden of these diseases is

observed in low-income countries (namely, in Africa, South-East Asia, and Eastern Mediterranean) and 40% among children under 5 years of age [52]. Other report by ECDC (European Centre for Disease Prevention and Control) and EFSA (European Food Safety Authority) reveals that, in 2021, were reported 4 005 foodborne outbreaks including 32 543 cases of disease, 2 495 hospitalizations, and 31 deaths among 27 European Union members and the United Kingdom (Northern Ireland) [53].

There are certain bacteria, that despite not being usually associated with disease in aquatic animals, can be found in these organisms and are important foodborne agents, responsible for several outbreaks in humans. Within these group of bacteria are *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus* [53]. Contamination of food products by these bacteria can occur through contaminated water (used in aquaculture settings, for irrigation in agriculture or for cleaning and processing of food), food handling, and inappropriate food transportation and storage [52].

1.3.1 *Salmonella* spp.

Salmonella genus is characterized by Gram-negative rods from *Enterobacteriaceae* family, facultative anaerobic, oxidase-negative, catalase-positive, and capable of adapting to several environmental conditions (growth temperatures ranging from 8 to 45ºC and pH tolerance ranging from 4.0 to 9.5) [54].

Although not usually associated with disease in fish and shellfish, these bacteria were already found in these products, as well as poultry meat, eggs, pork, beef, vegetables, and water. Being inhabitants of the gastrointestinal tract of warm-blooded animals and reptiles, *Salmonella* spp. are usually a sign of fecal contamination. Sometimes, serovars isolated from seafood differ from those found in gastrointestinal tract of warm-blooded animals and reptiles, which may suggest other reservoirs. In seafood and tropical waters, *Salmonella* serovars most frequently identified are *Salmonella* Weltevreden, *Salmonella* Senftenberg, *Salmonella* Saintpaul, *Salmonella* Rissen, *Salmonella* Lexington, *Salmonella* Newport, *Salmonella* Anatum and *Salmonella* Albany [55,56]. On the contrary, serovars *Salmonella* Enteritidis, *Salmonella* Typhimurium, *S.* Newport, and *Salmonella* Heidelberg are the most frequently isolated in cases of food poisoning in humans. The most common symptoms caused by an infection by *Salmonella* spp. are stomach ache and diarrhea, that sometimes can evolve to more severe infections like endocarditis, meningitis, osteitis, arthritis, and bacteremia [54]. Factors like sodC1, spvB, and SPI-2 are important in *Salmonella*'s virulence, namely in avoiding destruction by macrophages, interfering with actin polymerization, and modifying vesicular trafficking [17].

1.3.2 *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped, facultatively aerobic, motile or non-motile, non-spore forming, oxidase-negative, and catalase-positive bacteria, capable of reducing nitrates [57]. This member of the *Enterobacteriaceae* family is a commensal of the intestines of worm-blooded animals, thus the presence of these bacteria in food products is commonly associated with fecal contamination [58]. Like *Salmonella* spp., *E. coli* are not usually associated with disease in fish [36], nonetheless the consumption of food contaminated with this species can originate food poisoning outbreaks in humans [59,60].

Enteric *E. coli* can be classified into pathotypes, such as enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC)/ enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli*. Others can cause extraintestinal diseases (e.g., meningitis and sepsis), being classified as extraintestinal pathogenic *E. coli* (ExPEC). Pathotypes EPEC, STEC/EHEC, ETEC, EAEC and EIEC are frequently associated with food poisoning outbreaks. We highlight STEC pathotype, the fourth most reported zoonosis in the European Union, that can cause hemolytic uremic syndrome, hemorrhagic colitis, chronic post-infection sequelae (such as irritable bowel syndrome) and death. STEC strains have several virulence factors, such as Shiga toxins (Stx), hemolysin (*hly*) operon, translocated intimin receptor (Tir), adhesin (ToxB), and type III secretion system [53,58,61,62].

1.3.3 *Staphylococcus aureus*

Staphylococcus aureus are Gram-positive cocci belonging to the *Staphylococcaceae* family and considered commensals, not only in humans (namely in nasal mucosa and skin) but also in other homeothermic animals. These coagulase-positive staphylococci are facultative anaerobic, oxidase-negative, salt tolerant, and catalase-positive [63]. When present in food, these bacteria can cause a foodborne intoxication (named staphylococcal food poisoning) through their ability to produce staphylococcal enterotoxins. These toxins are responsible for a stimulation of T cells, resulting in the production of inflammatory cytokines, and are extremely resistant to freezing, heat, low pH, proteolytic enzymes, and drying. Twenty-two different staphylococcal enterotoxins (SEs) were already identified, being types A to E (SEA, SEB, SEC, SED, and SEE) the most commonly identified in staphylococcal food poisoning cases. Symptoms of staphylococcal food poisoning include abdominal pain, vomiting, nausea, and diarrhea [64,65].

1.4 Understanding the use of antibiotics in aquaculture

1.4.1 Why and how are antibiotics used?

As already explained in section 1.2, animals produced in aquaculture can suffer from several diseases that cause high morbidity and mortality and, consequently, severe economic losses. To control diseases caused by bacteria, antibiotics are still widely used. However, antibiotics are not only used to treat disease animals, but also for prophylaxis. In the latter, antibiotics are administrated to healthy animals to prevent the onset of bacterial diseases. Furthermore, therapeutics can lead to metaphylaxis, since the most common method of administration of antibiotics is mixed in food, meaning that not only disease but also healthy animals are exposed to these substances. Other methods of antibiotic administration are bath (occasionally used; antibiotics are added to the water), injection of the antibiotic directly in the diseased animal or topical application in open wounds or ulcers (rarely used). These last two methods are more efficient and healthy animals are not affected; however, these are not very feasible measures when you have large populations densities. In aquaculture, antibiotics are not usually used as growth promoters, since these substances do not seem to accelerate growth or improve feed efficiency in fish, as in livestock. Moreover, European Union banned the use of antibiotics as growth promoters since 2006, although they are still used in other countries [66–68].

The deficient report of antibiotics consumption in several countries, makes it difficult to assess the total volumes of antibiotics used in aquaculture farming. Nonetheless, the available information allows the observation of a great diversity in antibiotic use between countries. For example, China, the largest producer of aquaculture animals, authorized 13 different antibiotics for use in aquaculture (enrofloxacin, doxycycline, flumequine, norfloxacin, florfenicol, oxolinic acid, neomycin, sulfamethazine, sulfadiazine, sulfamonomethoxine, sulfamethoxazole, trimethoprim, and thiamphenicol), while in the United Kingdom only 5 are allowed (amoxicillin, oxytetracycline, oxolinic acid, trimethoprim-sulfadiazine, and sarafloxacin) [5]. In Europe, most of the countries authorized florfenicol, oxytetracycline, erythromycin, sarafloxacin, and sulphonamides in association with ormetoprim or trimethoprim for aquaculture production, however differences between countries are also observed [69,70]. Not only is there a great diversity in the classes of antibiotics used, but also in the amounts. According to a report from the European Medicines Agency (EMA), in 2021, the total sales of antimicrobial agents for food-producing animals (that includes aquaculture) in 31 European countries (including Portugal)

corresponded to 5 219.6 tons (of active ingredient), with penicillins and tetracyclines representing the most sold antibiotic classes (31.2% and 25.8%, respectively) [71]. However, unlike Europe, North America and Japan that have strict regulations for antibiotic use in aquaculture farming, most aquaculture producers (the vast majority, developing countries) lack of regulation and control in these sector [72]. Since 2003, China implemented several changes to improve food safety, however the detection of prohibit antibiotics, such as chloramphenicol, in seafood imported from this country to the European Union reveals a lack of control in the implementation of those measures. In India, the second largest aquaculture producer, antibiotic sales and usage are not regulated. While in Chile, which is only surpassed by Norway in salmon production, reported the use of 279 g of antimicrobials to produce 1 ton of salmon, whereas in Norway, just 4.8 g are used to produce the same quantity [67,69,73].

Several factors can influence the type, frequency, and amount of antibiotics used, namely the type of animals produced, farming environment and practices, production technology, the existence of food safety regulations, and accessibility of a veterinarian [74].

1.4.2 Impact of antibiotic use in aquaculture on human and animal health and on the environment

As already mentioned in section 1.4.1, in aquaculture farms, the most common method to administrate antibiotics to farmed animals is mixed in food. This means that healthy individuals are also exposed to antibiotics which may change normal microbiota present in these animals, selecting for resistant bacteria and possibly making them more susceptible to infections difficult to treat. Uneaten food with antibiotics accumulates in sediments from aquaculture farms and can be carried by water currents to other sites, exposing other animals to these substances. The same can happen to unabsorbed antibiotics or their by-products that are excreted in urine and feces possibly exerting a selective pressure in bacteria already present in water and sediments, since it is known that antibiotics and their by-products can remain active sometimes for several months in these environments (depending on their half-life, chemical structure, and environmental chemical and physical variables). Oxytetracycline, for example, can remain active in the sediments for months to more than a year. By-products of antibiotics can also be toxic not only to the environment but also to aquatic animals. Human health can be affected directly through the consumption or handling of aquatic animals or through direct contact with contaminated environments with antibiotic residues/by-products/resistant bacteria/resistance genes. Antibiotics and their by-products can be responsible for allergies and

toxicity in humans, and the long-term consequences of the ingestion of small quantities of these substances are unknown (potentially contributing to alteration of human normal microbiota). The problem of human infections caused by bacteria normally found in the aquatic environment was already described in sections 1.2 and 1.3. On the other hand, human activities (agriculture, industry, tourism, hospitals, sewage, and water from treatment plants, etc.) can also contribute to the contamination of aquatic environments, such as aquaculture farms, with antibiotics and their by-products or resistant bacteria/resistance genes. MGE (like integrons, transposons, and plasmids) and bacteriophages play an important role in the exchange of antibiotic resistance genes between the different sectors and are frequently found in aquatic environments (section 1.7) [73,75–77].

Therefore, we can conclude that animal, human, and environmental health are interconnected, and that resistant bacteria, resistance genes and antibiotics can be transmitted in any direction among these sectors (Figure 1.8). Any approach to minimize antibiotic resistance must include animal, human and environment (One Health approach) [69].

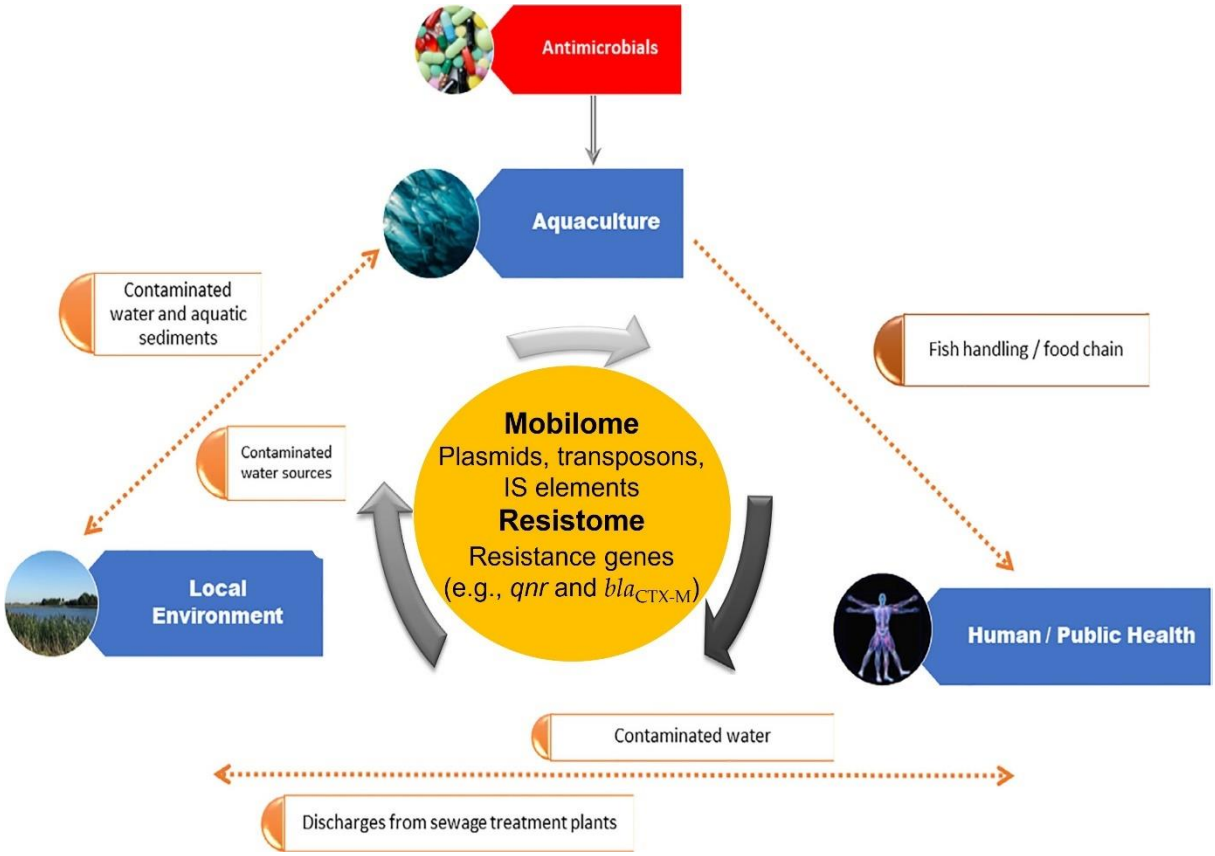

Figure 1.8 — Diagram representing the impact of the use of antibiotics in aquaculture on human/animal health and on the environment (adapted from [69]).

1.5 Mechanisms of action of antibiotics

Antibiotics are the most important medical discovery of the 20th century, allowing the cure of bacterial infections with previously high mortality rates (such as tuberculosis and pneumonia) and introducing profound changes in healthcare, enabling several medical procedures, such as organ transplants, other surgeries, and cancer treatment [78,79]. Antibiotics are defined as substances capable of inhibiting growth or killing bacteria and divided in several groups according to the mechanism of action (Figure 1.9) [80].

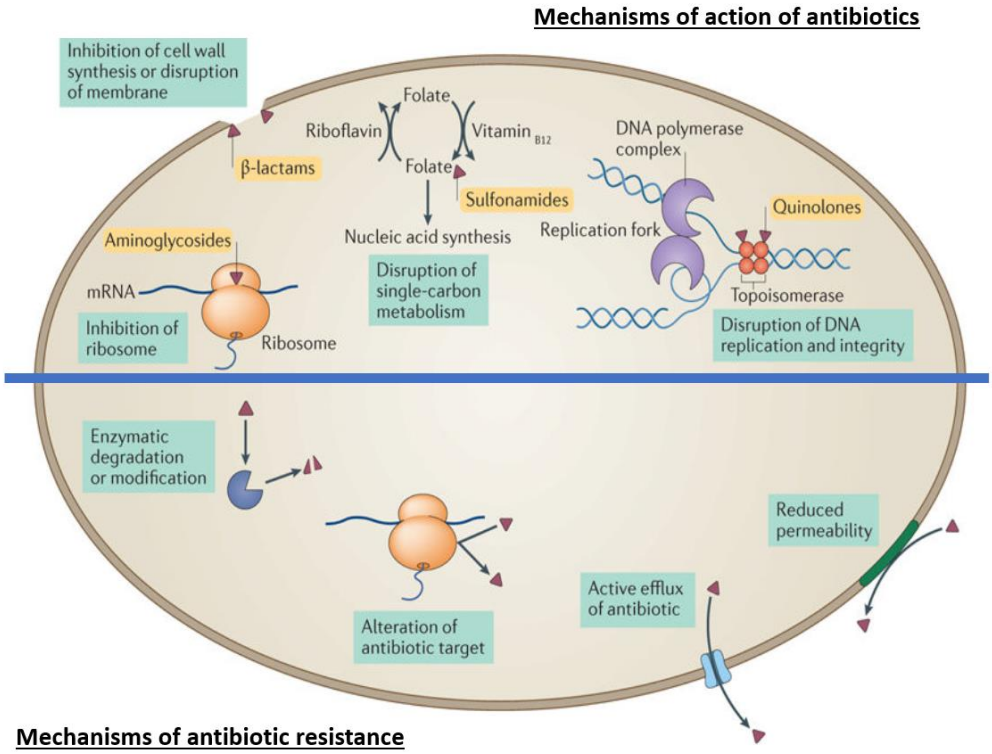

Figure 1.9 — Summary of antibiotic action (inhibition of the cell wall synthesis, disruption of the cell membrane, inhibition of nucleic acid and protein synthesis and folate antagonists) and resistance mechanisms (limitation of the uptake of an antibiotic, modification of an antibiotic target, inactivation of an antibiotic and active antibiotic efflux) (adapted from [81]).

1.5.1 Inhibition of the cell wall synthesis

Among the antibiotics that inhibit the cell wall synthesis in bacteria are β-lactams, fosfomycin, and glycopeptides.

The first β-lactam antibiotic (penicillin) was discovered in 1928, by Alexander Fleming, and introduced into clinical practice in the 1940s. β-lactams are constituted by a β-lactam ring that mimics D-alanine-D-alanine and binds to penicillin binding proteins (PBPs), that function

as transpeptidases. These enzymes catalyze the cross-linking of two D-alanine-D-alanine of *N*-acetylmuramic acid, responsible for the rigidity of the cell wall. By biding to PBPs, β-lactam antibiotics act as an alternative substrate, not allowing the participation of D-alanine-D-alanine in transpeptidation reactions and causing the acylation of PBPs. Therefore, the integrity of the cell wall is compromised, as well as the osmotic stability, resulting in cell lyses (bactericidal effect) [82]. Besides penicillins and derivates, this class of antibiotics includes cephalosporines, carbapenems, monobactams, and β-lactamase inhibitors. β-lactamase inhibitors (such as clavulanic acid, tazobactam, and sulbactam) have low antibacterial activity, but when combined with β-lactam antibiotics can improve their efficacy against β-lactamase producers [83].

Fosfomycin is a derivate of a phosphoric acid isolated in *Streptomyces* spp. in 1969 and was introduced in clinical practice in 1971. This broad-spectrum antibiotic mimics glycerol-3-phosphate and glucose-6-phosphate, entering in the bacterium through their membrane transporters, GlpT and UhpT, respectively. Inside the bacterial cell, fosfomycin binds covalently to the UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), thus inhibiting this enzyme. Once MurA is inhibited, the formation of *N*-acetylmuramic acid (the precursor of peptidoglycan) is interrupted, resulting in the loss of cell wall's integrity, consequent lysis, and death (bactericidal effect) [79,84,85].

Unlike β-lactams and fosfomycin, glycopeptides antibiotics do not inhibit the cell wall synthesis by binding to important enzymes, inactivating their active site, but they bind to the substrate. More specifically, glycopeptides bind to the D-alanine-D-alanine terminus of the lipid II monomer (cell wall precursors) through hydrogen bonds, interfering with transglycosylation reaction (i.e., extension of linear glycan chains) and subsequent cross-linking (transpeptidation reactions). The first glycopeptide introduced in clinical practice was vancomycin (1955), two years after its discovery in *Amycolatopsis orientalis* from a soil sample. Besides vancomycin, the most relevant antibiotics of this class are teicoplanin (also a natural product), as well as the semi-synthetics telavancin, oritavancin, and dalbavancin (all used in Gram-positive bacteria) [86,87].

1.5.2 Disruption of the cell membrane

Daptomycin is a lipopeptide antibiotic, discovered in 1987 and introduced in clinical practice in 2003. Its spectrum of activity includes Gram-positive bacteria, but the mechanism of action is not fully understood, although it is well accepted that calcium is essential in daptomycin action. Daptomycin forms a complex with calcium, whose insertion in the membrane

is calcium- and phosphatidylglycerol-dependent. Once inserted in the membrane, daptomycin oligomerizes and moves to the inner membrane leaflet. The theory most accepted is that the complexes of daptomycin form pore like channels in the membrane, resulting in ion leakage and dissipation of the membrane potential that can lead to cell death (bactericidal effect) [79,88].

Another antibiotic that acts disrupting the cell membrane is colistin (or polymyxin E), a narrow-spectrum antibiotic discovered in 1947 and used in clinical practice for the first time in the 1950s. Due to adverse effects, such as neurotoxicity and nephrotoxicity, the use of this antibiotic was abandoned in the 1970s. However, the emergence of multidrug and extensively drug resistant Gram-negative bacteria dictated its resurgence as a last-resort treatment. Like daptomycin, the mechanism of action by which this antibiotic exerts its bactericidal effect is not fully understood. The main action mechanism proposed is based on electrostatic interactions between colistin and the lipid A moiety of lipopolysaccharide (LPS) present in the outer membrane. Once bonded to the LPS, colistin, that has a higher affinity for LPS than divalent cations Mg^{2+} and Ca^{2+}, displaces these divalent cations from the negatively charged phosphate groups of membrane lipids. This action is responsible for the weakening and increased permeability of the cell membrane, leading to the loss of cellular contents, increased uptake of colistin, inner membrane lysis and cell death. Other alternative mechanisms of action proposed are vesicle-vesicle contact pathway, inhibition of respiratory enzymes, production of reactive oxygen species (ROS), superoxide, hydrogen peroxide, and hydroxyl radicals responsible for oxidative stress, and inhibition of the endotoxin activity [89,90].

1.5.3 Inhibition of nucleic acid synthesis

The quinolones class includes synthetic antibiotics whose spectrum of activity depends on the generation under analysis. Nalidixic acid, the first antibiotic of this class, was discovered in 1962 and introduced in clinical therapy in the following years. This antibiotic has a narrow spectrum of activity (only used to treat urinary tract infections caused by Gram-negative bacteria) and is considered, along with oxolinic acid, the first-generation of quinolones. Further improvements in quinolones structure, especially the addition of fluorine at position C6 and a piperazine ring at position C7, originated fluoroquinolones (e.g., ciprofloxacin, levofloxacin, and moxifloxacin). Therefore, new antibiotics emerged with a larger spectrum of activity (that includes not only Gram-negative but also Gram-positive and anaerobic bacteria), improved bioavailability, efficacy, and lower frequency of resistance. This class of antibiotics acts by interfering with DNA gyrase and topoisomerase IV enzymes, thus inhibiting DNA synthesis. DNA

gyrase is composed by four subunits, two A and two B, encoded by *gyrA* and *gyrB* genes, respectively. This enzyme is responsible for the introduction of supercoils into DNA through ATP (Adenosine triphosphate) hydrolysis, allowing chromosome condensation by establishing the super-helical density, the initiation of transcription, and reducing the torsional stress in front of replication forks and transcription complexes. On the other hand, topoisomerase IV (also composed by four subunits, two A and two B, encoded by *parC* and *parE* genes, respectively) is mainly responsible for the decatenation of newly replicated chromosomes, although it also plays a role in the establishment of the super-helical density and reduction of the torsional stress by the relaxation of positive supercoils. Once bound to these enzymes, quinolones stop replication process and, consequently, DNA synthesis and bacterial growth, and are responsible for chromosomal fragmentation that may lead to cell death (depending on the concentrations used) [91–93].

Ansamycin class of antibiotics was first discovered in 1959 and introduced into clinical therapy in the 1960s. Rifampicin belongs to this class of antibiotics, that demonstrate a broad-spectrum of activity against Gram-positive bacteria but a narrow spectrum against Gram-negative bacteria. This antibiotic acts by binding nearby the active site of RNA polymerase and prevents the formation of phosphodiester bonds in the RNA backbone and, consequently, RNA synthesis (bactericidal action) [94,95].

1.5.4 Inhibition of protein synthesis

There is a wide variety of antibiotics whose mechanism of action is the inhibition of protein synthesis, but each one acts in a different phase of translation (initiation, elongation, and termination).

Aminoglycosides are broad-spectrum antibiotics discovered and introduced into clinical practice in the 1940s. These antibiotics (such as gentamicin, netilmicin, tobramycin and amikacin) bind to phospholipids and teichoic acids of Gram-positive bacteria or to phospholipids and LPS in Gram-negative bacteria and cause the displacement of magnesium ions responsible for the stabilization of bacterial membrane. Therefore, an increased permeability allows the entry of aminoglycosides into the cytoplasm, where they bind to the A-site on the 16S ribosomal RNA of the 30S ribosome subunit and change its conformation. This change in conformation leads to codon misreading and an introduction of incorrect amino acids into polypeptides, creating proteins with possible changes in structure and function, which will, in turn, lead to cell death (bactericidal effect) [96].

Unlike aminoglycosides, phenicols, macrolides, lincosamides, and streptogramins are four classes of antibiotics that also inhibit protein synthesis but through the interaction with the 50S ribosome subunit of bacteria. Chloramphenicol was the first antibiotic to be described from the class of phenicols in 1947. This broad-spectrum antibiotic was introduced into clinical practice in 1949 and binds to A-site of the peptidyltransferase center (PTC) of 50S ribosome subunit. Other antibiotics belonging to this class are thiamphenicol and florfenicol. Streptogramins (e.g., pristinamycin and quinupristin-dalfopristin) were discovered in the 1950s, are mainly active against Gram-positive bacteria, and are composed by two structurally distinct molecules (A and B). Although both molecules bind to the 50S subunit, they do so in different places. The binding site of streptogramin B overlaps with the binding site of macrolides, a site nearby the PTC at the beginning of the nascent peptide exit tunnel. In 1952, erythromycin was the first antibiotic from macrolides class to be discovered and was introduced into clinical practice in the same year. Lastly, lincosamides were discovered in 1962, introduced into clinical practice in the following year, and are active against several Gram-positive and anaerobic bacteria. Examples of antibiotics from this class are clindamycin, pirlimycin and lincomycin. Like chloramphenicol, lincosamides also bind at the A-site of PTC. These four classes of antibiotics bind to their target interfering with the positioning of aminoacyl-transfer RNA (tRNA) or peptidyl-tRNA for peptide transfer or directly blocking certain actions necessary for peptide transfer, and thus inhibiting protein synthesis [79,97,98].

Another antibiotic that binds to the 50S ribosomal subunit is linezolid [97], which is synthetic, belongs to oxazolidinones class and was first approved to use in clinical practice in 2000. This antibiotic is active against Gram-positive bacteria and acts early in the process of protein synthesis by preventing the formation of the initiation complex and, consequently, reducing the length of peptide chains and the rate of translation [99].

Tetracyclines are broad-spectrum antibiotics (natural or semisynthetic) with a bacteriostatic effect. The first tetracycline, chlortetracycline, was discovered in 1948 and introduced clinically in the same year. Other antibiotics belonging to this class are oxytetracycline, doxycycline, minocycline, and tigecycline. Taking Gram-negative bacteria as an example, tetracyclines enter the cell through the outer membrane porins OmpC and OmpF as Mg^{2+} quelates. Once in the cytoplasm, tetracycline binds close to the A-site on the 16S ribosomal RNA of the 30S ribosome subunit, inhibiting the docking of aminoacyl-tRNA during the elongation process [79,100].

Discovered in 1971 and used in clinical practice since 1985, mupirocin is mostly active against Gram-positive pathogens, being bactericidal or bacteriostatic depending on the

concentrations used. This antibiotic competes with isoleucine and binds to its active site in isoleucyl-tRNA synthetase, inhibiting this enzyme, reducing the cellular levels of the isoleucine-charged tRNA and, therefore, leading to the blockage of protein and RNA synthesis [101].

The last example here presented of an antibiotic that inhibits the protein synthesis is fusidic acid, a substance mainly active against Gram-positive bacteria (specially staphylococci). Discovered in 1958 and introduced into clinical practice in 1962, fusidic acid binds to the elongation factor G (EF-G), producing a change in conformation that prevents the disconnection of EF-G from the ribosome (necessary to the binding of the next aminoacyl-tRNA unit) and thus blocking the elongation of the polypeptide chain [102].

1.5.5 Folate antagonists

Folate is vital in several reactions required for bacterial growth and survival. Unlike mammals, prokaryotes and protozoa have the ability to synthesize their own folate, which makes this biosynthesis system a good target for antimicrobial agents. In folate biosynthesis, molecules of pteridine and para-aminobenzoic acid (PABA) are used by dihydropteroate synthetase (DHPS) for the formation of dihydropteroic acid, to which glutamic acid is added, giving rise to dihydrofolate (DHF) which is then reduced to tetrahydrofolate (THF). During the folate cycle, THF is converted to DHF and further reduced after the reaction by dihydrofolate reductase (DHFR), to participate in further reactions. THF is responsible for the transportation of carbon fragments that will be used in the biosynthesis of nitrogenous bases, constituents of nucleic acids. Sulphonamides and trimethoprim target folate synthesis and cycle [87].

Sulphonamides is an antibiotic class discovered in 1932 and introduced into clinical practice in 1936 [79]. One of the most commonly used sulphonamides is sulfamethoxazole that mimics PABA, forms a complex with pteridine and binds to DPHS, inhibiting the formation of dihydropteroic acid [103].

Trimethoprim belongs to diaminopyrimidines class of antibiotics discovered in 1950 and used clinically for the first time in 1962 [79]. This antibiotic is an analogue of DHF and binds to DHFR, inhibiting the formation of THF. Typically used in combination with sulfamethoxazole, this combination has mainly a bacteriostatic effect [103].

1.6 Mechanisms of antibiotic resistance

Antimicrobial resistance is understood as a capacity of a microorganism to survive and/or reproduce in the presence of an antimicrobial agent (such as antibiotics) [104]. This

resistance can be intrinsic or acquired. Intrinsic resistance is a feature present in the genome of almost all bacteria of the same species, it is not acquired by horizontal gene transfer (HGT) and is independent of a previous selective pressure by an antibiotic. On the other hand, acquired resistance is a characteristic acquired by previously susceptible bacteria through spontaneous mutations that occur in genes located on the chromosome and that are later transmitted vertically during replication, or through the acquisition (permanent or temporary) of genetic material by HGT. Mechanisms of antibiotic resistance can be categorized in four groups: limitation of the uptake of an antibiotic; modification of an antibiotic target; inactivation of an antibiotic; and active antibiotic efflux (Figure 1.9) [105].

1.6.1 Limitation of the uptake of an antibiotic

This group of mechanisms of antibiotic resistance includes modifications in permeability of the cell wall or outer membrane, alterations in porins and the production of biofilms that limit the entry of an antibiotic into the bacterial cell [105].

S. aureus can develop an intermediate resistance to vancomycin (VISA: vancomycin-intermediate *S. aureus*) through the production of a thickened cell wall, usually after a prolonged selective pressure by this glycopeptide, restricting the entry of vancomycin into the bacterial cell [105]. Some studies indicate that the thickened cell wall of VISA strains can be a risk factor to the development of decreased susceptibility to daptomycin since it prevents daptomycin to reach to the cell membrane (site of action) [106]. Mutations in *yycG*/*walk* and *rpoB*/*rpoC* genes seem to be linked to this mechanism [107].

Alterations in membrane permeability to fosfomycin are also observed in *Acinetobacter baumannii* due the presence of the gene *abrp*. Mutations in *glpT* and *uhpT* genes are associated with fosfomycin resistance due to modifications in fosfomycin's transporters (GlpT and UhpT). Mutations in *ptsI* and *cyaA* genes are also responsible for fosfomycin resistance since they are associated with decreased expression of GlpT and UhpT transporters [85].

In Gram-negative bacteria, β-lactams antibiotics must cross the outer membrane through diffusion or porin channels to reach to PBPs in the inner membrane. Mutations or insertion sequences in the genes that encode for these outer membrane proteins (OMPs) can lead to conformational modifications or a decreased in their expression, thus originating decreased susceptibility to β-lactams. For example, the loss of OprD in *P. aeruginosa* is correlated with decreased susceptibility to meropenem and resistance to imipenem. This decreased in the expression of OMPs is also observed in some species of *Enterobacteriaceae* family resistant to carbapenems, as well as in *A. baumannii* resistant to meropenem and imipenem [83].

Decreased expression of OprD can also interfere with polymyxins resistance in *P. aeruginosa* [90]. Alterations in conformation and number of porins channels, as well as modifications in the LPS composition and structure can also decreased the uptake of quinolones into the bacterial cell, preventing the interaction of this antibiotic with their targets. OmpA, OmpC, OmpD, OmpF, Tsx and LamB are examples of porins whose loss or weakened expression can originate decreased susceptibility to quinolones [91].

Similar alterations in membrane permeability and transport are also observed in bacteria with decreased susceptibility to aminoglycosides, chloramphenicol, and trimethoprim [108–110].

Finally, the formation of biofilms can diminish the uptake of numerous antibiotics. This matrix constituted by proteins, DNA and polysaccharides forms an adhesive and dense barrier that interferes with the uptake of antibiotics into the bacterial cell [105]. Among bacteria that produce biofilms are methicillin resistant *S. aureus* (MRSA), *Enterococcus faecalis*, *P. aeruginosa*, *Klebsiella pneumoniae* and *E. coli* [111–115].

1.6.2 Modification of an antibiotic target

In this type of resistance mechanism, the target is modified thereby preventing the binding of the antibiotic.

The active sites of β-lactam antibiotics are PBPs. Changes in these structures, such as the altered PBP (PBP2a) produced by *S. aureus* after acquisition of *mecA* or *mecC* genes, can lower the affinity to penicillins and cephalosporins [82].

The target of fosfomycin is MurA. Mutations in the gene that encodes MurA (*murA*) are responsible for a modification in the structure of this enzyme (substitution of cysteine with aspartate), thus preventing the binding of fosfomycin [85].

Resistance to glycopeptides is due to the production of modified cell wall precursors with a D-alanine-D-lactate or a D-alanine-D-serine instead of D-alanine-D-alanine terminus. These modified cell wall precursors have lower affinity to glycopeptides antibiotics, such as vancomycin and teicoplanin. This type of resistance is encoded by *van* genes, with *vanA* being described in *S. aureus* and the most often found in *Enterococcus* genus [86].

The site of action of daptomycin is the cell membrane, thereby any modifications in this structure can originate decreased susceptibility to daptomycin. Among these modifications are the alterations in fatty acids composition that changes the fluidity of the membrane, a higher production of carotenoid pigment, and the increased of the positive charge of the membrane due to a greater synthesis of lysyl-phosphatidyl glycerol (owing to mutations in *mprF* gene or

dltABCD operon in *S. aureus*), that is transported to the outer layer of the membrane and causes an electric repulsion of daptomycin [107].

Modifications of the lipid A moiety of LPS are one of the main mechanisms of resistance to colistin. One of these modifications consist in the substitution of the phosphate groups of lipid A with the cationic 4-amino-4-deoxy-Larabinose (L-Ara4N) and/or phosphoethanolamine (PEtN) moieties. Mutations in the two-component regulatory systems responsible for these modifications can lead to an upregulation and, consequently, a decrease in the negative charge of the outer membrane, thus avoiding colistin binding. Some of the genes involved in the synthesis of LPS-modifying enzymes are the *pmrCAB* and *pmrHFIJKLM* operons, the *pmrE* gene (located in bacterial chromosomes) and *mcr* genes (plasmid-mediated resistance). Other modifications of lipid A include deacylation and hydroxylation through *lpxR*-like genes, also involved in altered permeability of the outer membrane to colistin. The decreased in the synthesis of LPS, caused by mutations in *lpxA*, *lpxC*, and *lpxD* genes in *A. baumannii* and *P. aeruginosa*, reduces the number of colistin targets and contributes to the resistance to this antibiotic [90].

Among the antibiotics that inhibit nucleic acid synthesis, resistance to quinolones may be due to changes in the conformation of DNA gyrase and topoisomerase IV enzymes that prevent the binding of these group of antibiotics. These changes are the result of mutations in quinolone resistance determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* genes. High levels of resistance are usually associated with mutations in both enzymes. Resistance to quinolones may also be associated with the acquisition of *qnr* genes, that encode proteins from the pentapeptide-repeat family capable of binding to DNA gyrase and topoisomerase IV and protecting them from quinolones action [91].

The main mechanism of resistance to rifampicin is the alteration of its binding site in RNA polymerase, due to mutations in the rifampicin resistance-determining region (RRDR) of the *rpoB* gene [116].

In resistance to aminoglycosides, target site modifications can be caused by chromosomal mutations or an enzyme. In the latest, an enzyme named 16S rRNA methyltransferase is responsible for the methylation of specific rRNA nucleotide residues (N7 position of nucleotide G1405 and N1 position of nucleotide A1408), thereby blocking aminoglycosides from binding to their site of action. There are several genes that encode these enzymes, namely *armA*, *rmtA*, *rmtB1*, *rmtB2*, *rmtC*, *rmtD*, *rmtD2*, *rmtE*, *rmtF*, *rmtG*, and *rmtH*, some located on plasmids [96].

Mechanisms of resistance common to macrolides, lincosamides, streptogramins, phenicols and linezolid are associated with mutations in the regions that encode the domain V of 23S RNA (that includes the region of PTC) and ribosomal proteins L3, L4 and L22, or the

synthesis of methyltransferases that modified the binding sites of this group of antibiotics. Genes *erm* (often associated with mobile genetic elements) encode methyltransferases responsible for the methylation of the adenine residue at position 2058 in the domain V of the 23S rRNA, conferring resistance to macrolides, lincosamides and streptogramin B antibiotics. Likewise, the rRNA methyltransferases encoded by *cfr* gene (already associated with a plasmid location) are responsible for the methylation of the adenine residue at position 2503 in the domain V of the 23S rRNA, conferring resistance to lincosamides, streptogramin A, phenicols and linezolid [97]. The same position in the domain V of the 23S rRNA is also methylated by an enzyme encoded by *rlmN* gene, originating linezolid resistance [117].

Resistance to another group of antibiotics that inhibit protein synthesis, tetracyclines, is related to mutations in 16S rRNA and *rpsJ* gene, that induce conformational changes thus lowering the affinity of this antibiotic to the 30S ribosomal subunit, or tetracycline ribosomal protection proteins (RPPs). RPPs are GTPases that catalyze the release of tetracyclines (namely, minocycline, tetracycline, and doxycycline) from the ribosome in a GTP-dependent reaction. The most studied RPPs are Tet(M) and Tet(O) [100].

Resistance to mupirocin can be classified into low-level (encoded on the chromosome) or high-level (usually encoded on plasmids). Low-level resistance is due to modifications of mupirocin's biding site in the isoleucyl-tRNA synthetase, blocking its action. These modifications are caused by mutations in the gene encoding this enzyme, *ileS*. High-level resistance is due to the acquisition of *mupA* or *mupB* genes, that encode different isoleucyl-tRNA synthetases with low-affinity to mupirocin [101].

Resistance to fusidic acid can be also located in the chromosome (*fusA* gene) or plasmids (*fusB* and *fusC* genes). Mutations in *fusA* gene results in modifications in EF-G that decreases the affinity to fusidic acid. On the other hand, metalloproteins, encoded by *fusB* and *fusC* genes, bind to EF-G allowing the disconnection of EF-G from the ribosome and, therefore, the protein synthesis to proceed [102].

Lastly, resistance to sulphonamides is related with mutations in gene *folP* that encodes for a chromosomal DHPS enzyme, or, more frequently, the acquisition of genes *sul* that encodes for DHPS variants with low affinity to sulphonamides [118]. Similarly, resistance to trimethoprim can be associated with mutations in the gene that encodes DHFR enzyme, acquisition of *dfr* genes that encode DHFR variants with low affinity to trimethoprim and overproduction of DHFR due to mutations in the promotor region [110].

1.6.3 Inactivation of an antibiotic

The inactivation of an antibiotic is possible through the action of enzymes that are able to transfer a chemical group altering the antibiotic's structure, or that directly degrade the antibiotic [105].

Enzymes able to hydrolyze the β-lactam ring of β-lactams antibiotics are named β-lactamases. This hydrolysis causes an opening on the ring preventing it to bind to PBPs. This mechanism of resistance is the most frequently described in Gram-negative bacteria [105]. β-lactamases can be classified according to their molecular characteristics (Ambler classification) or functional properties (Bush-Jacoby classification) and be located on the chromosome or associated with mobile genetic elements, such as plasmids and integrons. This work will use Ambler classification, that divides β-lactamases in four classes (A, B, C, and D). Enzymes from classes A, C and D have an amino acid serine in their active site (e.g., TEM, SHV, CTX-M, KPC, AmpC and OXA), whereas enzymes from class B (metallo-β-lactamases) have one or two Zn^{2+} ions associated with histidine/cysteine/aspartate residues in their active site and a different hydrolytic mechanism (e.g., VIM, IMP and NDM) [82,83].

Another enzyme capable of modifying an antibiotic is a flavin-dependent monooxygenase, encoded by the family of *tet(X)* genes, that adds a hydroxyl group between the C and B rings of the tetracycline core, inactivating this antibiotic. Genes *tet(X)* were already associated with plasmids and transposons [100].

Genes *fosA* and *fosB*, usually located in plasmids, encode for enzymes from the glyoxalase superfamily responsible for the modification and consequent inactivation of fosfomycin. FosA enzyme, most frequently found in Gram-negative bacteria, opens the epoxide group of fosfomycin and adds a sulphydryl group of the cysteine of tripeptide glutathione. FosB enzyme, most frequently found in Gram-positive bacteria, is responsible for the nucleophilic addition of l-Cys or bacillithiol (BSH) to fosfomycin [85].

Aminoglycoside-modifying enzymes are a very diverse group that can be divided in three categories, according to their capacity to phosphorylate, acetylate or adenylate amino or hydroxyl groups of aminoglycosides antibiotics: *O*-phosphotransferases (APHs), *N*-acetyltransferases (AACs) and *O*-nucleotidyltransferases (ANTs). These enzymes are commonly described on plasmids and originate changes in the antibiotic molecules, lowering the affinity of these substances to their targets [96]. An aminoglycoside acetyltransferase, AAC(6')-Ib-cr, is also responsible for the acetylation of the amino nitrogen of the piperazine ring of fluoroquinolones,

interfering with the binding of this antibiotic to DNA gyrase and topoisomerase IV enzymes [93].

The main mechanism of resistance to nonfluorinated phenicols is the inactivation by *O*-acetyltransferases (CATs), encoded by *cat* genes that can be found in both Gram-negative and Gram-positive bacteria and associated with mobile genetic elements. These enzymes are capable of transferring an acetyl group to the hydroxyl group at C3 position of the antibiotic molecule. In *Streptomyces venezuelae*, other inactivating chloramphenicol enzyme, *O*-phosphotransferase, was also discovered [97].

Streptogramins can also be inactivated by enzymes, namely acetyltransferases encoded by *vat* genes (for streptogramin A) and lactone hydrolases encoded by *vgb* genes (for streptogramin B). These genes were already found in the chromosome and plasmids [97].

1.6.4 Active antibiotic efflux

The last mechanism of antibiotic resistance addressed in this work is the active efflux through pumps encoded by genes located in the chromosome or plasmids. Usually, efflux pumps are not specific for antibiotics transportation, their main function is the transportation of several substances essential to the normal functioning of the bacterial cell. Efflux pumps can be classified, according to their energy source and structure, in five different families: the small multidrug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family, the ATP-binding cassette (ABC) family, the resistance-nodulation-cell division (RND) family, and the major facilitator superfamily (MFS) (Figure 1.10) [105].

SMR family is composed by pumps with four transmembrane segments and uses the proton-motive force (H^+) as a source of energy. These efflux pumps are responsible for resistance to β-lactams and aminoglycosides in, for example, *E. coli* (EmeR pump) and *Staphylococcus epidermidis* (SMR pump), and for resistance to fluoroquinolones in *Serratia marcescens* (SsmE pump) [105,119].

MATE family pumps have twelve transmembrane segments and uses a Na^+ gradient as a source of energy. These pumps are able to extrude several fluoroquinolones and some aminoglycosides (e.g., NorM pump in *V. parahaemolyticus*) [105].

Organized into six transmembrane segments and using energy from ATP hydrolysis, the ABC family of pumps is responsible for the transport of tetracyclines, fluoroquinolones (e.g., VcaM in *Vibrio cholerae* and SmdAB in *S. marcescens*), aminoglycosides and polymyxins (e.g., MacAB in *S. marcescens*) [105,119].

RND family of pumps uses a substrate/H^+ antiport mechanism to extrude not only antibiotics, but also solvents, detergents, dyes, and heavy metals. These pumps are formed by twelve transmembrane segments with two periplasmic loops between the segments 1 and 2, and the segments 7 and 8, and need an OMP and a periplasmic membrane fusion protein to function. Examples of efflux pumps from this family are Tet (resistance to tetracyclines), Mef (resistance to macrolides), MexAB-OprM (resistance to tetracyclines, fluoroquinolones, β-lactams, sulfamethoxazole, trimethoprim, and chloramphenicol in *P. aeruginosa*), MexXY-OprM (resistance to aminoglycosides, tetracyclines, macrolides and polymyxins in *P. aeruginosa*), AcrAB-TolC (resistance to fluoroquinolones, tetracyclines, chloramphenicol, penicillins and macrolides in *Enterobacteriaceae*) and OqxAB (resistance to quinolones, chloramphenicol, tetracyclines, and trimethoprim) [90,91,105,120].

MFS family is constituted by pumps with twelve or fourteen transmembrane segments and function through solute/H^+ antiport or solute/cation (Na^+ or H^+) symport. Examples of these pumps can be found in *E. coli*, with Fsr and QepA (resistance to trimethoprim, and fluoroquinolones, respectively), and in *S. aureus*, with LmrS (linezolid, chloramphenicol, erythromycin, and trimethoprim resistance) and NorA (resistance to quinolones) [105,120].

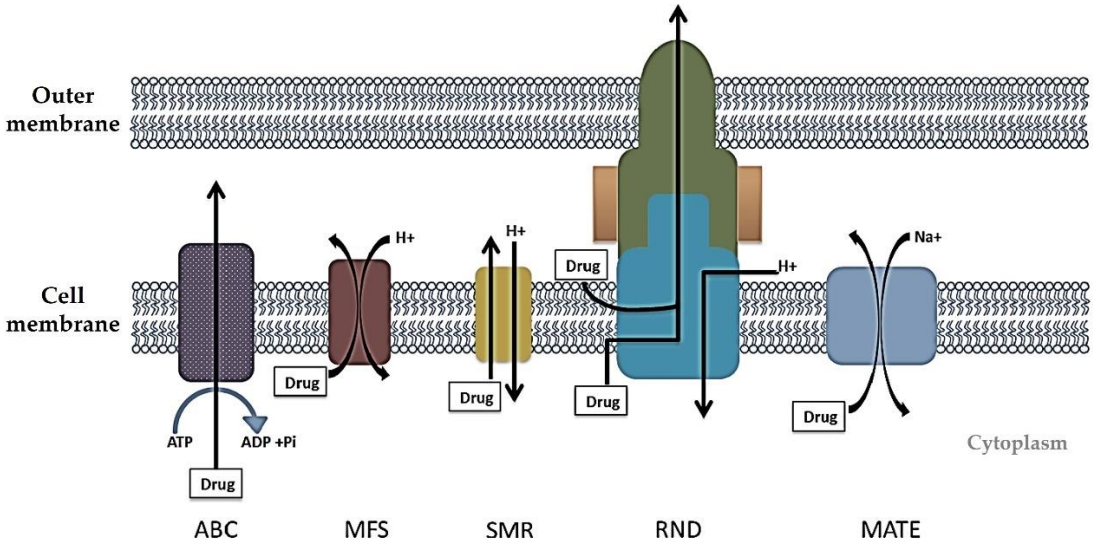

Figure 1.10 — General structure of the five different families of efflux pumps: ATP-binding cassette (ABC), major facilitator superfamily (MFS), small multidrug resistance (SMR), resistance-nodulation-cell division (RND) and multidrug and toxic compound extrusion (MATE) (adapted from [105]).

1.7 Importance of mobile genetic elements (MGE)

Throughout this introduction, several antibiotic resistance genes were mentioned and associated with different mobile genetic elements. These MGE are elements responsible for the

intracellular (within the same chromosome, plasmid or between plasmids from the same cell) and extracellular (between different bacterial cells from the same or different species) movement of DNA segments. Examples of MGE are insertion sequences (IS), transposons (Tn), insertion sequence common regions (IS*CRs*), gene cassettes (GC), integrons (In), miniature inverted-repeat transposable elements (MITEs), plasmids, and genomic islands [121].

IS are small DNA segments, usually with only one or two transposase genes, that have the ability to move to different locations, inactivating genes by direct insertion or changing the expression of nearby genes (e.g., IS*Aba1* located upstream of $bla_{\text{OXA-51}}$-like genes can increased their expression, thus originating resistance to carbapenems). When part of a composite transposon (region bounded by two copies of the same or related IS), IS can be involved in the movement of antibiotic resistance genes (e.g., Tn*9* with IS*1* are associated with the movement of *catA1* gene that confers resistance to chloramphenicol) (Figure 1.11). Other types of transposons are "unit transposons", which are larger than IS, carry a transposase gene and possibility antibiotic resistance genes, and are bounded by inverted repeats (IR) (e.g., Tn*551* normally associated with *erm(B)* gene in *Staphylococcus* spp., conferring resistance to macrolides, lincosamides and streptogramins) [121,122].

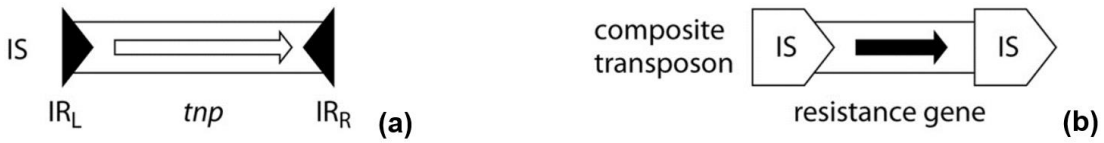

Figure 1.11 — General composition of an insertion sequence (IS) and a composite transposon. **(a)** IS with terminal inverted repeats (IR_L: inverted repeat left; IR_R: inverted repeat right) and a transposase gene (*tnp*). **(b)** Composite transposon bounded by two IS (adapted from [121]).

IS*CRs* are insertion sequences similar to IS*91*-like elements that replicate through the rolling circle mechanism and are delimited by an *ori*IS (origin of replication) and a *ter*IS (termination sequence). These elements are capable of mobilizing DNA fragments adjacent to *ter*IS, being therefore important in the propagation of antibiotic resistance genes like *sul2* (resistance to sulphonamides) and *tet(31)* (resistance to tetracycline) associated with IS*CR2* [123].

Gene cassettes are circular elements, normally with just one gene (without a promoter) and the *attC* recombination site. These nonreplicative elements are frequently found incorporated into integrons. In turn, integrons are constituted by an *intI* gene (encoding an integrase), an *attI* recombination site (located upstream the *intI* gene) and a promoter (Pc) responsible for the expression of gene cassettes (Figure 1.12). For example, integrons from class I were already

associated with genes encoding resistance to chloramphenicol, macrolides, β-lactams, and quinolones, among others [124].

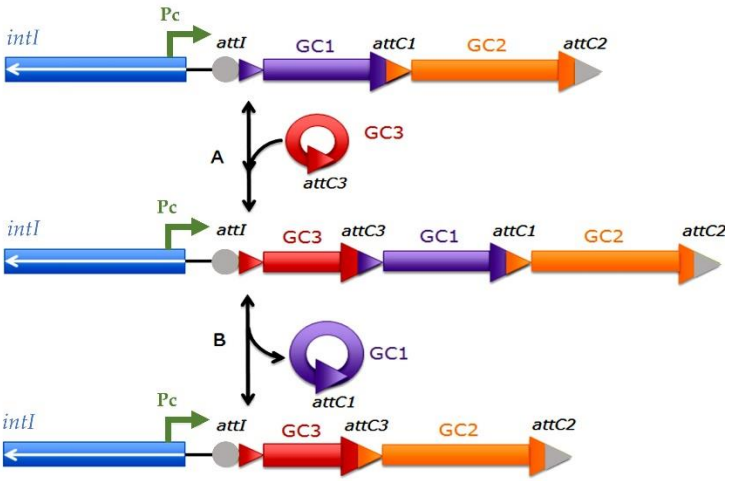

Figure 1.12 — General structure of gene cassettes (GC) incorporated into an integron. *intI*: gene that encodes for an integrase; Pc: promotor; *attI*: recombination site of the integron; *attC*: recombination site from GC. In situation **A** we can observe the insertion of GC3 into the integron. In situation **B** we observe the excision of GC1 from the integron (adapted from [125]).

MITEs derivate from transposons and IS but have lost some elements, such as the transposase gene, and are unable of self-transposition. An example of MITEs associated with resistance genes are the Tn*3*-derived inverted-repeat miniature elements (TIMEs) [121].

Plasmids are extrachromosomal DNA capable of autonomous replication and can be divided in three categories according to mobility: conjugative/self-transmissible, mobilizable, and nonmobilizable. Conjugative plasmids use their own machinery to self-transfer. On the other hand, mobilizable plasmids only have a relaxase gene, the relaxosomal components *oriT* and some auxiliar proteins, needing to use the mating pair formation complex of other genetic element of the host cell. Nonmobilizable plasmids cannot be included in any of the previous categories, do not have a relaxase (a crucial enzyme for conjugation) and spread by transduction or transformation processes. Plasmids are frequently found associated with other mobile genetic elements already described. Examples of plasmids associated with antibiotic resistance genes are F (FIIK associated with bla_{KPC} and IncFIIY with bla_{NDM}), R ($bla_{CTX\text{-}M\text{-}15}$, bla_{NDM}, bla_{VIM}, and bla_{KPC}; Figure 1.13) and ColE1-like (*qnrB19*) [121,126].

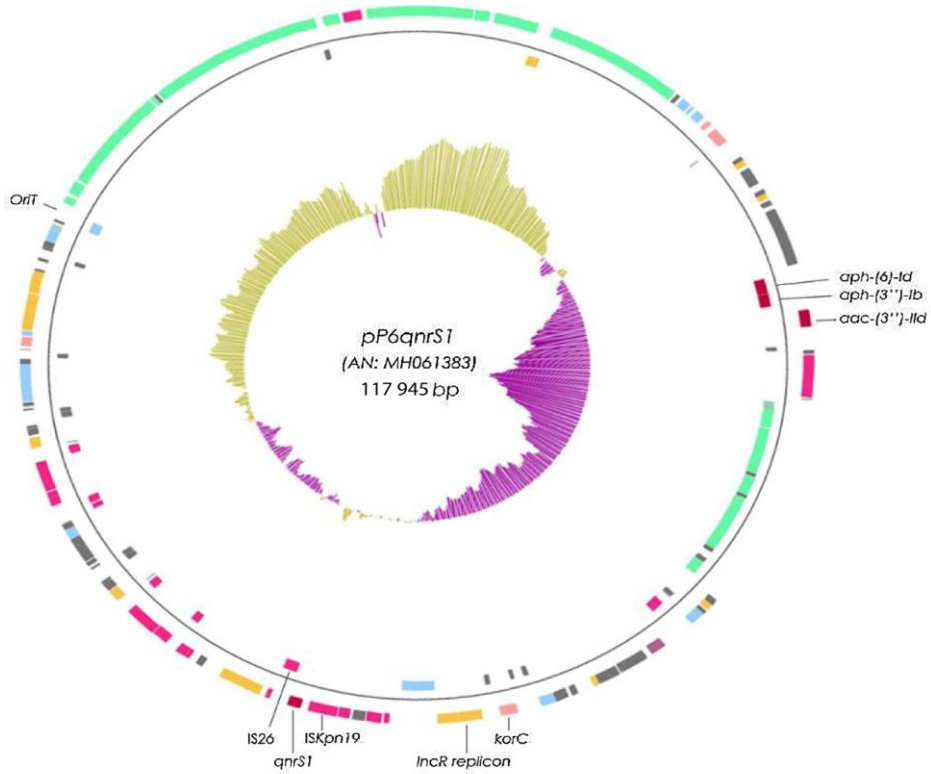

Figure 1.13 — Example of an IncR plasmid harboring antibiotic resistance genes and mobile genetic elements, like insertion sequences (IS) (adapted from [127]).

Genomic islands are DNA segments of different sizes from the bacterial chromosome, usually delimited by direct repeats and acquired by HGT. These elements can be classified according to the phenotype they encode: pathogenicity islands, if they carry virulence factors, or resistance islands, if they carry resistance genes. This group of MGE includes staphylococcal cassette chromosome elements (SCC*mec*), integrative conjugative elements, *S. aureus* pathogenicity islands (SaPI) and integrative mobilizable elements [121].

All MGE described above are involved in the acquisition of antibiotic resistance genes by bacteria through a process named HGT, that includes three different mechanisms: conjugation, transformation, and transduction (Figure 1.14). For conjugation to occur, bacterial cells must be physically connected through a pilus, DNA from the donor cell is copied and is sent to the recipient cell. In transformation, free DNA is incorporated by competent bacteria using membrane protein complexes. Bacteria can be naturally competent in a specific stage of their life cycle or receive extracellular stimulus to become competent. Transduction is the mechanism whereby bacterial DNA can be transferred and incorporated in the chromosome of a recipient cell by bacteriophages [128]. Therefore, HGT is an important process whereby bacteria acquire new genetic material that allows them to adapt and survive in different environments and to several selective pressures (such as the use of antibiotics) [129].

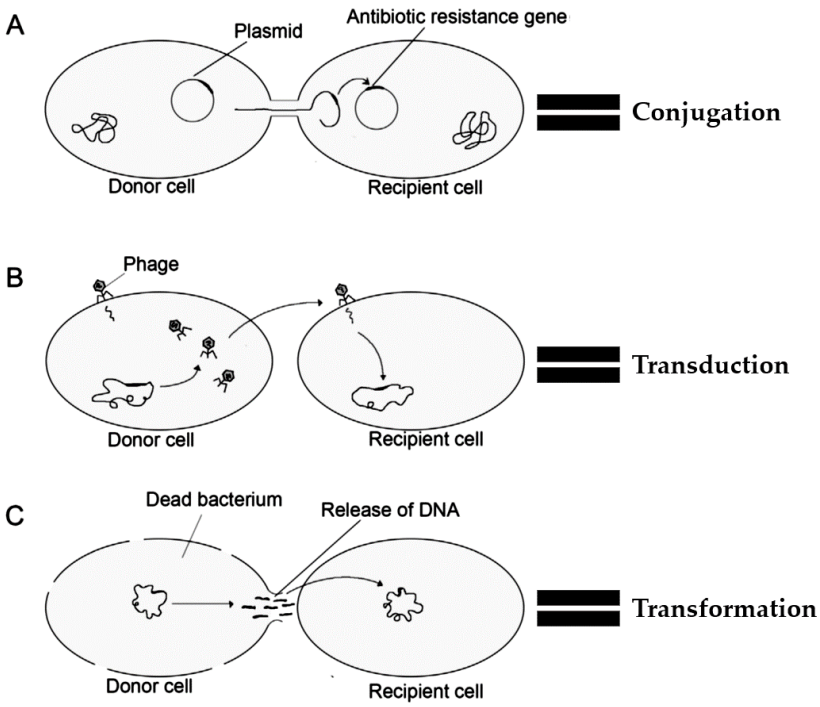

Figure 1.14 — Three different mechanisms for horizontal gene transfer (HGT): **(A)** conjugation, when DNA from the donor cell is copied and sent to the recipient cell through a pilus; **(B)** transduction, when bacterial DNA is transferred and incorporated in the chromosome of a recipient cell by bacteriophages; and **(C)** transformation, when free DNA is incorporated by competent bacteria using membrane protein complexes (adapted from [113]).

1.8 Next generation sequencing applied to antibiotic resistance

In the last few years, we have witnessed a development of new sequencing technologies that provide more faster and accurate results in the research for resistance and virulence genes in bacteria.

One example of that technologies is whole genome sequencing (WGS), whose ability to produce millions of reads in just one run became a strong advantage over traditional Sanger sequencing, which only allows to investigate small fragments of the bacterial genome each time (using specific primers). In just one run, WGS provides information for identification of bacteria, epidemiological typing, presence of MGE, resistance genes (to antibiotics, heavy metals, disinfectants, etc.) and virulence factors. Conventional molecular characterization methods (such as restriction fragment length polymorphism [RFLP], pulsed-field gel electrophoresis [PFGE], multilocus sequence typing [MLST], and matrix-assisted laser desorption ionization–time of flight [MALDI-TOF]) can be used to obtain this information but have lower taxonomic

resolution and are time-consuming. Nowadays, there are several platforms that allow the sequence of entire genomes, namely Illumina (with MiniSeq, MiSeq and NextSeq systems), Pacific Biosciences (with Sequel and RS II systems) and Oxford Nanopore Technologies (with MiniON Mk1 and PromethION systems). Whereas Illumina platforms use sequencing-by-synthesis technology (that relies on several library preparation steps, involving amplification of the initial DNA fragment), the most recent systems of the platforms Pacific Biosciences and Oxford Nanopore Technologies use single-molecule real-time sequencing technology (that abdicates the amplification step). These platforms have several differences among them, namely in performance (read length, output, coverage, number of reads and error rate), costs and run time. Although WGS has become less expensive in recent years, this technology still has disadvantages such as not giving information if the genes detected are being expressed, only provides information about cultivable bacteria, originates gaps in the assembled genome due to repeat regions, and still depends on specialize staff for data processing and interpretation that remain time-consuming (even though there are already several web-based tools that allow an easier search for, for example, resistance and virulence genes). Nevertheless, this technology has proven to be a major assistant in outbreaks, with the possibility to recreate transmission pathways and identify the source of the outbreak, also allowing the detection of micro-evolutionary changes over timescales of weeks or months, as well as helping in the development of new antibiotics (through, for example, the identification of novel antibiotic targets) and surveillance systems [50,130–132]. Using PFGE analysis to study a *Salmonella* Enteriditis outbreak in a restaurant, Vaughn et al. could not identify patients with common exposures. However, with WGS these authors could narrow the investigation to a single clade and identify additional patients and the source of the foodborne outbreak [133]. Another outbreak, this time in a hospital and caused by carbapenem producing *K. pneumoniae*, was studied through WGS by Benulič et al., who identified the possible source of the outbreak linked to a transmission of a plasmid carrying $bla_{\text{OXA-48}}$ gene and a second outbreak previously unidentified was also detected [134].

Other technology, metagenomics, solves one of the problems of WGS, since it does not require cultivation of the microorganisms. In this technology, DNA is extracted directly from a sample and later two methods can be applied. The first one, 16S sequencing, analyzes the 16S rRNA gene and provides information about the taxonomy of the microorganisms present in a sample and their relative abundance. 16S rRNA gene exists in nearly all bacteria and is highly conserved with only some variable regions specific to certain species or genus, thus allowing an identification of the bacterium by comparison with sequences already described. The

second method, whole metagenome sequencing (WMS), analyzes all genes present in a genome through DNA fragmentation and sequencing, right after extraction without the amplification step. This method allows not only to establish relative abundance of taxa but also to identify resistance, virulence, and functional genes, as well as mobile genetic elements (Figure 1.15). Therefore, metagenomics is an extremely useful method to analyze samples (like environmental samples) where culture-based methods cannot give all information. Some disadvantages of metagenomics are common to WGS technology, namely the costs and the need for specialized staff and equipment to analyze the amount of data generated [135,136]. The work developed by Li et al. used a metagenomic approach to characterize the bacterial community and antibiotic resistance genes of 18 ready-to-eat (RTE) food (including meat, vegetables, and fruit). They revealed that the most abundant phyla were *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, *Bacteroidetes* and *Actinobacteria* and detected 18 types of antibiotic resistance genes, most of them associated with β-lactams, chloramphenicol, aminoglycosides, tetracycline, bacitracin and macrolide-lincosamide-streptogramin resistance [137]. This type of analysis can also be applied to aquaculture [138].

Other technologies, such as metatranscriptomics (that uses mRNA to study gene expression), metaproteomics (that studies protein expression) and metabolomics (that studies intracellular metabolite concentrations), can be used to complement the information provided by genomic methods [139].

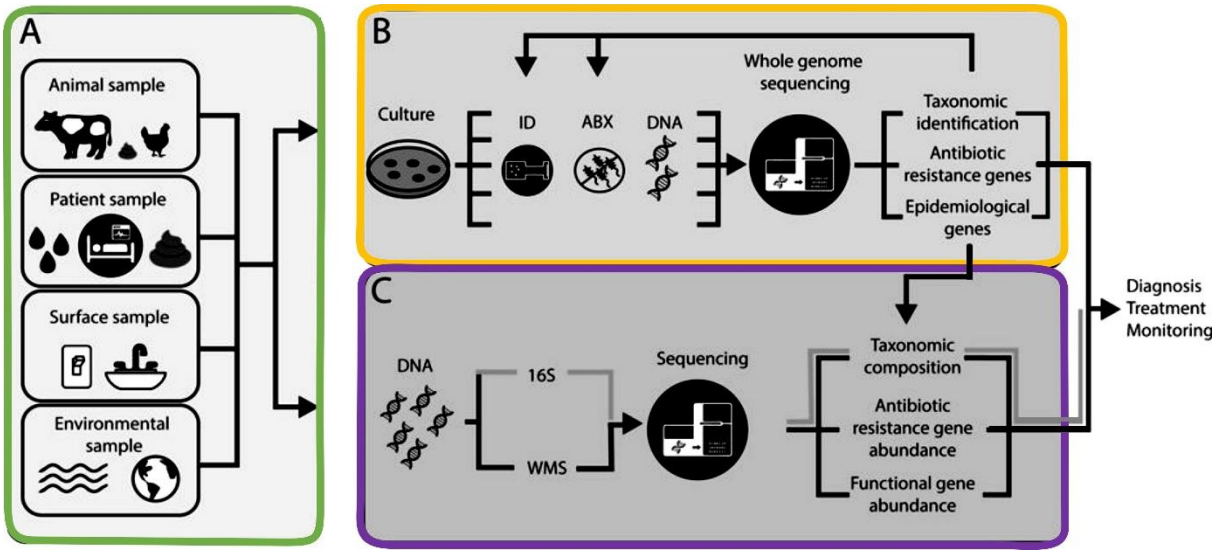

Figure 1.15 — Schematic representation of the application of whole genome sequencing (WGS) and metagenomic approaches. **(A)** Different samples can be studied. **(B)** Before implementing WGS, samples can be cultured and bacteria isolated, identified, tested for antibiotics (ABX) susceptibility and DNA must be extracted. WGS results allow taxonomic determination and identification of resistance and epidemiological genes. These results can be compared

to phenotypic characterization. **(C)** In metagenomic technology, DNA is extracted directly from a sample and later two methods can be applied. The first one, 16S sequencing, provides information about the taxonomy of the microorganisms. The second method, whole metagenome sequencing (WMS), allows not only to establish relative abundance of taxa but also to identify resistance, virulence, and functional genes, as well as mobile genetic elements (adapted from [135]).

2

AIM OF THE THESIS

As we have seen, the use of antibiotics in aquaculture can result in the emergence of reservoirs of antibiotic resistant bacteria in farmed fish and other animals, as well as in the aquatic environment.

Our main hypothesis is that the microbiome of different aquatic farmed animals reflects the environment in which they live regarding antibiotic resistance and virulence factors (VFs), which, in turn, may have severe consequences not only for their own health, with implications at industry's economic level, but also for the health of the human populations. Although, in recent years, some studies have been developed to understand how the different environmental conditions shape animal's microbiome regarding the presence of specific pathogens, antibiotic resistance determinants, and VFs in aquaculture [138,140], there are still many gaps to fill, especially in Portugal [141–144]. Thus, this thesis aimed to contribute to the knowledge of aquaculture's microbiome and how the resistance mechanisms are being promoted in these environments, thereby allowing for antibiotic resistance to develop, and spread via food and environment.

Specific objectives of the different studies included:

1. to explore the diversity of bacterial composition of different animals produced in different aquaculture environments;
2. to understand which antibiotic resistances, molecular mechanisms and VFs are being promoted in these animals and environments, namely through MGE;
3. to characterize in more detail specific strains through whole genome sequence (WGS), namely the genetic environment of resistance genes, to better understand how these genes are being selected and disseminated.

The information obtained, will help to address how the different ecosystems may be influencing the composition of the different microbiomes, and how this directs the potential parallel evolution of VFs and antibiotic resistance genes. Ultimately, this work will highlight specific research that could provide the basis of science-based policies for the suitable use of antibiotics in aquaculture.

3

BACTERIAL DIVERSITY AND ANTIBIOTIC SUSCEPTIBILITY OF *SPARUS AURATA* FROM AQUACULTURE

This research paper was published as:

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

Personal contributions:

Salgueiro, V. contributed to acquisition of laboratory data, data curation and formal analysis, investigation, writing of the original draft, and final approval of manuscript.

Note:

This work was partially presented at the Congress of Microbiology and Biotechnology 2019 (MICROBIOTEC 19) in Coimbra, Portugal.

3.1 Abstract

In a world where the population continues to increase and the volume of fishing catches stagnates or even falls, the aquaculture sector has great growth potential. This study aimed to contribute to a deeper knowledge of the diversity of bacterial species found in *Sparus aurata* collected from a fish farm and to understand which profiles of diminished susceptibility to antibiotics can be found in these bacteria that might be disseminated in the environment. One hundred thirty-six bacterial strains were recovered from *S. aurata* samples. These strains belonged to *Bacillaceae*, *Bacillales* Family XII. *Incertae Sedis*, *Comamonadaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Erwiniaceae*, *Micrococcaceae*, *Pseudomonadaceae* and *Staphylococcaceae* families. *Enterobacter* sp. was more frequently found in gills, intestine, and skin group than in muscle group ($p \leq 0.01$). Antibiotic susceptibility tests found that non-susceptibility to phenicols was significantly higher in gills, intestine, and skin samples (45%) than in muscle samples (24%) ($p \leq 0.01$) and was the most frequently found non-susceptibility in both groups of samples. The group of *Enterobacteriaceae* from muscles presented less decreased susceptibility to florfenicol (44%) than in the group of gills, intestine, and skin samples (76%). We found decreased susceptibilities to β-lactams and glycopeptides in the *Bacillaceae* family, to quinolones and mupirocin in the *Staphylococcaceae* family, and mostly to β-lactams, phenicols and quinolones in the *Enterobacteriaceae* and *Pseudomonadaceae* families. Seven *Enterobacter* spp. and five *Pseudomonas* spp. showed non-susceptibility to ertapenem and meropenem, respectively, which is of concern because they are antibiotics used as a last resort in serious clinical infections. To our knowledge, this is the first description of species *Exiguobacterium acetylicum*, *Klebsiella michiganensis*, *Lelliottia* sp. and *Pantoea vagans* (excluding cases where these bacteria are used as probiotics) and of plasmid-mediated quinolone resistance *qnrB19*-producing *Leclercia adecarboxylata* associated with *S. aurata*. The non-synonymous G385T and C402A mutations at *parC* gene (within quinolone resistance-determining regions) were also identified in a *Klebsiella pneumoniae*, revealing decreased susceptibility to ciprofloxacin. In this study, we found not only bacteria from the natural microbiota of fish, but also pathogenic bacteria associated with fish and humans. Several antibiotics for which decreased susceptibility was found here are integrated into the World Health Organization list of "critically important antimicrobials" and "highly important antimicrobials" for human medicine.

Keywords: *Sparus aurata*, aquaculture, antibiotic resistance, *qnrB19*, One Health.

3.2 Introduction

In the last 30 years, global production of the aquaculture sector has increased and today represents nearly half of the fish consumed worldwide, with China as the main producer. This was a consequence of higher demand due to the reduction or stagnation of fishing catches and an increasing world population [2].

Portugal is the third major consumer of fish, in Europe [145]. Although aquaculture represents a small portion of this consumption, this sector has grown in the last decades and is expected to increase in the coming years. This is a country with favorable conditions for aquaculture, where the main production is of bivalve mollusks, and extensive and intensive systems are predominant. Tanks for fish production represent about 5% of all Portuguese aquaculture infrastructures (in 2012), and turbot (*Scophthalmus maximus*), European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) are the main species produced [10]. *S. aurata* is a species belonging to the *Sparidae* family and *Perciformes* order [12], is often found in shallow waters and is sensitive to water temperature. Gilthead seabream has great commercial importance in Europe, representing one of the main species cultivated in this continent [146].

However, despite all the advantages, aquaculture production can have a negative impact, specifically in degradation of natural resources and rising of antibiotic use [2,76]. A higher density of fish in a specific area is usually related to an increase in stress conditions, leading to a predisposition for infectious diseases and a higher antibiotic consumption. Therefore, these environments can function as a reservoir of antibiotic resistance and/or antibiotic resistance genes. In Europe, antibiotics for growth promotion are not authorized, and only eight antibiotics are allowed for prophylaxis and therapeutics: ampicillin, oxytetracycline, florfenicol, flumequine, oxolinic acid, sarafloxacin, erythromycin and sulphonamides associated with trimethoprim or ormetoprim. These antibiotics are mostly administered by oral-medicated feed or bath, which are the easiest methods to apply but expose both sick and healthy individuals to antibiotics and allow the accumulation of these substances in sediments and water. This contributes to selective pressure in bacteria from these environments [68,69].

In this context, this study aimed to contribute to a deeper knowledge of the diversity of bacterial species found in *S. aurata* from aquaculture and the respective antibiotic susceptibilities that can be disseminated in their environment. The correlation of antibiotic resistance with the WHO list of "critically important antimicrobials" and "highly important antimicrobials" for human medicine will also be established.

3.3 Materials and methods

3.3.1 Sample collection and preparation

Five commercial-size *S. aurata* (500–1500 g) were collected in March 2018 in a land tank from a fish farming pilot station in the south of Portugal by the Portuguese Institute for the Sea and Atmosphere. This station is located in the Ria Formosa Natural Park and is an integrated multi-trophic aquaculture with a semi-intensive system. The weight, furcal length, total length, and condition index of the 5-gilthead seabream were measured (Table 3.1). Each fish was divided into 4 samples (gills, intestine, muscle, and skin), that were frozen and transported on ice to the National Institute of Health Dr. Ricardo Jorge, where they were analyzed. This study includes the results of the testing for 5 samples of muscle (from fish 1, 2, 3, 4 and 5) and the gills, intestine, and skin samples from fish 1 that were treated separately (except for some results presentation) (Figure S 1).

Table 3.1 — Weight, furcal length, total length, and condition index of the five *Sparus aurata* collected in March 2018, as well as the water temperature of the land tank from fish farming pilot station in the south of Portugal.

S. aurata	Weight (g)	Furcal Length (cm)	Total Length (cm)	Condition Index[1]	Water Temperature
Fish 1	1303	37.7	42.3	1.72	17.5 °C
Fish 2	1201	34.4	38.9	2.04	
Fish 3	977	35.6	39.7	1.56	
Fish 4	1076	36.2	40.1	1.67	
Fish 5	1197	37.4	41.1	1.72	

[1]Condition index allows the assessment of the health of the fish through the relationship between its weight and length (values greater than 1 indicate a good condition of the fish) [147].

3.3.2 Bacterial isolation and identification

Ten grams of each sample were homogenized in peptone water (Stomacher 80 Biomaster®, Seward, UK), incubated for 12 to 18 h at 37°C and further diluted [148,149]. Each dilution was plated in selective media (MacConkey agar, Mannitol salt agar and UriSelect™4 chromogenic agar) and incubated for 18 to 20 h at 37°C. Colonies with different morphology (to avoid duplications) were selected and DNA extracted, according to manufacturer's instructions (MagNA Pure 96 Instrument, Roche, Manheim, Germany). Strains were identified by

VITEK® 2 (BioMérieux, Marcy-l'Étoile, France) and amplification of the 16S rRNA gene, as already described [150].

3.3.3 Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by disk diffusion (Bio-Rad, Marnes-la-Coquette, France) and minimum inhibitory concentration (MIC) by in-house broth microdilution and E-test® (BioMérieux, Marcy l'Etoile, France). Different bacterial families were tested for different antibiotics (Table 3.2). For *Enterobacteriaceae* and *Erwiniaceae*, the antibiotics tested were amoxicillin/clavulanic acid, aztreonam, cefepime, cefotaxime, cefoxitin, ceftazidime, ertapenem, imipenem, meropenem, piperacillin/tazobactam, ciprofloxacin, trimethoprim/sulfamethoxazole, gentamicin, chloramphenicol, florfenicol, flumequine and oxytetracycline. For *Pseudomonadaceae*, the antibiotics tested were aztreonam, cefepime, ceftazidime, doripenem, ertapenem, imipenem, meropenem, piperacillin/tazobactam, ciprofloxacin, levofloxacin, amikacin, gentamicin, netilmicin, tobramycin, chloramphenicol, florfenicol, flumequine and oxytetracycline. On the other hand, for *Staphylococcaceae*, the antibiotics tested were cefoxitin, ciprofloxacin, levofloxacin, moxifloxacin, rifampicin, mupirocin, fusidic acid, daptomycin, linezolid, teicoplanin and vancomycin. For *Enterococcaceae*, the antibiotics tested were ampicillin, high concentration (HC) gentamicin, HC streptomycin, linezolid, teicoplanin and vancomycin. For *Bacillaceae*, the antibiotic tested was vancomycin.

For Gram-negative bacteria, the antibiogram was completed with 1) disc combination test (DCT or combined disk test, CDT) [151], which is based on the comparison between zone diameters of one disc of antibiotic alone and another with an inhibitor, here using cefotaxime (30 µg) and cefotaxime/clavulanic acid (30 µg + 10 µg) to search for the presence of extended-spectrum β-lactamase (ESBL-positive if there is a difference of ≥ 5 mm); 2) DCT to compare zone diameters of meropenem and meropenem/dipicolinic acid (1000 µg), to search for the presence of Metallo-β-lactamase (MBL-positive if ≥ 5 mm) [152]; 3) DCT with amoxicillin and amoxicillin/clavulanic acid plus cloxacillin (500 µg), to search for the presence of AmpC (positive for cephalosporinases if ≥ 5 mm) [151]; 4) double disc synergy test (DDST) with two discs containing predefined amounts of the β-lactam and the inhibitor, placed close to each other, here using boronic acid (300 µg) and carbapenems to search for any class A carbapenemase (positive if a synergy is observed between carbapenems discs) [152]; 5) DDST to search for MBL when a synergy is observed between dipicolinic acid and carbapenems [152]; 6) DDST to detect

Table 3.2 — Antibiotics used for antibiotic susceptibility testing and respective concentrations and breakpoints by bacterial family.

Family	Method	Antibiotics Tested (Concentration)	Breakpoints
Bacillaceae	MIC by E-test®	VA (0.016–256 µg/L)	CLSI M45
Enterobacteriaceae *Erwiniaceae*	Disk diffusion	AMC (20 + 10 µg), AZT (30 µg), FEP (30 µg), CTX (5 µg), FOX (30 µg), CAZ (10 µg), ERT (10 µg), IMP (10 µg), MEM (10 µg), PTZ (36 µg), CIP (5 µg) SXT (25 µg), GEN (10 µg)	EUCAST
	MIC by broth microdilution	CHL, FLO, OTC FMQ	CLSI VET08 CASFM VET 2019
Enterococcaceae	Disk diffusion	AMP (2 µg), HC GEN (30 µg), HC STR (300 µg)	EUCAST
	MIC by E-test®	LNZ, TP, VA	
Pseudomonadaceae	Disk diffusion	AZT (30 µg), FEP (30 µg), CAZ (10 µg), DOR (10 µg), ERT (10 µg), IMP (10 µg), MEM (10 µg), PTZ (36 µg), CIP (5 µg), LEV (5 µg), AN (30 µg), GEN (10 µg), NET (10 µg), TMN (10 µg)	EUCAST
	MIC by broth microdilution	CHL, FLO, FMQ, OTC	CLSI M100[1]
Staphylococcaceae	Disk diffusion	FOX (30 µg), CIP (5 µg), LEV (5 µg), MOX (5 µg), RIF (5 µg), MUP (200 µg), FUS (10 µg)	EUCAST
	MIC by E-test®	DPC (0.016–256 µg/mL), LNZ (0.016–256 µg/mL), TP (0.016–256 µg/mL), VA (0.016–256 µg/mL)	

[1]Breakpoints for CHL were used for FLO as well; breakpoints for CIP were used for FMQ; and breakpoints for tetracycline were used for OTC. Abbreviations — AN: amikacin; AMC: amoxicillin/clavulanic acid; AMP: ampicillin; AZT: aztreonam; FEP: cefepime; CTX: cefotaxime; FOX: cefoxitin; CAZ: ceftazidime; CHL: chloramphenicol; CIP: ciprofloxacin; DPC: daptomycin; DOR: doripenem; ERT: ertapenem; FLO: florfenicol; FMQ: flumequine; FUS: fusidic acid; GEN: gentamicin; HC GEN: high concentration gentamicin; HC STR: high concentration streptomycin; IPM: imipenem; LEV: levofloxacin; LNZ: linezolid; MEM: meropenem; MOX: moxifloxacin; MUP: mupirocin; NET: netilmicin; OTC: oxytetracycline; PTZ: piperacillin/tazobactam; RIF: rifampicin; TP: teicoplanin; TMN: tobramycin; SXT: trimethoprim/sulfamethoxazole; VA: vancomycin; CASFM VET: Comité de l'antibiogramme de la Société Française de Microbiologie Recommandations Vétérinaires; CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing.

AmpC when a synergy is observed between boronic acid and third-generation cephalosporins and/or cloxacillin and cefoxitin/ceftazidime [151]; 7) temocillin disc to indicate the presence of an OXA-48 carbapenemase; and 8) faropenem disc to indicate the presence of carbapenemases, confirming the results from 4), 5) and 7) tests.

MIC_{50} and MIC_{90} were calculated for *Enterobacteriaceae* since it was the most represented family, as reported elsewhere [153]. MIC_{50} represents the MIC value that inhibits 50% of the strains tested (and is equivalent to the median MIC value), whereas MIC_{90} represents the MIC value that inhibits 90% of the strains tested. *Escherichia coli* strain ATCC 25922 was used as quality control for Gram-negative bacteria, whereas *Staphylococcus aureus* strain ATCC 25923 and *Enterococcus faecalis* ATCC 29212 were used for Gram-positive bacteria.

3.3.4 Statistical analysis

Statistical analysis of the results was performed to detect positive or negative associations between fish samples (muscle vs. gills, intestine, and skin) and each bacterial family/species and non-susceptibility to different antibiotic's class (only factors identified as statistically significant are shown). Fisher exact test was used to assess differences in bacterial families/species/non-susceptibility to different antibiotic's class between fish samples, and one-tailed *p*-values of ≤ 0.05 were considered to be statistically significant. Associations were established by calculation of odds ratios with 95% confidence intervals. The null hypothesis was rejected for *p*-values of ≤ 0.05. All statistical analysis was calculated using OpenEpi software, v. 3.01 [154].

3.3.5 Detection of antibiotic resistance genes

The interpretation of the antibiotic susceptibility testing results guided the research of resistance genes by PCR (Polymerase Chain Reaction), and all the positive results were sequenced, as described elsewhere [150].

The genes $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV} and $bla_{OXA\text{-}1}$-type were investigated for Gram-negative strains that showed decreased susceptibility to β-lactams and/or demonstrated a positive result for the DCT and DDST [155,156]. The presence of genes $bla_{OXA\text{-}48}$, bla_{VIM}, $bla_{IMP\text{-}1}$-type, bla_{NDM}, bla_{KPC}, bla_{GES} and bla_{SME} was studied for Gram-negative bacteria with decreased susceptibility to carbapenems and/or that demonstrated a positive result for the DCT and DDST, using primers described in this study for the first time (Table 3.3), and others previously described [157–161]. All Gram-negative strains with decreased susceptibility to quinolones were tested

for *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib* and *qepA* genes (Table 3.3) [162–166]. For one *Klebsiella pneumoniae* strain with decreased susceptibility to ciprofloxacin, which tested negative for the genes described previously, we searched for mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes (Table 3.3) [167–169]. All Gram-negative bacteria with decreased susceptibility to quinolones but negative results for *qnr*, *aac(6')-Ib* and *qepA* genes were investigated for the presence of *oqxAB* genes, with primers and PCR reactions conditions described elsewhere [150]. All Gram-negative strains were investigated for the presence of *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, and *mcr-5* genes as previously described [170].

Apart from the antibiotic susceptibility testing results, all *Staphylococcus* spp. were tested for the presence of *mecA*, *mecC*, *vanA*, *vanB* and *vanD* genes (Table 3.3) [171]. On the other hand, all *Enterococcus* spp. and three *Bacillus* spp. resistant to vancomycin were investigated for the presence of *vanA*, *vanB* and *vanD* genes. The cycling conditions of the PCR multiplex for detection of *vanA*, *vanB* and *vanD* genes were as follows: 1 cycle of denaturation at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min and elongation at 72°C for 1 min, and a final cycle of elongation at 72°C for 10 min.

Table 3.3 — Primers used in the detection of resistance genes that were described in this study for the first time.

Gene	Forward Primer Sequence (5′ → 3′)	Reverse Primer Sequence (5′ → 3′)	AT[2]	PCR[3]
bla_{OXA-48}	GACTATATTATTCGGGCTAA	ACCACTTCTAGGGAATAATT	58°C	140 bp
bla_{NDM}	GTTTGATCGTCAGGGATGGC	AACGGTGATATTGTCACTGGT	56°C	359 bp
bla_{GES}	AAAGCAGCTCAGATCGGTGT	TCTCTCCAACAACCCAATC	56°C	707 bp
bla_{SME}	CAGATGAGCGGTTCCCTTTA	AACCCAATCAGCAGGAACAC	56°C	509 bp
qnrB [1]	ATGACGCCATTACTGTATAA	CTAACCAATCACCGCGATGC	49°C	697 bp
qnrC	AACGTACGATCAAATTG	TCCACTTTACGAGGTTCT	55°C	560 bp
gyrB	GGACAAAGAAGGCTACAGCA	CGTCGCGTTGTACTCAGATA	55°C	880 bp
vanA	AAGGTCTGTTTGAATTGTCCG	CGACTTCCTGATGAATACGA	55°C	417 bp
vanB	CCATACTCTCCCCGGATAGG	TTGACCTCATTTAGAACGATGC	55°C	721 bp
vanD	ATTGGAATCACAAAATCCG	GGCTGTGCTTCCTGATG	55°C	626 bp

[1]Primers used for sequencing. [2]Annealing temperature. [3]Polymerase chain reaction (PCR) product.

3.4 Results

3.4.1 Bacterial diversity in *S. aurata* samples

One hundred thirty-six bacterial strains were recovered from the total of *S. aurata* samples. Eighty-eight were Gram-negative bacteria, and 48 were Gram-positive bacteria. The results of VITEK® 2 and amplification of the 16S rRNA gene revealed that the majority of strains belonged to the *Enterobacteriaceae* family (55% in muscle samples and 60% in gills, intestine and skin samples), followed by *Staphylococcaceae* in muscle samples (16%) and *Bacillaceae* in gills, intestine and skin samples (17%) (Table 3.4). *Bacillales* Family XII. *Incertae Sedis*, *Comamonadaceae* and *Micrococcaceae* families were only found in muscle samples, whereas *Erwiniaceae* family was only found in gills samples. Nevertheless, these results do not represent statistically significant differences.

Table 3.4 — Bacterial families of the 136 strains recovered from muscle, gills, intestine, and skin samples.

Bacterial Family	Fish Farm			
	Muscle (n = 5)		Gills, Intestine and Skin (n = 1)[1]	
	No. of Strains	%	No. of Strains	%
Bacillaceae	9	10%	7	17%
Bacillales Family XII. *Incertae Sedis*	1	1%	0	0%
Comamonadaceae	2	2%	0	0%
Enterobacteriaceae	52	55%	25	60%
Enterococcaceae	4	4%	2	5%
Erwiniaceae	0	0%	1	2%
Micrococcaceae	4	4%	0	0%
Pseudomonadaceae	7	7%	1	2%
Staphylococcaceae	15	16%	6	14%
Total	94	100%	42	100%

[1]Results from gills, intestine and skin samples were treated jointly.

Within the most represented family, *Enterobacteriaceae*, we observed the following species of bacteria: *Enterobacter cloacae*, *Enterobacter hormaechei*, *Enterobacter* sp., *Klebsiella michiganensis*, *K. pneumoniae*, *Leclercia adecarboxylata*, *Lelliottia* sp. (in both groups of samples), *Citrobacter freundii*, *Citrobacter freundii* complex (only in muscle samples) and *E. coli* (only in gills sample). *Staphylococcaceae* family included *S. aureus*, *Staphylococcus haemolyticus*, *Staphylococcus pasteuri* (in both groups of samples), *Staphylococcus capitis*,

Staphylococcus epidermidis, *Staphylococcus petrasii*, *Staphylococcus saprophyticus* and *Staphylococcus* sp. (only in muscle samples). *Bacillaceae* included *Bacillus cereus*, *Bacillus* sp. (in both groups of samples), *Bacillus pumilus*, *Bacillus thuringiensis* (only in muscle samples), *Bacillus amyloliquefaciens* and *Bacillus subtilis* species (only in gills, intestine, and skin group of samples). *Pseudomonadaceae* included *Pseudomonas stutzeri* (in both groups of samples) and *Pseudomonas putida* (only in muscle samples). *Enterococcaceae* included *Enterococcus hirae* (in both groups of samples) and *Enterococcus faecalis* (only in gills sample). *Bacillales* Family XII. *Incertae Sedis* included *Exiguobacterium acetylicum* species. *Comamonadaceae* included *Comamonas aquatica* species. *Micrococcaceae* included *Kocuria rhizophila* species. Finally, *Erwiniaceae* family included *Pantoea vagans* species. Despite appearing in both groups of samples, *Enterobacter* sp. are more frequently found in gills, intestine and skin group than in muscle group ($p \leq 0.01$; Table 3.5 and Table S 1). Statistically significant associations were not found for the other species analyzed.

Table 3.5 — Odds ratio (OR) and 95% confidence intervals (CI) ($p \leq 0.05$) from the analysis of negative and positive correlations between fish samples (muscle vs. gills, intestine, and skin) and each bacterial species and non-susceptibility to different antibiotic classes.

Fish Sample	Bacterial Species[1]	Antibiotic's Class[2]	OR[3]	95% CI	*p* Value
Muscle	ALL	Phenicols	0.3921 (P)	0.1701–0.912	≤ 0.01
Gills, intestine, and skin	ALL	Phenicols	2.55	1.096–5.879	≤ 0.01
Muscle	*Enterobacter* sp.	-	0.1648 (P)	0.02645–0.7834	≤ 0.01
Gills, intestine, and skin	*Enterobacter* sp.	-	6.067	1.277–37.8	≤ 0.01

Only significant associations are presented: *p*-values ≤ 0.05 and confidence limits excluding null values (0, 1, or [*n*]). [1]ALL include all species identified in the study, described in point 3.4.1 of results. [2]Antibiotic classes tested were glycopeptides, mupirocin, phenicols, quinolones, and β-lactams. [3](P) indicates an OR value for a protective or negative association; otherwise, values should be interpreted as a positive association.

3.4.2 Phenotypic characterization of the bacterial strains

In this study, non-susceptibility to phenicols was the most frequently found in both groups of samples, being significantly higher in gills, intestine and skin samples (45%; Table 3.6) than in muscle samples (24%; Table 3.6) ($p \leq 0.01$; Table 3.5). Non-susceptibility to β-lactams is the second most prevalent in both groups of samples (13% in muscles and 10% in gills, intestine, and skin samples; Table 3.6). Decreased susceptibility to quinolones was also found

in this study (7% in muscle and 5% in gills, intestine, and skin samples). On the other hand, decreased susceptibility to glycopeptides and to mupirocin was only found in muscle samples.

Table 3.6 — Antibiotic susceptibility testing results of the 136 strains found in this study (these results do not include known intrinsic non-susceptibilities).

Antibiotic's Class	Fish Farm			
	Muscle (*n* = 94)		Gills, Intestine and Skin (*n* = 42)	
	R/I (%)	S (%)	R/I (%)	S (%)
Aminoglycosides	0 (0)	94 (100)	0 (0)	42 (100)
Fusidanes	0 (0)	94 (100)	0 (0)	42 (100)
Glycopeptides[1]	3 (3)	91 (97)	0 (0)	42 (100)
Lipopeptides	0 (0)	94 (100)	0 (0)	42 (100)
Mupirocin	1 (1)	93 (99)	0 (0)	42 (100)
Oxazolidinones	0 (0)	94 (100)	0 (0)	42 (100)
Phenicols[2]	23 (24)	71 (76)	19 (45)	23 (55)
Quinolones[3]	7 (7)	87 (93)	2 (5)	40 (95)
Rifampicin	0 (0)	94 (100)	0 (0)	42 (100)
Tetracyclines	0 (0)	94 (100)	0 (0)	42 (100)
Trimethoprim/sulfamethoxazole	0 (0)	94 (100)	0 (0)	42 (100)
β-lactams[4]	12 (13)	82 (87)	4 (10)	38 (90)

[1]Vancomycin. [2]Chloramphenicol and florfenicol. [3]Ciprofloxacin, flumequine and levofloxacin. [4]Amoxicillin/clavulanic acid, aztreonam, cefepime, cefotaxime, cefoxitin, ceftazidime, ertapenem, meropenem and piperacillin/tazobactam. R: resistant. I: intermediate. S: susceptible.

Among *Enterobacteriaceae* strains, no differences were observed in MIC_{50} and MIC_{90} between the two groups of samples for the four antibiotics tested (Table 3.7). For each antibiotic, slight differences were registered between MIC_{50} and MIC_{90} (just 1-fold dilution). When comparing the decreased susceptibilities between the two groups of samples (muscle vs. gills, intestine and skin), a major difference was found only in florfenicol, with the group of *Enterobacteriaceae* from muscles presenting 44% of non-susceptible strains, while in the group of gills, intestine and skin samples, the values were higher, with 76% (Table 3.7).

Table 3.7 — MIC_{50} and MIC_{90} for *Enterobacteriaceae* strains (*n*=77).

Antibiotic	*Enterobacteriaceae*											
	Muscle (*n*=52)						Gills, Intestine and Skin (*n*=25)					
	MIC_{50}	MIC_{90}	Range	S%	I%	R%	MIC_{50}	MIC_{90}	Range	S%	I%	R%
Flumequine	0.5	1	0.125-2	100	NA	0	0.5	1	0.25-4	100	NA	0
Chloram-phenicol	4	8	1-32	96	2	2	4	8	2-32	92	4	4
Florfenicol	8	16	1-32	56	37	7	8	16	1-32	24	60	16
Oxytetracy-cline	2	4	0.5-4	100	0	0	2	4	0.5-4	100	0	0

NA: not applicable, because intermediate category does not exist for flumequine. R: resistant. I: intermediate. S: susceptible.

In Table 3.8 is registered the decreased susceptibility profile for the 61 strains that had a non-susceptibility result for at least one antibiotic (including intrinsic non-susceptibilities). In Gram-positive bacteria, we found decreased susceptibilities to β-lactams and glycopeptides in the *Bacillaceae* family and to quinolones and mupirocin in the *Staphylococcaceae* family. In Gram-negative bacteria, *Enterobacteriaceae* family showed several decreased susceptibility profiles, with non-susceptibilities to β-lactams, phenicols and quinolones; the same non-susceptibilities to these antibiotic classes were found in strains from the *Pseudomonadaceae* family. Non-susceptibilities to ertapenem and to the antipseudomonal carbapenem (meropenem) were found in seven *Enterobacter* spp. and five *Pseudomonas* spp., respectively.

Table 3.8 — Phenotypic profile of the 61 strains that revealed decreased susceptibility to at least one antibiotic, including intrinsic non-susceptibilities.

Family	Species	Decreased Susceptibility Profile	No. of Strains
Bacillaceae	*Bacillus cereus*	VA	1
	Bacillus sp.	VA	2
Enterobacteriaceae	*Citrobacter freundii*	AMC, FOX, FLO	1
	Citrobacter freundii complex	AMC, FOX, FLO	1
	Enterobacter cloacae	AMC, FOX, CHL, FLO	2
		AMC, FOX, FLO	4
		AMC, AZT, FEP, CTX, FOX, CAZ, ERT, FLO, PTZ	1
		AMC, AZT, CAZ, ERT, FOX	1
	Enterobacter hormaechei	AMC, FOX, FLO	12
		AMC, FOX	5

		AMC, AZT, FEP, CTX, FOX, CAZ, ERT, FLO, PTZ	1
		AMC, AZT, FEP, CTX, FOX, CAZ, ERT, FLO, PTZ	1
	Enterobacter sp.	AMC, FOX, FLO	6
		AMC, FOX, FLO, ERT	1
		AMC, FOX, CAZ	1
		AMC, AZT, FEP, CTX, FOX, CAZ, CHL, ERT, FLO, PTZ	1
		AMC, AZT, CTX, FOX, CAZ, ERT, FLO	1
	Escherichia coli	AMC, CHL, FLO	1
		AMC, FLO	2
	Klebsiella pneumoniae	CIP, FLO	1
		FLO	5
	Leclercia adecarboxylata	CIP, FLO	1
Pseudomonadaceae	*Pseudomonas putida*	AZT, ERT, MEM	2
		AZT, CHL, ERT, FLO, FMQ, MEM	2
	Pseudomonas stutzeri	AZT, CHL, ERT, FLO, FMQ	3
		AZT, CIP, ERT, FLO, FMQ, MEM	1
Staphylococcaceae	*Staphylococcus petrasii*	CIP, LEV, MUP	1
Total			61

AMC: amoxicillin/clavulanic acid; AZT: aztreonam; FEP: cefepime; CTX: cefotaxime; FOX: cefoxitin; CAZ: ceftazidime; CHL: chloramphenicol; CIP: ciprofloxacin; ERT: ertapenem; FLO: florfenicol; FMQ: flumequine; LEV: levofloxacin; MEM: meropenem; MUP: mupirocin; PTZ: piperacillin/tazobactam; VA: vancomycin.

3.4.3 Genotypic characterization

The search for resistance genes by PCR revealed a *qnrB19* gene in a *L. adecarboxylata* strain, with decreased susceptibility to ciprofloxacin (zone diameter=24 mm). The analyses of the mutations present in *gyrA*, *gyrB*, *parC* and *parE* genes of a *K. pneumoniae* strain with decreased susceptibility to ciprofloxacin (zone diameter=25 mm) showed the presence of non-synonymous mutations G385T (Ala129Ser in protein) and C402A (Ser134Arg in protein) in *parC* gene. No other resistance genes were identified in the studied bacteria among all the searched genes.

3.5 Discussion

The aquaculture sector has experienced a strong growth in recent decades and with it the number of studies to answer some concerns about the quality and safety of its products.

Some studies have focused on the search of antibiotic residues in waters and/or sediments from fish farms, while others have focused on the search for specific pathogens, such as *E. coli*, not only in water and/or sediments but also in fish and shellfish [77,142,172–175]. This study aims to provide data, not only on the bacterial diversity in *S. aurata* from aquaculture, but also on antibiotic resistance genes that circulate in these environments. This information is crucial in the construction of science-based policies for the suitable use of antibiotics in aquaculture.

In this study, we found a very diverse bacterial population with 31 species that belonged to nine different families. Microbiota present in fish depends not only on the fish genetics and diet but is also determined by microbiota present in their environment, such as water and sediments. Microbiome composition is normally different between individual fish belonging to the same species but also varies between healthy and sick individuals, wherein the healthy individuals seem to have a higher diversity of bacterial population [35]. The condition index of fish belonging to this study varied between 1.56 and 2.04 (Table 3.1), meaning that these *S. aurata* were healthy (values higher than 1), possibly explaining the diversity found.

Of the species found in this study, only *Pseudomonas* spp. and *K. rhizophila* appeared to frequently cause diseases in fish, *K. rhizophila* being an emerging pathogen [30,176]. *Pseudomonas* spp. are ubiquitous in nature, and *P. fluorescens* is the most important species in fish infections (not found in this study), although *P. putida* had already been found in internal organs of fish and *P. stutzeri* in sediments of marine waters [177]. This genus is responsible for strawberry disease and septicemia in some fish species [30]. *P. putida* and *P. stutzeri* are also opportunistic pathogens in humans and were already associated with bacteremia, endocarditis, keratitis, meningitis, pneumonia, skin and soft tissue infections and urinary tract infections [178–182]. *Kocuria* spp. are Gram-positive and coccoid bacteria isolated from numerous environments: skin of mammals, marine sediments, soil, and food [183–187]. Specifically, *K. rhizophila* causes a variety of lesions in fish and is responsible for a 50% mortality rate: exophthalmia, skin petechiae, increased skin melanization, liver congestion, inflammation of the intestine and hemorrhages [30]. Reports revealed that this species was already responsible for some infections in humans, mainly catheter-related bacteremia [188].

Other bacterial species from this study are known to be opportunistic pathogens in fish, some already described in *S. aurata*: *C. freundii* complex, *Staphylococcus* spp., *K. pneumoniae*, *E. faecalis* and *E. cloacae* [43,189–192]. All these species have pathogenic significance for humans, causing foodborne diseases, meningitides, wound and urinary tract infections, bacteremia, bone and joint infections, endocarditis, among others [42,43,112,193,194].

Bacillus spp., *E. coli* and *E. hormaechei* were already collected from fish, although they were not associated with fish diseases [195–197]. These three species are responsible for human diseases like food poisoning (especially, *B. cereus* and *E. coli*), bacteremia, meningitis, brain abscesses, endophthalmitis, pneumonia and sepsis [198–200].

There is a single report about the association of *L. adecarboxylata* with fish, more specifically in the oral cavity of sharks [201]. However, this species is frequently found in water environments. It can cause endocarditis, bacteremia, and peritonitis in human hosts [202,203]. Some studies indicate that *E. hirae* could be a part of the natural microbiota of fish. There is no literature involving this bacterium in fish infections, but this species was well characterized in human infections, such as endocarditis, pyelonephritis, acute pancreatitis, and septic shock, representing 1 to 3% of infections caused by *Enterococcus* spp. in clinical practice [204].

To our knowledge, this study seems to represent the first description of the species *E. acetylicum*, *K. michiganensis*, *Lelliottia* sp. and *P. vagans* associated with *S. aurata* (excluding cases where these bacteria are used as probiotics). These are environmental species, frequently found in soil, water, air, plants, and insects [205–211]. Some of these species have been associated with infections in humans, namely immunocompromised individuals, but rarely [212–217].

Together with *E. coli*, *E. faecalis* is also an indicator of fecal contamination [218,219]. Species like *Bacillus* spp., *E. acetylicum* and *Enterococcus* spp. can be used as probiotics in aquaculture to reduce antibiotic consumption and avoid the spread of antibiotic resistance genes [197,220,221]. We could not obtain information if this was the case of this fish farming pilot station, in Portugal, possibly justifying the presence of these bacteria in our samples.

β-Lactam antibiotics are used in aquaculture in many countries, especially amoxicillin [68]. Several studies revealed a high prevalence of non-susceptibility to this class of antibiotic in fish, shrimps, and water samples from aquaculture farms. These studies reported the discovery of bla_{SHV} and bla_{TEM} genes, associated with β-lactam non-susceptibility [222–225]. In our study, β-lactam resistance genes were not detected. The non-susceptibilities found here were probably related to genes or other resistance mechanisms not studied (e.g., efflux pumps). Likewise, some of the non-susceptibilities found to β-lactams are intrinsic, such as the resistance to ERT in *Pseudomonas* spp. [226]. Non-susceptibility to AMC, FOX, AZT, FEP, CTX, CAZ and PTZ in *Enterobacter* spp., *E. coli* and *Citrobacter* spp. could be explained by chromosomal AmpC β-lactamase or AmpC hyperproduction [227]. Worryingly, our study revealed decreased susceptibilities to carbapenems of *Enterobacter* spp. (ertapenem), usually associated with acquired resistance, as well as in *Pseudomonas* spp. (meropenem), which are not used in

aquaculture and are considered last-resort antibiotics to treat serious infections caused by multidrug-resistant bacteria in humans [228].

Interestingly, although banned for use in food-producing animals in many countries (since 1994 in Europe [229]), decreased chloramphenicol susceptibility continues to be described, not only in the present study (with MIC of 32 to > 64 mg/L), but also in others, with the presence of *cat* genes [218,230]. Chloramphenicol can occur naturally, produced by soil organisms such as *Streptomyces venezuelae*. The persistence of decreased susceptibility to this antibiotic may be due to intrinsic mechanisms of resistance as possibly in *P. putida* and *P. stutzeri* from our study or co-selection with other antibiotics and/or heavy metals [229,231,232]. On the other hand, florfenicol is widely used in aquaculture [69]. In a review article, Miranda et al. [233] compiled several studies in Chilean salmon farms that revealed a high frequency of decreased susceptibility to florfenicol, such as in our samples. However, there are further studies that described lower rates [234,235].

Another antibiotic class commonly used in aquaculture is quinolones, specifically flumequine and oxolinic acid [68,76]. In this study, we found low frequency of decreased susceptibility to quinolones (7% in muscles and 5% in gills, intestine, and skin group). These results are confirmed by other works in *S. aurata* samples [174] and other fish species [236], while others revealed higher frequencies, also in fish samples [237]. Of the nine strains with decreased susceptibility to quinolones in this study, we found genes that justify that resistance in only two; the others may have untested resistance mechanisms. *qnrB* genes are frequently found in aquaculture environments [238,239], but to our knowledge, this is the first description of *qnrB19* gene in an *L. adecarboxylata* strain. This strain had decreased susceptibility to ciprofloxacin and was susceptible to flumequine (MIC=4 mg/L). The *qnrB19* gene had already been reported in food-producing animals, such as pigs, poultry, and veal calves, sometimes associated with mobile genetic elements, like plasmids and insertion sequences, demonstrating a potential for spreading [164,240]. Moreover, *qnrB19* gene was also found in *Enterobacteriaceae* from human clinical samples [241–243]. *K. pneumoniae* strain with decreased susceptibility to ciprofloxacin and susceptibility to flumequine (MIC=1 mg/L) revealed two non-synonymous mutations in *parC* gene within the QRDR, one of them (G385T) already described in a *K. pneumoniae* strain from nosocomial origin [244]. It is known that mutations in QRDR of *parC* gene can result in structural changes in topoisomerase IV, reducing the affinity of this enzyme to fluoroquinolones [245].

Vancomycin and mupirocin are not used in aquaculture [68]. Decreased susceptibilities to these antibiotics were found only in muscle samples in this study. *Bacillus* spp. resistance to

vancomycin probably does not represent an intrinsic resistance since several studies indicated the susceptibility of these bacteria to this glycopeptide [198]. The search for *vanA*, *vanB* and *vanD* genes was negative, so this non-susceptibility could be explained by the presence of other genes not studied, like *vanC*, *vanE* and *vanG*, or mutations in *walKR*, *vraRS* and *graRS* genes involved in cell wall metabolism and cellular response to cell wall damage [246]. To our knowledge, this is the first description of *Bacillus* sp. strains resistant to vancomycin in *S. aurata* from aquaculture. This resistance was already found in this environment but in *Enterococcus* species [218]. Acquired reduced susceptibility to mupirocin in *S. petrasii* could be related to mutations in the *ileS* gene and, more rarely, to the acquisition of plasmid-mediated *mupA* gene, like in *S. aureus* [247]. There are no data available about the frequency of detection of decreased susceptibility to mupirocin in aquaculture.

As we demonstrated, some antibiotics used in food-producing animals are the same, or belong to the same class, as antibiotics used in humans [80]. Several antibiotics for which decreased susceptibility was found in this study are integrated into the WHO list of "critically important antimicrobials" and "highly important antimicrobials" for human medicine. This means that for some bacterial infections, these antibiotics are the only therapy available, and that they are also used in humans to treat infections caused by bacteria/resistance genes that may be transmitted from non-human sources (namely food-producing animals) [104].

3.6 Conclusions

This study unraveled some information about bacterial diversity and antibiotic resistance genes that circulate in *S. aurata* raised in aquaculture.

It is noteworthy the difficulty of characterizing the strains as susceptible or non-susceptible due to the absence of breakpoints to some of the species found in these aquatic environments and to antibiotics commonly used in aquaculture. Further research is needed in this area, also in relation to intrinsic resistances since opportunistic environmental bacteria can represent a health threat and a reservoir of antibiotic-resistant bacteria/resistance genes.

In the current study, we can observe protective associations in muscle samples, while positive correlations were found in gills, intestine, and skin group of samples. Indeed, being part of the gills, intestine and skin group represents a risk factor for the presence of *Enterobacter* sp. and non-susceptibility to phenicols.

Antibiotics are used in humans and aquaculture to treat infections caused by bacteria. Furthermore, it was already described that antibiotic residues can remain in fish tissues for long

periods of time, increasing exposure of commensal bacteria and/or fish pathogens, along with aquatic bacteria, to these antibiotics, enhancing the development of resistance [69]. Therefore, it is fundamental a "One Health" approach, combining the efforts of human and veterinary medicine, along with agriculture sector, to reduce the spread of resistant bacteria and/or bacterial resistance genes.

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4

ASSESSING THE BACTERIAL COMMUNITY COMPOSITION OF BIVALVE MOLLUSKS COLLECTED IN AQUACULTURE FARMS AND RESPECTIVE SUSCEPTIBILITY TO ANTIBIOTICS

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Personal contributions:

Salgueiro, V. contributed to acquisition of laboratory data, data curation and formal analysis, investigation, writing of the original draft, and final approval of manuscript.

Note:

This work was partially presented at the Congress of Microbiology and Biotechnology 2019 (MICROBIOTEC 19) in Coimbra, Portugal and at the 31st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2021), online event.

4.1 Abstract

Aquaculture is a growing sector, providing several products for human consumption, and it is therefore important to guarantee its quality and safety. This study aimed to contribute to the knowledge of bacterial composition of *Crassostrea gigas*, *Mytilus* spp. and *Ruditapes decussatus*, and antibiotic resistances/resistance genes present in aquaculture environments. Two hundred and twenty-two bacterial strains were recovered from all bivalve mollusks' samples belonging to the *Aeromonadaceae*, *Bacillaceae*, *Comamonadaceae*, *Enterobacteriaceae*, *Enterococcaceae*, *Micrococcaceae*, *Moraxellaceae*, *Morganellaceae*, *Pseudomonadaceae*, *Shewanellaceae*, *Staphylococcaceae*, *Streptococcaceae*, *Vibrionaceae*, and *Yersiniaceae* families. Decreased susceptibility to oxytetracycline prevails in all bivalve species, aquaculture farms and seasons. Decreased susceptibilities to amoxicillin, amoxicillin/clavulanic acid, cefotaxime, cefoxitin, ceftazidime, chloramphenicol, florfenicol, colistin, ciprofloxacin, flumequine, nalidixic acid and trimethoprim/sulfamethoxazole were also found. This study detected six *qnrA* genes among *Shewanella algae*, ten *qnrB* genes among *Citrobacter* spp. and *Escherichia coli*, three *oqxAB* genes from *Raoultella ornithinolytica* and $bla_{\text{TEM-1}}$ in eight *E. coli* strains harboring a *qnrB19* gene. Our results suggest that bacteria and antibiotic resistances/resistance genes present in bivalve mollusks depend on several factors, such as host species and respective life stage, bacterial family, farm's location, and season, and that is important to study each aquaculture farm individually to implement the most suitable measures to prevent outbreaks.

Keywords: bivalve mollusks, aquaculture, antibiotic resistance, oxytetracycline, PMQR.

4.2 Introduction

Aquaculture is an ancient activity, practiced since the Roman Empire (140 B.C.) in Europe. It has developed over the centuries, but it was in the last three decades that it experienced its greatest growth, pressured by increased demand [2,4].

Bivalve mollusks are known to be rich in proteins, vitamin D, long-chain omega-3 fatty acids, iodine and selenium, contributing to a healthy diet [17]. These organisms represent the main aquaculture production in Portugal and, in 2012, 95.2% of the active establishments were for bivalve mollusks production [10]. In 2015, the main species produced were *Ruditapes decussatus*, with 2300 tons, *Mytilus* spp., with 1200 tons, and *Crassostrea gigas* and *Ostrea edulis*,

with 650 tons. Other important species are *Cerastoderma edule* (264 tons). The central and southern regions of Portugal (regions B and A, respectively) are the most relevant in the national production of bivalve mollusks [9,10].

C. gigas (common name: Japanese oyster) is the mollusk most consumed worldwide. Along with *Mytilus* spp. (common name: mussels), they have a global geographical distribution, facilitated by features such as high fertility, rapid growth, and resistance to environmental variations (salinity, temperature, etc.). These are euryhaline species, whose natural habitat is in the lower limit of the intertidal zone until the subtidal (about 15 m) in estuaries and coastal lagoons for *C. gigas*, and in the high intertidal to subtidal regions in estuarine areas to oceanic seawaters for *Mytilus* spp. [4,19]. On the other hand, *R. decussatus* (common name: clams) are mostly cultivated in Portugal, Spain, the Atlantic coast of France and in the Mediterranean basin. This species is usually found in shallow waters, burrowed in sand and silty mud. These bivalve species feed by filtration of phytoplankton and organic matter (detritus) from the surrounding water [4,21,28]. This type of feeding allows an accumulation of numerous contaminants in these animals, such as toxins, antibiotic residues, bacteria, viruses, and protozoa. Therefore, bivalve mollusks can suffer from numerous infectious diseases, especially if cultured in high densities, that cause high mortality rates and have a significant commercial impact [32]. Among the most frequent diseases are those caused by bacteria, which leads to an increase in antibiotic consumption to treat and prevent the spread of these diseases. Moreover, the accumulation of antibiotic residues can submit commensal and pathogenic bacteria of these organisms and bacteria from the aquatic environment to a selective pressure, contributing to the rise of antibiotic resistance [21]. Among the most frequently found bacteria in bivalve mollusks are those belonging to the *Proteobacteria* phylum [140,248]. In this phylum, we can find normal commensal bacteria (e.g., *Bacillus* spp., *Vibrio* spp. and *Aeromonas* spp.) and non-commensal bacteria (e.g., *Shewanella algae*). Bacteria from both groups can become pathogenic to these organisms [32,249]. Previous studies detected antibiotic resistance, namely to amoxicillin and quinolones, in bivalve mollusks [250,251]. Other studies estimate 700 000 deaths per year around the world due to antibiotic-resistant bacteria [78]. Antibiotic resistance is a growing and global threat, reaching not only human, but also veterinary medicine, since there are studies that indicate the transfer of antibiotic resistance genes between these two reservoirs [252,253]. Bacteria present in bivalve mollusks (e.g., *Vibrio* spp. and *Photobacterium damselae*) can be responsible for infections in humans through the consumption (e.g., gastroenteritis) or handling of these organisms (e.g., wound infections that can evolve to necrotizing fasciitis with multiple organ failure and septicemia) [254–256].

Given this scenario, we designed a study to understand the diversity of antibiotic-resistant bacterial species present in the three mainly produced bivalve mollusks (*R. decussatus*, *Mytilus* spp. and *C. gigas*) in two locally distant regions of Portugal, and the molecular mechanisms of antibiotic resistance that are circulating in these aquaculture environments.

4.3 Materials and methods

4.3.1 Sample characterization

The Portuguese Institute for the Sea and Atmosphere provided the bivalve samples used in this study. In summer of 2019, were collected one sample of clams (*R. decussatus*) and one sample of Japanese oyster (*C. gigas*) in aquaculture farm 1 from region A (south of Portugal); three samples of mussels (*Mytilus* spp.) from aquaculture farms 2, 3 and 4, also in region A; and one sample of Japanese oyster collected from aquaculture farm 5 in region B (central region of Portugal). The aquaculture farms from region A present in this study are distributed along its coastline.

In autumn of 2019, the sampling previously described was repeated for both regions, except for the sample of Japanese oyster in region B, which was collected in a different aquaculture (farm 6).

All samples were frozen and transported on ice to the National Institute of Health Dr. Ricardo Jorge, where they were analyzed. In this study, one sample corresponds to 3 to 10 individuals, depending on the species (minimum 50 g, for each sample).

4.3.2 Bacterial isolation and identification

Fifty grams of each sample were homogenized in peptone water (Stomacher 80 Biomaster®, Seward, UK), making a 1:10 dilution, and incubated for 12 to 18 h at 37°C. Each dilution was plated in selective media, containing specific concentrations of different antibiotics (allowing an initial screening of decreased susceptibilities), and incubated for 18 to 20 h at 37°C. Aeromonas agar, MacConkey agar and Thiosulfate Citrate Bile Salts Sucrose Agar contained the following standard antibiotic concentrations to select antibiotic resistant strains: 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. Mannitol salt agar and UriSelect™4 chromogenic agar contained 8 mg/L of oxytetracycline. Plates with and without antibiotic were used as controls. Colonies with different morphology (to avoid duplications) were selected and

DNA extracted, according to manufacturer's instructions (MagNA Pure 96 Instrument, Roche, Manheim, Germany). Strains were identified by MALDI-TOF (BioMérieux, Marcy-l'Étoile, France) and amplification of the 16S rRNA gene, as previously described [150].

4.3.3 Statistical analysis of results

Statistical analysis was performed to detect positive or negative associations between bivalve species and each bacterial family, bivalve species/bacterial family and season, *C. gigas*/bacterial family and location, bivalve species/bacterial family and nonsusceptibility to different classes of antibiotics (using the results from the initial screening in selective media). Only factors identified as statistically significant are shown. Fisher's exact test was used to assess differences in bacterial families/season/location/nonsusceptibility to different classes of antibiotics between bivalve species and one-tailed *p* values of ≤ 0.05 were considered to be statistically significant. Associations were established by calculation of odds ratios with 95% confidence intervals. The null hypothesis was rejected for *p* values of ≤ 0.05. All statistical analysis was calculated using OpenEpi software, version 3.01 [154].

4.3.4 Molecular detection of resistance genes

All Gram negative strains were investigated for the presence of $bla_{\text{OXA-48}}$, bla_{VIM}, $bla_{\text{IMP-1}}$-type, bla_{NDM}, bla_{KPC}, bla_{GES}, bla_{SME} (β-lactams resistance genes), *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6')-Ib*, *qepA* (quinolones resistance genes), *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5* and *mcr-9* genes (colistin resistance genes) through Polymerase Chain Reaction (PCR), using primers already reported [257], with the exception of *mcr-9* primers. Primers and conditions for the search of *mcr-9* gene are here described for the first time (mcr9-F, 5'–TTCCCTTTGTTCTGGTTG-3', and mcr9-R, 5'–GGATTATAGACGCTGGTG-3'; initial denaturation at 94°C for 7 min, followed by 30 cycles of 94°C for 45 s, 55.6°C for 45 s and 72°C for 1 min and 45 s with a final extension at 72°C for 10 min). For 5 strains recovered from MacConkey agar with cefotaxime, we investigated the presence of $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, and $bla_{\text{OXA-1}}$-type (β-lactams resistance genes) [257]. Furthermore, the presence of *oqxAB* gene (a quinolones resistance gene) was investigated for 20 strains recovered from Aeromonas/MacConkey agar with nalidixic acid [257]. Four more strains were searched for bla_{TEM}, bla_{SHV}, and $bla_{\text{OXA-1}}$-type genes according to the antibiotic susceptibility testing (see next section): two with an intermediate phenotype to ceftazidime and the other two with positive results in DCT.

All *Staphylococcus* spp. were tested for the presence of *mecA*, *mecC*, *vanA*, *vanB* and *vanD* genes [257], whereas all *Enterococcus* spp. were studied for the presence of *vanA*, *vanB* and *vanD* genes.

4.3.5 Antibiotic susceptibility testing of strains with resistance genes

Antibiotic susceptibility was studied by disk diffusion (Bio-Rad, Marnes-la-Coquette, France) and MIC by in-house broth microdilution for nineteen strains that revealed the presence of resistance genes. Antibiotics tested and respective concentrations and breakpoints are listed in Table 4.1. The antibiogram was completed with DCT, DDST, faropenem (10 µg) and temocillin (30 µg) to search for ESBL, MBL, AmpC cephalosporinases and carbapenemases, as already reported [257]. The strains were considered multidrug resistant if they presented resistance to three or more structurally unrelated antibiotics. EUCAST species-specific intrinsic resistances were considered (https://www.eucast.org/expert_rules_and_intrinsic_resistance/; accessed on Apr 13, 2021).

Table 4.1 — Antibiotics, respective concentrations and breakpoints used, by bacterial family.

Bacterial Family	Method	Antibiotics Tested (Concentration)	Breakpoints
Enterobacteriaceae	Disk diffusion	AMC (20 + 10 µg), AZT (30 µg), FEP (30 µg), CTX (5 µg), FOX (30 µg), CAZ (10 µg), ERT (10 µg), IMP (10 µg), MEM (10 µg), PTZ (36 µg), CIP (5 µg), SXT (25 µg), GEN (10 µg)	EUCAST[1]
	MIC	CHL, FLO, OTC FMQ CIP	CLSI VET08[2] CASFM VET 2019[3] EUCAST
Shewanellaceae	Disk diffusion	AZT (30 µg), FEP (30 µg), CAZ (10 µg), IMP (10 µg), MEM (10 µg), PTZ (36 µg), CIP (5 µg), LEV (5 µg), AN (30 µg), GEN (10 µg), NET (10 µg), TMN (10 µg)	EUCAST[4]
	MIC	CHL, FLO, OTC, CIP, FMQ	CLSI M100[4,5]

AMC: amoxicillin/clavulanic acid; AN: amikacin; AZT: aztreonam; CAZ: ceftazidime; CHL: chloramphenicol; CTX: cefotaxime; CIP: ciprofloxacin; ERT: ertapenem; FEP: cefepime; FLO: florfenicol; FMQ: flumequine; FOX: cefoxitin; GEN: gentamicin; IPM: imipenem; LEV: levofloxacin; MEM: meropenem; NET: netilmicin; OTC: oxytetracycline; PTZ: piperacillin/tazobactam; SXT: trimethoprim/sulfamethoxazole; TMN: tobramycin; CASFM VET: Comité de l'antibiogramme de la Société Française de Microbiologie Recommandations Vétérinaires. CLSI: Clinical and Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing. [1]https://www.eucast.org/clinical_breakpoints/ (accessed on Apr 13, 2021). [2]https://clsi.org/ (accessed on Apr 13, 2021). [3]https://www.sfm-microbiologie.org/2019/07/09/casfm-veterinaire-2019/ (accessed on Apr 13, 2021). [4]Breakpoints for *Shewanella* spp. were not available, therefore breakpoints from EUCAST and CLSI M100 for *Pseudomonas* spp.

were used, as reported elsewhere [258]. [5]Breakpoints for CHL were used for FLO as well; breakpoints for CIP were used for FMQ; and breakpoints for tetracycline were used for OTC.

4.4 Results

Overall, after the initial screening with selective media containing antibiotics (amoxicillin, cefotaxime, chloramphenicol, colistin, nalidixic acid and/or oxytetracycline), two hundred and twenty-two bacterial strains were recovered from the bivalve mollusks' samples included in this study. One hundred and ninety-two were Gram-negative bacteria, whereas only thirty were Gram-positive bacteria. Gram-negative bacteria prevail in all three species of bivalve mollusks, when compared with Gram-positive bacteria. All bacterial families and respective species found in this study are listed in Table 4.2.

Table 4.2 — Bacterial families and respective species found in our study.

Bacterial Family	Bacterial Species	Bivalve Species
Aeromonadaceae	*Aeromonas punctata*	*R. decussatus*
	Aeromonas sp.	*Mytilus* spp.
Bacillaceae	*Bacillus* sp.	*R. decussatus*
	Bacillus cereus group	*C. gigas*, *Mytilus* spp. and *R. decussatus*
Comamonadaceae	*Comamonas aquatica*	*R. decussatus*
Enterobacteriaceae	*Citrobacter werkmanii*	
	Pseudocitrobacter faecalis	
	Enterobacter cancerogenus	*Mytilus* spp.
	Escherichia fergusonii	*C. gigas*
	Raoultella ornithinolytica	*Mytilus* spp. and *R. decussatus*
	Citrobacter braakii	*C. gigas* and *R. decussatus*
	Klebsiella aerogenes	
	Enterobacter spp. (*E. hormaechei*, *E. kobei*)	*C. gigas* and *Mytilus* spp.
	Klebsiella spp. (*K. pneumoniae, K. oxytoca*)	
	Citrobacter freundii	*C. gigas*, *Mytilus* spp. and *R. decussatus*
	Enterobacter cloacae	
	Escherichia coli	
Enterococcaceae	*Enterococcus* spp. (*E. faecalis*, *E. hirae*)	
	Enterococcus faecium	*C. gigas*
	Vagococcus fluvialis	*Mytilus* spp.
Micrococcaceae	*Micrococcus luteus*	

Moraxellaceae	*Acinetobacter* spp. (*A. beijerinckii*, *A. junii*, *A. pittii*, *A. ursingii*)	*C. gigas*
	Moraxella osloensis	
Morganellaceae	*Morganella morganii*	*C. gigas* and *R. decussatus*
	Proteus hauseri	*Mytilus* spp.
	Proteus vulgaris	*C. gigas*, *Mytilus* spp. and *R. decussatus*
	Providencia spp. (*P. rettgeri*, *P. stuartii*)	
Pseudomonadaceae	*Pseudomonas mendocina*	*Mytilus* spp. and *R. decussatus*
	Pseudomonas putida	*C. gigas* and *Mytilus* spp.
Shewanellaceae	*Shewanella algae*	
Staphylococcaceae	*Staphylococcus pasteuri*	*Mytilus* spp. and *R. decussatus*
	Staphylococcus warneri	*Mytilus* spp.
	Staphylococcus xylosus	*R. decussatus*
Streptococcaceae	*Lactococcus garvieae*	
Vibrionaceae	*Photobacterium damselae*	*C. gigas* and *Mytilus* spp.
	Vibrio alginolyticus	*C. gigas*, *Mytilus* spp. and *R. decussatus*
	Vibrio fluvialis	*Mytilus* spp.
	Vibrio spp. (*V. furnissii*, *V. vulnificus*)	*R. decussatus*
Yersiniaceae	*Serratia marcescens*	*C. gigas*

4.4.1 Bacterial diversity in clams' samples

In clams' samples we identified ten different families of bacteria (Figure 4.1, Figure 4.2 and Table S 2): *Aeromonadaceae*, *Comamonadaceae*, *Enterococcaceae* (only in summer), *Bacillaceae*, *Staphylococcaceae*, *Streptococcaceae* (only in autumn), *Enterobacteriaceae*, *Morganellaceae*, *Pseudomonadaceae* and *Vibrionaceae* (in both seasons). In summer, *Morganellaceae* was the most frequent bacterial family (38.1%). In fact, *Morganellaceae* appears more in this season in clams than in other bivalves studied (p=0.02; Table 4.3). However, in autumn the results differ, with *Enterobacteriaceae* representing the most frequently isolated bacterial family (52.4%).

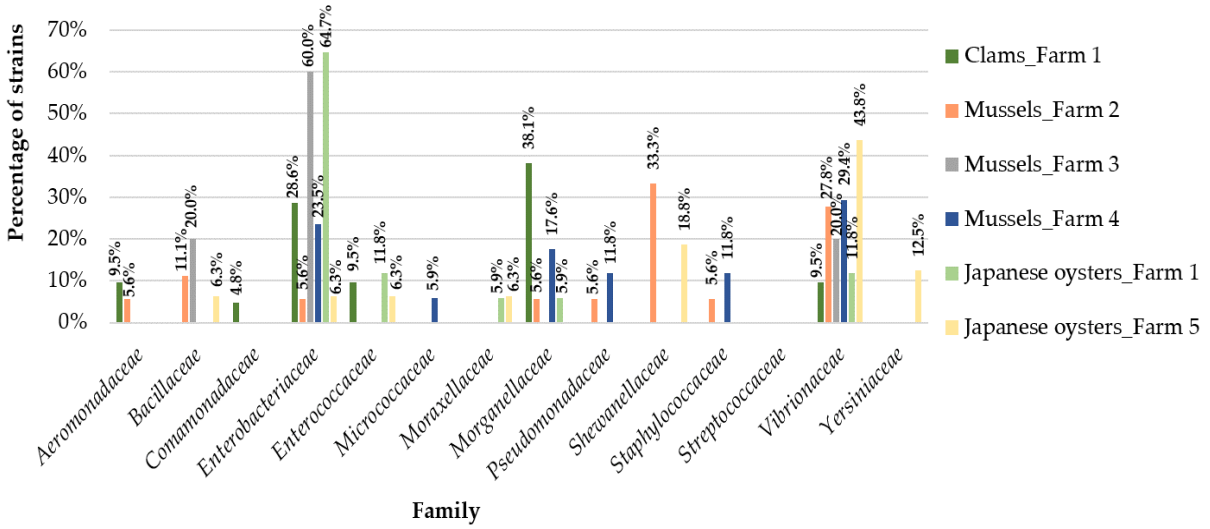

Figure 4.1 — Distribution of the bacterial families among the five aquaculture farms in the summer.

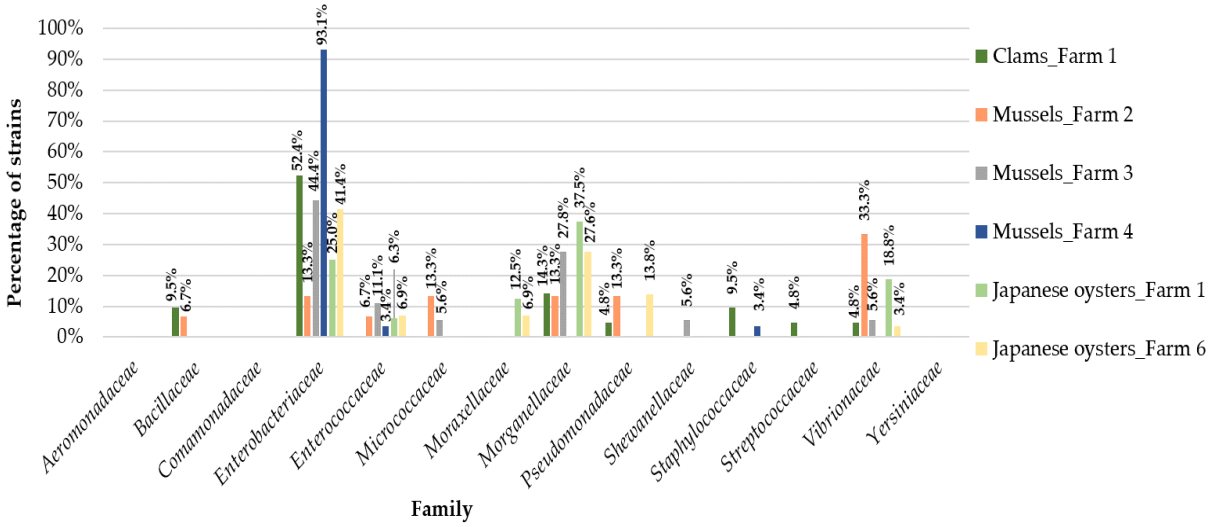

Figure 4.2 — Distribution of the bacterial families among the five aquaculture farms in the autumn.

Table 4.3 — Odds ratio (OR) and 95% confidence intervals (CI) ($p \leq 0.05$) from the analysis of positive and negative associations between bivalve species and each bacterial family, bivalve species/bacterial family and season, *C. gigas*/bacterial family and location, bivalve species/bacterial family and nonsusceptibility to different antibiotic's class (using the results from the initial screening in selective media).

Bivalve's Common Name	Bivalve Species	Bacterial Family	Season	Collection Site	Antibiotic	OR[1]	95% CI	*p* Value
Clams	*R. decussatus*/All	*Morganellaceae*	Summer	All	NA	6.933	1.02–54.16	0.02
Mussels	*Mytilus* spp./All	*Enterobacteriaceae*	Summer	All	NA	0.1908 (P)	0.05897–0.597	≤0.01
	Mytilus spp./All	*Enterobacteriaceae*	Autumn	All	NA	5.242	1.675–16.96	≤0.01
	Mytilus spp.	*Enterobacteriaceae*	Summer	All	NA	0.1689 (P)	0.0584–0.4595	≤0.01
	Mytilus spp.	*Enterobacteriaceae*	Autumn	All	NA	5.92	2.176–17.12	≤0.01
	Mytilus spp.	*Morganellaceae*	All	All	NA	0.437 (P)	0.1842–0.983	0.02
	Mytilus spp.	*Shewanellaceae*	Summer	All	NA	10.76	1.202–503	≤0.01
	Mytilus spp.	*Vibrionaceae*	Summer	All	NA	3.54	1.058–12.75	0.02
Japanese oysters	*C. gigas*/All	*Enterobacteriaceae*	Summer	All	NA	9.429	2.263–45.46	≤0.01
	C. gigas	*Morganellaceae*	Summer	All	NA	0.0692 (P)	0.001588–0.5202	≤0.01
	C. gigas	*Morganellaceae*	Autumn	All	NA	14.45	1.922–629.7	≤0.01
	C. gigas	All	All	All	OTC	0.4167 (P)	0.2293–0.7527	≤0.01
	C. gigas	All	Summer	All	OTC	2.786	1.061–7.35	0.02

Only significant associations are presented: *p* values ≤ 0.05 and confidence limits excluding null values (0, 1, or [*n*]). [1]Odds Ratio. (P) indicates an OR value for a protective or negative association; otherwise, values should be interpreted as a positive association. CI: Confidence intervals. NA: not applicable. OTC: oxytetracycline.

4.4.2 Bacterial diversity in mussels' samples

Ten different families of bacteria were found among mussels' samples: *Aeromonadaceae* (only in summer), *Enterococcaceae* (only in autumn), *Bacillaceae*, *Enterobacteriaceae*, *Micrococcaceae*, *Morganellaceae*, *Pseudomonadaceae*, *Shewanellaceae*, *Staphylococcaceae* and *Vibrionaceae* (in both seasons). In aquaculture farm 2, the most frequently found bacterial family in summer was *Shewanellaceae* (33.3%), whereas in autumn the most frequently found was *Vibrionaceae* (33.3%). In aquaculture farm 3, *Enterobacteriaceae* predominated in both seasons (with 60.0% in summer and 44.4% in autumn). In aquaculture farm 4, the most frequently found bacterial family in summer was *Vibrionaceae* (29.4%) and in the autumn it was *Enterobacteriaceae* (93.1%) (Figure 4.1, Figure 4.2 and Table S 2). Overall, we verified that *Enterobacteriaceae* appears more in autumn in mussels than in other bivalves ($p \leq 0.01$) and was most frequent in autumn than in summer among mussels' samples ($p \leq 0.01$). Our statistical

analysis showed that *Morganellaceae* were not usually associated with mussels' samples (protective association; p=0.02). In this bivalve species, *Shewanellaceae* and *Vibrionaceae* were most frequently found in summer than in autumn ($p \leq 0.01$ and p=0.02, respectively) (Table 4.3).

4.4.3 Bacterial diversity in Japanese oysters' samples

In Japanese oysters' samples we identified nine different bacterial families: *Bacillaceae*, *Shewanellaceae*, *Yersiniaceae* (only in summer), *Pseudomonadaceae* (only in autumn), *Enterobacteriaceae*, *Enterococcaceae*, *Moraxellaceae*, *Morganellaceae* and *Vibrionaceae* (in both seasons). In this group of samples, in aquaculture farm 1 *Enterobacteriaceae* was the most frequently found bacterial family in summer (64.7%; Figure 4.1, Figure 4.2 and Table S 2). Indeed, this family appears more in summer in Japanese oysters than in the other bivalves studied ($p \leq 0.01$; Table 4.3). In autumn, *Morganellaceae* was the most common family (37.5%) in aquaculture farm 1. In fact, *Morganellaceae* is more frequently found in autumn than in summer on all aquaculture farms studied ($p \leq 0.01$). In farm 5 (only samples collected in summer), *Vibrionaceae* was the most frequently found bacterial family (43.8%), while in farm 6 (only samples collected in autumn), *Enterobacteriaceae* predominated (41.4%).

4.4.4 Initial evaluation of decreased susceptibilities

Initial screening with selective media containing antibiotics allowed the identification of decreased susceptibilities. Figure 4.3 (Figure S 2 and Table S 3) presents the results of this screening (eliminating known intrinsic resistances for the analysis). Decreased susceptibility to oxytetracycline prevails in all bivalve species, aquaculture farms and seasons.

In clams, no decreased susceptibility to colistin was found and decreased susceptibility to chloramphenicol was only identified in samples collected in summer (15.4%).

In mussels, no reduced susceptibility to cefotaxime was found. In contrast, amoxicillin and oxytetracycline reduced susceptibilities were present in all farms and seasons. Autumn was the only season where reduced susceptibility to chloramphenicol was found (16.7% in farm 3 and 18.5% in farm 4). However, reduced susceptibility to colistin was only found in a sample from farm 2, collected in summer (16.7%). Reduced susceptibility to nalidixic acid was not found in farm 3.

Although, in Japanese oysters, only decreased susceptibility to oxytetracycline was found in all farms and seasons, this decreased susceptibility was not so frequent in these

bivalve species (protective association) when compared to other species of bivalve analyzed in this study ($p \leq 0.01$; Table 4.3). Furthermore, this decreased susceptibility appears more associated with summer than autumn in these bivalves (p=0.02; Table 4.3). Decreased susceptibilities to amoxicillin and colistin were only found in samples from region B (farms 5 and 6), whereas decreased susceptibility to cefotaxime was only recovered in samples from region A (farm 1). Decreased susceptibility to nalidixic acid was only observed in samples collected in autumn (31.3% in farm 6 and 10.0% in farm 1).

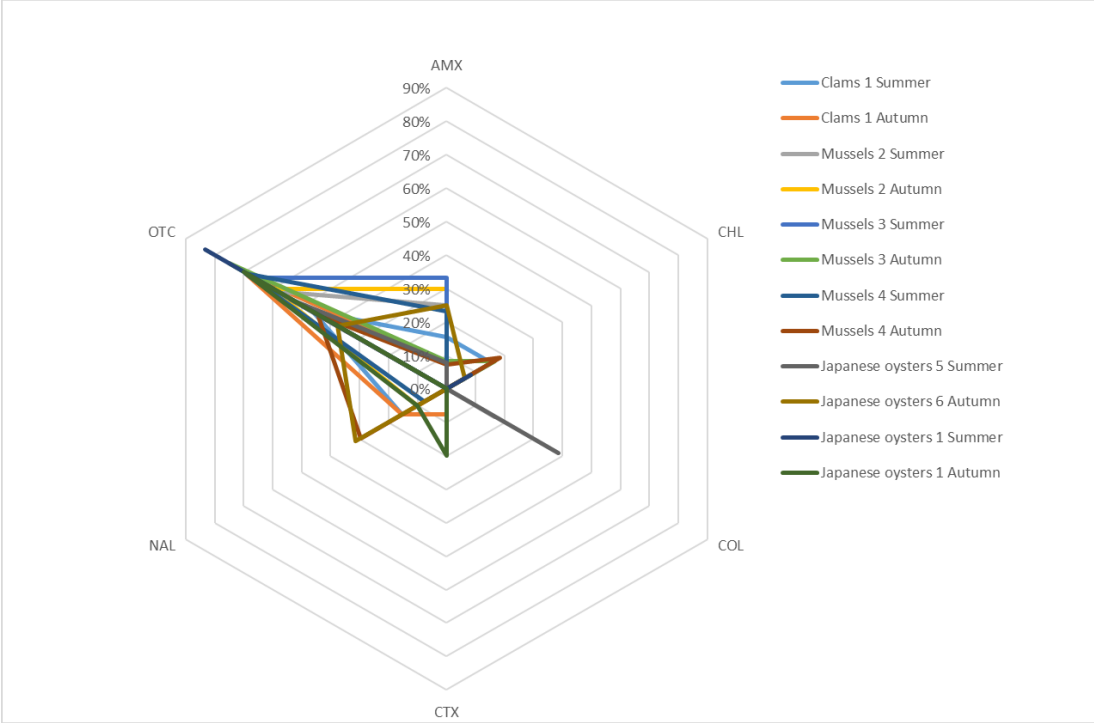

Figure 4.3 — Decreased susceptibilities found in bivalves' samples. These results were obtained through the initial screening with selective media containing antibiotics and do not include known intrinsic resistances. AMX: amoxicillin; CHL: chloramphenicol; COL: colistin; CTX: cefotaxime; NAL: nalidixic acid; OTC: oxytetracycline.

4.4.5 Antibiotic susceptibility of β-lactamase- and plasmid-mediated quinolone resistance (PMQR)-producing strains

Investigation of resistance genes by PCR revealed six *qnrA* genes among *S. algae*, ten *qnrB* genes among *C. braakii*, *C. freundii* and *E. coli*, and three *oqxAB* genes from *R. ornithinolytica* strains (Table 4.4). *qnrA* genes were found in aquaculture farms from region A and B, in both seasons, although they predominated in summer. However, *qnrB* and *oqxAB* genes were only found in aquaculture farms from region A in autumn. Three PMQR-producing strains (*C.*

braakii, *C. freundii* and *S. algae*) revealed a susceptibility profile to all quinolones tested, with zone diameters ranging from 31 to 33 mm (disk diffusion) and concentrations of <0.015 to 0.125 mg/L (MIC) for ciprofloxacin, concentrations of 0.5 to 2 mg/L for flumequine, and a zone diameter of 27 mm for levofloxacin. The remaining sixteen PMQR-producing strains revealed a decreased susceptibility to at least one quinolone tested, with concentrations of 0.5 to >16 mg/L for ciprofloxacin and 2 to 64 mg/L for flumequine. Non-susceptibilities to amoxicillin, colistin and oxytetracycline (MIC concentrations of 8 to 64 mg/L for the last one) were also found among *S. algae* strains. All *R. ornithinolytica* harboring an *oqxAB* gene were also resistant to oxytetracycline, with concentrations ranging from 16 to 64 mg/L. Decreased susceptibility to β-lactam antibiotics among *Citrobacter* spp. was mostly intrinsic. This genus also revealed decreased susceptibility to phenicols and oxytetracycline, with a concentration of 32 mg/L for chloramphenicol (*C. braakii*), 8 to 16 mg/L for florfenicol and 8 mg/L for oxytetracycline. *E. coli* harboring *qnrB19* and $bla_{\text{TEM-1}}$ genes revealed non-susceptibility to β-lactam antibiotics as well, in addition to non-susceptibilities to chloramphenicol, florfenicol, ciprofloxacin, flumequine, oxytetracycline and trimethoprim/sulfamethoxazole. Our study detected 8 multidrug resistance strains (8 *E. coli* isolated in mussels).

Table 4.4 — Phenotype and genotype profile of the nineteen β-lactamase- and PMQR-producing strains.

Bacterial Species	Farm (No. of Strains)	Bivalve Mollusk Species	Season	Decreased Susceptibility Profile	AR Genes
C. braakii	1 (*n*=1)	*R. decussatus*	A	AMX, AMC, FOX, CHL, FLO, OTC	*qnrB*-type[1]
C. freundii	1 (*n*=1)	*C. gigas*	A	AMC, CAZ, FOX, FLO, OTC	*qnrB44*
E. coli	4 (*n*=8)	*Mytilus* sp.	A	(AMX), AMC, (CAZ), (CIP), CHL, FLO, (FMQ), OTC, SXT	*qnrB19*, $bla_{\text{TEM-1}}$
R. ornithinolytica	4 (*n*=3)	*Mytilus* sp.	A	(CIP), FMQ, NAL, OTC	*oqxAB*
S. algae	2 (*n*=3)	*Mytilus* sp.	Su	(AMX), (FMQ), OTC	*qnrA3*
	2 (*n*=1)	*Mytilus* sp.	Su	CIP, FMQ, OTC	*qnrA11*
	5 (*n*=1)	*C. gigas*	Su	COL	*qnrA12*
	3 (*n*=1)	*Mytilus* sp.	A	FMQ, OTC	*qnrA2*

[1]This *qnrB* sequence contains a premature stop codon due to naturally occurring deletion (suggesting a non-functional protein), so it was not possible to assign an allele number (accession number GenBank MW183827). AMX: amoxicillin; AMC: amoxicillin/clavulanic acid; FOX: cefoxitin; CAZ: ceftazidime; CIP: ciprofloxacin; CHL: chloramphenicol; COL: colistin; FLO: florfenicol; FMQ: flumequine; NAL: nalidixic acid; OTC: oxytetracycline; SXT: trimethoprim/sulfamethoxazole; AR: antibiotic resistance; A: Autumn; Su: summer. Variable presence of nonsusceptibility phenotype is indicated by parentheses.

4.5 Discussion

As we observed in our study, bivalve mollusks usually concentrate a great diversity of bacterial species/families, which makes them susceptible to various diseases and may represent a risk to human health, since some of these bivalves are eaten raw (e.g., oysters). Indeed, statistically significant differences in bacterial composition between bivalve species from the same aquaculture farm and season (clams and Japanese oysters from farm 1, region A) were detected. Within the same bivalve species, we also observed variations between farms from the same region (mussels in region A) and different regions (Japanese oysters in region A and B), although these variations were not statistically significant. Fernández et al. detected variations in bacterial composition of post larvae and adult stages of *Crassostrea corteziensis*, *C. gigas* and *Crassostrea sikamea* at different cultivation sites [248]. They concluded that the most frequent phyla were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* (in that order), using a high-throughput sequencing approach (pyrosequencing). In a different study, Pierce and Ward evaluated the gut microbiome from *Crassostrea virginica* and *Mytilus edulis* and confirmed that these species had similar (but not identical) gut microbiomes that vary with the seasons [140]. The most abundant phyla were *Proteobacteria*, *Tenericutes*, *Verrucomicrobia*, *Bacteroidetes*, *Cyanobacteria*, *Planctomycetes*, *Actinobacteria*, *Firmicutes*, and *Fusobacteria*. In our study, the most frequently identified phylum was also *Proteobacteria*, which comprised the following families: *Aeromonadaceae*, *Comamonadaceae*, *Enterobacteriaceae*, *Moraxellaceae*, *Pseudomonadaceae*, *Shewanellaceae*, *Vibrionaceae*, and *Yersiniaceae*. These findings agree with the hypothesis previously proposed that bacterial composition in bivalve mollusks is influenced by host species and respective life stage, diet, rearing conditions, bacterial composition of the aquatic habitat, salinity, and temperature [32,259,260].

Bacteria found in this study belonging to the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Micrococcus*, *Photobacterium*, *Pseudomonas*, and *Vibrio* are ubiquitous in the water environments and commensal microbiota of bivalves [32]. Some of these bacteria, especially those belonging to *Proteobacteria* phylum, are important for bivalve mollusks' metabolism, since they are able to fix nitrogen in the gastrointestinal tract of these organisms and degrade cellulose and agar (the main elements of the food ingested by bivalve mollusks) [259]. Species belonging to the genera *Aeromonas*, *Pseudomonas* and *Vibrio* are also responsible for diseases in bivalve larvae [32]. Information about the frequency and pathogenicity of other genera found in this study in bivalve mollusks is scarce. However, there are several studies reporting human infections caused by all seven genera described above, namely wound infections,

foodborne diseases (by ingestion of raw seafood), myonecrosis, septicemia, necrotizing fasciitis, empyema, bacteremia, endocarditis, severe respiratory, urinary and biliary tract infections, meningitis, and keratitis [32,254–257,260–264].

In addition to commensal microbiota of bivalve mollusks, we also found non-commensal bacteria, some already reported in bivalve mollusks, others with no information about their presence in these organisms. The genera *Enterobacter*, *Enterococcus*, *Klebsiella*, *Proteus*, *Providencia*, and *Staphylococcus*, as well as the species from *Citrobacter freundii* complex, *Escherichia coli*, *Morganella morganii*, *Shewanella algae* and *Vagococcus fluvialis*, found in this study, were already reported in bivalve mollusks (especially clams, mussels and oysters) [249,250,265–273]. These groups of bacteria, some of which are fish pathogens, are commonly found in aquatic environments [249,257,271,272,274,275]. All these bacteria were already associated with human infections, such as ear and eye infections, osteomyelitis, infective arthritis, endocarditis, bacteremia, meningitis, intestinal and urinary tract infections, brain abscess, peritonitis, enteritis and septicemia, and some are recognized as important agents in nosocomial infections [43,249,250,271,274,276–285].

To our knowledge, this is the first report associating *Comamonas aquatica*, *Escherichia fergusonii*, *Lactococcus garvieae*, *Moraxella osloensis*, *Pseudocitrobacter faecalis*, *Raoultella ornithinolytica*, and *Serratia marcescens* with bivalve mollusks from aquaculture. These bacteria were already recovered from a wide range of environments, such as water, soil, plants, fish, insects, milk, cheese, sugar cane, mango, and the feces of warm-blooded animals, among others [48,281,286–292]. Furthermore, they are responsible for bacteremia, septic shock, biliary, gastrointestinal, urinary tract and wound infections, meningitis, infective endocarditis, lumbar osteomyelitis, and hepatic abscess in humans [48,288,291–293].

Non-commensal bacteria of bivalve mollusks such as *Enterobacter* spp., *Enterococcus faecalis*, *Enterococcus faecium*, *E. coli*, *Klebsiella aerogenes*, *K. pneumoniae*, *M. osloensis*, *M. morganii*, and *Providencia rettgeri* could be indicators of fecal contamination, since these bacteria are widely found in the gastrointestinal tract of humans and several other animals [250,257,269,271,272,277,282,284,287]. These and other bacteria can enter aquaculture farms through runoff from land (especially during periods of high precipitation), sewage, maritime traffic and birds or marine mammals. Fecal material from land and sewage can concentrate a high bacterial diversity, as well as several heavy metals, antibiotics, and organic substances, promoting a selective pressure on bacteria normally present in an aquatic environment [250].

The initial screening with selective media containing antibiotics and, subsequently, the MIC results revealed the prevalence of decreased susceptibility to oxytetracycline in all bivalve

species, aquaculture farms and seasons. This prevalence could be explained by the high frequency of prescription of this antibiotic in aquaculture, due to broad spectrum of activity, low cost, and potency [21]. With one exception, we could not establish an association between an antibiotic and a specific bivalve species, location, or season. The exception was the decreased susceptibility to oxytetracycline that was statistically associated (p=0.02) with summer in Japanese oysters.

Decreased susceptibility to β-lactams was also found in the present study. This antibiotic class is frequently used in aquaculture in several countries [257] and high resistance rates are usually observed in bivalve mollusks (especially regarding amoxicillin), often associated with bla_{TEM} and bla_{CTX-M} [250]. In our study, we also detected the bla_{TEM-1} gene in eight multidrug resistant *E. coli* strains with decreased susceptibilities to amoxicillin, amoxicillin/clavulanic acid and/or ceftazidime. Noteworthy, strains with non-susceptibilities to β-lactams might have other resistance mechanisms not studied here (e.g., efflux pumps).

The other decreased susceptibility detected in this study was to chloramphenicol, although this antibiotic has already been banned for use in food-producing animals in Europe since the 1990s. This decreased susceptibility can persist in the environment due to co-selection with other antibiotics (especially florfenicol, which is widely used in aquaculture, and decreased susceptibility to this antibiotic was also found in this study) and/or heavy metals. Moreover, there are soil bacteria that are capable of producing this substance [257]. Other studies also confirmed the presence of this antibiotic resistance in bivalve mollusks [250,294].

Worryingly, our study detected decreased susceptibility to colistin, an antibiotic of last resort against multidrug resistant Gram-negative bacteria in human medicine. This antibiotic is also used in veterinary medicine, including aquaculture, the latter hypothesized by some authors as the source of certain colistin resistance genes [295,296]. In our study, fourteen strains revealed decreased susceptibility to this antibiotic. Of these, seven, identified as *S. algae* and *P. damselae*, had non-intrinsic resistance and were isolated from mussels collected in aquaculture farm 2 and Japanese oysters collected in aquaculture farm 5 (in regions A and B, respectively). These results may reflect a selective pressure in these regions that facilitates the dissemination of strains with decreased susceptibility to colistin. No plasmid-mediated colistin resistance-encoding genes were detected, which suggested that other resistance mechanisms not studied here are responsible for the decreased susceptibility to this antibiotic (other *mcr*-variant genes; efflux pumps; or *pmrC*, *pmrE*, *mgrB* genes, among others genes and operons that play a role in lipopolysaccharide modification and consequent decreased susceptibility to

colistin) [89]. Previous studies had already detected decreased susceptibility to colistin and *mcr-1* gene in clams and mussels, respectively [296,297].

Our study revealed a low prevalence of decreased susceptibility to nalidixic acid (13%) in all bivalve samples analyzed. This antibiotic belongs to a class commonly used in aquaculture, quinolones [257]. Former studies in bivalve mollusks, also detected low levels of decreased susceptibility to quinolones [250,251]. Of the twenty strains with decreased susceptibility to nalidixic acid, only three strains of *R. ornithinolytica* revealed a positive result for the *oqxAB* gene. These three strains also revealed decreased susceptibility to other quinolones, such as flumequine and ciprofloxacin. Investigation of quinolones resistance genes by PCR in all Gram-negative strains, regardless their phenotype, revealed the presence of six *qnrA*-type genes among *S. algae* and ten *qnrB*-type genes among *C. braakii*, *C. freundii* and *E. coli*. Interestingly, not all strains harboring a *qnr* gene revealed a resistance phenotype to quinolones (one *C. braakii* with a *qnrB*-type, one *C. freundii* with a *qnrB44* and one *S. algae* with a *qnrA12*). This may be caused by non-functional proteins, such as in the case of *C. braakii* with a deletion in *qnrB* gene that originated a premature stop codon, or a low expression of these genes, difficult to detect by phenotypic methods [164]. These results highlight the importance of using both phenotypic and genotypic methods in research of antibiotic resistances/resistance genes, since there is not always phenotypic and/or genotypic expression. Although *qnrA*, *qnrB* and *oqxAB* genes are frequently found in aquaculture environments [143,257,298], little information is known about their frequency in bivalve mollusks, thus this study can contribute to better knowledge in this field.

All *E. coli* strains that harbor a *qnrB19* gene had a decreased susceptibility to quinolones (ciprofloxacin and/or flumequine) and also presented resistance to the combination trimethoprim/sulfamethoxazole (also used in aquaculture [257]). This resistance was previously reported in bivalve mollusks [299] and could be associated with the acquisition of genes *sul1* and *sul2* (for sulfamethoxazole resistance) and *dhfr1* and *dhfr2* (for trimethoprim resistance), causing the alteration of the antibiotic target [300].

In this study, we observed low resistance rates/few resistance genes to the antibiotics tested (except for oxytetracycline). However, it is important to implement surveillance plans in aquaculture farms since this environment can be a reservoir of antibiotic resistance and/or antibiotic resistance genes. The implementation of measures that help to prevent outbreaks is also crucial, because fighting an outbreak is more difficult and expensive. Examples of such measures are limiting stock movements, avoiding exposure to elevated temperatures and high or low salinity, strict hygiene measures, and decreasing stock densities, among others. In the

presence of an outbreak, it is important to identify the pathogen responsible and, if possible, to test its susceptibility to antibiotics, so that veterinarians can use a narrow-spectrum antibiotic at the correct concentration. Whenever possible, antibiotic administration by bath or feed should be avoided, giving preference to more individual methods to prevent the exposure of healthy individuals and the aquatic environment to a selective pressure. Investment in alternatives to antibiotics should be made, such as antimicrobial peptides (produced by several species of bivalve mollusks), bacteriophages, probiotics, and vaccines, always considering animal welfare and the product's safety for human consumption [32,73].

4.6 Conclusions

In recent years, there has been an increase in studies on microbiota and antibiotic resistances/resistance genes present in aquaculture, mainly on fish. This study presents an important contribution to fill the gaps in the knowledge of bacterial diversity and antibiotic resistance mechanisms in bivalve mollusks. We could observe a great variety of bacterial species and antibiotic resistances among clams, mussels and Japanese oysters, seasons, and locations. This fact highlights the need to study and adapt the surveillance plans and measures to prevent the spread of antibiotic resistance to each specific location and animal species. Therefore, bivalve mollusks can play an important role in monitoring these aquaculture environments, since their filter feeding habits make them excellent indicators of environmental pollution [250].

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5

GENETIC RELATEDNESS AND DIVERSITY OF *STAPHYLOCOCCUS AUREUS* FROM DIFFERENT RESERVOIRS: HUMANS AND ANIMALS OF LIVESTOCK, POULTRY, ZOO, AND AQUACULTURE

This research paper was published as:

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Personal contributions:

Salgueiro, V. contributed to acquisition of laboratory data, data curation and formal analysis, investigation, writing of the original draft, and final approval of manuscript.

Note:

This work was partially presented at the One Health EJP Annual Scientific Meeting 2020, online event.

5.1 Abstract

The main aim of this study was the characterization of antibiotic resistance mechanisms in 82 *Staphylococcus aureus* strains isolated from humans and animals. Antibiotic susceptibility testing was performed on all *S. aureus* isolates accordingly, and antibiotic resistance genes were investigated by genotypic methods. The genetic diversity of *S. aureus* was studied through *spa*, MLST, and *agr* typing methods. The majority of *S. aureus* from human sources were resistant to cefoxitin (and harbor the *mecA* gene) and fluoroquinolones, whereas only four strains of *S. aureus* from animal sources revealed resistance to ciprofloxacin. In the set of *S. aureus* isolated from humans, the most frequent *spa*, MLST, and *agr* group were t032, ST22, and I, respectively. In strains from animal origin the most common *spa*, MLST, and *agr* group found were t2383, ST398, and III/not typable, respectively. *S. aureus* from humans and animals were identified either in clonal complexes CC5, CC30, and CC398, suggesting that they have the same putative founder in their evolution. Considering the three CCs encompassing strains from human and animal reservoirs with different *spa* types, we can hypothesize that this might reflect an adaptation to different phylogenetic lineages in those reservoirs (host species) probably associated to genetic diversification of pre-existing strains.

Keywords: MRSA, ST398, Portugal, animals, human isolates.

5.2 Introduction

Staphylococcus aureus has a great capacity of dissemination, acquisition of new antibiotic resistances and production a variety of virulence factors, such as toxins (responsible for food poisoning). These virulence factors are controlled by *agr* gene, a central transcription regulator that responds to cell density and can be divided in four specificity groups [301–303].

Methicillin resistance is commonly observed worldwide among *S. aureus* isolated in hospitals (MRSA) [304]. This resistance is usually encoded by *mecA* gene, which is responsible for the resistance to all β-lactam antibiotics, except for the fifth generation cephalosporins. The *mecA* gene encodes for PBP2a, with low affinity for β-lactam antibiotics. A *mecA* homolog gene, the *mecC* gene, which also encodes a modified PBP, has been described in *S. aureus* [302,305]. Vancomycin is usually the antibiotic chosen for the treatment of infections caused by MRSA [306]. However, strains of *S. aureus* resistant to vancomycin (VRSA) have already been

described. VRSA, with high-level MICs, is mediated by a *vanA* gene cluster, which is transferred from vancomycin-resistant *Enterococcus*. However, *S. aureus* low-level vancomycin resistant strains, non-*vanA* mediated, are more common. hVISA (heterogeneous-Vancomycin-Intermediate *S. aureus*) are characterized by a vancomycin susceptible MIC when tested by routine methods but with a subpopulation of cells that can grow in the presence of ≥ 2 mg/L of vancomycin. This heterogeneous phenotype appears to be related to a thickening of the cell wall and an increase of unbound peptidoglycan precursors, which bind to glycopeptide antibiotics and prevent their interaction with the precursors located at the cell wall [307,308].

In addition to being a commensal and an opportunistic pathogen in humans [301,309], these bacteria can also colonize birds and fish, and be maintained in the environment (water, air, and manure) [310]. In the last decade, MRSA has emerged as a significant animal health problem worldwide, representing an important economic burden, mainly in cattle, poultry, and pigs [309]. Likewise, these bacteria have been found in fish and shrimp from aquaculture origin, raising additional food safety concerns [311]. Several studies have reported possible transmissions from animal to man or vice versa [312–314]. These transmissions can occur through direct contact with animals or their products [310].

In this study, we aimed to identify *S. aureus* strains isolated from humans and animals (from livestock, poultry, zoo, and aquaculture) and to characterize their antibiotic resistance against structurally unrelated antibiotics (β-lactams, glycopeptides, and fluoroquinolones). The genetic relatedness and diversity of these bacteria within these two environments was also evaluated.

5.3 Materials and methods

5.3.1 Bacterial isolates

This study included 58 *S. aureus* isolated from humans (with community and nosocomial origins), randomly selected from the strain collection of the National Reference Laboratory of Antibiotic Resistances and Healthcare Associated Infections in Lisbon. Strains were isolated from pus (*n*=10), blood/cerebrospinal fluid (*n*=6), exudates (*n*=9), urine (*n*=2), respiratory secretions (*n*=14), ascitic fluid (*n*=2), and unknown samples (*n*=15).

Twenty-four isolates of *S. aureus* were collected approximately in the same period (between 2008 and 2018) from animal sources (bird, *n*=1; bovine, *n*=1; dolphin, *n*=1; duck, *n*=2; goat, *n*=2; ovine, *n*=1; rabbit, *n*=6; swine, *n*=2; waterbuck, *n*=1; and gilthead seabream from

aquaculture, *n*=7), at National Institute of Agrarian and Veterinary Research and at Portuguese Institute for the Sea and Atmosphere. Results were compared with the *S. aureus* isolates from humans.

All strains were identified by VITEK® 2 (BioMérieux, Marcy-l'Étoile, France) and amplification of the 16S rRNA gene, as previously described [150].

5.3.2 Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by disk diffusion (Bio-Rad, Marnes-la-Coquette, France) and E-test® (BioMérieux, Marcy l'Etoile, France), according to EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/; accessed on Jul 29, 2020). The antimicrobials tested were cefoxitin (FOX; 30 µg), ciprofloxacin (CIP; 5 µg), vancomycin (VA; 0.016-256 µg/mL), and teicoplanin (TP; 0.016-256 µg/mL) for all *S. aureus*. *S. aureus* strain ATCC 25923 was used as a quality control.

The isolates were considered multidrug resistant if they presented resistance to three or more structurally unrelated antibiotics.

5.3.3 Glycopeptide resistance detection (GRD)

All strains resistant or with borderline breakpoints to vancomycin or teicoplanin were subjected to a GRD test (BioMérieux, Marcy-l'Étoile, France), according to manufacturer's instructions, for identification of hGISA (heterogeneous Glycopeptide-Intermediate *S. aureus*). The test was considered positive when the result was ≥ 8 µg/mL for either vancomycin or teicoplanin, and standard vancomycin MIC < 4 µg/mL.

5.3.4 DNA extraction

DNA was extracted using MagNa Pure 96 Instrument (Roche, Manheim, Germany), according to manufacturer's instructions, or using lysostaphin as previously described [315].

5.3.5 Detection of *mec* genes

S. aureus that demonstrated resistance to cefoxitin were investigated for the presence of *mecA* and *mecC* genes by multiplex PCR. A 23 µL final reaction mixture included: buffer (1×, Qiagen, Hilden, Germany), dNTPs (0.5 mM of dATP, dCTP, dGTP and dTTP, Roche Diagnostics, Basel, Switzerland), $MgCl_2$ (3 mM, Qiagen, Hilden, Germany), Q solution (1×, Qiagen, Hilden, Germany), primers (0.4 µM; [171]), Taq polymerase (1 U, Qiagen, Hilden, Germany), and sterile

double distilled water. Two microliters of DNA were added to this final reaction mixture and the PCR reactions conditions were as follows: initial denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 30 s, 59ºC for 1 min, and 72ºC for 1 min with a final extension at 72ºC for 10 min.

5.3.6 *spa* typing

spa typing was performed to all *S. aureus* using the aforementioned PCR reaction and primers described by others [316]. The PCR products (5 µL) were purified using illustra™ Exo-ProStar™ 1-Step and the following thermal cycling conditions: 37ºC for 15 min, followed by 80ºC for 15 min. The purified products were sequenced with the automatic sequencer ABI PRISM® 3100 (Applied Biosystem, Waltham, MA, USA). The sequences were analyzed with the software BioNumerics© Applied Maths and the *spa* types were identified using the database available at http://spatyper.fortinbras.us/ (accessed on Jul 29, 2020). New *spa* types were submitted and accepted in the database Ridom SpaServer (http://spaserver.ridom.de/; accessed on Jul 29, 2020).

5.3.7 Multilocus sequence typing (MLST)

The seven housekeeping genes (*arcC*, *aroE*, *glpF*, *pta*, *gmk*, *tpi*, and *yqiL*) were amplified for all *S. aureus* isolates, as already described [317]. The PCR products were purified, sequenced, and analyzed as described above. The database available at https://pubmlst.org/ saureus/ (accessed on Jul 29, 2020) was consulted for the determination of the sequence type (ST) and submission of new ST.

5.3.8 *agr* typing

The accessory gene regulator (*agr*) was also studied for all *S. aureus* isolates. The PCR reaction mixture had the same composition as that described above, using the primers described by others [318]. The thermal cycling conditions differed from those used for *mec* genes only in the annealing temperature that was 59.8ºC for 30 s.

5.3.9 Minimum spanning tree

A minimum spanning tree was built based on eight genes (*arcC*, *aroE*, *glpF*, *pta*, *gmk*, *tpi*, *yqiL*, and *spa*) of all *S. aureus* isolates with the software PHYLOViZ Online (available at: https://online.phyloviz.net/index; accessed on Jul 29, 2020), which uses the goeBURST

algorithm [319] and its expansion for representing the possible evolutionary relationships between strains.

5.4 Results

5.4.1 Phenotypic analysis

The majority (69.0%) of *S. aureus* from human sources were resistant to cefoxitin (with diameter of inhibition zone ranging from 6 to 21 mm), which classifies them as MRSA, and presented a high percentage of resistance to ciprofloxacin (81.0%; with diameter of inhibition zone ranging from 6 to 20 mm). Furthermore, human strains also showed decreased susceptibility to teicoplanin (1.7%), with MIC of 6 μg/mL (Table 5.1). One *S. aureus* (1.7%) isolate was multidrug resistant and characterized as hGISA, with a positive result of ≥ 8 μg/mL for teicoplanin and standard vancomycin MIC of 2 μg/mL.

On the contrary, the 24 *S. aureus* isolated from animals revealed susceptibility profiles to almost all antibiotics tested, except for four strains recovered from rabbits, which showed resistance only to ciprofloxacin, with diameter of inhibition zone ranging from 6 to 10 mm (Table 5.1).

Table 5.1 — Antibiotic susceptibility results from *S. aureus* isolated from humans and animals.

Antibiotic	*S. aureus* No. (%)			
	Humans (*n*=58)		Animals (*n*=24)	
	R	S	R	S
FOX	40 (69.0)	18 (31.0)	0 (0.0)	24 (100.0)
CIP	47 (81.0)	11 (19.0)	4 (16.7)	20 (83.3)
TP	1 (1.7)	57 (98.3)	0 (0.0)	24 (100.0)
VA	0 (0.0)	58 (100.0)	0 (0.0)	24 (100.0)

FOX: cefoxitin; CIP: ciprofloxacin; TP: teicoplanin; VA: vancomycin. R: resistant. S: susceptible.

5.4.2 *mec* genes in *S. aureus*

Of the 40 strains resistant to cefoxitin from human sources, 39 were positive for the *mecA* gene, thus confirming this phenotype. The exception was a cefoxitin resistant strain,

which showed a negative result for both *mecA* and *mecC* genes. The *mecC* gene was not found in any of the *S. aureus* isolates.

5.4.3 Genetic relatedness and diversity of *S. aureus*

Regarding *agr*-typing, among all *S. aureus* isolated from humans, 31 (53.4%) belonged to group I, 19 (32.8%) to group II, and 3 (5.2%) to group III. No strains belonging to group IV were detected and five isolates were negative for the presence of any of the four groups. The search of *agr* gene in the *S. aureus* from animal sources revealed 3 (12.5%) strains belonging to group I, 3 (12.5%) to group II, 7 (29.2%) to group III, and 4 (16.7%) to group IV. Seven strains (29.2%) from aquaculture were *agr* non-typable.

Twenty-nine different *spa* types were identified for *S. aureus* from human origin and two types were here described for the first time (t14878 and t14933). For the strains from animal origin were detected 12 different types of *spa*, among which one new type (t15307). In the group of isolates from human sources, the most frequently identified type of *spa* was t032, whereas in the group isolated from animal sources the most abundant was t2383. The *spa* type t571 was found in both reservoirs.

MLST revealed 13 different ST among the strains collected from human sources and 11 ST among the strains from animal sources (ST3254, ST3269, and ST3270 were here described for the first time). The ST5, ST34, and ST398 were found in both reservoirs. The most frequently identified ST in *S. aureus* from human origin was ST22, and ST398 was the most frequent in *S. aureus* from animal origin (Figure 5.1).

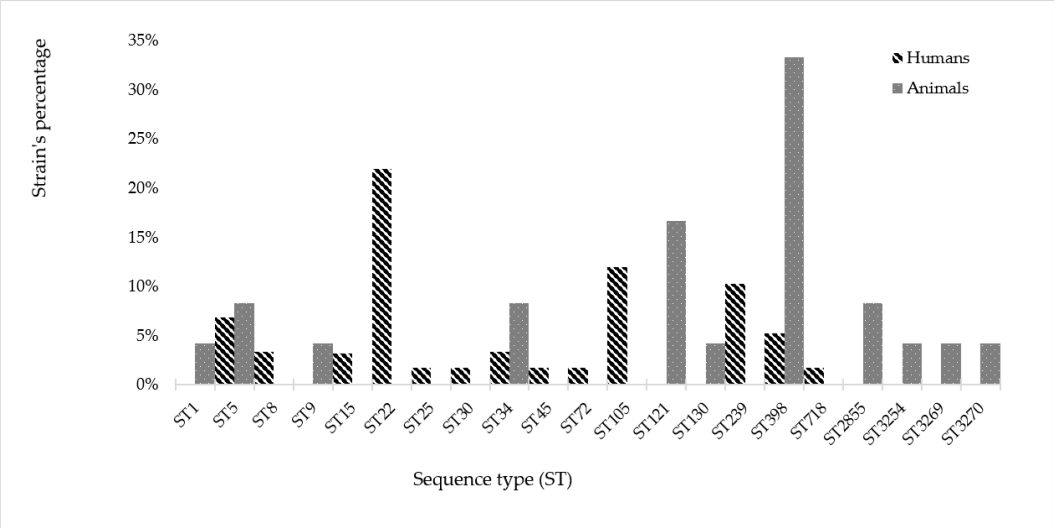

Figure 5.1 — Distribution of *S. aureus* sequence typing (ST) clonal lineages of human and animal origin.

Table 5.2 shows the various genes studied for *S. aureus* bacteria and the respective phenotypes.

In isolates of human origin, the resistance to cefoxitin and ciprofloxacin were widely dispersed by the various *spa* types, ST, and *agr* groups. On the contrary, teicoplanin resistance was found only associated with *spa* t002, ST105, and *agr* group II. As for the new *spa* types, t14878 was associated to one strain susceptible to all antibiotics tested and t14933 was related with a cefoxitin and ciprofloxacin resistant strain. Furthermore, in the group of the isolates from humans, two strains whose *spa* gene could not be amplified belonged to ST105 and *agr* group II and revealed only resistance to ciprofloxacin.

In strains of animal origin, resistance to ciprofloxacin was associated with two *spa* types, t1190 and t645, two ST, ST2855 and ST121, and two *agr* groups, III and IV. The only new *spa* in this group, t15307, was associated with a new ST, ST3270, belonged to *agr* group I and was susceptible to all antibiotics tested. The other two new ST, ST3254, and ST3269, belonged to different *spa* types (t748 and t1166, respectively), different *agr* groups (III and I, respectively) and had susceptibility phenotypes to all antibiotics tested.

Table 5.2 — Association between the genotype and the phenotype of the 82 strains of *S. aureus* from two different reservoirs (humans and animals).

spa Type	MLST	*agr* Group	*mecA*	Resistance Profile
***S. aureus* from humans (*n*=58):**				
t002 (*n*=6)	ST5, ST105	II	+	FOX, CIP, (TP)
t008 (*n*=1)	ST8	I	+	FOX, CIP
t020 (*n*=1)	ST22	I	+	FOX, CIP
t022 (*n*=2)	ST22	I	+	FOX, CIP
t030 (*n*=1)	ST239	I	-	CIP
t032 (*n*=8)	ST22	I	+	FOX, CIP
t037 (*n*=3)	ST239	I	+	FOX, CIP
t062 (*n*=1)	ST5	II	-	-
t078 (*n*=1)	ST25	NT	-	-
t084 (*n*=2)	ST15	II	-	(FOX), CIP
t104 (*n*=1)	ST8	I	+	FOX, CIP
t132 (*n*=1)	ST45	NT	-	-
t148 (*n*=1)	ST72	I	-	-
t179 (*n*=1)	ST5	II	+	FOX, CIP
t571 (*n*=3)	ST398	NT	-	-
t688 (*n*=1)	ST105	II	-	CIP
t718 (*n*=1)	ST22	I	+	FOX, CIP

t747 (*n*=4)	ST22	I	+/-	(FOX), CIP
t910 (*n*=3)	ST22, ST34	I, III	+/-	(FOX), (CIP)
t932 (*n*=1)	ST239	I	+	FOX, CIP
t1094 (*n*=3)	ST5, ST105	II	+	FOX, CIP
t1223 (*n*=1)	ST239	I	-	CIP
t1442 (*n*=1)	ST718	II	-	-
t2357 (*n*=2)	ST22	I	+	FOX, CIP
t4903 (*n*=1)	ST30	III	-	-
t5624 (*n*=1)	ST22	I	+	FOX, CIP
t10682 (*n*=2)	ST105	II	+	FOX, CIP
t14878[1] (*n*=1)	ST34	III	-	-
t14933[1] (*n*=1)	ST22	I	+	FOX, CIP
NT (*n*=2)	ST105	II	-	CIP
***S. aureus* from animals (*n*=24):**				
t045 (*n*=2)	ST5	II	-	-
t337 (*n*=1)	ST9	II	-	-
t414 (*n*=2)	ST34	III	-	-
t571 (*n*=1)	ST398	I	-	-
t645 (*n*=4)	ST121	IV	-	(CIP)
t693 (*n*=1)	ST1	III	-	-
t748 (*n*=1)	ST3254[2]	III	-	-
t843 (*n*=1)	ST130	III	-	-
t1166 (*n*=1)	ST3269[2]	I	-	-
t1190 (*n*=2)	ST2855	III	-	CIP
t2383 (*n*=7)	ST398	NT	-	-
t15307[1] (*n*=1)	ST3270[2]	I	-	-

[1]*spa* type first identified in this study; [2]ST first identified in this study; NT: not typable; -: Susceptible to all antibiotics studied; and variable presence of nonsusceptibility phenotype is indicated by parentheses.

The minimum spanning tree based on the *spa*-types, ST and *agr*-types found in this study (Figure 5.2) shows that both clonal complexes CC5 and CC30 grouped strains from animal and human origins. Furthermore, the seven ST398 strains from aquaculture origin differed from the remaining four (one from animal and three from human origin) by *spa*-type.

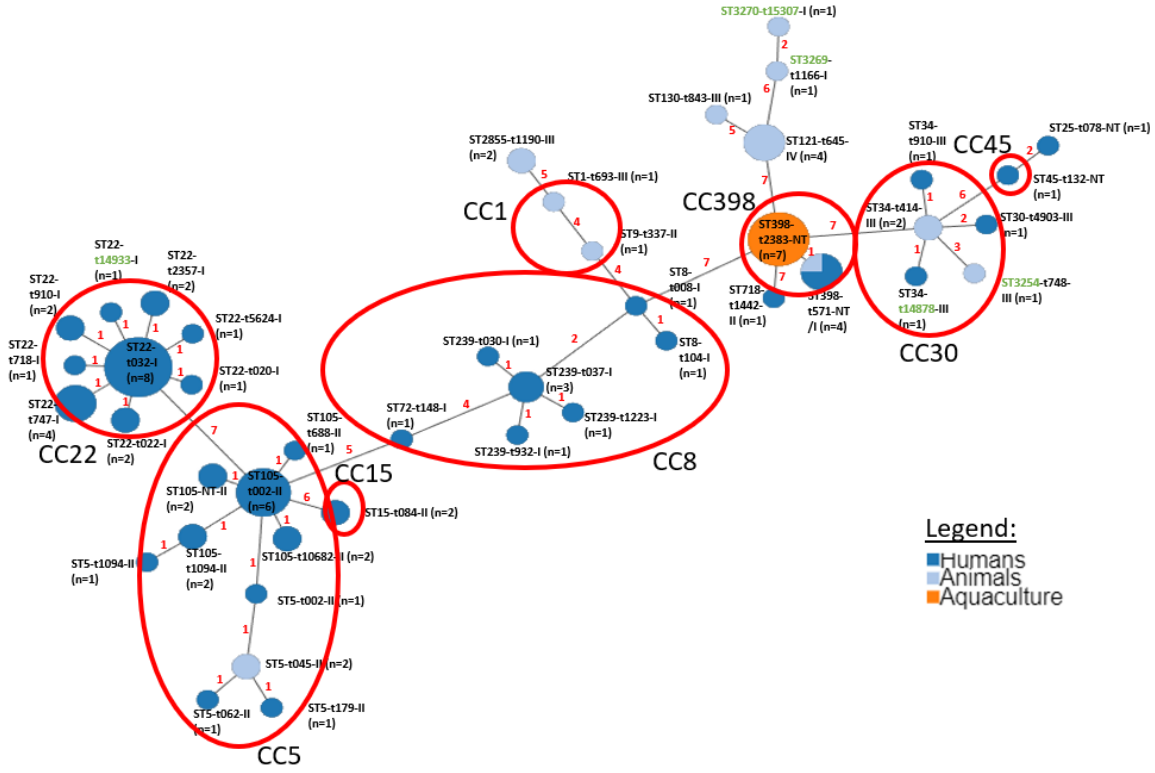

Figure 5.2 — Minimum spanning tree based on the various *spa*, ST, *agr*, and clonal complexes (CC) types found in different reservoirs, in this study (humans, animals, and aquaculture; here aquaculture is in a different color from the other animals, to highlight that all these strains belong to ST398 and *spa* type t2383). ST and *spa* types in green represent those described here for the first time. CC are shown by red circles. Numbers in red corresponds to the link length, which is proportional to the number of differences by its scalability (scaling factor: 7).

5.5 Discussion

Antibiotic resistance is a complex phenomenon involving several resistance mechanisms and affecting different bacterial species and genera in the most diverse environments, from hospitals to communities and animals, thus becoming a growing public health problem. Given this scenario, it is indispensable to monitor and collect information on resistance genes in the various reservoirs, and to try to contain or even prevent eventual dissemination; this is the reason that led to the elaboration of this study.

The high prevalence of cefoxitin and ciprofloxacin resistances in human strains (Table 5.1) agrees with other Portuguese studies [320], as well as other countries [321,322]. On the contrary, countries like Gambia have much lower rates [323]. Of the 40 strains resistant to cefoxitin, 39 had the *mecA* gene and only one was negative for both *mecA* and *mecC*. In this last case, cefoxitin resistance may be due to a high yield of a penicillinase capable of slowly degrading these antibiotics or to changes in the genes coding for PBP, leading to amino acid

substitutions in the transpeptidase domain [324]. An alternative explanation is related to mutations in the hybridization zone of the primers that prevented the amplification of these genes.

In the present work, no MRSA were found among strains from animals, but other studies have already described them, namely in pigs [325] and calves [326] in Portugal, in wild animals such as deer, goats, vultures, and wild boars in Spain [327] and in sheep and goats, as well as their milk, in Nigeria [328]. These cases, although MRSA prevalence rates have been low, are a concern not only for animal health but also for human health since these animals can serve as reservoirs of resistance genes that can be disseminated among different settings.

In relation to infections caused by MRSA and in patients unable to tolerate vancomycin, fluoroquinolones (that belong to the third most commonly antibiotic class prescribed in Portugal [329]) have become one of the treatment options [320,330]. The resistance mechanisms to ciprofloxacin were not the aim of this study but others indicate that in *S. aureus* the primary target is topoisomerase IV (more specifically mutations in the *parC* gene), which normally leads to moderate levels of resistance. Usually, these mutations precede other mutations in *gyrA* gene. Another resistance mechanism to this class of antibiotics in *S. aureus* is the expression of a chromosome encoded MFS family efflux pump, NorA, which has the ability of extrusion of these antibiotics; thus, conferring a low-level resistance [330]. Indeed, our strains showed a large range of diameter of inhibition zone for resistance to fluoroquinolones (from 6 to 20 mm), which might be related with both of those mechanisms, singly or in association.

Teicoplanin is often used in cases of septic arthritis and osteomyelitis caused by MRSA. The resistance to teicoplanin found in the present study may be related with the presence of *tca*RAB (a teicoplanin resistance operon) or the inactivation of *tca*A. This strain is also hGISA, thus probably registering alterations in the cell wall, as already described here. These heterogeneous populations represent a clinical challenge since they can lead to treatment failure. Sometimes, cross-resistance between teicoplanin and vancomycin can be observed [331].

In the group of strains isolated from humans, t032 (ST22) was the most frequent *spa* type followed closely by t002 (ST5/ST105). These findings agree with the global frequencies of these *spa* types (10.10% for t032 and 6.59% for t002; http://www.spaserver.ridom.de/; accessed on Jul 29, 2020) and Portuguese studies in the community [332] and in hospitals [320]. Portuguese hospitals have so far described several stages in the clonal dissemination of MRSA: first, in 1992 and 1993, the Iberian clone ST247-t008/t051 replaced the Portuguese clone ST239-t421. In 1994 and 1995, occurred the rapid spread of the multidrug resistant Brazilian clone ST239-t037 (found in three strains collected in hospitals, in our study); later, this clone was replaced by the ST22-t032 epidemic clone (in eight strains from our study). Shortly afterwards,

the New Yorker/Japanese clone ST5-t067 and, more recently, the ST105-t002 clone appeared as the second most prevalent clones [333] (which was found in five strains from our study). For the other clones in this study, the ST-*spa* association had already been described for several cases: the clone ST8-t008 in humans and pigs in Norway; the ST22-t020 and the ST5-t179 in the community in Portugal; the ST239-t932 in Malaysia; the ST5-t062 in Brazil; the CC45-t132 and CC25-t078 in Lebanon; the ST239-t030 in China; the ST8-t104 in Angola; the ST15-t084 in Iran associated with hospitals; the ST22-t022 in USA, Canada, Europe, Middle East, Asia, and Australia/New Zealand; the CC5-t688 in USA associated with the community; ST22-t747/t910/t2357 and CC5-t10682 in Portugal associated with hospitals and the community; the CC5-t1094 in rabbits in Portugal; and the ST72-t148 in humans, gorillas, and chimpanzees in Gabon [302,304,320,332,334–343]. Community-based strains used to be associated with ST1, ST8, ST30, ST72, and ST80. However, except for ST1 and ST80, the other ST were found in this study in hospital-acquired strains. In fact, a 2013 study in Portugal showed that barriers between hospital and community are becoming smaller and the ST most frequently found in the community are also identified in hospitals, especially ST22 and ST5/105 [344].

In *S. aureus* strains isolated from animals, there were some ST-*spa* associations previously described, such as the ST5-t045 clone in dogs and cats in the USA [345], although in this study it was found in goats; the ST9-t337 in pigs in Thailand [346], as in the strains studied here; the ST398-t571 in pigs and humans in Korea and the USA, respectively [347,348], having been found in a dolphin and humans in our study; the CC398-t2383 in pigs and humans from Denmark and Netherlands, respectively [349,350], while in this study this clone was found in all strains from gilthead seabream from aquaculture origin; the CC130-t843 in humans in the United Kingdom, France, and Spain, and in dogs in the United Kingdom and Germany, in cattle in the United Kingdom, Denmark, and France, among others [351], having been found here in a bird of a zoo; CC133-t1166 in a goat in Tunisia [352], having been identified in a duck in our sample; and ST2855-t1190 in hares [353], associated with rabbits in this study. The exceptions were the clone ST3270-t15307, because it was described here for the first time associated with a duck, and the *spa* type t748 (collected in a pig) associated with a new ST, the ST3254, which were previously associated with ST239 in hospitals in China [338]. In the present study, in *S. aureus* from animals we identified three important clones known to cause infections in humans and animals, ST398-t571/t2383 and ST130-t843. Effectively, ST398 is one of the most worrying ST, since it become a rapidly emerging cause of human infections, most often associated with livestock exposure. In Portugal, CC398 has already been identified in MRSA strains responsible for community acquired infections [344], and animals such as in breeding calves [326],

companion animals [354], wild animals [315], and among healthy pigs [355]. It is not only the MRSA isolates from this clone that cause important infections, because cases of septicemia and infections of the skin and soft tissues caused by methicillin-susceptible *S. aureus* (MSSA) belonging to ST398 have also been registered [356]. All seven *S. aureus* from gilthead sea-bream belonged to ST398. To our knowledge, this is the first description of ST398 in fish from aquaculture, being mostly described in livestock, poultry and wildlife [310], and associated to a high virulence [357] is of concern.

In this study, the resistance to glycopeptides (hGISA strain included) was exclusively associated with clone ST105-t002-*agr* type II, as in previous studies, like Sakoulas et al. [358], Moise-Broder et al. [359], and Purrello et al. [360]. The mechanisms by which this happens are still unclear, but there seems to be a selective advantage of some clones associated with *agr* group II towards a selective pressure by the presence of glycopeptides [359].

For two strains isolated in a hospital, it was not possible to amplify the XR region of protein A encoded by the *spa* gene. This has already been described and may be due to a complete absence of the *spa* gene or to deletions/insertions in the region encoding the IgG binding domain of the protein A. This region is upstream of the XR region where the primer forward hybridizes; thus, preventing the amplification.

The *agr* locus acts in the presence of a high extracellular concentration of the called Autoinducing Peptide (AIP), this concentration being proportional to the bacterial population density. Interestingly, AIP segregated by a group, can inhibit the expression of an *agr* from a different group, which may lead to cooperation between strains belonging to the same *agr* group and competition between strains with different *agr* groups. Strains of *S. aureus* belonging to the same *agr* group are thus considered to have similar biological properties and a close genetic relationship [358,360]. In strains collected in humans, group IV was not found and group I was the most prevalent, as in a study by Azmi et al. [111]. A distinct distribution was observed in strains collected from animals, since here the predominant group was III, unlike other studies such as Smyth et al. [361], and there were representatives of all four groups in this samples. Other studies have demonstrated the predominance of group III, but in strains of MRSA from deer [362], sheep [363], and rabbit [336].

The minimum spanning tree analysis (Figure 5.2) shows that CC5 (ST5 and ST105) and CC30 (ST30, ST34, and ST3254) grouped strains from both animal and human origins. In CC5, ST5 strains from human and animal origin have the same putative founder ST105-t002-II. Lowder et al. have suggested a recent human-to-poultry host jump of this lineage, favored by a close contact between these two hosts, showing that ST5 is a well-adapted lineage to

humans, often related with community- and hospital-associated MRSA [364,365]. In the adaptation to this recent host (poultry), this lineage lost the function of several genes implicated in pathogenesis of human infections and acquired MGE. This lineage is known for a great capacity of acquiring MGE which contributes to the adaption to new hosts like poultry and pigs [364,365]. The eventual transmission between reservoirs (e.g., human–animal–human) might be confirmed by genomic approaches. Furthermore, as belonging to the same *agr* group II, it might also be considered that ST5 strains have related biological properties and a close genetic relationship. Regarding CC30, the ST30 and its single locus variant (SLV) ST34 from human origin, and the new ST3254 from animal origin (double locus variant of ST30), might have the same founder ST34-t414-III, from animal origin. All belong to the *agr* group III. The seven ST398 strains from aquaculture origin had different *spa*-types and *agr* groups (mostly non-typable or from group I) when compared with the other ST398 strains (one from animal and three from human origins). Considering the three CCs that encompass strains from human and animal reservoirs with different *spa*-types, we can hypothesize that this might reflect an adaptation to different phylogenetic lineages in those reservoirs (host species), eventually associated to genetic diversification of pre-existing strains, which have consequences in the control of propagation of these strains between reservoirs.

5.6 Conclusions

Overall, in this study we assessed the susceptibility to four antibiotics belonging to three different classes of 82 *S. aureus* strains collected in humans and animals and evaluated the genetic relatedness and diversity of these bacteria within several environments.

In *S. aureus* from human origin, resistance to fluoroquinolones and cefoxitin predominated, the latter caused by expression of the *mecA* gene. Portugal, despite a decreased of MRSA in the years of 2015–2018, still has the highest rates among European Union/European Economic Area countries [366]. Effective strategies are needed not only in hospitals, but also in health care facilities and veterinary institutions to reduce the dissemination of this bacterium, which is a public health problem today. Reduced susceptibility to teicoplanin was also registered, which although poorly disseminated is of concern, namely after the first identification of VRSA in Europe, in a Portuguese hospital [367]. Two new *spa* types (t14878 and t14933) were found in *S. aureus* strains from humans and one new *spa* type (t15307) and three new ST (ST3254, ST3269, and ST3270) in strains collected from animals. The same types of *spa* and ST found in both humans and animals demonstrate the spread of clones between different

reservoirs. We highlight the first description of ST398 in fish from aquaculture. These findings, together with reports of MRSA CC398 from livestock, healthcare-associated or hospital-acquired MRSA, and community-associated MRSA [365,368], show that CC398 is being increasingly disseminated, including in animal reservoirs beyond livestock, such as in aquaculture. It is increasingly important to distinguish between the different reservoir-adapted clades of MSSA and MRSA to better control the spread paths and to implement more focused measures. High throughput sequencing (such as whole genome sequencing) will be helpful to elucidate eventual re-adaptation of MSSA and/or MRSA to different reservoirs and if transmission is human–animal–human or other, as in the case of ST5 from CC5 (Figure 5.2). This study highlights the increasing importance of controlling the spread of *S. aureus* between several reservoirs.

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6

FIRST COMPARATIVE GENOMIC CHARACTERIZATION OF THE MSSA ST398 LINEAGE DETECTED IN AQUACULTURE AND OTHER RESERVOIRS

This research paper was published as:

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Personal contributions:

Salgueiro, V. contributed to microbiological and molecular experiments, data analysis, namely bioinformatics, writing of the original draft, revision, and final approval of the definitive version of the manuscript.

Note:

This work was partially presented at the 31st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2021), online event.

6.1 Abstract

Staphylococcus aureus ST398 can cause diseases in several different animals. In this study we analyzed ten *S. aureus* ST398 previously collected in three different reservoirs in Portugal (humans, gilthead seabream from aquaculture and dolphin from a zoo). Strains tested against sixteen antibiotics, by disk diffusion or minimum inhibitory concentration, showed decreased susceptibility to benzylpenicillin (all strains from gilthead seabream and dolphin) and to erythromycin with an $iMLS_B$ phenotype (nine strains), and susceptibility to cefoxitin (methicillin-susceptible *S. aureus*, MSSA). All strains from aquaculture belonged to the same *spa* type, t2383, whereas strains from the dolphin and humans belonged to *spa* type t571. A more detailed analysis using single nucleotide polymorphisms (SNPs)-based tree and a heat map, showed that all strains from aquaculture origin were highly related with each other and the strains from dolphin and humans were more distinct, although they were very similar in ARG, VF and MGE content. Mutations F3I and A100V in *glpT* gene and D278E and E291D in *murA* gene were identified in nine fosfomycin susceptible strains. The *blaZ* gene was also detected in six of the seven animal strains. The study of the genetic environment of *erm(T)*-type (found in nine *S. aureus* strains) allowed the identification of MGE (rep13-type plasmids and IS*431R*-type), presumably involved in the mobilization of this gene. All strains showed genes encoding efflux pumps from major facilitator superfamily (e.g., *arlR*, *lmrS*-type and *norA/B*-type), ATP-binding cassettes (ABC; *mgrA*) and multidrug and toxic compound extrusion (MATE; *mepA/R*-type) families, all associated to decreased susceptibility to antibiotics/disinfectants. Moreover, genes related with tolerance to heavy metals (*cadD*), and several VF (e.g., *scn*, *aur*, *hlgA/B/C* and *hlb*) were also identified. Insertion sequences, prophages, and plasmids made up the mobilome, some of them associated with ARG, VF and genes related with tolerance to heavy metals. This study highlights that *S. aureus* ST398 can be a reservoir of several ARG, heavy metals resistance genes and VF, which are essential in the adaption and survival of the bacterium in the different environments and an active agent in its dissemination. It makes an important contribution to understanding the extent of the spread of antimicrobial resistance, as well as the virulome, mobilome and resistome of this dangerous lineage.

Keywords: *Staphylococcus aureus*, ST398, WGS, animals, humans, resistome, virulome, mobilome.

6.2 Introduction

Staphylococcus aureus can cause diseases in humans and animals constituting an important clinical and public health problem [104].

Firstly described in livestock, *S. aureus* ST398 has proven to be able to break barriers and become a successful bacterium in several environments and countries from all over the globe. This ST was previously described in numerous diseased and healthy mammal species, birds, and fish, as well as in humans [310,369]. Antimicrobial resistance (AMR) in *S. aureus* is frequent, especially methicillin-resistant *S. aureus* (MRSA) [104]. Infections in humans can range from localized and a lower degree of severity, such as skin and soft tissue infections, to severe invasive illnesses, such as bloodstream infection and pneumonia, either associated with MRSA or with methicillin-sensitive *S. aureus* (MSSA) [357,370]. Previous studies suggest that MRSA does not necessarily replace infections caused by MSSA, but causes additional infections [371]. In fact, some studies suggest that MSSA is responsible for most infections related to healthcare settings and community worldwide [370,372], with an increase in reports associated with invasive infections caused by CC398 lineage in patients with no livestock contact [373]. However, little attention has been given to MSSA molecular epidemiology and mechanisms of pathogenicity [370], not only regarding human but also animal reservoirs, namely in environments such as aquaculture. Studies with retail foods [374] and pig farms [375] uncover MSSA ST398 harboring multiple virulence and antibiotic resistance genes, some of which with a multidrug-resistant phenotype, confirming that MSSA ST398 can represent a health hazard. *Staphylococcus* spp. are not considered part of the commensal fish microbiota. Few studies have focused on *Staphylococcus* spp. as the etiological agent of infection in fish, however this genus was already associated with exophthalmia and hemorrhages in these animals (often resulting in death) [376–378]. On the contrary, there are several studies on outbreaks of human food poisoning caused by the consumption of infected fish [64,379,380]. Closer proximity among humans and animals can promote the dissemination of pathogens between the two reservoirs, as already described for *S. aureus* ST398 [381].

A better understanding of genetic diversity, ARG, VF, and MGE present in ST398 lineage from different reservoirs, is crucial to understand its importance, possible transmission routes and prevent its dissemination. Whole Genome Sequencing (WGS) gives a more complete and discriminative information than traditional methods, like Multilocus Sequence Typing (MLST) and *spa* type, making it suitable for this type of studies [381,382]. Aquaculture has received very little attention when compared to terrestrial animals, so this study may show important

characteristics of this reservoir as a potential danger to human health. Thus, using a WGS approach, our study intended to contribute to clarify the severity associated with the potential for spread of the ST398 lineage, also demonstrating the presence of determinants such as MGE and different ARG and VF circulating in different environments, and exploring how they are genetically related.

6.3 Materials and methods

6.3.1 Study design and bacterial identification

We analyzed ten *S. aureus* ST398 previously collected in three different reservoirs in Portugal: humans (*n*=3; from pus, respiratory secretions, and unknown samples collected between 2015 and 2017), gilthead seabream from aquaculture (*n*=6; 4 from muscles, 1 from skin, and 1 from gills samples collected in 2018) and a dolphin (*n*=1; from a bronchoalveolar washing collected in 2011) [369]. Human strains belonged to a collection of the National Institute of Health Dr. Ricardo Jorge (INSA) compiled in region A. On the other hand, the dolphin strain was collected in region B and belong to the collection of the National Institute of Agrarian and Veterinary Research. Regions A and B are approximately 550 km apart. Gilthead seabream samples were collected by the Portuguese Institute of Sea and Atmosphere in an aquaculture tank exposed to seawater, located in region B, and sent to INSA where preparation and bacterial isolation procedures were performed, as described elsewhere [257]. Bacterial species identification was performed by VITEK® 2 and amplification of the 16S rRNA gene, as previously described [150].

6.3.2 Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by: (1) disk diffusion (Bio-Rad, Marnes-la-Coquette, France) for the following antibiotics: cefoxitin (FOX; 30 µg), ciprofloxacin (CIP; 5 µg), levofloxacin (LEV; 5 µg), moxifloxacin (MOX; 5 µg), rifampicin (RIF; 5 µg), mupirocin (MUP; 200 µg), and fusidic acid (FUS; 10 µg); (2) minimum inhibitory concentration (MIC) through E-test® (BioMérieux, Marcy-l'Étoile, France) or in-house broth microdilution for the following antibiotics: daptomycin (0.016–256 µg/mL), linezolid (0.5–2 µg/mL), teicoplanin (0.016–256 µg/mL), and vancomycin (0.016–256 µg/mL); (3) E-test® for benzylpenicillin (0.016–256 µg/mL); and (4) VITEK® 2 (BioMérieux, Marcy-l'Étoile, France) for the following antibiotics: erythromycin (1–8 µg/mL), tetracycline (0.5–2 µg/mL), tigecycline (0.25–1 µg/mL), and fosfomycin (8–32

µg/mL). VITEK® 2 was also used to detect inducible clindamycin resistance. All antibiotic susceptibility tests were performed and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2022) guidelines, except for mupirocin (EUCAST 2016) (https://www.eucast.org/clinical_breakpoints/; accessed on May 24, 2022).

6.3.3 Whole genome sequencing

Genomic DNA was extracted with MagNA Pure 96 Instrument (Roche, Manheim, Germany) and quantified by Qubit Fluorometric Quantitation (Thermo Fisher Scientific, Waltham, MA), according to manufacturer's instructions. Libraries from 1 ng of genomic DNA were prepared, in two different sets, using the dual-indexed Nextera XT Illumina library preparation, before cluster generation and paired-end sequencing (2×150 bp) on a NextSeq 550 Illumina platform (Illumina Inc., San Diego, CA), according to manufacturer's instructions.

6.3.4 Genome annotation and resistome, virulome and mobilome analysis

Sequence reads were trimmed and filtered (primers and adapters sequence removal, and a minimum size cut-off of 50 bp), according to quality criteria (limit=0.05), and assembled *de novo* using CLC Genomics Workbench version 21.0.3 (QIAGEN, Aarhus, Denmark), with default parameters, as previously described [383]. Online tools and databases available at the Center for Genomic Epidemiology (CGE; https://www.genomicepidemiology.org/; accessed on May 24, 2022) were used to confirm bacterial species (KmerFinder 3.2 [384]), predict multilocus sequence type (MLST 2.0 [385]) and *spa* type (spaTyper 1.0 [386]), investigate the presence of antibiotic and disinfectant resistance genes (ResFinder 4.1 [387]), virulence genes (VirulenceFinder 2.0 [388]), plasmids (PlasmidFinder 2.1 [389]), and other mobile genetic elements (MobileElementFinder version 1.0.3 [390], SCCmecFinder 1.2 [391]), and to estimate bacteria's pathogenicity towards human hosts (PathogenFinder 1.1 [392]). The Comprehensive Antibiotic Resistance Database (CARD [393]) was also used to investigate the presence of antibiotic resistance genes. PHASTER and ISsaga search web tools allowed the identification and annotation of prophage sequences and insertion sequences, respectively [394,395]. All analysis were performed using default parameters. A phylogenetic tree was constructed with 50 *S. aureus* ST398 strains (10 from our study and 40 from NCBI database; Table S 4), based on single nucleotide polymorphisms (SNPs), using CSI Phylogeny 1.4 web tool with default parameters [396]. The same strains were used to perform a heat map representing the alignment percentage (AP) of these strains, using CLC Genomics Workbench version 21.0.3 with Euclidean

distance and complete linkage parameters. CLC Genomics Workbench version 21.0.3 was also used to search for integrons, heavy metals tolerance genes, other VF, and *agr*-type, as well as to study the genetic environment of antibiotic resistance genes. Online tool RFPlasmid was used to predict chromosomal or plasmid location of the previously identified genes [397].

6.3.5 Nucleotide sequence accession numbers

The genomes of the ten strains included in this study were deposited in GenBank under BioProject number PRJNA795413 and accession numbers JAKFBA000000000 (INSaAq36), JAKFAZ000000000 (INSaAq61), JAKFAY000000000 (INSaAq69), JAKFAX000000000 (INSaAq83), JAKFAW000000000 (INSaAq134), JAKFAV000000000 (INSaAq156), JAKFAU000000000 (LV31741/11), JAKFAT000000000 (INSa869), JAKFAS000000000 (INSa910) and JAKFAR000000000 (INSa934). More information about number of reads/bases/contigs, consensus length, average coverage and contigs N50 is available in Table S 5.

6.4 Results and discussion

Our results show that all *S. aureus* ST398 strains studied, from different reservoirs, are very similar to each other, not only regarding ARG and VF, but also MGE, demonstrating the importance of the comparative study of different compartments. In Table 6.1, we can see that all *S. aureus* ST398 from aquaculture belong to the same *spa* type, t2383, already isolated in food-producing animals (pigs and calves) and in humans, associated with an outbreak in a residential care facility [350,398–400]. All the human and the dolphin strains belonged to the same *spa* type: t571. This ST-*spa* association is frequent in MSSA strains and was already described in several human samples, medical device surfaces, food producers, domestic animals, and retail food [348,374,401]. Considering that ST398-t571 association is commonly found in humans and that the dolphin included in this study was from a zoo, with the present data we can hypothesize that a possible human to animal transmission may have occurred. For nine of the 10 strains studied, *agr* locus was non-typable, which is consistent with other studies [374]. For a more detailed analysis of relatedness, we constructed a SNPs-based tree (Figure 6.1), as well as a heat map (Figure 6.2), with the 10 strains from our study and 40 from NCBI database belonging to different reservoirs (Table S 4). Interestingly, SNPs analysis revealed a wide range of SNPs values among strains from ST398 lineage (minimum: 0; maximum: 16292; Table S 6), as already described by other studies [370,402]. As we can see in Figure 6.1, the SNPs-based tree divided the 10 strains from our study into three distinct clusters: one grouping all strains

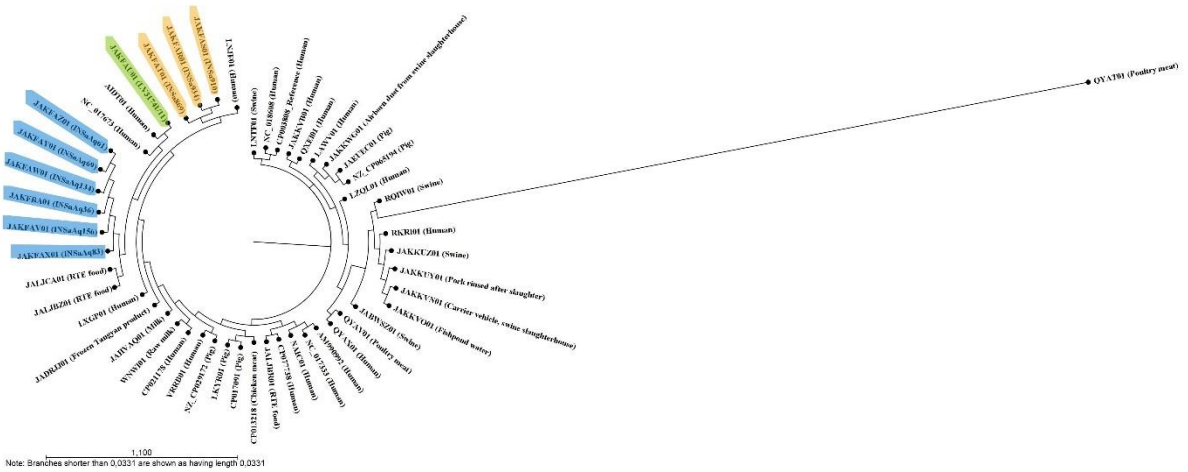

Figure 6.1 — Single nucleotide polymorphism (SNPs)-based tree, constructed with CSI Phylogeny 1.4 [396], showing the relationship between 50 *S. aureus* ST398 (10 from our study and 40 from NCBI database; Table S 4). Strains from this study are highlighted in different colors according to the origin of the samples (humans in orange, dolphin from a zoo in green and gilthead seabream from aquaculture in blue). This analysis divided the 10 *S. aureus* ST398 from this study into three distinct clades: one encompassing all strains from human origin that were more closely related to LNJF01 strain also from human origin; the second with the dolphin' strain and two animal-independent MSSA isolated in humans (AIDT01 and NC_017673); and the third with strains from aquaculture that cluster together with two strains isolated in Russia from ready-to-eat (RTE) food (JALJCA01 and JALJBZ01).

from human origin that were cluster together with LNJF01 strain (isolated from a human infection in France); the second with the dolphin' strain and two animal-independent MSSA isolated in humans from Dominica and United States (AIDT01 and NC_017673, respectively); and the third with strains in aquaculture that cluster together with two strains isolated in Russia from ready-to-eat (RTE) food (JALJCA01 and JALJBZ01). This information is confirmed through the analysis of the heat map (Figure 6.2), where we can observe a high percentage of alignment (AP) between the strains previously mentioned adding LXGP01 (isolated in a human with bloodstream infection in France). The human strains from our study differ 148 to 231 SNPs from LNJF01 and LXGP01 strains (Table S 6). The *S. aureus* ST398 isolated in one dolphin differs 77 to 88 SNPs from AIDT01 and NC_017673 strains, respectively. On the other hand, our strains isolated in gilthead seabream from the same aquaculture farm differ 109 to 119 SNPs from JALJCA01 and JALJBZ01 strains. There is no consensus on the SNPs cut-off to define whether strains are related or not, with studies considering less than 15 SNPs to define that certain strains are related [403], others consider less than 40 [404,405] or even 50 SNPs [402] (all these cut-offs are highlighted in different colors in Table S 6). Using any of the criteria, *S. aureus* ST398 from humans in this study are very distinct from each other, which were collected in

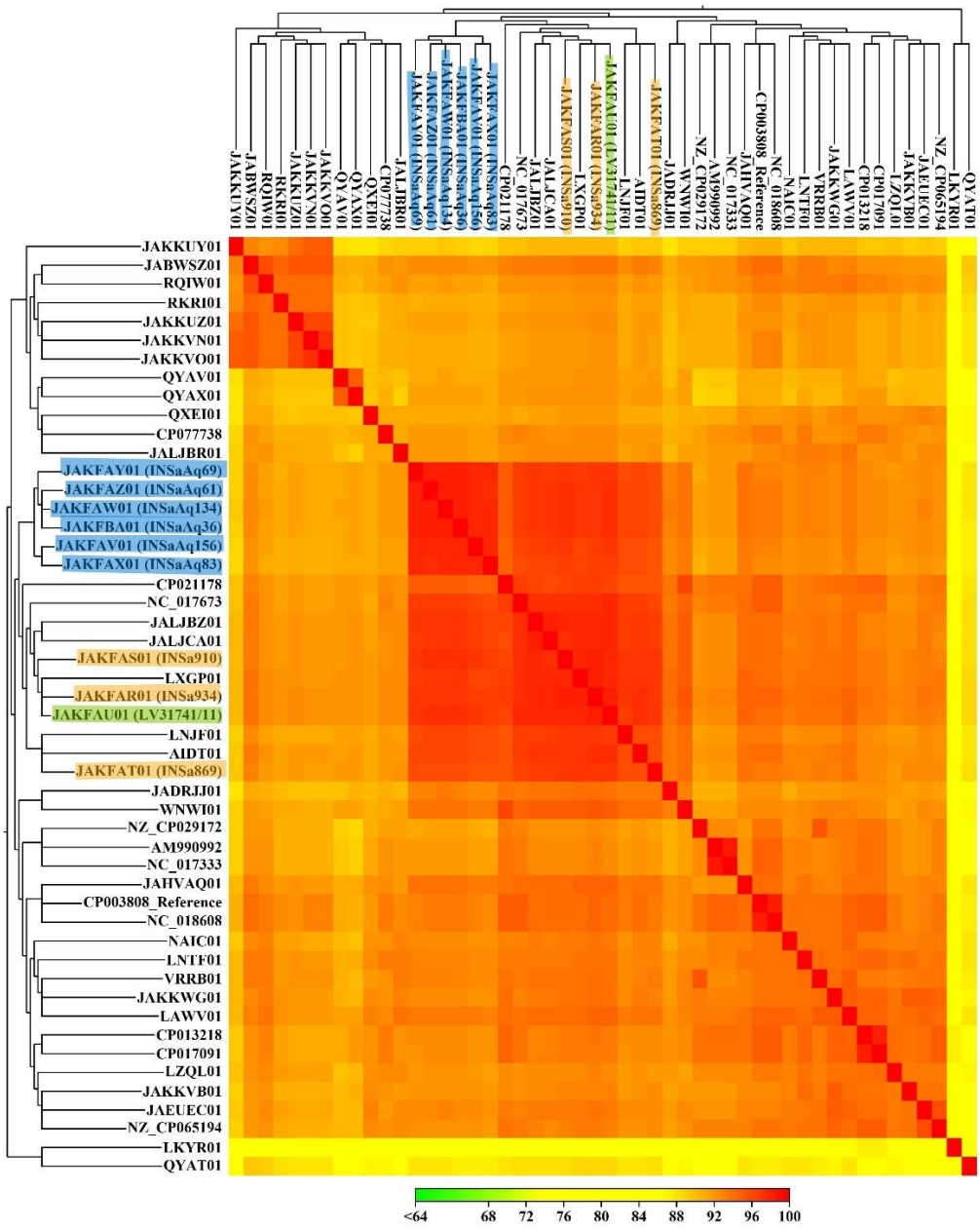

Figure 6.2 — Heat map representing the alignment percentage (AP) between 50 *S. aureus* ST398 strains (10 from our study and 40 from NCBI database; Table S 4), constructed with CLC Genomics Workbench version 21.0.3, using Euclidean distance and complete linkage parameters. *S. aureus* from this study are highlighted according to the origin of the samples (humans in orange, dolphin in green and seabream from aquaculture in blue).

different years (minimum SNPs difference: 97 between INSa869 and INSa910; maximum SNPs difference: 149 between INSa869 and INSa934), and from the strains recovered from the dolphin and gilthead seabreams (minimum SNPs difference: 169 between INSa910 and INSaAq83; maximum SNPs difference between INSa934 and LV31741/INSaAq69) (Table S 6). The strain isolated in a dolphin was also very distinct from the strains from gilthead seabream collected

in an aquaculture farm (minimum SNPs difference: 178 with INSaAq83; maximum SNPs difference: 191 with INSaAq36). None of the strains with origin in the same aquaculture farm were indistinguishable, with a minimum SNPs difference of 3 (between INSaAq61 and INSaAq69/134 isolated in muscle and skin samples from 3 gilthead seabream) and a maximum SNPs difference of 33 (between INSaAq36 and INSaAq83 isolated both in muscle samples but from 2 different fish). Using the narrowest criteria (less than 15 SNPs), INSaAq83 is more closely related with INSaAq156 and the remaining strains from aquaculture with each other. Using the largest criteria (less than 50 SNPs), all strains from aquaculture origin are closely related. All these strains were from 5 gilthead seabream collected in the same aquaculture farm and possibly have a very recent common ancestor.

Erythromycin and penicillins are considered by the World Health Organization (WHO) as "critically/highly important antimicrobials" and commonly used in humans, aquaculture settings and veterinary medicine [69,104,400]. In our study, seven strains were resistant to benzylpenicillin and nine out of 10 had an $iMLS_B$ phenotype (with resistance to erythromycin plus inducible resistance to clindamycin) and the *erm(T)*-type gene. The genetic environment of *erm(T)*-type gene in all the positive strains was the same (Figure 6.3): upstream of *erm(T)*-type gene, the genetic environment was composed by a *rep* gene (involved in replication), a gene encoding a metalloregulator ArsR/SmtB family transcription factor (involved in tolerance/resistance to heavy metals, such as zinc, cadmium, cobalt, arsenic and antimony), *cadD* gene (that encodes for cadmium resistance transporter CadD) and *ermCL* gene (that regulates the expression of *erm* genes); downstream the gene, a plasmid truncated replication protein was identified [406–408]. The only distinct feature was in *S. aureus* ST398 recovered from a dolphin (LV31741/11) that presented an IS*431R* upstream the *rep* gene. All *erm(T)*-type genes were predicted to be located in plasmids, that may have played a role in the transmission between the different host species and environments. Six of the seven benzylpenicillin resistant strains with animal origin were positive for *blaZ* gene (except for INSAq83 isolated from muscle of gilthead seabream) and just one was predicted to be located in a plasmid (INSaAq156 isolated from gills of gilthead seabream). When analyzing the contigs that contained *blaZ* gene, we verified that the only difference between the strains from the gilthead seabream (excepting for INSaAq156) and the dolphin was the presence of phage's genes in strains from aquaculture origin, namely Staphy_3MRA for INSaAq36, INSaAq61 and INSaAq134, and Staphy_53 for INSaAq69 (Table 6.1). The examination of the genetic environment nearby the *blaZ* gene (Figure 6.4) allow us to identify, upstream the gene in all strains, the two regulatory genes *blaI* and

blaR1 controlling the *blaZ* expression [409]. All strains were susceptible to cefoxitin (values ranging from 26 to 35 mm in disk diffusion), thus were considered MSSA.

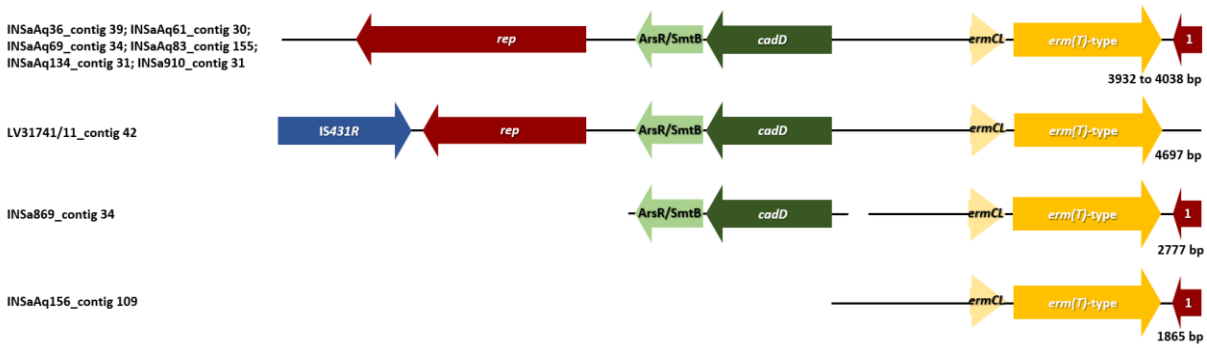

Figure 6.3 — Genetic environment of *erm(T)*-type gene found in 9 out of 10 *S. aureus* ST398 strains (see Table 6.1: the exception was INSa934 isolated in humans). Arrows are drawn to scale. Genes in blue are associated with mobile genetic elements. Genes in red are associated with replication. Genes in green with tolerance to heavy metals. Genes in yellow are related with antibiotic resistance. 1-Truncated replication protein for plasmid. *erm(T)*-type genes had the same genetic environment in all strains, being the only distinct feature encountered in *S. aureus* ST398 recovered from one dolphin (LV31741/11) that presented an IS*431R* upstream the *rep* gene.

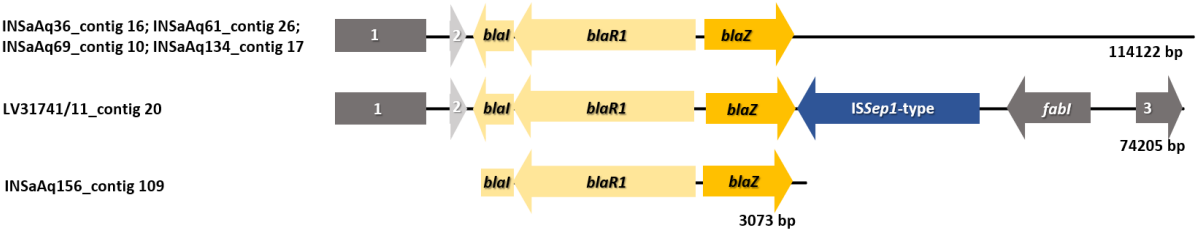

Figure 6.4 — Genetic environment of *blaZ* gene identified in 6 out of 7 strains with animal origin (except for INSAq83 isolated from muscle of gilthead seabream). Arrows are drawn to scale. Genes in dark grey correspond to normal functions of the bacterial cell. Genes in yellow are related with antibiotic resistance. Genes in blue are associated with mobile genetic elements. 1-Lactonase family protein. 2-Hypothetical protein. 3-YoID-like family protein.

Although most of *S. aureus* studied (excepting INSa934 from human origin) presented mutations F3I and A100V in *glpT* gene and D278E and E291D in *murA* gene (usually related with fosfomycin resistance), all were susceptible to fosfomycin (MIC values ≤16 mg/L) as described by others [410,411]. More studies are needed to understand the mechanisms of fosfomycin resistance in Gram-positive bacteria, namely *S. aureus* [412]. No resistance was detected among the other 13 antibiotics tested.

Excepting for one *S. aureus* from humans and another from aquaculture, all strains presented the same genes that encode efflux pumps from major facilitator superfamily (MFS; *arlR*, *mgrA*, *lmrS*-type and *norA*/*B*-type), ATP-binding cassette (ABC; *mgrA*) and multidrug and toxic

compound extrusion (MATE; *mepA*/*R*-type) families, not only responsible for the decreased susceptibility to antibiotics/disinfectants, but also tolerance to heavy metals. These genes were all predicted to be in the chromosome.

Likewise, the virulome's composition was very similar in all *S. aureus* ST398 studied and comprised genes related to host immune evasion (*scn* and *chp*), exoenzymes (*aur*) and toxins (*hlgA*/*B*/*C*, *hlb*, *pvl*-type and *cidA*/operon *cid*-type) production and adherence (*eno*-type). Genes *scn* and *chp* (located upstream *scn* gene) compose the immune evasion cluster (IEC) from type C [413]. Some studies suggest that IEC is a human-specific characteristic, with type B as the most frequently found among clinical human isolates of *S. aureus* [413,414]. IEC type C was present in 8 of the 10 strains studied, with INSaAq83 and INSaAq156 strains with only *scn* gene, which encodes the staphylococcal complement inhibitor (SCIN), responsible for preventing opsonophagocytosis and killing of *S. aureus* by neutrophils [414]. This data suggest that *S. aureus* isolated in dolphin and gilthead seabream could have a human origin; the close contact between dolphins and humans in a zoo can explain this data, whereas the aquaculture setting may include intense human activity due to the exposure of the tanks to seawater, which can carry several bacteria and resistance determinants from different locations [415,416]. Birds can also play a role in the transmission of bacteria and resistance determinants between different environments, including aquaculture [417,418]. Price et al. also suggests that CC398 can be originated in humans as MSSA and subsequently disseminated to livestock. It is though that *S. aureus* has undergone some changes to adapt to this new host, such as the loss ϕSa3 prophage and the acquisition of the SCC*mec* cassette and *tet(M)* gene, conferring methicillin and tetracycline resistance, respectively, due to the use of broad-spectrum cephalosporins and tetracycline antibiotics in food producing animals. Posteriorly, this lineage was reintroduced in humans, followed by the reacquisition of ϕSa3 prophage, which usually harbors the genes encoding IEC [381]. However, the boundaries between animal and human CC398 lineages are fading, with recent studies detecting livestock-associated MRSA (LA-MRSA) isolated in humans and positive for IEC genes. These observations may also indicate that the high evolutionary rate of MRSA in terms of virulence and genome content may cause the emergence and spread of more human-adapted strains with more virulent characteristics, which may be happening in MSSA as well [419,420]. Prophage Staphy_StauST398_4, found in all strains from our study in the same contig as IEC type C (except for strains INSaAq83 and INSaAq156), was already associated with IEC genes [421]. Additionally, all strains were considered pathogenic to humans with mean values of 98% (Table 6.1).

The mobilome was formed by several insertion sequences, prophages, and plasmids (Table 6.1), some associated with ARG, VF and genes related with tolerance to heavy metals (Figure 6.3 and Figure 6.4). MGE can be involved in the acquisition of ARGs by horizontal gene transfer [121], just like plasmids with *erm(T)*-type gene found in this study. This acquisition of new genetic material allows bacteria to adapt and survive in different environments and to the selective pressures exerted by the use of antibiotics [129].

To our knowledge no other country reported the presence of the *S. aureus* ST398 lineage in aquaculture, namely in the perspective of this study, using WGS to compare different molecular characteristics between three reservoirs in Portugal: humans, aquaculture gilthead seabream and dolphin from a zoo. With this study we can conclude that for this lineage of *S. aureus*, the human, animal, and environmental health are linked, and that antibiotic resistant bacteria and ARG can be transmitted in different directions among these reservoirs. We also highlight that MGE and bacteriophages are found in aquatic environments and that *S. aureus* ST398 may harbor several heavy metals resistance genes and VF, playing an important role in their dissemination between different reservoirs. This study using 10 strains in a One Health approach (human and animal/aquatic environments), as well as the WGS as a high-throughput technology, makes an important contribution to the scientific community and clinical practitioners to understand the extent of the spread of AMR, and the virulome, mobilome and resistome of this dangerous bacterium. The results obtained can help to recognize ways to break transmission routes and prevent the spread of *S. aureus* ST398 in various reservoirs, apparently related or not. We also show that aquaculture has received very little attention when compared to terrestrial animals, however it may pose a potential danger to human health, demonstrated here in relation to the spread and/or acquisition of clinically relevant bacterial determinants.

Table 6.1. — Summary of the results of the investigation of *spa* type, *agr*, resistance profile, antibiotic/disinfectant resistance genes, virulence genes, plasmids, other mobile genetic elements, prophages, and bacteria's pathogenicity towards human hosts, using several online tools and databases.

Reservoir	*spa*	*agr*	SP	AR genes	Efflux pumps related with AR and tolerance to heavy metals	Virulence factors	Plasmids	Prophages	Insertion sequences	HPP (%)
Aquaculture (*n*=6)	t2383 (*n*=6)	NT (*n*=6)	BPN, ERY (*n*=7)	*erm(T)*-type (P), *glpT*-type* (C), *murA** (C) (*n*=7), *blaZ* (C, *n*=5; P, *n*=1)	*arlR* (C), *mgrA* (C), *mepA*-type (C), *mepR*-type (C), *lmrS*-type (C), *norA*-type (C) (*n*=6), *norB*-type (C; *n*=5)	*scn* (C/P), *aur* (C), *hlgA*-type (C), *hlgB* (C), *hlgC* (C), *hlb* (C), *eno*-type (C), *pvl*-type (C), *cidA-type* (C), *operon cid*-type (C) (*n*=10)	rep13-type (*n*=7)	Staphy_StauST398_4 (*n*=6), Staphy_3MRA (*n*=4), Staphy_53 (*n*=2)	IS*Sau1*-type (*n*=1), IS-*Sau2*-type (*n*=4), IS*Sau3*-type (*n*=3), IS*Sau5*-type (*n*=6), IS*Sau8*-type (*n*=4)	98 (*n*=6)
Dolphin (*n*=1)	t571 (*n*=4)	I (*n*=1)			*arlR* (C), *mgrA* (C), *mepA*-type (C), *mepR*-type (C), *norA*-type (C), *norB*-type (C), *lmrS*-type (C) (*n*=3)			Staphy_StauST398_4 (*n*=4)	IS*Sep1*-type, IS*Sau2*-type, IS*431R*-type (*n*=1)	98.3 (*n*=3)
Humans (*n*=3)		NT (*n*=3)	ERY (*n*=2)	*erm(T)*-type (P), *glpT*-type* (C), *murA** (C) (*n*=2)			rep13-type (*n*=2), rep24b-type (*n*=1)		IS*Sau1*-type (*n*=2), IS-*Sau2*-type (*n*=3), IS*Sau3*-type (*n*=2), IS*Sau5*-type (*n*=1), IS*Sau8*-type (*n*=2)	
			ND (*n*=1)	ND (*n*=1)	*mepA*-type (C), *norA*-type (C), *norB*-type (C), *lmrS*-type (C) (*n*=1)		ND (*n*=1)			98.2 (*n*=1)

*These genes presented mutations F3I, A100V (in *glpT*), D278E, and E291D (in *murA*) usually related with fosfomycin resistance (detected by CARD). SP: susceptibility profile; AR: antibiotic resistance; HPP: human pathogen probability; NT: not typable; ND: not detected; BPN: benzylpenicillin; ERY: erythromycin; (P): predicted plasmid location; (C): predicted chromosomal location.

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7

AQUACULTURE AS A HOTSPOT FOR MGE, VIRULENCE AND RESISTANCE GENES (ANTIBIOTICS, DISINFECTANTS AND HEAVY METALS) AND THEIR CONTRIBUTION TO THE ENVIRONMENTAL RESISTOME

This research paper was submitted as:

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Personal contributions:

Salgueiro, V. contributed to methodology, formal analysis, investigation, writing - original draft, preparation, writing – review and editing, and final approval of the definitive version of the manuscript.

Note:

This work was partially presented at the 4th International Caparica Conference in Antibiotic Resistance 2021 (IC^2AR 2021), Caparica, Portugal, at the Congress of Microbiology and Biotechnology 2019 (MICROBIOTEC 19) in Coimbra, Portugal, at the 31st European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2021), online event and at the 32nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2022), Lisbon, Portugal.

7.1 Abstract

Aquaculture environments can be hotspots of 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 mollusks cultivated in aquaculture farms, using a Whole Genome Sequencing approach. Sixty-six Gram-negative strains (*Aeromonadaceae*, *Enterobacteriaceae*, *Hafniaceae*, *Morganellaceae*, *Pseudomonadaceae*, *Shewanellaceae*, *Vibrionaceae*, and *Yersiniaceae* families) were selected for genomic characterization. Species and MLST were determined, and antibiotic/disinfectants/heavy metals resistance genes, virulence determinants, mobile genetic elements (MGE) and pathogenicity to humans were investigated. Our study revealed new sequence types (e.g., in *Aeromonas* spp. ST879, ST880, ST881, ST882, ST883, ST887, ST888; in *Shewanella* spp. ST40, ST57, ST58, ST60, ST61, ST62; in *Vibrio* spp. ST206, ST205). More than 140 different genes were identified in the resistome of seabream and bivalve mollusks, encompassing genes associated with β-lactams, tetracyclines, aminoglycosides, quinolones, sulfonamides, trimethoprim, phenicols, macrolides and 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, among others; 74.2% of the strains analyzed were considered pathogenic to humans. We explored the genetic environment of some antibiotic resistance genes (e.g., $bla_{\text{TEM-1B}}$, $bla_{\text{FOX-18}}$, *aph(3")-Ib*, *dfrA*-type, *aadA1*, *catA1*-type, *tet(A)/(E)*, *qnrB19* and *sul1/2*), highlighting the MGE in their vicinity (e.g., integrons, plasmids and Tn*As*) that could be involved in resistance dissemination between bacteria from different environments. This study reviews and deepens the diversity of resistance genes that can be transmitted to humans and to the environment, and which unquestionably have the environment but also aquaculture as intermediaries in a complex chain.

Keywords: seabream, bivalve mollusks, Gram-negative bacteria, resistome, virulome, mobilome.

7.2 Introduction

Water is a rich environment encompassing several organisms (such as fish, bivalve mollusks, and algae) and their microbiomes, but it is also a point of confluence for bacteria originated from other environments (human and animal) [415]. Aquaculture farms are examples of such diverse environments, where the use of antibiotics can increment selective pressure among commensals as well as pathogenic bacteria [66,67].

In aquaculture settings, antibiotics are usually administrated mixed in food, exposing disease and healthy animals to these substances [2,3]. Uneaten food with antibiotics as well as unabsorbed antibiotics or their by-products that are excreted in urine and feces can be carried by water currents to other sites, 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), marine birds or mammals, and may concomitantly contain various bacteria, as well as various heavy metals, antibiotics, and organic substances, promoting selective pressure on bacteria normally present in the aquatic environment. For that reason, marine bivalve mollusks are possible indicators of multidrug-resistant *Escherichia coli* and other species of the *Enterobacteriaceae* family [250]. 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, such as aquaculture farms [66,67]. The statistically significant decrease in susceptibility to oxytetracycline found during the summer in Japanese oysters, which could be directly influenced by the factors described above, such as bird migration, was previously demonstrated by us [422]. Salgueiro et al. also illustrated that *Staphylococcus aureus* ST398 isolated in gilthead seabream from aquaculture, could had an environmental origin due to the exposure of the tank to seawater and, consequently, to antibiotics, bacteria, and resistance determinants from different locations [423].

It is known that antibiotics, even in sublethal concentrations, interfere in 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 it happens in bivalve mollusks, due to their filter feeding habit, as well as in biofilms which are important in bacterial endurance in water environments. The presence of heavy metals or disinfectants can also select for antibiotic resistance through events of cross-resistance, since these elements are used as substrates to efflux pumps and the genes responsible for tolerance to heavy metals and disinfectants can also be located in the same genetic elements as antibiotic resistance genes (ARG) [424,425]. Several theories support that some of the ARG frequently

found in clinical isolates were originated in aquatic bacteria, such as *qnrA* (conferring quinolones resistance) and *bla*$_{\text{OXA-48}}$ (conferring carbapenems resistance) genes that probably derived from the chromosome of *Shewanella* spp., a bacterium typical from freshwater and marine environments [426,427]. Likewise, plasmid *bla*$_{\text{AmpC}}$ genes may had their origin in other aquatic bacteria such as *Hafnia alvei* and *Aeromonas hydrophila* or in bacteria that circulate between a wide range of environments (like water and gut), such as *Enterobacter asburiae* and *Citrobacter freundii* [425]. The hypothesis is that these genes jumped from the chromosome of these aquatic species due to the pressure of antibiotic application and with the help of mobile genetic elements (MGE) (such as insertion sequences [IS] and plasmids) and disseminated into bacteria most frequently found in clinical settings [425–427]. Acquired antibiotic resistance may be due to mutations in genes already present in bacterial chromosome or through the acquisition of genetic material by horizontal gene transfer (HGT), using the three mechanisms, conjugation, transformation, and transduction [428]. Due to the highly abundance of phages in water environments, transduction represents an important vehicle for ARG acquisition [429]. Thus, aquaculture environments can be reservoirs of antibiotic resistance [428].

Whole Genome Sequencing (WGS) represents one of the most comprehensive technologies nowadays. Therefore, this study used WGS in bacteria isolated in fish and bivalve mollusks from aquaculture production, collected directly in aquaculture farms and market, to identify which resistance/virulence determinants and MGE are circulating in this environment and to understand the contribution of aquaculture sources in the emergence and spread of antibiotic resistance. Thus, the study search and analyzes in a very deep way 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, and not only on the metabolism of fish or bivalve mollusks [430]. In fact, this is the first study in Portugal that deepens the diversity of resistance genes that can be transmitted to humans and to the environment, and which have the environment but also aquaculture as intermediaries in a complex chain.

7.3 Materials and methods

7.3.1 Characterization of the bacterial collection

In 2018 and 2019, *Sparus aurata* (muscle, gills, skin, intestine) samples were collected in aquaculture farm 7 (from southern region of Portugal) and in a market. On the other hand, in 2019, bivalve mollusks samples were collected in 6 Portuguese aquaculture farms: *Crassostrea gigas* in aquaculture farms 1 (southern region), 5 and 6 (central region); *Mytilus* sp. in aquaculture farms 2, 3 and 4 from southern region; and *Ruditapes decussatus* in aquaculture farm 1. *Mytilus* sp. were also purchased in a market (central region), imported from Spain but with the depuration process performed in Portugal. All analyzes were performed in the National Institute of Health Dr. Ricardo Jorge. Sample preparation and bacterial isolation procedures were already described elsewhere [257,422] and included 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 different morphology were selected so that no duplications were included.

A total of 66 Gram-negative strains were selected for genomic characterization. Of 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 aim of understanding whether there were any ARG related to antibiotics 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/market) are present in Table S 7.

7.3.2 Identification of bacterial strains and antibiotic susceptibility testing

At the time of strains isolation, bacterial species identification was performed by MALDI-TOF (Bruker, Germany) or amplification of the 16S rRNA gene, as previously described [150]. Posteriorly, antibiotic susceptibility testing was performed by disk diffusion (Bio-Rad, Marnes-la-Coquette, France) and minimum inhibitory concentration (MIC) by in-house broth microdilution. The different antibiotics tested, and respective breakpoints are described in Table S 8. Strains were considered multidrug resistant if they presented a resistant phenotype to three or more structurally unrelated antibiotics.

7.3.3 Genomic DNA extraction and preparation

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

7.3.4 Whole genome sequencing

Libraries from 1 ng of genomic DNA were prepared using the dual-indexed Nextera XT Illumina library preparation, before cluster generation and paired-end sequencing (2×150bp for half of the strains and 2×250bp for the other half; more detail in Table S 9) on a MiSeq Illumina platform (Illumina Inc., San Diego, CA), according to manufacturer's instructions.

7.3.5 Data analysis and molecular characterization

Sequence reads were trimmed and filtered, according to quality criteria, and assembled *de novo* 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 [384]), predict multilocus sequence type (MLST), whenever available (MLST 2.0 [385]), investigate the presence of antibiotic and disinfectant resistance genes (ResFinder 4.1 [387]), virulence genes (VirulenceFinder 2.0 [388]), plasmids (PlasmidFinder 2.1 [389]), and other MGE, as well as their relation to ARG and virulence factors (MobileElementFinder version 1.0.3 [390]), and to estimate bacteria's pathogenicity towards human hosts (PathogenFinder 1.1 [392]). New sequence types (ST) were submitted at the respective database in PubMLST (https://pubmlst.org/; accessed on 25 July 2022). A Minimum Spanning Tree (MST) was constructed with CLC Genomics Workbench version 21.0.3 based on MLST, for *Aeromonas* spp., *Citrobacter* spp., *Enterobacter cloacae* complex, *Shewanella* spp. and *Vibrio* spp. that presented new ST, to understand 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; [396]), to construct a phylogenetic tree and a SNPs matrix with all *E. coli* detected in this study. The phylogenetic tree image was edited with CLC Genomics Workbench version 21.0.3. The Comprehensive Antibiotic Resistance Database (CARD [393]) was also used to investigate the presence of ARG; and PHASTER search web tool allowed the identification and annotation of prophage sequences [395]. The online tool RFPlasmid was

used to predict chromosomal or plasmid location of the previously identified genes [397]. BLAST Ring Image Generator (BRIG, v. 0.95 [431]) was used to generate an image that allows a circular comparison between all contigs harboring *qnrB19* genes detected in this study as having a plasmid location. The closest plasmid sequences obtained using the NCBI Microbial genomes BLAST analysis were used as reference plasmids. ISsaga [394], was used to complement the search of MGE, such as insertion sequences. 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 [432]), as well as to study the genetic environment of ARG.

7.3.6 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 S 9. 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*$_{\text{FOX-18}}$ from INSAq178), MZ359745 (*bla*$_{\text{TER-3}}$ from INSAq228), MZ092824 (*bla*$_{\text{CTX-M-246}}$ from INSAq229), MZ359742 (*bla*$_{\text{PLA-7}}$ from INSAq240), MZ092826 (*bla*$_{\text{FOX-19}}$ from INSAq241), MZ092827 (*bla*$_{\text{OXA-960}}$ from INSAq243), MZ092828 (*bla*$_{\text{OXA-961}}$ from INSAq334), MZ092822 (*bla*$_{\text{CMY-175}}$ from INSAq424), MZ092830 (*bla*$_{\text{OXA-963}}$ from INSAq494) and MZ092832 (*bla*$_{\text{OXA-965}}$ from INSAq497).

7.4 Results and discussion

7.4.1 Identification of bacterial strains

The online tool KmerFinder 3.2 was used to confirm the species of all 66 Gram-negative strains selected for this study, previously identified by MALDI-TOF or amplification of the 16S rRNA gene. Results revealed the misidentification of 18 strains, belonging to *Aeromonadaceae*, *Enterobacteriaceae*, *Hafniaceae*, *Morganellaceae*, and *Shewanellaceae*, and allowed the identification of 7 strains to the species level (within the genus *Aeromonas*, *Enterobacter*, *Hafnia*, *Serratia*, and *Vibrio*) (Table S 10). The identification of 6 strains by KmerFinder 3.2 was later corrected by Average Nucleotide Identity (ANI) when submitted in GenBank (1 *Aeromonas veronii* was changed to *Aeromonas allosaccharophila*; 1 *Proteus vulgaris* to *Proteus terrae*; and 3 *C. freundii*/*C. freundii* complex were changed to *Citrobacter portucalensis*; Table S 10). This

confirms the difficulties already described in other works to identify closely related members from these genera to the species level [41,433–437]. Only identifications provide by KmerFinder 3.2 and confirmed by ANI will be used throughout this work.

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). Except *P. terrae*, all the other species were already associated at some point with water environments and/or aquatic animals [257,297,422,433,438–446]. Nonetheless, to 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.

7.4.2 Genetic diversity

The MLST was predicted for 40 strains (Table 7.1 and Table 7.2), namely *Aeromonas* spp., *C. freundii* complex, *E. cloacae* complex, *E. coli*, *K. pneumoniae*, *Shewanella* spp. and *Vibrio* spp. Within the genus *Aeromonas*, one strain of *A. caviae* (isolated from mussels acquired in the market) was identified as belonging to ST368, while the other 7 strains (from gilthead seabream's muscle and mussels acquired in the market) presented new sequence types due to new alleles: ST879, ST880, ST881, ST882, ST883, ST887, and ST888. In the MST from Figure 7.1 A.1 (and Figure S 3 A), we can observe that the new ST879, ST880, ST882, ST883 and ST888 have the same putative founder, the ST1251 belonging to *A. caviae* isolated in drinking water from Bangladesh, in 2013 (PubMLST database id 1182), from which they differ in 6 loci. The ST closest to ST883 is ST193 (isolated in vegetables from Italy, in 2011; PubMLST database id 213), from which it differs in 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

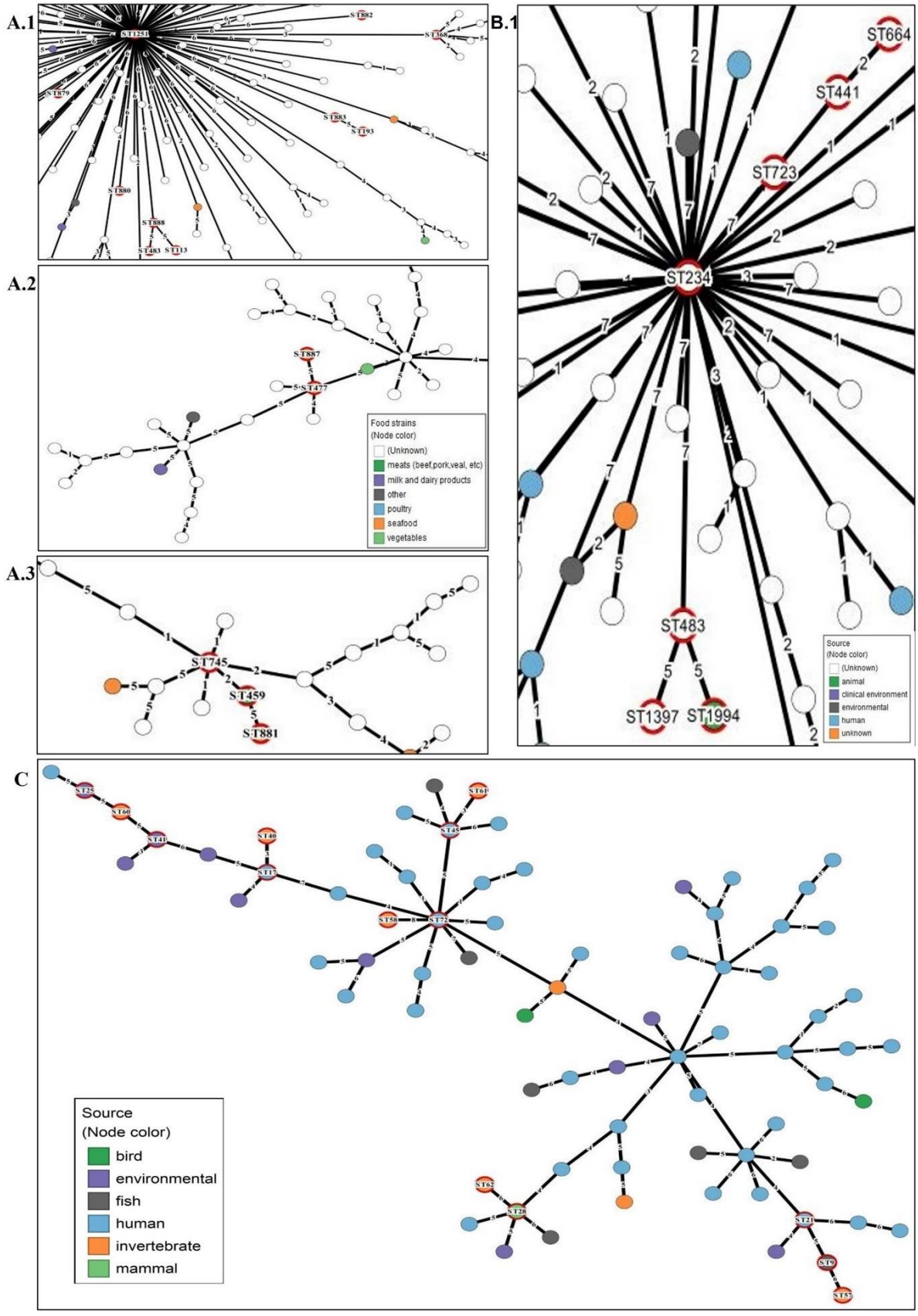

Figure 7.1 — Minimum spanning trees (MST) constructed with CLC Genomics Workbench version 21.0.3, using MLST schemes to understand possible evolutionary relationships among the new ST detected in: **(A)**

Aeromonas spp.; **(A.1)** Zoom of the MST from Figure S 3 A, highlighting the new ST879, ST880, ST882, ST883 and ST888; **(A.2)** Zoom of the MST from Figure S 3 A, highlighting the new ST887; **(A.3)** Zoom of the MST from Figure S 3 A, highlighting the new ST881; **(B.1)** *Enterobacter cloacae* complex, zoom of the MST from Figure S 3 B, highlighting the new ST1994; **(C)** *Shewanella* spp., with the new ST found in this study (ST40, ST, 57, ST58, ST60, ST61 and ST62). ST found in this study and their closest neighbors are highlighted with a red circle.

to ST888, from which they differ in 5 loci. In Figure 7.1 A.2 (and Figure S 3 A), we can see the new ST887, that differ in 5 loci from its putative founder, ST477, belonging to *A. hydrophila* isolated in seafood from China, in 2014 (PubMLST database id 541). Finally, the other new ST identified in this family, ST881, differs in 5 loci from ST459 (Figure 7.1 A.3 and Figure S 3 A) already found in *A. veronii* isolated in meat samples from China, in 2014 (PubMLST database id 524). *A. caviae* ST368 was previously isolated from human blood in Spain, in 2014 (PubMLST database id 424).

C. freundii isolates belonged to 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 associated with a *C. freundii* harboring genes $bla_{\text{VIM-1}}$ and $bla_{\text{KPC-2}}$ collected in a patient admitted to a hospital [447]; ST169, previously associated with a carbapenemase (OXA-48 and VIM-1) producer *C. freundii* isolated in the digestive tract of patients with acute leukemia [448], in our study was found in mussels acquired in a market. The three strains of *C. portucalensis* 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 from Figure S 4 A, we can observe the new ST971 clustering together with ST343 (identified in *Citrobacter* sp. isolated in food samples from China, in 2017; PubMLST database id 315) and ST834 (of unknown origin), from which it differs in 5 loci.

All strains from the *E. cloacae* complex (four *E. hormaechei* and one *E. asburiae*) found in this study were isolated in gilthead seabream from farm 7, only two belonged to the same ST and one is new due to new alleles (ST1994). Already found in *Citrobacter* sp. isolated in humans (United States of America, USA, in 2018; PubMLST database id 415), ST483 is the putative founder of ST1994 (Figure 7.1 B.1 and Figure S 3 B), from which it differs in 5 loci. *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 found in muscle samples from distinct fish. To our knowledge, this is the first description of these three ST in aquaculture reservoirs. However, ST190 was already found

in other studies associated with human clinical samples, namely by Lin et al., that identified this ST in a *E. cloacae* with 19 resistance genes (such as *mcr-9*, *catA1* and *dfrA1*) [449], and by Liu et al., that linked this ST to non-susceptible carbapenemase isolates of *E. cloacae* complex producers of KPC-2 and IMP carbapenemases [450], among others.

The 11 strains of *E. coli* studied belonged to three different ST according to their origin: ST10 (*n*=8) isolated in mussels from farm 4, ST58 (*n*=1) in the intestine of one gilthead seabream acquired in the market, and ST2607 (*n*=2) isolated in the gills of a gilthead seabream from farm 7. ST10, a well-known ST among *E. coli* responsible for human extraintestinal infections all over the world [451], was also described in Venus clam (*Ruditapes philippinarum*), acquired in a market, associated with a bla_{VIM-1} [452]; and with poultry, vegetables and water sources associated with CTX-M extended-spectrum β-lactamases (ESBL) [453]. Although belonging to the same ST, these eight *E. coli* ST10 are not indistinguishable, with minimum SNPs difference of 6 between INSAq316 and INSAq319, and INSAq317 and INSAq324 (Figure S 5 and Table S 11). These *E. coli* ST10 also present some differences, such as regarding ARG, virulence factors, heavy metal resistance genes, MGE, and others (discussed in the next chapters). According to the relatedness criteria for *E. coli* SNP typing scheme of Schürch et al. (≤10 SNPs) [403], INSAq316 is closely related with INSAq317 and INSAq319; additionally, INSAq317 is closely related with INSAq324; lastly, INSAq321 is closely related to INSAq354. ST58 is disseminated across the world and was collected from a variety of sources, such as clam *Chamelea gallina* [454], humans, cattle, swine, poultry, birds, companion animals, water, and store-bought produce [455]. We could not find any literature regarding ST2607.

The two strains of *K. pneumoniae* isolated in muscle and intestine of fish from the same farm (7) belonged to ST134. This ST was already identified in strains of *K. pneumoniae* recovered from patients at hospitals in China and Japan and associated with several resistance genes, such as bla_{KPC-2}, bla_{IMP-1}, *qnrA1*, *qnrB6*, *sul1*, and *tet(B)* [456,457].

Within the *Shewanella* genus, only two strains belonged to the same new ST (ST57; isolated in mussels from farm 2). In Figure 7.1 C, we can observe that ST57 differs in 6 loci from ST9, which belonged to *S. algae* isolated in fish from France, in 1980 (PubMLST database id 9). The other five strains, isolated in mussels from market and farms 2 and 3 (southern region of Portugal) and Japanese oysters from farm 5 (central region of Portugal), also revealed ST here described for the first time (ST40, ST58, ST60, ST61, and ST62). According to MST in Figure 7.1 C, ST40 differs only in 3 loci when compared to ST17. Interestingly, ST17 was detected in *S. chilikensis* isolated in human samples from Spain (in 2012; PubMLST database id 17), which is consistent with the *Shewanella* species that harbor 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 (belonging to *S. algae* isolated in human from Taiwan, in 2016; PubMLST database id 104), although differing from this ST in the 8 loci. In the same Figure 7.1 C, we can observe that ST60 differs in 5 loci from ST25 (*S. indica* isolated in sea sediments from USA, in 1992; PubMLST database id 25) and ST41 (*S. indica* isolated also in sediments from India, in 2006; PubMLST database id 63); ST61 differs in 3 loci from ST45 (*S. algae* isolated in human from Norway, in 1997; PubMLST database id 70); and ST62 differs in 6 loci from ST28 (*S. algae* isolated in *Neophocaena phocaenoides* from South Korea, in 2017; PubMLST database id 28).

Vibrio spp. were all isolated in mussels acquired in the market. One *V. alginolyticus* belonged to the ST183, which was previously identified in human clinical samples in Norway, as well as in marine animals and water in China (PubMLST database). One *V. diabolicus* and one *V. antiquarius* presented new STs (ST205 and ST206, respectively). The MST in Figure S 6 A shows ST205 with just 1 locus of difference compared to ST63 (*V. alginolyticus/ diabolicus* isolated in live marine animals from Italy, in 2007; PubMLST database id 70). These ST clustered together with ST32 (PubMLST database id 33) and ST57 (PubMLST database id 64), both isolated in live marine animals, in the same species, country and year of ST63. The other new ST, ST206, differs in 2 loci from ST574 (identified in *V. alginolyticus/ diabolicus* isolated in *Panaeus vannamei* from Italy, in 2011; PubMLST database id 1188), as we can see in Figure S 6 B. Together with ST143 (identified in *Vibrio chagasii* from unknown origin; PubMLST database id 175) and ST567 (identified in *V. alginolyticus/ diabolicus* isolated in *Hymenopenaeus muelleri* from Italy, in 2011; PubMLST database id 1181), ST206 and ST574 form a cluster, with ST574 as the putative founder.

More studies are needed to comprehend the specificities of each ST regarding hosts and environments, but we can recognize that these STs succeed in diverse environments and infected different hosts, from fish and bivalve mollusks to humans.

Table 7.1 — 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[1]	-	*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, PTZ	*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		*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), *mdtE/G/H* (C), *msbA* (C)	-		-	IS*3*-type MITEEc1-type	3	94.4
***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 allosaccharophila* (*n*=1)**										

INSAq178	Muscle	ST881[1]	ERT	*bla*$_{FOX-18}$[2] (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[1]	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[1]	-	-	-	-	-	IS*Aeme1*-type IS*As2/7/13/18/31*-type, IS*As9*/21, IS*Ec28*-type, IS-*Kpn3/10*-type, MITEAeme2	2	47.1
INSAq180	Muscle	ST882[1]	-	-	-	-	-	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[1]	-	*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[1]	-	*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			AMC, FLO						-	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
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[2] (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[2] (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

[1]New MLST profiles found in this study. [2]New alleles found in this study. 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 less than 100% coverage and/or identity.

Table 7.2 — 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[2] (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[1]	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/qac-delta1/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), *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),		*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					Col156-type, Col(BS512), IncFIB(AP001918)-type, IncFII-type, IncQ1-type		1	93.1

INSAq322 (Farm 4)	*Mytilus* sp.	ST10	AMC, CAZ, CHL, CIP, FLO, FMQ, OTC, SXT	*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)			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(AP001918)-type, IncFII-type, IncQ1-type		1	93.1
INSAq354 (Farm 4)	*Mytilus* sp.	ST10	AMC, CHL, FLO, FMQ, OTC, SXT						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[1]	AMX, FMQ, OTC	*bla*$_{OXA-SHE-type}$ (C), *qnrA3* (C)	-	-	-	IS*Kpn2*-type, IS*Slo2*-type	-	30.7
INSAq494 (Farm 2)	*Mytilus* sp.	ST61[1]	FMQ, OTC	*bla*$_{OXA-963}$[2] (C), *qnrA3* (P),	-	-	-	-	-	28.5
INSAq495 (Farm 2)	*Mytilus* sp.	ST57[1]	OTC	*bla*$_{OXA-964}$ (C), *qnrA3* (C)	-	-	-	IS*Kpn2*-type, IS*Slo2*-type	-	29.1
INSAq497 (Farm 5)	*Crassostrea gigas*	ST62[1]	COL	*bla*$_{OXA-965}$[2] (C), *qnrA12* (P)	-	-	-	IS*Vpa2*-type	-	39.1
***Shewanella chilikensis* (*n*=1)**										
INSAq334 (Farm 2)	*Mytilus* sp.	ST58[1]	FMQ, OTC	*bla*$_{OXA-961}$[2] (C), *qnrA11* (P)	-	-	-	-	-	30.7
***Shewanella indica* (*n*=1)**										
INSAq347 (Farm 3)	*Mytilus* sp.	ST60[1]	FMQ, OTC	*bla*$_{OXA-962}$ (C), *qnrA2* (P)	-	-	-	IS*Shes3*-type	-	35.5
B) **Market**										
***Aeromonas allosaccharophila* (*n*=1)**										
INSAq241	*Mytilus* sp.	ST888[1]	ERT, IMP, MEM	*bla*$_{FOX-19}$[2] (C), *bla*$_{OXA-959}$ (C), *cphA7*-type (C)	-	-	-	IS*Ahy2*-type, IS*As7/34*-type	1	42
***Aeromonas caviae* (*n*=1)**										

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*$_{PLA-7}$[2] (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[1]	-	*bla*$_{OXA-960}$[2] (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[1]	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[1]	AMP	*bla*$_{CARB-57}$ (C)	-	-	-	-	-	66.6

[1]New MLST profiles, found in this study. [2]New alleles found in this study. 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 less than 100% coverage and/or identity.

7.4.3 Resistome

The investigation of genes associated with antibiotic/disinfectant/heavy metals resistance revealed more than 140 different genes (Table 7.1 and Table 7.2; Figure 7.2 and Figure S 7) and gave us a snapshot of the resistome of seabream and bivalve mollusks raised in aquaculture farms.

In *Aeromonadaceae* family, genes associated with β-lactams resistance predominated (e.g., *bla*$_{\text{TEM-1B}}$, *bla*$_{\text{FOX-18/19}}$), followed by genes associated with tetracyclines (e.g., *tet(A)*, *tet(E)*-type) and aminoglycosides resistance (e.g., *aph(3'')-Ib*, *aph(6)-Id*). We highlight *bla*$_{\text{FOX-18}}$ and *bla*$_{\text{FOX-19}}$ genes here described for the first time. The gene *bla*$_{\text{FOX-18}}$ has 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*$_{\text{FOX-3}}$, sharing 95.5% of identity with this gene. On the other hand, *bla*$_{\text{FOX-19}}$ has 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 distinguish this gene from *bla*$_{\text{FOX-7}}$ with which it shares 95.8% of identity. These genes were found in 2 strains of *A. allosaccharophila* isolated in gilthead seabream (*bla*$_{\text{FOX-18}}$; INSAq178) and mussels (*bla*$_{\text{FOX-19}}$; INSAq241) acquired in different markets, that were also positive for *bla*$_{\text{OXA-958/959}}$ and *cphA*-type genes, as well as included in the category "Susceptible, increased exposure" of EUCAST concerning carbapenems (ertapenem for INSAq178; ertapenem, imipenem and meropenem for INSAq241) (Table 7.1 and Table 7.2). Genes *bla*$_{\text{FOX}}$ were already described by Maravić et al. in *A. caviae* isolated from wild-growing *Mytilus galloprovincialis* in Croatia [458] and in water and sediments collected in aquaculture ponds in China [459]. To our knowledge, this is the first description of *bla*$_{\text{OXA-958}}$ in gilthead seabream and *bla*$_{\text{OXA-959}}$ in mussels from aquaculture. These *bla*$_{\text{OXA}}$ genes were already described in *Aeromonas* spp. isolated from feces of dairy cattle and wastewater treatment plant effluent (accession numbers: LKKI01000011 and AP022264, respectively). Two susceptible strains had a *cphA5*-type gene (intrinsic in some species of *Aeromonas*) but were susceptible to carbapenems. This was already described and can be related with a low enzymatic activity of this metallo-β-lactamase [460]. Most ARG detected in this family were previously associated with aquaculture activities [422,461–464].

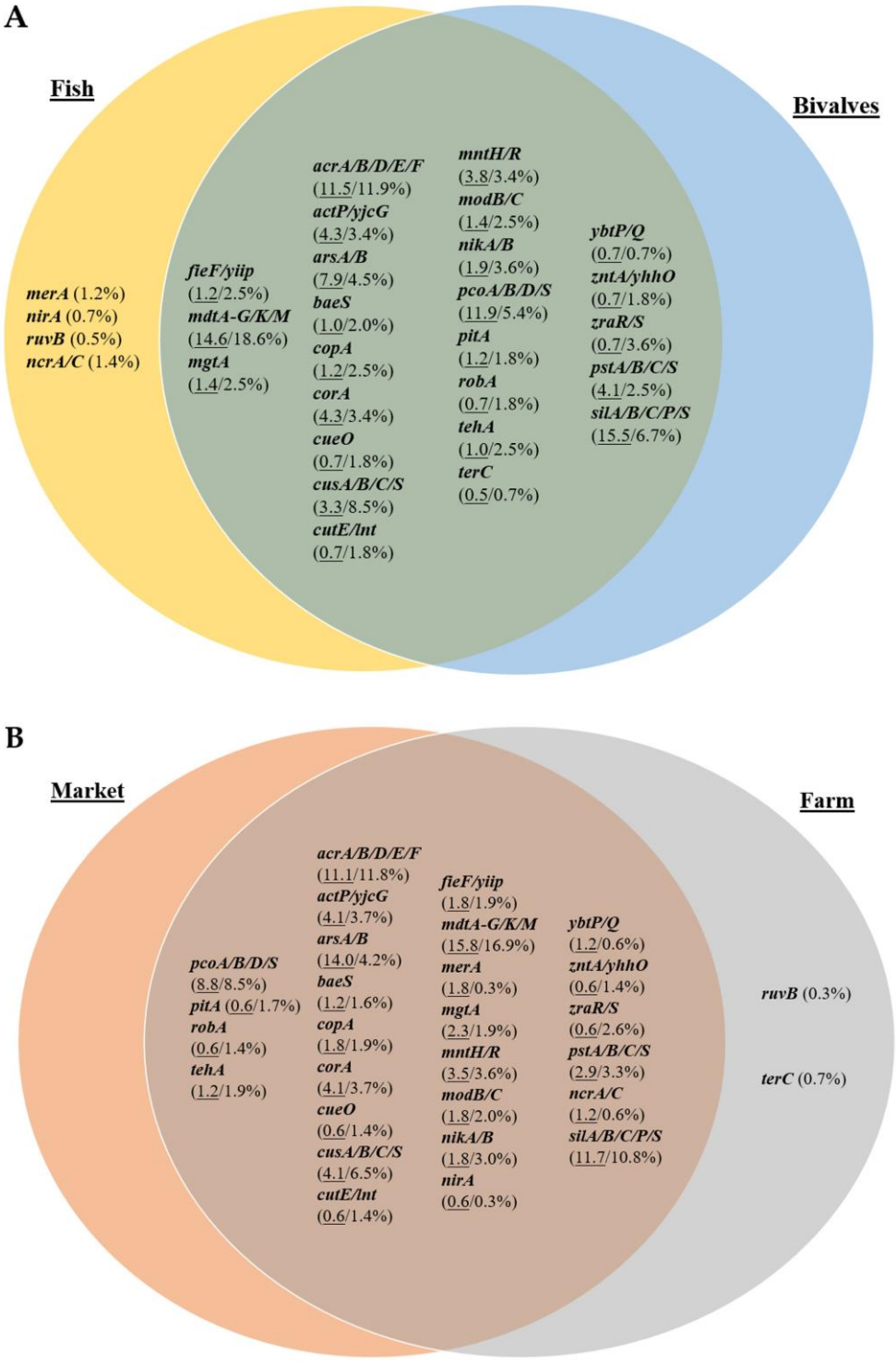

Figure 7.2 — Venn diagrams representing genes (and their prevalence in parentheses) found in this study associated with tolerance to heavy metals in: **(A)** Fish vs. bivalve samples; **(B)** Market vs. farm samples. In the group of genes found in both reservoirs, the underlined percentages correspond to the prevalence of that gene in fish/market (A/B) samples and the non-underlined ones correspond to bivalve/farm (A/B) samples.

Enterobacteriaceae represented the family with the most diverse ARG (Table 7.1 and Table 7.2), mostly associated with efflux systems that are commonly found in aquatic

environments, possibly due to their wide variety of substrates, including substances essential for cellular functions [232]. These efflux pumps 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]) and were 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 ARG were associated with decreasing the uptake of an antibiotic (e.g., *marA* for several antibiotics), modification of the antibiotic target (e.g., *dfrA1*-type for trimethoprim; *sul1/2* for sulfonamides) and inactivation of an antibiotic (e.g., bla_{SHV-60} and bla_{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_{TER-3}, $bla_{CTX-M-246}$, bla_{PLA-7} (coding for class A β-lactamases each) and $bla_{CMY-175}$ (coding for a class C β-lactamase) were found in this study for the first time and are usually related to β-lactams resistance. Gene bla_{TER-3} from one *R. terrigena* isolated in gilthead seabream' skin from market has 1 nonsense mutation (G160A) and 2 missense mutations (A350G and G351A) compared to bla_{TER-2}, with which it shares a homology of 99.3%. This class A β-lactamase is characteristic of the chromosome of *R. terrigena* that is mainly found in water environments [465]. Also identified in gilthead seabream' skin from market, $bla_{CTX-M-246}$ from *K. intermedia* has 99.1% of homology with $bla_{CTX-M-10}$, from which it differs due to 3 missense mutations (G367A, A483C and A636G), belonging to CTX-M-1 group. It is widely accepted that bla_{CTX-M} genes originated from chromosomal *bla* genes from *Kluyvera* spp., that were mobilized to other species and disseminated worldwide [466]. Genes bla_{CTX-M} were found by other authors in *E. coli* and *K. pneumoniae* strains isolated in aquaculture environments, namely by Said et al. in gilthead seabream [467] (just like in our study) and Sivaraman et al. in samples of shrimp, water, and sediment [466], where CTX-M-1 group predominated. Chromosomal bla_{PLA-7} isolated in *R. planticola* from mussels acquired in the market demonstrated 4 missense mutations compared with bla_{PLA-1a} (G578A, A644C, A793G and G845C) and bla_{PLA-2a} (G578A, A644C, A793G and G841A) genes, with which it shares 99.0% of homology. No literature was found regarding the frequency of bla_{PLA} genes in aquaculture. The last new allele found in *Enterobacteriaceae*, $bla_{CMY-175}$ (from *C. portucalensis* isolated in Japanese oysters from farm 1), has 99.7% of homology with bla_{CMY-34} with only 1 missense mutation (C602A). Genes bla_{CMY} were already identified in other studies encompassing several fish species, namely in *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 in *E. coli* isolated in freshwater *Perca fluviatilis* in Switzerland [468,469]. 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 had susceptible increased exposure for ertapenem. These last 3 strains of *E. hormaechei* (INSAq99, INSAq107 and INSAq140; Table 7.2) were positive for the presence of *bla*$_{\text{ACT-17}}$ and *bla*$_{\text{ACT-89}}$ genes that can only confer resistance to carbapenems if associated with other mechanisms, such as the loss of outer membrane porins [227]. Genes weakly expressed, namely *bla*$_{\text{OXY-1-3}}$ in *K. michiganensis*, *bla*$_{\text{TER-3}}$ in *R. terrigena*, *bla*$_{\text{CTX-M-246}}$ in *K. intermedia*, and *bla*$_{\text{PLA-7}}$ in *R. planticola*, can explained the susceptible phenotypes, just like in the studies developed by Cantón et al. for *bla*$_{\text{CTX-M}}$ [470], González-López et al. for *bla*$_{\text{OXY}}$ [471] and Walckenaer et al. for *bla*$_{\text{TER}}$ and *bla*$_{\text{PLA}}$ [465,472]. Nine *E. coli* were classified as multidrug resistant: one recovered in gilthead seabream from the market and eight isolated in mussels from farm 4. Most ARG found in this bacterial family were punctually described in a few previous studies related with different aquaculture reservoirs [143,257,422,466,468,473–482]. Nonetheless, our study allowed a more complete approach, covering not only acquired, but also chromosomal genes (less studied), identifying several new alleles and genes described for the first time in aquaculture reservoirs (e.g., *bla*$_{\text{ORN-1}}$, *bla*$_{\text{OXY-1-3}}$, *qnrB38*, *mdtE*, *mdtN*, *mdtO*, *mdtP*, *baeS*, *eptA*, *emrA*, *emrK*, *yojI*, and *kdpE*).

All strains from the *Hafniaceae* family analyzed in this study revealed at least one antibiotic resistance gene, *bla*$_{\text{ACC-1a/1c/1d/3-type}}$, 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 that was "susceptible, increased exposure" for ceftazidime). Genes *bla*$_{\text{ACC}}$ are ubiquitous in the chromosome of *Hafnia* spp. and encode a class C β-lactamase that can be classified into two groups: low-level inducible cephalosporinases (susceptible to ceftazidime) and high-level constitutive cephalosporinases (resistant to ceftazidime, just like seven strains in our study; Table 7.2) [483,484]. To our knowledge, there is no studies identifying a chromosomal class C β-lactamase in *O. proteus*, but due to their high homology with *Hafnia* genus [485], it is plausible that this species also harbors a similar β-lactamase, encoded by *bla*$_{\text{ACC}}$-like genes, such as the ones found in our four strains of *O. proteus* (Table 7.2). Genes *bla*$_{\text{ACC}}$ were detected in previous studies, namely associated with blue mussels and Venus clams (acquired in a market; in *H. alvei* and *E. coli*, respectively), although the authors do not specify whether the samples originate from aquaculture farms [486]. Three strains of *H. paralvei* (from gills and muscle samples of gilthead seabream purchased in a market) harbored the plasmid-mediated quinolone resistance (PMQR)

determinant *qnrD2* gene, although these strains had a susceptible phenotype to all quinolones tested. PMQRs are known for a low-level resistance to quinolones [487]. The classic phenotypic methods are usually unable to identify a diminished susceptibility to these antibiotics. Although relatively rare, *qnrD* genes have been found in food sources, such as lettuce and broiler samples [383,488], as well as in aquaculture reservoirs, namely in shrimp intestinal tract, shrimp pond waters and sediments samples [489], and in effluents of coastal aquaculture of *Paralichthys olivaceus* [490]. However, some studies do not specify the *qnrD* variant, so we could not confirm if our study represents the first description of *qnrD2* in aquaculture reservoirs. Although, to our knowledge, this is the first description of *qnrD2* in *H. paralvei*. Gene *qnrD2* was firstly described in a human isolate of *Salmonella enterica* serovar Hadar [491] and, according to NCBI database, was also detected in *Providencia rettgeri* (NZ_ABFNOU020000056), *Proteus mirabilis* (NZ_JAAATT010000048) and *Morganella morganii* (NZ_JADNHI010000046) isolated in human samples.

The only strain of *Morganellaceae* family analyzed in this study, a *P. terrae* isolated in mussels from the market, had a positive result for the ARG *tetH*-type and *hugA*-type, associated with resistance to tetracyclines and β-lactams, respectively. This strain was also resistant to chloramphenicol (ARG not detected), thus being classified as multidrug resistant. Gene *tetH* was already found in effluents of coastal aquaculture of *P. olivaceus* in south Korea [490] and in sediments from aquaculture fish farms (rainbow trout and common white fish) in the northern Baltic Sea [492]. On the other hand, to our knowledge, this is the first description of *hugA*-type gene in mussels from aquaculture origin. This chromosomal *hugA* gene was identified in *Plesiomonas shigelloides* in wild-caught fish [493] and in *Proteus* spp. in cecum samples from chicken slaughterhouses [494].

In *Shewanellaceae* family, all 7 strains analyzed harbored a gene encoding a class D β-lactamase (*bla*$_{\text{OXA-960/961/962/963/964/965}}$, or *bla*$_{\text{OXA-SHE}}$-type) and one variant of the PMQR gene *qnrA* (*qnrA1*, *qnrA2*, *qnrA3*, *qnrA11* or *qnrA12*). New genes *bla*$_{\text{OXA-960/961/963/965}}$ were here identified for the first time, coding for class D β-lactamases that belong to the OXA-55 family. Gene *bla*$_{\text{OXA-960}}$ was found in *S. chilikensis* from mussels acquired in the market and has 93.1% of identity with *bla*$_{\text{OXA-55}}$, from which it differs in 20 missense mutations (A98C, C117A, T121G, C137T, T138C, A199T, G212T, C292A, G316C, T383C, G514A, A592C, G593A, G713A, G716A, T717A, G781A, T792G, G796A, and A857T). Gene *bla*$_{\text{OXA-961}}$, identified also in *S. chilikensis*, but from mussels originated in farm 2, has 17 missense mutations (A98C, T121G, C134T, C292A, G316C, T383C, A470C, A471G, A517G, A592C, G593A, G713A, G716A, T717A, C764T, G781A, and G796A) concerning *bla*$_{\text{OXA-55}}$, with which it shares 94.9% of identity. Gene *bla*$_{\text{OXA-963}}$ belong

to one *S. algae* strain isolated in the same sample as *bla*$_{OXA-961}$ and shares 98.7% of identity with *bla*$_{OXA-55}$, with only 5 missense mutations (T121A, C292A, T383C, G499C and A584T) separating these two genes. The last new *bla*$_{OXA}$ found in this family, *bla*$_{OXA-965}$, was identified in *S. algae* isolated in Japanese oysters from farm 5 and has 97.7% of homology with *bla*$_{OXA-55}$, with which it differs in 9 missense mutations (T121A, A128G, C292A, T383C, G520A, G560A, A561G, G629T and G781A). Genes *bla*$_{OXA-962}$ and *bla*$_{OXA-964}$ are here described for the first time in mussels from aquaculture origin (*S. indica* from farm 3 and *S. algae* from farm 2, respectively). The only descriptions regarding these two genes were found in NCBI database, with *bla*$_{OXA-962}$ associated with *S. chilikensis* from unspecified origin in Japan (accession number: AP024611) and *bla*$_{OXA-964}$ associated with *S. algae* isolated in *Neophocaena phocaenoides* in South Sea of South Korea (accession number: CP033575). All OXA detected in this family were predicted to have a chromosomal location and were not able to hydrolyze carbapenems tested. All *qnrA* variants found in this family were already described in aquaculture reservoirs, such as water columns, sediments [132,495], mussels and Japanese oysters [422]. Tomova et al. studied water and sediments samples of aquaculture origin, as well as urinary tract isolates from people living in the aquaculture area and found identical *qnrA1* in putative uncultured marine bacteria and in urinary tract isolate *E. coli*, suggesting that HGT can occur between marine bacteria and human pathogens [495]. One *S. algae*, isolated from mussels collected in farm 2, was considered multidrug resistant, although only *qnrA3* and *bla*$_{OXA-SHE}$-type genes were found.

The three strains of *Vibrionaceae* family analyzed were resistant to ampicillin and harbored a variant of *bla*$_{CARB}$ gene (*bla*$_{CARB-56/57}$). Studies developed by Zhang et al. [496] and Hossain et al. [497] detected *bla*$_{CARB}$ genes in *E. coli* isolated in water from fishponds and in *Vibrio parahaemolyticus* isolated in shrimp from aquaculture, respectively. Some studies, such as the one developed by Zhang et al. did not identify the variant of the *bla*$_{CARB}$ genes [496], thus making it impossible to compare with the variants found in our study. However, according to NCBI database, *bla*$_{CARB-57}$ was already detected in *V. diabolicus* (just like in our study) isolated in *Epinephelus coioides* (accession number: AMPD01000002). One *V. antiquarius* revealed also a *fos*-type gene, normally associated with fosfomycin resistance.

No antibiotic or disinfectant resistance genes were detected in the only strain from the *Yersiniaceae* family (*S. liquefaciens*) and in the two strains from *Pseudomonadaceae* family (*P. stutzeri*), isolated in gilthead seabream from market and farm 7, respectively. The later *P. stutzeri* had a resistance phenotype to ertapenem, chloramphenicol, florfenicol (intrinsic resistances) and flumequine, meaning that the methods used did not detect all the resistance genes present in these strains. Both *P. stutzeri* and *S. liquefaciens* species were already detected

in aquaculture environments, such as biofilters in a recirculating aquaculture system in France for seabass [498] and in salmon farms in Chile [499].

Disinfectant resistance genes were detected only in *Aeromonadaceae* and *Enterobacteriaceae* families (Table 7.2). Two strains of *Aeromonas* genus (*A. media* and *A. salmonicida* from *S. aurata* acquired in the market) were positive for *qacE*-type or *sitABCD*-type genes, encoding 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 was already described in bacteria isolated from a tilapia aquaculture system [477]. Genes *sitABCD*-type and *qacE*-type were also identified in our study 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). A *formA*-type gene had already been identified in another study carried out by this team, in one *Enterobacter ludwigii* isolated in *S. aurata* from aquaculture [480]. These three genes are associated with resistance to disinfectants frequently used in aquaculture settings. Quaternary ammonium compounds, such as benzalkonium chloride, are responsible for the disruption of the cell membrane, destruction of enzymes involved in energy production and denaturation of vital proteins [500]. Aldehydes disinfectants (such as formaldehyde) are used to control ectoparasites in fish raised in aquaculture farms [501], which 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 the formation of free hydroxyl radicals that cause destruction in DNA, membrane lipids and other cell components crucial for bacteria survival [502,503]. Some of these genes are not exclusively related to disinfectants resistance, as in the case of the *sitABCD* operon (encoding an ABC cassette-ATPase type transporter) that contributes also to the transport of iron and manganese, as well as to virulence in *E. coli* and *S. enterica* serovar Typhimurium [504–506]. In fact, the detoxification of free radicals and protection against oxidative damage caused by hydrogen peroxide is associated with the acquisition of iron and manganese by the bacteria [504]. This system can be very important in water environments, where the concentrations of manganese can be low [507].

Heavy metals found in aquaculture settings, as well as in the animals farmed, 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) [508,509]. Moreover, heavy metals, such as copper and zinc, can be incorporated in fish feed as preservatives and/or additives or used as anti-fouling agents in fish cages [510]. Globally, we found a great variety

of heavy metals resistance/tolerance genes, with the group of *mdt* genes being the most frequent (16.6%), followed by *acr* genes group (11.7%) and *sil* genes (11.0%) (Figure S 7). These genes were involved in resistance to copper and zinc (*mdt* and *acr* genes), arsenic (*acr* genes) and silver (*sil* genes), mainly through efflux systems [511–513]. Other resistance/tolerance genes found 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*) [514–520]. Bacteria isolated from both fish and bivalves revealed a great diversity of heavy metals resistance/tolerance genes (Figure 7.2 A). The top five of the genes most found in both reservoirs were similar, with *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 mollusks. Furthermore, genes *merA*, *nirA*, *ruvB*, and *ncr* were only present in fish samples (Figure 7.2 A).

In the perspective of market vs. aquaculture farm, a great variety was also found in both reservoirs (Figure 7.2 B). The top five of the genes most frequently found were *mdt* (15.8%) > *ars* (14.0%) > *sil* (11.7%) > *acr* (11.1%) > *pco* (8.8%) in market and *mdt* (16.9%) > *acr* (11.8%) > *sil* (10.8%) > *pco* (8.5%) > *cus* (6.5%) in aquaculture farms. Genes *ruvB* and *terC* were only present in aquaculture farm samples (Figure 7.2 B). Several studies were already developed with the objective of quantifying heavy metals and/or heavy metal resistance in aquaculture samples [521–524], however few studies have focused on the investigation of the genes that cause that resistance, as in our study [132,479]. Zago and colleagues studied the resistome, mobilome and virulome of 12 multidrug-resistant strains from *Vibrionaceae* and *Shewanellaceae* families, isolated in aquaculture fish farms in Italy. These authors identified 26 different genes related with resistance to 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*) [132]. On the other hand, Zhou and colleagues collected soil, water, and sediment samples from three duck-fish farms in China and concluded that metal resistance genes were more abundant in sediment samples than in soil samples. These authors found genes related to copper resistance, *pcoA* and *pcoD*, in all samples analyzed. Gene *zntA*, associated with zinc resistance, was also detected and was the most abundant metal resistance gene in the majority of the samples analyzed [479]. However, Zou et al. only studied zinc and copper resistance genes.

Heavy metals can persist in the environment, resulting in modifications of bacterial communities, favoring the selection of heavy metals resistance/tolerance genes [522]. Some of these metals, such as iron, copper, and zinc, are vital for bacterial functions, however if in excess can have a toxic effect [521]. The same is true for aquatic animals and humans since metals can

accumulate in the bodies and cause severe complications in animal and human health [509,525].

7.4.4 Virulome

Aquaculture's virulome investigation made possible the identification of 25 different genes in *Aeromonadaceae* and *Enterobacteriaceae*, associated in the literature with an increase in virulence (Table 7.1 and Table 7.2).

In *Aeromonadaceae* family, 13 genes were identified in only one strain of *A. media* (INSAq193 from *S. aurata* acquired in the market), associated with production of toxins (*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) [526–535]. To our knowledge, this is the first description of *cia*, *cvaC*, *etsC*, *iucC*, *mchF*, *hlyF*, *iroN* and *iss* genes in aquaculture. Some of these genes were found in fishery products, just like in the studies developed by Massella et al. [536] and Zhang et al. [537], however they do not specify the origin of the samples. These seven genes were already found in other reservoirs, such as drinking water, marine and freshwater, dog, seal, birds, and South Australian grey-headed flying fox pups [538–541]. Genes *iutA*, *traT* and *ompT* were identified in aquaculture reservoirs, namely in *K. pneumoniae* from shrimp aquaculture farms [466], in *Acinetobacter johnsonii* isolated in cultured *Oreochromis niloticus* [542] and in *Vibrio ichthyoenteri* from *Paralichthys olivaceus* cultured in a marine farm [543].

In *Enterobacteriaceae*, 66.7% of the strains (22/33) had a positive result for virulence genes, having been identified in gilthead seabream acquired in farm 7 and market, clams, and mussels from farms 1 and 4, respectively. All genes found in *A. media* INSAq193 (described above) were common to the *Enterobacteriaceae* family adding other virulence genes related 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*) [544–551]. To our knowledge, of the virulence genes detected in our study, only *terC* and *papC* genes had already been described in aquaculture, namely in *Pangasianodon hypophthalmus* in Vietnam [552] and *S. aurata* in Portugal [480]. Genes *astA*, *lpfA*, *iha* and *irp* were identified by Bueris et al. in wild-caught seafood (more specifically, marine bivalves) in Brazil [553]. Genes *mchB*, *mchC* and *gad* were already associated to *E. coli* isolated from water, sediments, and seal samples [539,554]. Another study by Foysal et al. detected *sigA* gene in *Shigella* spp. isolated in hisla fish (*Tenualosa ilisha*) acquired in the market (unknown origin) [555]. Genes *hra*, *capU* and *fyuA* were previously detected in *E. coli* strains isolated from surface

water of agricultural drainage in Mexico [556] and coastal marine sediments from the Adriatic Sea [557].

In general, *traT* was the most identified virulence gene (9.0%), followed by *iutA* (8.3%). Most studies conducted so far focused on virulence genes in specific bacteria responsible for disease in animals produced in aquaculture. Deng et al. studied 70 strains belonging to nine *Vibrio* species and analyzed their prevalence in disease marine fish from an aquaculture area in South China, as well as the presence of ARG and virulence factors [558]. On the other hand, Nhinh et al. studied the prevalence of infection, ARG and virulence factors of *A. hydrophila* isolated in diseased fish (tilapia, channel catfish and carp) from several farms in Vietnam [559]. Our study represents a more complete approach, encompassing several genera and species of Gram-negative bacteria, isolated not only in fish (*S. aurata*), but also in three species of bivalve mollusks (*C. gigas*, *Mytilus* spp., and *R. decussatus*), as well as the investigation of virulence genes present in the chromosome or in plasmids. In contrast to other studies involving animals produced in aquaculture [558,560], all *Vibrio* spp. identified in our work were negative for the presence of virulence genes, like *flaC* (flagella C), *tdh* (thermostable direct hemolysin), *vvh* (hemolysin), and *trh* (TDH-related hemolysin). These differences may be explained by the complexity of virulence expression, dependent on several factors, such as environmental conditions (e.g.: water temperature, salt conditions), the presence of competitors (bacteria from the same species or not), and hosts [561–563]. Likewise, in *Aeromonadaceae* family, only one strain had virulence genes (Table 7.1), not corresponding to the common virulence genes studied in this family, namely *hlyA* (hemolysin), *act* (cytotoxic heat-labile enterotoxin), *ast* (cytotonic heat-stable enterotoxin), *aerA* (aerolysin), and *alt* (cytotonic heat-labile enterotoxin) [559,564]. The fact that all genes detected here, excepting *ompT* gene, were predicted to have a plasmid location suggest that these genes may have been acquired through MGE from other bacterial species, namely from *Enterobacteriaceae* family.

7.4.5 Mobilome and its importance in the spread of resistance

Our study revealed a great number and diversity of MGE distributed across the 8 bacterial families analyzed (Table 7.1 and Table 7.2). These 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. Col[pHAD28] predominated among the plasmids (13.5%), IS*26* among the IS (5.6%), Tn*3* among the transposons (50.0%), cn_3415_IS*Apu1* among the composite transposons (33.3%), and MITEEc1 among MITEs (60.0%).

In the particular case of prophages, they were present in 72.7% of the 66 strains analyzed (with values ranging from 1 to 8 prophage regions), being the most frequent Entero_Mu and Escher_HK639 (7.3% each) (Figure S 8). Mu phages are known to infect several Gram-negative bacteria and to be involved in the dissemination of bacterial transposable elements [565] and Escher_HK639 prophage was already described in a carbapenem-resistant *C. freundii* isolated from a patient admitted in a Hospital in South Africa [566]. Fish had a greater diversity of prophages than bivalves, with Escher_HK639 as the most frequent (9.9%; Figure S 9 A). In bivalve samples, Entero_Mu was the most frequent prophage (21.1%) (Figure S 9 A). Only prophages 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 (Figure S 9 A). Both market and farms reservoirs revealed a great diversity of prophages, with Klebsi_phiKO2 and Vibrio_12B12 as the most frequent in market (7,3% each one) and Entero_Mu as the most frequent in aquaculture farms (14,8%) (Figure S 9 B). Prophage Klebsi_phiKO2 was previously associated with human clinical isolates of *K. pneumoniae* [567], whereas Vibrio_12B12 was already isolated in a *Vibrio penaeicida* from a Japanese shrimp farm [568]. 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 reservoirs (market and farms; Figure S 9 B). Our investigation confirms that water environments and, consequently, the animals that inhabit them, have a great abundance and diversity of phages, that can also participate in the spread of ARG and virulence factors through generalized or specialized transduction. These elements can integrate bacterial chromosomes (becoming known as prophages, representing up to 20% of the bacterial genome), taking with them antibiotic resistance and/or virulence genes that will benefit the bacteria under certain environmental conditions, such as the exposure to sub-inhibitory concentrations of antibiotics. Among the examples of genes acquired via prophages integration in bacterial genomes are *stx* (Shiga toxin) in *E. coli* O157:H7 and bla_{TEM} in *E. coli* [569,570].

The MGE described above can be responsible for the spread of resistance genes between bacteria and the environment, therefore we investigate the genetic environment of several ARG. In the next lines, we highlight the most significant findings of contigs with a plasmid location, as predicted *in silico*.

The bla_{TEM-1B} genes (β-lactams resistance) from one *E. coli* isolated in a gilthead seabream acquired in the market and eight *E. coli* found in mussels from farm 4 had a recombinase family protein upstream and a transposase zinc-binding domain-containing protein downstream, similar to *A. media* from gilthead seabream acquired in the market (Figure 7.3 A and

B). Transposons from the Tn*3* family (Tn*As1*) are widespread in many phyla of bacteria, associated with several ARG, such as *bla*$_{TEM-1}$, including in aquatic environments [164,571,572]. As in our study (Figure 7.3 B), it's not uncommon to find integrated in these elements other MGE like class 1 integrons and IS [571,573]. It was already recognized the important role of IS*26* in the spread of multidrug resistance genes, especially in Gram-negative bacteria, and their association with class 1 integrons, where they can insert at distinct sites, causing several rearrangements of antibiotic resistance loci [121,574]. The arrangement and orientation of IS*26* and *dfrA*, *bla*$_{TEM-1B}$ genes observed in this study (Figure 7.3 B) was already detected in other works. Kubomura et al. identified IS*26* and *dfrA* gene in enteropathogenic and enteroaggregative *E. coli* (EPEC/EAEC) [575], and Gomes-Neves et al. identified IS*26* and *bla*$_{TEM-1B}$ in strains of *Salmonella enterica* from slaughtered swine samples [576].

The *dfrA*-type genes from *A. media* and *E. coli* isolated in gilthead seabream acquired in the market are not only downstream the integrase gene *intI1*, but also to the transposon Tn*As1* (Tn*3* family), as shown in Figure 7.3 B, C and D. In addition, *A. media* has an IS*26* downstream the *dfrA5*-type gene, a recombinase family protein and *bla*$_{TEM-1B}$.

In Figure 7.3 D, E and F, we can observe the alignment of all *sul2*, *aph(3'')-Ib*, *aph(6)-Id* genes (sulphonamides and aminoglycosides resistance) and their respective genetic environment. The presence of an essential gene for plasmid replication (*repA*) in strains from groups D and E confirms the possible association of these ARG with a plasmid [577]. All *E. coli* from group D (found in mussels from farm 4) presented other ARG in this plasmid, such as: *dfrA1*-type, *aadA1*, *sul1* and *mph(B)* (trimethoprim, aminoglycosides, sulphonamides and macrolides resistance, respectively); disinfectants and heavy metals resistance genes, such as *qacEΔ1* and *mer* operon; and additional mobile genetic elements, like class 1 integrons, with *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 are found in association with *aadA*, *dfrA* and *mph(B)* genes [232,578,579]. The ARG *sul*, *dfrA* and *mph(B)* are involved in resistance to antibiotics frequently used in aquaculture, being often responsible for a selective pressure enabling the survival of bacteria with these genes and the promotion of HGT and recombination [69,70,580]. Other genes, like *aadA*, can thrive in these environments due to co-selection [232].

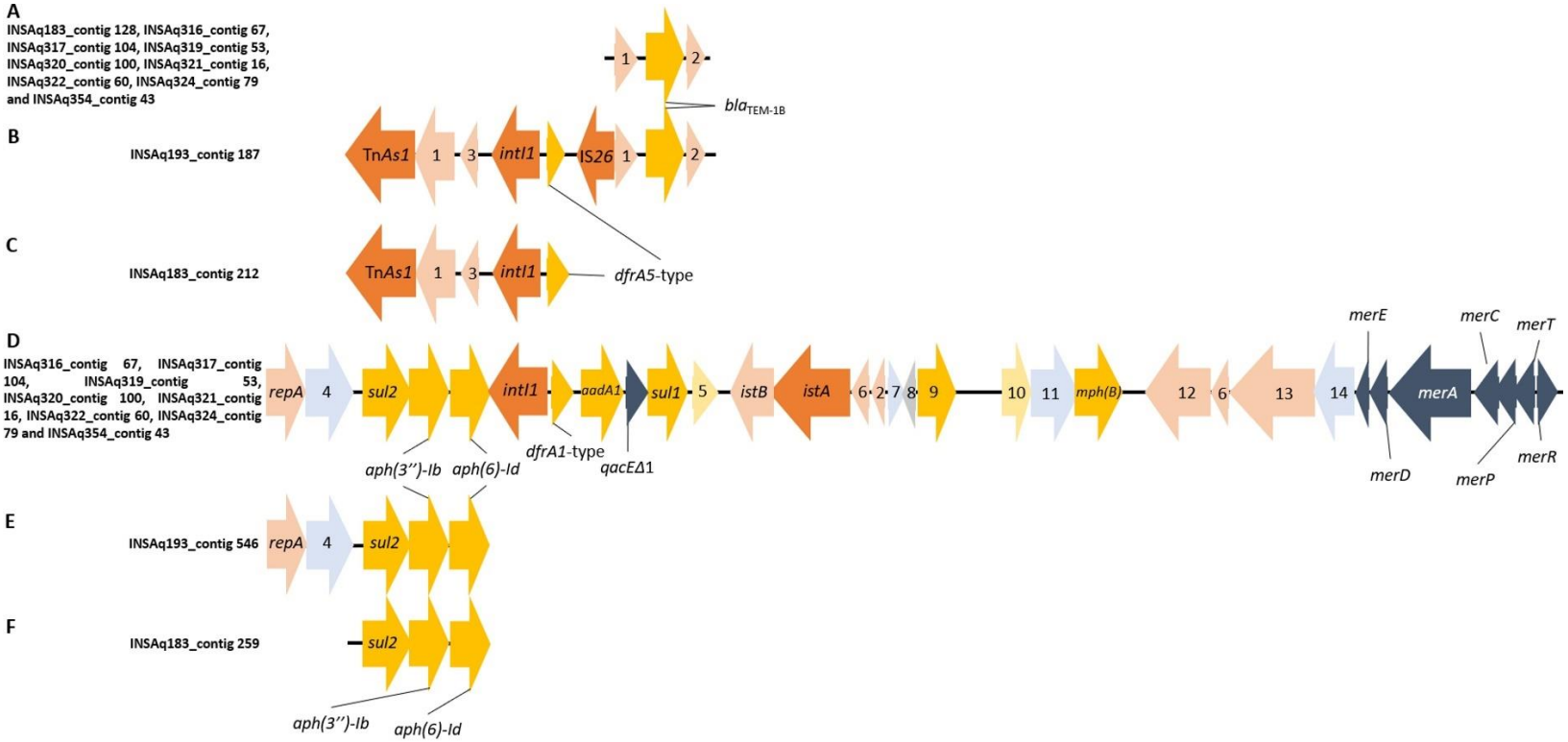

Figure 7.3 — (A) and **(B)** Alignment of all *bla*$_{TEM-1B}$ found in this study and their respective genetic environments. **(B)**, **(C)** and **(D)** Alignment of all *dfrA*-type found in this study and their respective genetic environments. **(D)**, **(E)** and **(F)** Alignment of all *sul2*, *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-β-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.

Noteworthy, an IS*As12* was detected 4864 bp upstream of a major facilitator superfamily (MFS) transporter and the *bla*$_{FOX-18}$ in a *A. allosaccharophila* from gilthead seabream acquired in the market (Figure 7.4 A). Although normally encoded in the chromosome of *Aeromonas* spp. [458], *bla*$_{FOX}$ genes were already associated with a plasmid location in this genus (as in our study), isolated in active sludge from a urban wastewater treatment plant in Poland [581].

All eight *E. coli* found in mussels from farm 4 had a *catA1*-type gene (phenicols resistance) with the same genetic environment (Figure 7.4 B). This gene is located upstream a GNAT family *N*-acetyltransferase and the transposon Tn*As3*, a transposon that also belongs to Tn*3* family and was previously associated with *cat* genes [582,583]. Once again, we prove that transposons from Tn*3* family are associated with several ARG and may play an essential role in their mobilization.

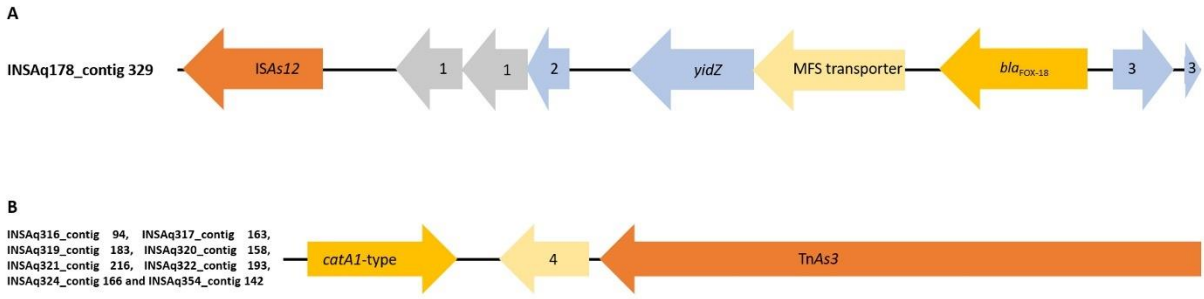

Figure 7.4 — (A) Gene *bla*$_{FOX-18}$ and its respective genetic environment detected in *Aeromonas allosaccharophila* isolated in the muscle of a gilthead seabream acquired in the market. **(B)** Gene *catA1*-type and its respective genetic environment detected in eight *Escherichia coli* isolated 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.

In Figure 7.5 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)* (that codes for repressor TetR, responsible for the regulation of *tet(A)* mRNA expression [584]) 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 with a IS*1* downstream the *tet(A)* gene and the EamA family transporter, and *E. coli* INSAq183 with Tn*As1* upstream the relaxase and the *tetR(A)* gene. *A. media* INSAq193 proved to be an important reservoir of ARG, as besides *tet(A)*, this strain had another ARG associated with tetracyclines resistance, *tet(E)* (Figure 7.5 B), upstream a *tetR* gene and

downstream a hypothetical protein and the IS*3*. Tn*As1* was observed in other studies in association with *tet(A)* gene, isolated in human samples with diarrhea and located in a plasmid along with *mcr-1* gene [585]. For this study, we did not find any reference to the association of IS *1* and *tet(A)*, as well as IS*3* with *tet(E)* in the literature (Figure 7.5). However, it is noteworthy that tetracyclines resistance genes are often associated with plasmids and transposons [586].

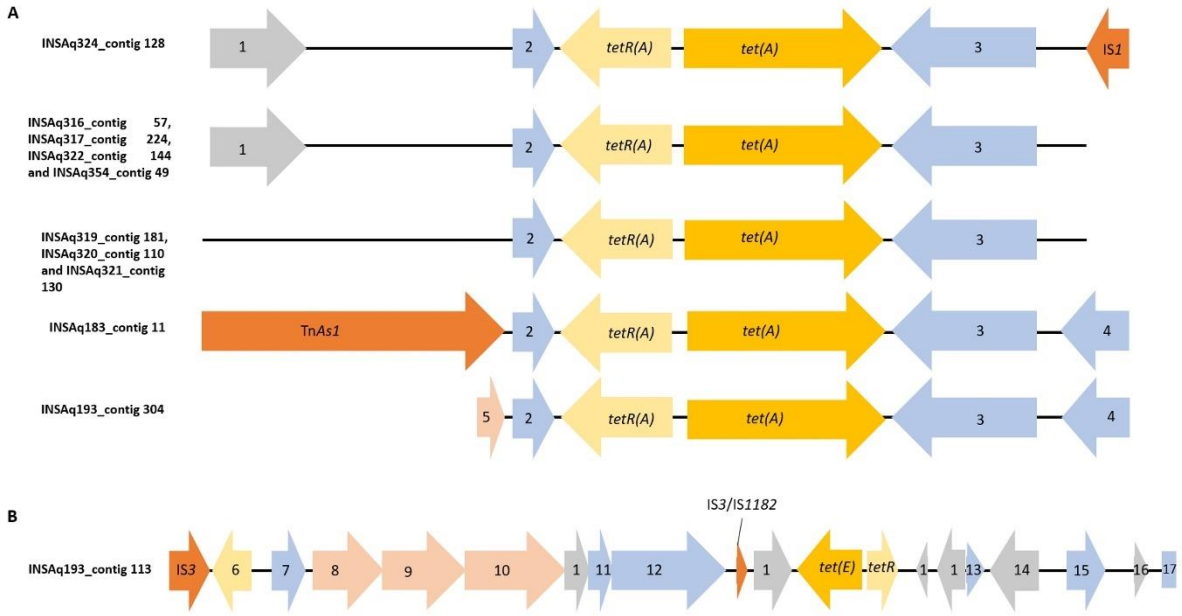

Figure 7.5 — (A) Genes *tet(A)* and their respective genetic environment detected in our study. INSAq183 (*Escherichia coli*) and INSAq193 (*Aeromonas media*) were isolated in intestine and muscle of gilthead seabream from market, respectively. All other strains (*E. coli*) were isolated in mussels from 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 functions. 1-Hypothetical protein. 2-Relaxase. 3-EamA family transporter. 4-Cysteine hydrolase. 5-Transposase. 6-LuxR C-terminal-related transcriptional regulator. 7-Singe-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.

All *qnrB19* genes found in this study were in small plasmids with approximately 3000 bp. In Figure 7.6, we can observe a comparative analysis between the *qnrB19*-harboring contigs found in our study and *qnrB19*-harboring reference plasmids (accession numbers: JN979787 and NC_019086.1). We concluded that the INSAq183 and INSAq193 *qnrB19*-harboring plasmids (one *E. coli* and one *A. media* isolated in the intestine and muscle of a gilthead seabream acquired in the market, respectively) have a greater similarity (99.6%) with the *qnrB19*-

harboring small ColE1-type plasmid (JN979787), isolated from one clinical *E. coli* isolate in Argentina [587]. All other *qnrB19*-harboring contigs found in our study (INSAq143 represents one *L. adecarboxylata* from gilthead seabream collected in farm 7; INSAq316, INSAq317, INSAq319, INSAq320, INSAq321, INSAq322, INSAq324 and INSAq354 are eight *E. coli* from mussels collected in farm 4) have a higher identity (99.8%) with other small ColE-type plasmid isolated in a *E. coli* from a fecal sample, collected from a healthy child in Peru (NC_019086.1; [588]). The genetic environment of *qnrB19* genes is identical in all strains analyzed, with *pspF* (phage shock protein operon transcriptional activator for *pspABCE*) gene, disrupted by an IS*Ecp1C*-like, upstream the *qnrB19* gene. Some authors propose that IS*Ecp1C*-like may have played an important role in the mobilization of *qnrB19* gene [587]. Gene *qnrB19* can be observed in large and small plasmids. Small plasmids, like those found in this study, were already described in other studies, encompassing different bacterial species within the *Enterobacteriaceae* family, several host species (e.g., horse, chicken, pigs, wild birds, humans, and environmental samples) and geographical regions [243,587,588]. This suggests that this type of small plasmids could be responsible for the dissemination of *qnrB19* gene in different reservoirs by HGT. Indeed, Moreno-Switt et al. demonstrated that the transfer of this small plasmids is possible, from *Salmonella* Heildelberg to *Salmonella* Typhimurium, through the action of a P22 bacteriophage (transduction) [243].

MGE play a fundamental role in the adaptation and evolution of bacteria to different environments: from the simplest elements, such IS, with the ability to move to several locations, changing the expression of nearby genes, to more complex MGE, such as plasmids, that can incorporate in their structure numerous other MGE (like transposons and integrons) and resistance/virulence genes, and be transmitted to other bacteria (through HGT) from the same or different species, genus, or family [121,582].

7.4.6 Pathogenicity to humans

All genes described above, since the antibiotic/disinfectant/heavy metals resistance genes to virulence factors (such as those related with adherence, fimbriae, toxins secretion, efflux proteins and other features essential to the survival and spread of bacteria into a human host), prophages and MGE, contribute to the pathogenicity of these strains. The analysis of the 66 strains studied, allowed to classify 49 (74.2%) as pathogenic to humans, with values ranging from 54.8% to 94.5% (Table 7.1 and Table 7.2). This is a worrying 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, exposing humans to potential risk of infection.

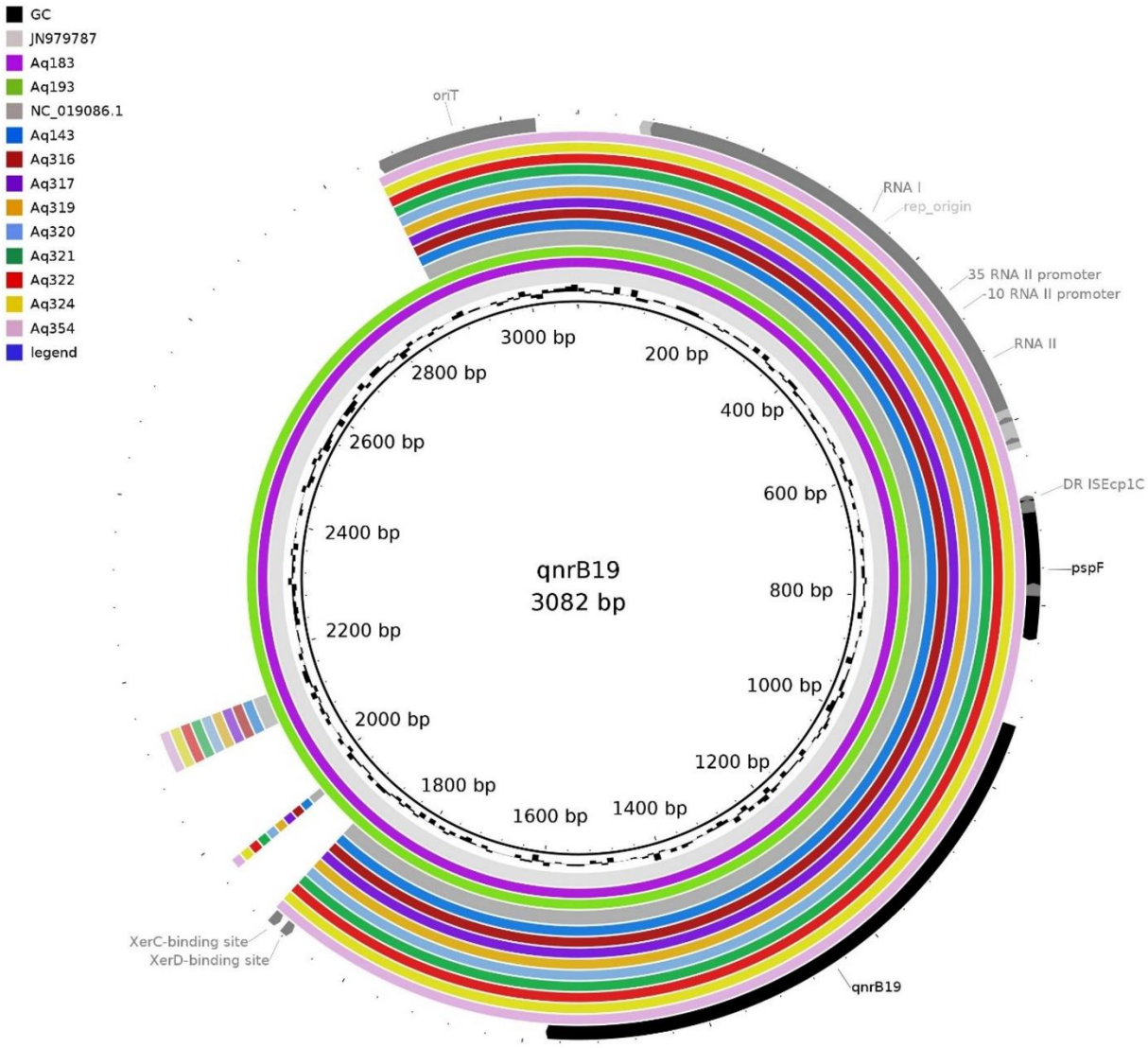

Figure 7.6 — Circular comparison, generated by BLAST Ring Image Generator (BRIG, v. 0.95 [431]), between all contigs harboring *qnrB19* genes detected in this study, as having a plasmid location. Plasmids used as reference are the closest plasmid sequences obtained using the NCBI Microbial genomes BLAST analysis.

7.5 Conclusions

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

New ST were described in *Aeromonas* spp., *C. portucalensis*, *E. asburiae*, *Shewanella* spp. and *Vibrio* spp., and their possible evolutionary relationships with other ST studied, contributing to the knowledge of the bacterial diversity in aquatic farmed animals. The great variety of antibiotic/disinfectants/heavy metals resistance genes found (e.g., bla_{TEM}, *sul2*, *qnrB19*, *qnrD2*, *catA1*-type, *tet*, *sitABCD*-type, *merA* and *copA*) confirms aquaculture environments as hotspots of resistance genes, many of which common to clinical human isolates. MGE, like class 1 integrons, plasmids and Tn*As*, proved to have an important role in the dissemination of tetracyclines, trimethoprim, β-lactams, phenicols, sulfonamides, quinolones, aminoglycosides, macrolides, disinfectants (like quaternary ammonium compounds), and heavy metals (such as mercury) resistances. Antibiotics, disinfectants, and heavy metals can persist in the environment for long periods, submitting bacterial communities to selective pressures and changing their numbers and composition. Under the right conditions, these genes can jump to new reservoirs and can become a hazard to human health [589]. This highlights the fact that two-way traffic of antibiotic resistant bacteria between the aquaculture and the environment is extremely likely and worrying, and could have consequences on human health, which is why it is important to implement One Health strategies to monitor these reservoirs and surrounding environment.

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8

GENOMIC ANALYSIS OF A *MCR-9.1*-HARBOURING INCHI2-ST1 PLASMID FROM *ENTEROBACTER LUDWIGII* ISOLATED IN FISH FARMING

This research paper was published as:

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Personal contributions:

Salgueiro, V. contributed to methodology, formal analysis, investigation, and writing-review and editing.

Note:

This work was partially presented at the One Health EJP Annual Scientific Meeting 2020, online event and at the 4th International Caparica Conference in Antibiotic Resistance 2021 (IC^2AR 2021), Caparica, Portugal.

8.1 Abstract

This study analyzed the resistome, virulome and mobilome of an MCR-9-producing *Enterobacter* sp. identified in a muscle sample of seabream (*Sparus aurata*), collected in a land tank from multi-trophic fish farming production. Average Nucleotide Identity analysis identified INSAq77 at the species level as an *Enterobacter ludwigii* that was resistant to chloramphenicol, florfenicol and fosfomycin and was susceptible to all other antibiotics tested. *In silico* antimicrobial resistance analyses revealed genes conferring *in silico* resistance to β-lactams (bla_{ACT-88}), chloramphenicol (*catA4*-type), fosfomycin (*fosA2*-type) and colistin (*mcr-9.1*), as well as several efflux pumps (e.g., *oqxAB*-type and *mar* operon). Further bioinformatics analysis revealed five plasmid replicon types, including the IncHI2/HI2A, which are linked to the worldwide dissemination of the *mcr-9* gene in different antibiotic resistance reservoirs. The conserved nickel/copper operon *rcnR-rcnA-pcoE*-IS*Sgsp1-pcoS*-IS*903-mcr-9-wbuC* was present, which may play a key role in copper tolerance under anaerobic growth and nickel homeostasis. These results highlight that antibiotic resistance in aquaculture are spreading through food, the environment, and humans, which places this research in a One Health context. In fact, colistin is used as a last resort for the treatment of serious infections in clinical settings, thus *mcr* genes may represent a serious threat to human health.

Keywords: aquaculture, *mcr-9* gene, seabream, One Health.

8.2 Introduction

The emergence of colistin resistance in the last years is a serious threat to the treatment of infections caused by multidrug-resistant bacteria in human medicine. Consequently, colistin, a last resort antibiotic, is categorized by the World Health Organization (WHO) as one of the highest priority critically important antibiotics for human medicine. The use of colistin in veterinary medicine has been prohibited in various countries. However, colistin is still an antibiotic extensively used in veterinary medicine for infections caused by *Enterobacterales* [590]. In aquatic animal species, colistin is also used to treat bacterial infections. Nevertheless, its application in aquaculture is much less common than that used in terrestrial animals [590].

Acquired resistance to colistin has been mostly due to chromosomal mutation in the PmrA/PmrB and PhoP/PhoQ, two-component regulatory systems, or through the increased

production of capsular polysaccharide [90,591]. Since the first report of the plasmid mediated colistin resistance (PMCR) gene *mcr-1* in an *Escherichia coli* strain isolated from a pig in China, we have seen an increase in the number of cases worldwide, in different bacterial species [592,593]. To date, ten PMCR genes (*mcr-1* to *mcr-10*), including variants, have been described, mainly in *Enterobacterales* from animals, humans, and the environment [594].

Originally, MCR-producing bacteria were mostly isolated from animals, including pigs [595]. The exception are the *mcr-9* and *mcr-10* that were identified in human isolates: *Salmonella enterica* serotype Typhimurium and *Enterobacter roggenkampii*, respectively [596,597]. Unlike the situation in terrestrial animals, PMCR in aquaculture has largely been ignored [296]. However, colistin-resistant bacteria have also been found in aquaculture [598,599], being considered by some authors as a major source of colistin resistance genes. Recent studies in China showed the presence of MCR-1- and MCR-3-producing bacteria isolated in the food chain, such as in aquaculture fish and shrimp [600] and in *E. coli* recovered from grass carp fish farms [601].

In Vietnam, *mcr-1* was detected in *E. coli* isolated from striped catfish grown in ponds [602,603] and a study from Spain reported *mcr-1* in *S. enterica* serovar Rissen isolated from mussels [604]. Recently, *mcr-1* was detected in *E. coli* isolated from fish guts of rainbow trout in Lebanon [605]. In Czech Republic, *mcr*-type genes were detected in colistin-resistant *Enterobacterales* and *Acinetobacter* strains isolated from aquaculture products (frog legs, crab meat and pangasius meat) originating from Vietnam [606]. Shen and co-workers also demonstrated the association between aquaculture and a high incidence of *mcr-1*-positive *E. coli* carried by humans [607]. Furthermore, a comparative analysis of the resistome of integrated and monoculture aquaculture ponds using metagenomics suggest that freshwater aquaculture is rich in opportunistic pathogen-associated taxonomic groups that may host antibiotic-resistant genes (including *mcr*) associated with critically important antibiotics used in human medicine [138]. Indeed, it has been proposed that some PMCR genes may have originated in aquatic environments, since MCR-3, MCR-4 and MCR-7 proteins showed an elevated level of homology to phosphoethanolamine transferases found in aquatic bacteria [296,598].

The *mcr-9* gene is an emerging variant of the PMCR determinants, which was first identified in 2019, in a *S. enterica* isolated from a human patient in the USA [596]. Among the *mcr*-like genes, *mcr-9*, along with *mcr-1*, is the most widely disseminated [595]. The *mcr-9* gene can be found worldwide in different reservoirs (human, animal, food, and environment) and in various species of *Enterobacteriaceae* [608], which makes this resistance mechanism a problem under the perspective of One Health.

This study aimed to analyze the resistome, virulome and mobilome of an MCR-9-producing *Enterobacter* sp. isolated from farmed *Sparus aurata* and, to our knowledge, this is the first description of the colistin resistance *mcr-9* gene in the aquaculture environment.

8.3 Materials and methods

8.3.1 Study design and bacterial identification

MCR-9-producing *Enterobacter* sp. INSAq77 was isolated from a seabream (*S. aurata*) of commercial-size (500–1500 g), which was collected in March 2018 in a land tank from a fish multi-trophic farming [257]. This station is in the Ria Formosa Natural Park (south of Portugal) with a semi-intensive production system. Animal welfare was safeguarded during production and transport accordingly with the European Commission SANTE/2016/G2/009 recommendations [609]. Species identification was performed by VITEK®2 Automated Identification System (BioMérieux, Marcy-l'Étoile, France), using GN ID card and by amplification of the 16S rRNA gene, as previously described [257].

8.3.2 Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by disk diffusion (amoxicillin/clavulanic acid, aztreonam, cefepime, cefotaxime, cefoxitin, ceftazidime, ciprofloxacin, ertapenem, gentamicin, imipenem, meropenem, piperacillin/tazobactam and trimethoprim/sulfamethoxazole; Bio-Rad, Marnes-la-Coquette, France) and minimum inhibitory concentration (MIC) by in house broth microdilution (colistin, chloramphenicol, florfenicol, flumequine and oxytetracycline) and E-test® (fosfomycin; BioMérieux, Hazelwood, MO, USA), as previously described [257].

8.3.3 Whole genome sequencing

DNA was extracted from freshly grown overnight culture (Magna Pure 96 Instrument, Roche, Manheim, Germany) and was quantified using Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Dual-indexed Nextera XT kit was used to library preparation followed by paired-end sequencing (2 x 250 bp) on a MiSeq Illumina platform (Illumina Inc., San Diego, CA, USA), according to the manufacturer's instructions.

8.3.4 Genome annotation and analysis

Genomes were *de novo* assembled using the INNUca v4.2.2 pipeline (https://github.com/B-UMMI/INNUca; accessed on Jan 4, 2022): after quality control analysis performed by FastQC v0.11.5 and cleaning (Trimmomatic v0.38), genomes were assembled with SPAdes 3.14.0 and subsequently improved using Pilon v1.23. *In silico* multilocus sequence type (MLST) prediction was performed using the MLST v2.19.0. A Prokka v1.13.3 was utilized to annotate the assemblies. Average Nucleotide identity (ANI) was performed at NCBI to confirm the INSAq77 bacterial species [610]. All *de novo* contigs were BLAST searched against GenBank's non-redundant nucleotide collection (nr/nt) [611]. Qiagen CLC Genome Finishing Module v.20.0.1 (Qiagen, Aarhus, Denmark) was used for visual inspection and manual editing by the alignment of contigs using BLAST against the contigs themselves, allowing contig joining and scaffolding.

8.3.5 Phylogenomic analysis of *E. ludwigii* genomes

All *E. ludwigii* genomes (*n*=76, Table S 12) available at NCBI library were used on the genomic comparison process. Single nucleotide polymorphisms (SNPs) phylogenetic analysis was performed by using CSI Phylogeny v1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/; accessed on Jan 4, 2022) with default options (reference strain NZ_CP017279). Phylogenetic tree image was visualized and edited by FigTree v1.4.4 (https://tree.bio.ed.ac.uk/software/figtree/; accessed on Jan 4, 2022).

8.3.6 Resistome, virulome and mobilome analysis

Online bioinformatics tools and databases available at the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org; accessed on Jan 4, 2022) were used to investigate the presence of antimicrobial resistance genes (ResFinder 4.1), virulence factors (VirulenceFinder 2.0), plasmids (PlasmidFinder 2.1 and pMLST 2.0 IncHI2 DLST configuration), mobile genetic elements (MobileElementFinder v1.0.3) and pathogenicity (PathogenFinder 1.1). The Comprehensive Antibiotic Resistance Database (CARD) with the "perfect", "strict" and "loose" default settings were also used to characterize antibiotic resistance [393]. ISsaga was used for the identification and annotation of insertion sequences [394]. PHASTER search web tool (https://phaster.ca; accessed on Jan 4, 2022) was applied to detect, identify, and annotate prophage sequences [395]. All analyses were performed using default parameters.

8.3.7 Plasmid characterization

BRIG v.0.95 was used to perform a circular comparison between the complete sequence of INSAq77 *mcr-9*-harbouring contig and the highly similar plasmids detected by performing BLAST against the Microbial Nucleotide BLAST database for complete plasmids (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on Jan 4, 2022). The genetic environment of the *mcr-9* gene was manually revisited using CLC Genomics Workbench v20.0.4 (Qiagen, Aarhus, Denmark). EasyFig v2.2.5 was used for the visualization and comparison of *mcr-9* genetic environment [612].

8.3.8 Data availability

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files. The *E. ludwigii* whole genome shotgun (WGS) project has the project accession JABRPH000000000. The new bla_{ACT}-type nucleotide sequence was submitted to the GenBank Database as $bla_{ACT\text{-}88}$ with accession number MW887657, after request of the new allele number to NCBI (https://www.ncbi.nlm.nih.gov/pathogens/submit-beta-lactamase/; accessed on Apr 7, 2021).

8.4 Results and discussion

The *mcr-1* and *mcr-9* variants are the most widespread *mcr*-family genes. The *mcr-9* gene was identified in 40 countries through six continents, with 61.5% of the *mcr-9*-positive strains isolated in the United States [595]. In that study, *S. enterica* was the most common host species, especially in turkeys and chickens. Furthermore, other systematic reviews showed that isolates carrying *mcr-9* were detected in 21 countries through six continents, mainly from Europe. *mcr-9*-positive isolates were disseminated by various genera and species of *Enterobacteriaceae* isolates among which *Enterobacter* spp. were predominant (37.0%) [608]. More than 50% of the isolates were from human origin, being 29.0%, 3.6% and 2.9% from animal, environmental and food, respectively.

Here, an *mcr-9*-producing isolate (INSAq77) identified in a muscle sample of a commercial size *S. aurata*, collected during the winter season (March 2018), in a land tank from a fish multi-trophic farming, in the south of Portugal is described. To our knowledge is the first description of *mcr-9* gene in the aquaculture environment.

Average Nucleotide Identity (ANI) analysis performed by NCBI identified INSAq77 at the species level as *E. ludwigii*. The genome sequences of *E. cloacae* are 98.98% identical by ANI to the *E. ludwigii*, with 82.5% coverage of the genome. Indeed, INSAq77 isolate was identified as *E. cloacae* by the VITEK®2 automated identification system (BioMérieux, Marcyl'Étoile, France) and sequencing of the 16S rRNA gene. However, it is well known that precise species identification for the taxonomy of *Enterobacter* is complex [435] and that *hsp60* gene sequencing showed a higher species diversity than MALDI-TOF [613]. Seven species have been grouped within the *E. cloacae* complex: *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *E. ludwigii*, *Enterobacter mori* and *Enterobacter nimipressuralis*, which share at least 60% similarity in their genome with *E. cloacae* [614].

E. ludwigii was first described as a novel *Enterobacter* species in 2005 [615]. All strains are naturally resistant to ampicillin, amoxicillin/clavulanic acid, and cefoxitin due to the production of a chromosomal AmpC β-lactamase. Antibiotic-resistant *E. ludwigii* has been found mainly in clinical samples [616], although a CTX-M-producing *E. ludwigii* was recently described in an environmental isolate collected from a wastewater treatment plant in India [617]. Recently, antibiotic-resistant *E. ludwigii* was also identified in India, in moribund goldfish collected from ornamental fish farms [618]. On the other hand, *E. ludwigii* has been suggested as a potential probiotic microorganism in agriculture and aquaculture [619,620].

INSAq77 strain was resistant to chloramphenicol (MIC 32 mg/L), florfenicol (32 mg/L) and fosfomycin (64 mg/L) but was susceptible to all other antibiotics tested; cefoxitin and amoxicillin/clavulanic acid are intrinsic resistances. The colistin MIC for MCR-9-producing *E. ludwigii* as 1 mg/L, within susceptible EUCAST breakpoint. Indeed, other studies have demonstrated that the presence of an MCR-9 enzyme not always is associated with colistin resistance [596,621,622]. Nevertheless, recent studies showed that *mcr* genes might enhance the survival ability of bacteria under clinical colistin pressure, thereby potentially leading to treatment failure [623,624].

This study also analyzed the resistome, virulome and mobilome of this MCR-9-producing *E. ludwigii* isolated from farmed *S. aurata*. The analysis of WGS yielded 225 contigs, ranging from 237 to 244,787 bp. The draft genome contained a total assembly length of 5,276,953 bp, with estimated depth coverage of 30.7x; the GC content was 54.1%.

The MCR-9-producing INSAq77 *E. ludwigii* isolate belonged to the ST1342 lineage, first reported here. The whole-genome SNP-based phylogenetic tree using the 75 *E. ludwigii* genomes indicated that INSAq77 is not closely related to the other studied isolates (Figure 8.1).

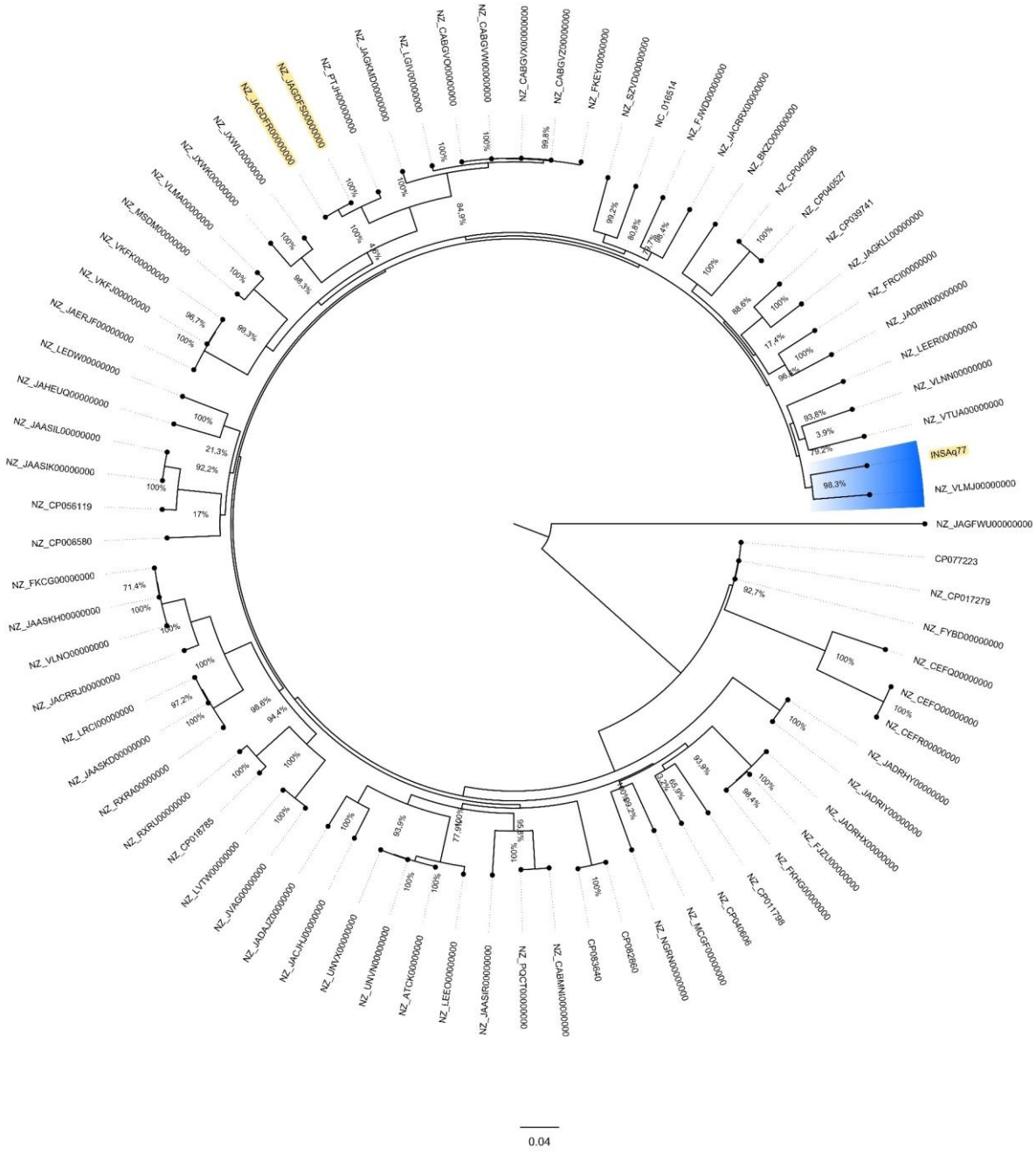

Figure 8.1 — Whole-genome SNP-based phylogenetic tree showing the relationship between 75 *E. ludwigii* genomes. The scale bar indicates 4% of nucleotide sequence divergence. The numbers at the nodes indicate percentage bootstrap replicates of 100. Sequences in the tree are indicated as GenBank accession number. Strain of the present study and the other MCR-9-producing isolates are highlighted in yellow. Blue color indicates cluster containing INSAq77.

Indeed, INSAq77 has 12% of nucleotide sequence divergence with the closest strain (NZ_VLMJ00000000), an *E. ludwigii* isolated from the lung of a clinical patient, in 2016, in the USA (PRJNA553678) [625]. The two other MCR-9-producing isolates (NZ_JAGDFR00000000 and NZ_JAGDFs00000000) were grouped into another cluster.

In silico antimicrobial resistance analyses using ResFinder 4.1, with a threshold of 90% identity and a minimum length of 60%, revealed acquired genes conferring resistance to β-lactams (bla_{ACT-88}, here firstly identified), fosfomycin (*fosA2*-type) and colistin (*mcr-9.1*). Furthermore, a total of 21 genes were detected *in silico* by CARD RGI perfect, strict, and loose algorithms, involved in efflux, transport and permeability, which might justify the florfenicol and chloramphenicol resistance identified by phenotypic methods (Table 8.1 and Table S 13).

CARD loose algorithm (match bitscore less than the curated one blastp bitscore) [393] identified that INSAq77 also harbors a *catA4*-type gene (64.8% of identity), which might infer resistance to chloramphenicol. However, the low percentage of identity it is not enough to assure the phenicol resistance causality of *catA4*-type gene. Other resistance mechanisms might be involved; indeed, the multiple antibiotic resistance (*mar*) locus, a resistance-nodulation-cell division (RND) antibiotic efflux pump detected *in silico* by CARD RGI strict algorithm, has been reported to contribute to chloramphenicol resistance [626]. Furthermore, the multiple antibiotic resistance *oqxAB*-type locus, another RND multidrug efflux pump operon was detected, which has been reported to contribute to multidrug resistance [627]. Diminished susceptibility to different antibiotic classes (e.g., aminoglycosides, fluoroquinolones, and tetracycline) were bioinformatically predicted (e.g., *rsmA*, *adeF*, *ramA*, *acrABR* and *soxAS*), although the isolate was phenotypically susceptible; this can be explained by the fact that efflux pumps are frequently associated with a low decrease in antibiotic susceptibility, which may not translate to a change in phenotype [628].

The acquired disinfectant resistance gene *formA*-type, a plasmid-mediated formaldehyde resistance mechanism [629], was also identified. The ability to survive aldehyde disinfection processes is clinically significant, with possible cross-resistance to antibiotics [630]. Furthermore, the INSAq77 isolate carried the *terC* virulence gene, commonly associated with IncHI2 plasmids and conferring resistance to tellurium, where soluble salts, especially potassium tellurite, were used clinically in humans as antimicrobial agents [631].

Table 8.1 — Perfect and strict best-hit results by predicted gene, obtained using the Resistance Gene Identifier (RGI).

Contig	RGI Criteria	ARO Term	Detection Criteria Model	AMR Gene Family	Drug Class	Resistance Mechanism	% Identity Matching Region	% Length Reference Sequence
INSAq77p_155	Perfect	mcr-9.1	Protein homolog	MCR phosphoethanolamine transferase	Peptide antibiotic	Antibiotic target alteration	100.0	100.0
INSAq77p_4	Strict	CRP	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, penam	Antibiotic efflux	99.1	100.0
INSAq77p_10	Strict	ACT-12	Protein homolog	ACT β-lactamase	Carbapenem, cephalosporin, cephamycin, penam	Antibiotic inactivation	98.7	100.0
INSAq77p_1	Strict	FosA2	Protein homolog	Fosfomycin thiol transferase	Fosfomycin	Antibiotic inactivation	98.6	100.0
INSAq77p_4	Strict	*Escherichia coli* EF-Tu mutants (R234F)	Protein variant	Elfamycin-resistant EF-Tu	Elfamycin antibiotic	Antibiotic target alteration	98.5	96.3
INSAq77p_82	Strict	baeR	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminoglycoside antibiotic, aminocoumarin antibiotic	Antibiotic efflux	95.8	100.0
INSAq77p_37	Strict	H-NS	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump, resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, cephalosporin, cephamycin, penam, tetracycline antibiotic	Antibiotic efflux	95.6	100.0
INSAq77p_47	Strict	msbA	Protein homolog	ATP-binding cassette (ABC) antibiotic efflux pump	Nitroimidazole antibiotic	Antibiotic efflux	94.7	100.0
INSAq77p_3	Strict	emrR	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone antibiotic	Antibiotic efflux	94.3	100.0
INSAq77p_25	Strict	*Escherichia coli* UhpT mutant (E350Q)	Protein variant	Antibiotic-resistant UhpT	Fosfomycin	Antibiotic target alteration	93.7	100.0
INSAq77p_21	Strict	marA	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump, General Bacterial Porin with reduced permeability to β-lactams	Fluoroquinolone antibiotic, monobactam, carbapenem, cephalosporin, glycylcycline, cephamycin, penam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, triclosan, penem	Antibiotic efflux, reduced permeability to antibiotic	93.6	99.2

INSAq77p_3_7	Strict	*Klebsiella pneumoniae* KpnH	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Macrolide antibiotic, fluoroquinolone antibiotic, aminoglycoside antibiotic, carbapenem, cephalosporin, penam, peptide antibiotic, penem	Antibiotic efflux	92.2	100.6
INSAq77p_11	Strict	oqxA	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, glycylcycline, tetracycline antibiotic, diaminopyrimidine antibiotic, nitrofuran antibiotic	Antibiotic efflux	91.1	100.0
INSAq77p_21	Strict	*Escherichia coli* marR mutant conferring antibiotic resistance	Protein overexpression	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, cephalosporin, glycylcycline, penam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, triclosan	Antibiotic target alteration, antibiotic efflux	91.0	100.0
INSAq77p_34	Strict	*Klebsiella pneumoniae* KpnF	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Macrolide antibiotic, aminoglycoside antibiotic, cephalosporin, tetracycline antibiotic, peptide antibiotic, rifamycin antibiotic	Antibiotic efflux	89.0	100.0
INSAq77p_3	Strict	rsmA	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, diaminopyrimidine antibiotic, phenicol antibiotic	Antibiotic efflux	85.3	100.0
INSAq77p_13	Strict	*Escherichia coli* ampH β-lactamase	Protein homolog	ampC-type β-lactamase	Cephalosporin, penam	Antibiotic inactivation	85.2	100.8
INSAq77p_34	Strict	*Klebsiella pneumoniae* KpnE	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Macrolide antibiotic, aminoglycoside antibiotic, cephalosporin, tetracycline antibiotic, peptide antibiotic, rifamycin antibiotic	Antibiotic efflux	82.0	83.3
INSAq77p_11	Strict	adeF	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, tetracycline antibiotic	Antibiotic efflux	60.9	99.2
INSAq77p_1	Strict	*Haemophilus influenzae* PBP3 mutant (D350N, S357N)	Protein variant	Penicillin-binding protein mutations conferring resistance to β-lactam antibiotics	Cephalosporin, cephamycin, penam	Antibiotic target alteration	53.1	96.4
INSAq77p_9	Strict	adeF	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone antibiotic, tetracycline antibiotic	Antibiotic efflux	41.2	97.9

ARO: Antibiotic Resistance Ontology. AMR: antimicrobial resistance.

PathogenFinder predicted the strain as being "human pathogenic" with a probability of 77.7% due to the presence of 74 genes belonging to known pathogenic protein families (Table S 14). Indeed, in addition to the known *E. cloacae* complex genes encoding pathogenic proteins, homologous sequences of pathogenic proteins from *Citrobacter koseri*, *Enterobacter* spp., *E. coli*, *S. enterica* and *Shigella* spp. were found in the study. Mobile genetic elements (MGE), such as plasmids, prophages, and transposons among others, are main drivers for the spread of antibiotic resistance [121]. In this study, nine insertion sequences (IS) were found using MobileElementFinder tool: IS*26*, IS*Kpn28*, IS*Sen4*-type, IS*30*-type, IS*Ecl1*-type, IS*Kpn43*-type, IS*Kpn24*-type, IS*100*-type, and IS*Ppu12*-type.

A total of eleven prophage regions were also identified using PHASTER tool (Figure S 10), of which two regions were intact (PHAGE_Salmon_SEN4_NC_029015 and PHAGE_ Entero_ HK542_NC_019769), eight regions were incomplete, and one region was questionable (PHAGE_Shigel_SfIV_NC_022749). Figure 8.2 shows the schematic representation of the phage-related proteins identified in the intact and questionable prophages. The size of the three prophages ranged from 16.7 Kb to 32.6 Kb with an average GC content of 52.7%. These prophages were firstly described in *S. enterica* subspecies *salamae* collected in the Czech Republic [632], *E. coli* isolated in Hong Kong and *Shigella flexneri* collected in Bangladesh [633], corroborating that MGE can be excised and integrated from the chromosome and MGE into each other. Indeed, several studies have already shown the presence of *mcr*-type genes in prophages [634,635], indicating the role of these MGE in the dissemination of antibiotic resistance. Furthermore, two cryptic prophages were detected by PathogenFinder (CP4-6 and CP4-57) which, although they do not form active phage particles or lyse their captors, can be considered the relatively permanent reservoirs of antibiotic resistance genes [636].

Further bioinformatics analysis revealed the presence of five plasmid replicon types: ColE10, Col(pHAD28)-type, IncFIA(HI1)-type, IncR-type, IncHI2 and IncHI2A, the last two linked to the worldwide dissemination of *mcr-9* gene [595,608]. The *mcr-9* gene can be found in different reservoirs (human, animal, food, and environment), in various species of *Enterobacteriaceae* strains, mostly associated with IncHI2/IncHI2A plasmid replicons [608]. Indeed, as observed in our study, several works demonstrated the prevalence of *mcr-9*-harboring IncHI2/IncHI2A plasmids among *Enterobacteriaceae* isolates: e.g., Carroll et al., in 2019, detected 59/65 assemblies where IncHI2 and/or IncHI2A plasmid replicon were present on the same contig as *mcr*-9 [596].

Figure 8.2 — Schematic representation of phage-related proteins identified in the intact and questionable prophages by PHASTER prophage database (https://phaster.ca; accessed on Jan 4, 2022) [395]. The arrows indicate the sequence orientation (5′ to 3′ above the black line and 3′ to 5′ under it). The abbreviations are: Att (phage attachment site), Coa (phage coat protein), Fib (phage tail fiber), Int (phage integrase), Hyp (hypothetical protein), Pla (phage plate protein), PLP (phage-like protein), Por (portal protein), Sha (phage tail shaft protein) and Ter (terminase).

The *mcr-9* gene was found in a 30,314 bp length contig, manually assembled after visual inspection and alignment of contigs (Aq77p_57, Aq77p_155, Aq77p_191, Aq77p_196, Aq77p_213) against themselves using CLC Genomics Finishing Module v.20.0.1 (Qiagen, Aarhus, Denmark); the GC content was 47.6%. The analysis of *mcr-9*-harbouring contig using the Microbial Nucleotide MegaBLAST analysis against the complete plasmids database revealed nine *mcr-9*-carrying IncHI2 plasmid sequences (>99.9% identity, >97% query coverage and e-value 0.0) from multiple species, collected in different antibiotic resistance reservoirs worldwide, including human clinical/colonization samples (Table 8.2). Of notice, three plasmids of sequence type 1 (ST1), accordingly with the IncHI2 pDLST scheme, were collected from environmental samples during an extended bla_{IMP-4}-associated carbapenemase outbreak in an Australian hospital [637].

Table 8.2 — Comparison of the INSAq77 *mcr-9*-containing contigs with the top nine IncHI2 *mcr-9*-harboring plasmids showing the highest identities (>99.0%, e-value 0.0, query coverage >94.0%).

Plasmid (bp)	Strain	Isolation Source/Country/Year	Identity (%)	Query Cover (%)	pMLST[b]	Acquired Antibiotic and Disinfectant Resistance Genes[c]	GenBank Acc. No.
INSAq77 IncHI2 (30,314)[a]	*E. ludwigii* INSAq77	Seabream (*Sparus aurata*)/Portugal/2018	-	-	DLST1	***mcr-9.1***	JABRPH000000000
pSPRC-Echo1 (339,920)	*E. hormaechei* C15117	Burns unit/Australia/2007	99.99%	99.0%	DLST1	*aac(6')-IIc*, *aph(3'')-Ib*-type, *aph(6)-Id*, bla_{SHV-12}, bla_{TEM-1B}, *catA2*-type, *dfrA19*, *mcr-9*, *qacE*, *qnrA1*-type, *sul1*, *sul2*, *tet(D)*	NZ_CP032842
p525011-HI2 (354,045)	*C. freundii* 525011	Unknown/China/2017	100.00%	97.0%	untyped, Nearest STs: 7,1,4,15	*aac(3)-IId*-type, *aac(6')-aph(2'')*, *aadA5*, *armA*, bla_{TEM-1B}, *catA2*-type, *dfrA1*-type, ***mcr-9***, *mph(E)*, *msr(E)*, *qacE*, *qnrA1*-type, *sul1*, *sul2*	NZ_MF344582
pOSUKPC4 (351,806)	*E. hormaechei* OSUKPC4_L	Animal/USA/2016	100.00%	98.0%	DLST1	*aadA1*, *aph(3'')-Ib*-type, *aph(6)-Id*, bla_{KPC-4}, $bla_{OXA-129}$, *dfrA21*, ***mcr-9***, *qacE*, *sul1*, *tet(B)*	NZ_CP024910
pOSUEC_D (354,256)	*E. hormaechei* OSUVMCKPC4-2	Animal/USA/2016	100.00%	98.0%	DLST1	*aadA1*, *aph(3'')-Ib*-type, *aph(6)-Id*, bla_{KPC-4}, $bla_{OXA-129}$, *dfrA21*, ***mcr-9***, *qacE*, *sul1*, *tet(B)*	NZ_CP029248
pK29 (269,674)	*K. pneumoniae* NK29	Human/Taiwan/2001	100.00%	98.0%	DLST1	*aadA2*, bla_{CMY-8}, $bla_{CTX-M-62}$-type, *catB2*, ***mcr-9***, *qacE*, *sul1*	NC_010870
pE1_001 (357,530)	*L. adecarboxylata* E1	Burns Unit Shower/Australia/2012	100.00%	98.0%	DLST1	*formA*-type, ***mcr-9***	NZ_CP042506
pE11_001 (339,433)	*C. freundii* E11	Burns Unit Shower/Australia/2012	100.00%	98.0%	DLST1	*formA*-type, ***mcr-9***	NZ_CP042525
pE61_001 (357,530)	*L. adecarboxylata* E61	Burns Unit Shower/Australia/2014	100.00%	98.0%	DLST1	*formA*-type, ***mcr-9***	NZ_CP042494
p565_1 (263,189)	*C. freundii* 565	Human Stool/Spain/2014	99,68%	95,0%	DLST1	*aac(6')-Ib-cr*, *aadA1*, *aadA2b*-type, $bla_{CTX-M-9}$, bla_{SHV-12}, bla_{VIM-1}, *catA1*-type, *dfrA16*, ***mcr-9*-type**, *qacE*, *qnrA1*-type, *sul1*	NZ_CP038657

[a]Length of the *mcr-9*-containing contig. [b]Plasmid pMLST-2.0 Server. [c]ResFinder-4.1 (Selected %ID threshold: 90%; selected minimum length: 60%). Acc. No.: accession number.

In INSAq77 IncHI2 plasmid and in all others studied here, except p565_1 from a *C. freundii* strain (NZ_CP038657, Figure 8.3), the *mcr-9* gene was surrounded upstream by an IS*903* element and downstream by a *wbuC* family gene, encoding a cupin fold metalloprotein, followed by IS*26*.

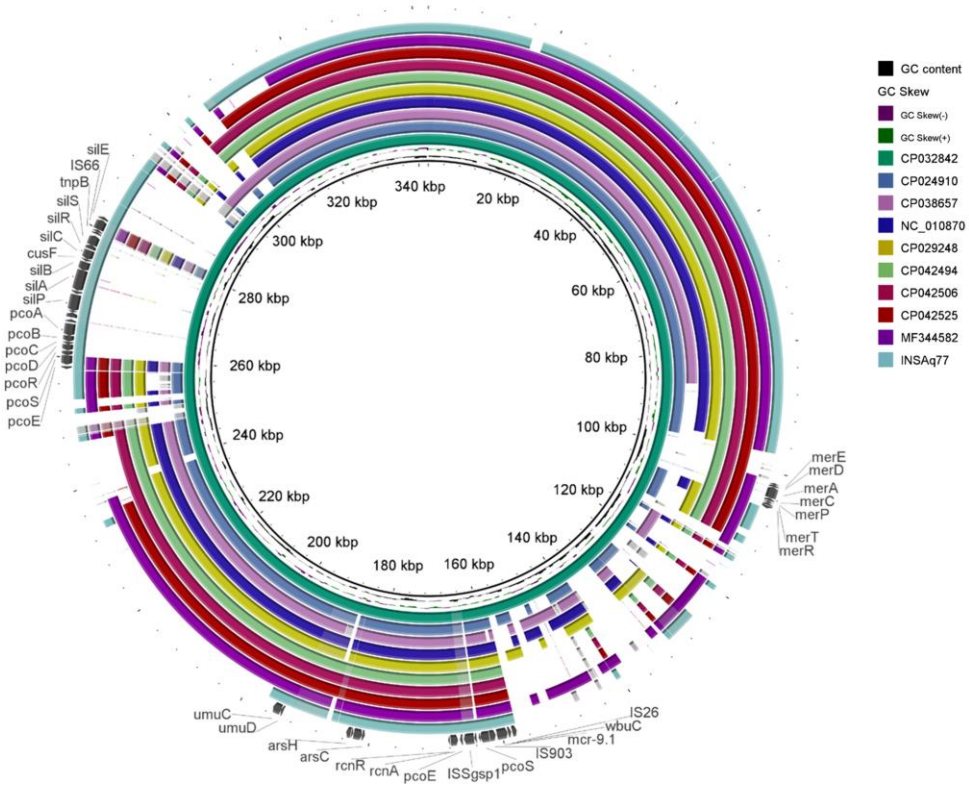

Figure 8.3 — Comparative analysis of INSAq77 *mcr-9.1*-containing contig with nine closely related IncHI2 *mcr-9*-harboring plasmids using the BLAST Ring Image Generator.

The *mcr-9* gene is frequently associated with the *wbuC* gene in different bacterial species, suggesting an essential role of this *wbuC* gene for the activity of the MCR-9 enzyme. Indeed, this gene was proposed to have transferred together with *mcr-9* as a whole fragment from *Buttiauxella* spp. [638]. On the other hand, *qseB*/*qseC* two-component regulators were absent, which could explain the susceptibility to colistin of INSAq77 isolate. Indeed, based on previous studies, in the presence of subinhibitory concentrations of colistin, the *qseB*/*qseC* regulatory system can induce the expression of the *mcr-9* gene, which results in an increase in MIC values [622,638,639]. The *mcr-9* gene is being described as part of a *mcr-9* cassette containing the *rcnR-rcnA-pcoE-pcoS*-IS*903-mcr-9-wbuC* core structure (Figure 8.3) [608].

As shown in Figure 8.3 and Figure 8.4, the genetic background immediately upstream of *mcr-9* was consistent among IncHI2 *mcr-9*-bearing plasmids. The exception is the presence of an IS*Sgsp1* element from the IS*66* family in the INSAq77 *mcr-9*-harbouring contig, showing

100% of identity with an isolate of *Klebsiella pneumoniae* collected, in 2012, from a human patient in the USA (CP007734). Furthermore, in this study, the presence of the conserved nickel/copper operon (i.e., *rcnA*, *rcnR*, *pcoE* and *pcoS* genes), which plays a key role in copper tolerance under anaerobic growth and nickel homeostasis in bacteria, was also detected. Downstream of the *mcr-9* gene, the nucleotide sequences, including that of INSAq77, were genetically diverse (Figure 8.3). Indeed, different regions were present among the IncHI2 plasmids also analyzed in this study, namely regions involved in resistance to copper (*pcoABCDRSE*), silver (*silESRCBAP*), arsenic (*arsCBRH*) and/or mercury (*merEDACPTR*).

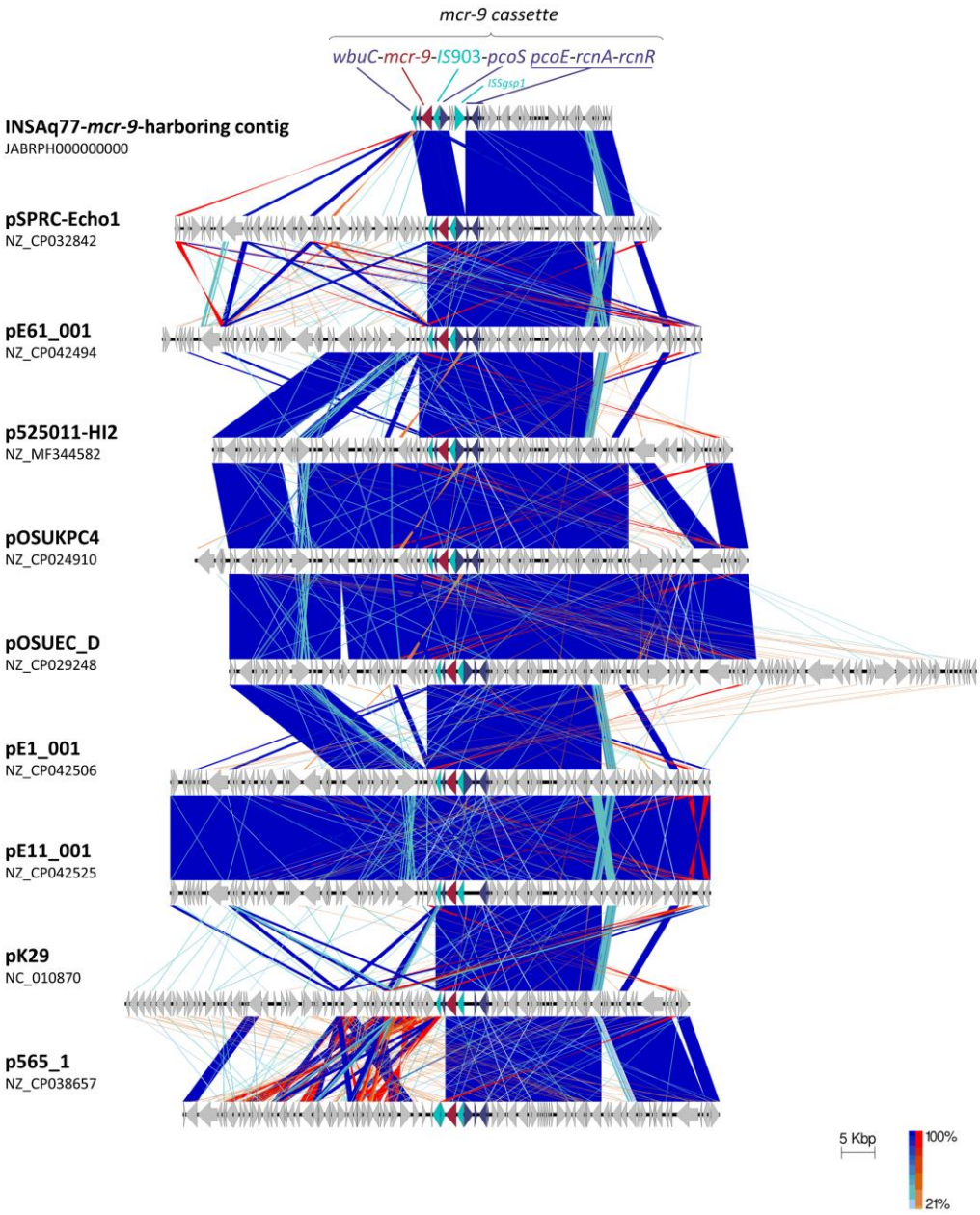

Figure 8.4 — Schematic representation of the genetic environment of INSAq77 *mcr-9.1*-containing contig with others IncHI2 *mcr-9*-harboring plasmids. Boxed arrows indicate direction of transcription for all genes. Blue bars: normal

tblastx matches; red: inverted matches; depth of shading: percentage blast match. Color-coding for the genes inside *mcr-9* cassette: dark red, *mcr-9* gene; cyan, mobile DNA; purple, other genes; grey, other CDSs. The scale is represented in base pairs.

The abundance of *mcr* variants and alleles in bacteria isolated from aquatic reservoirs suggests that these enzymes may play another role, namely a defense system against natural peptides and/or bacteriophages [640]. *mcr-9* gene was firstly described in the USA, in a clinical *S.* Typhimurium isolate, which demonstrates the high transmission potential of this colistin resistance determinant and places this research in a One Health context [641].

8.5 Conclusions

This work reinforces the knowledge that water environments play a crucial role in the spread of antibiotic resistance and that important antibiotic resistance mechanisms, such as *mcr* genes conferring low- or medium/high-level resistance to colistin, are also present in aquaculture. This fact, allows antibiotic-resistant bacteria to spread through food and through the environment, resulting in serious threats to human health [642].

The use of phenotypic methods to determine susceptibility to antibiotics may be a limitation, as they may not identify the low expression associated with the presence of a particular gene, as in this case. Thus, the implementation of high throughput methods in laboratories, such as the WGS, will make an important contribution to the detection of under-expressed genes, mostly when they are of clinical importance. Thus, the presence of antibiotic-susceptible isolates in different settings, such as the INSAq77 *mcr-9*-carrying strain isolated in aquaculture, highlights the risk of the silent dissemination of important resistance determinants, among which, in fact, the genes encoding such PMCR are an important example. Of concern is also the possible co-selection of antibiotic-resistant bacteria when exposed to heavy metals (copper and zinc), often used as growth promoters in aquaculture and terrestrial animal farms.

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9

GENERAL DISCUSSION AND CONCLUSIONS

Bacteria are extraordinary organisms with a remarkable ability to adapt to the most diverse environments and hosts, since the Antarctic ice, hydrothermal vents, and acidic hot springs to the gut of humans and several other animals, where they help in the digestion process [643,644]. The acquisition of new genetic information by HGT (transformation, conjugation, and transduction) plays an important role in this adaptation, namely in environments where the presence of antibiotics causes selective pressure in the bacterial communities [425,643]. Antibiotic resistance is one of the biggest threats to human health in the 21st century and aquatic environments are considered hotspots of recombination and genetic exchange events and to be the origin of several antibiotic resistance genes relevant in human medicine [425–428]. Therefore, throughout this Ph.D. thesis we deepen the study of the microbiome, resistome, virulome and mobilome of some of the most consumed fish and bivalve mollusks in Portugal from aquaculture origin. This work contributed to answer to the questions: which bacteria are circulating in aquaculture environments? Which resistance genes do they have? How different are these bacteria and resistance genes when compared to other reservoirs (such as humans and other animals)? Do aquaculture activities contribute to the emergence and spread of resistance genes? Which MGE are associated with this dissemination? Our contribution in answering these questions can be found in chapters 3 to 8, related to research papers, where we start from the analysis of 441 strains (including Gram-negative and Gram-positive bacteria), isolated from gilthead seabream (*S. aurata*), mussels (*Mytilus* sp.), clams (*R. decussatus*) and Japanese oysters (*C. gigas*) collected directly in aquaculture facilities or in the market (but with an aquaculture origin). Each research paper/chapter has a specific discussion; thus, this chapter (9, General discussion and conclusions) will focus on the main findings, that will be connected and discussed in the next lines.

In chapters 3 and 4, we started deciphering the bacterial composition of fish and bivalve mollusks' microbiome through bacteria's identification to the species level, whenever possible, as well as the study of phenotypic and genotypic characteristics of these individual strains by using culture-based and molecular methods, respectively. Gram-negative bacteria predominated in both reservoirs, wherein *Enterobacteriaceae* was the most frequently identified family in seabream, with samples collected in winter season, and in clams (farm 1), mussels (farm 4) and Japanese oysters (farm 6) in samples collected in autumn. The exceptions of *Enterobacteriaceae* predominance in the cold seasons were, for mussels' samples, in farm 2 where *Vibrionaceae* was the most frequently found in autumn and in farm 3 with *Enterobacteriaceae* predominating in both autumn and summer, and, for Japanese oysters, in farm 1 with *Enterobacteriaceae* most frequently found in summer. In the summer, *Morganellaceae* (in clams from farm 1), *Shewanellaceae* (in mussels from farm 2) and *Vibrionaceae* (in mussels and Japanese oysters from farms 4 and 5, respectively) predominated. *Enterobacteriaceae* is a very diverse family encompassing species from the most different environments, including water and aquatic animals [43,189,250,266]. Moreover, some members of this family are associated with fecal contamination (e.g., *E. coli*) and used as indicators of microbiology quality [219]. Winter and autumn are usually associated with high precipitation index, that favor the occurrence of runoff from land (e.g., agriculture activities, sewage) and consequent contamination of aquaculture settings [250]. 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. Besides being pathogenic to aquatic animals and cause huge economic losses [376], some members of *Enterobacteriaceae* family are currently one of the greatest threats in human health [645]. On the other hand, members of *Morganellaceae*, *Shewanellaceae* and *Vibrionaceae* families are well-known pathogens of fish and shellfish [376]. Furthermore, this study describes for the first time the bacteria *Exiguobacterium acetylicum*, *Klebsiella michiganensis*, *Lelliottia* sp. and *Pantoea vagans* associated with seabream and *Comamonas aquatica*, *Escherichia fergusonii*, *Lactococcus garvieae*, *Moraxella osloensis*, *Pseudocitrobacter faecalis*, *Raoultella ornithinolytica* and *Serratia marcescens* in bivalve mollusks from aquaculture, contributing to a better understanding of aquaculture's microbiome (Table S 15). Thus, we can infer that the composition of aquaculture's microbiome depends on several factors such as host species and conditions of the aquatic habitat [32,259,260]. For example, a study conducted by Bereded et al. [646], revealed differences in bacterial composition and diversity in the gut of Nile tilapia (*Oreochromis niloticus*) according to seasons and habitat types (fish reared in farms vs. fish in wild).

Monitoring antibiotic resistances is one of the most effective ways to understand how aquaculture environments are being affected by the use of antibiotics and several other contaminants. In chapters 3 and 4, we also studied antibiotic susceptibility of the bacteria isolated in seabream and bivalve mollusks against antibiotic classes important in aquaculture and human medicine (β-lactams, quinolones, tetracyclines, phenicols, glycopeptides, sulfonamides, trimethoprim, aminoglycosides, oxazolidinones, rifampicin, mupirocin, fusidanes, lipopeptides and polymyxins). Non-susceptibility to phenicols prevailed in seabream samples, followed by non-susceptibility to β-lactams (both classes include antibiotics used in aquaculture [68,69]). Indeed, non-susceptibility to carbapenems was detected in strains of *Enterobacter* (ertapenem) and *Pseudomonas* (meropenem) isolated in seabream samples. The use of carbapenems in food-producing animals is prohibited in the European Union, since these antibiotics are in the WHO's list of "Critically Important Antimicrobials in human medicine" and are used as a last-resort, mostly in hospitals, to treat infections caused by multidrug-resistant Gram-negative bacteria [104,400]. Contamination from other sources or co-selection events may be associated with this worrying finding. In bivalves' samples, the initial screening of antibiotic non-susceptibilities revealed a prevalence of non-susceptibility to oxytetracycline in all species of bivalves, farms, and seasons. These results can be consequences of the widespread use of oxytetracycline in aquaculture [21]. Moreover, this non-susceptibility is less frequent in Japanese oysters than in the other bivalves studied and is more associated with summer than autumn in oysters. Another worrying result concerns the discovery of non-susceptibility to colistin in bivalves samples, a last-resort antibiotic also used to treat infections caused by multidrug-resistant Gram-negative bacteria, namely carbapenem-resistant *Enterobacteriaceae* [90]. Some countries use colistin in aquaculture, although that is not the case of European countries [69,70,296]. Noteworthy, to our knowledge, was here described for the first time *Bacillus* spp. resistant to vancomycin in seabream from aquaculture (Table S 15). Non-susceptibility to quinolones was found in both reservoirs. Indeed, with the exception of eight $bla_{\text{TEM-1}}$ identified in *E. coli* isolated from bivalve mollusks, the only other resistance genes detected by PCR, in this work, were associated with reduced susceptibility to quinolones, more specifically six *qnrA* (in *S. algae* isolated in bivalve mollusks [two species were corrected for *S. chilikensis* and *S. indica* by ANI; see chapter 7, section 7.4.1 and Table S 10]), eleven *qnrB* (in *L. adecarboxylata* isolated in seabream and in *E. coli*, *C. braakii* and *C. freundii* isolated in bivalve mollusks [in the last two cases, the species identification was corrected for *C. portucalensis* by ANI; see chapter 7, section 7.4.1 and Table S 10]) and three *oqxAB* (in *R. ornithinolytica* from bivalve mollusks). To the best of our knowledge, *qnrB19* is here described for the first time associated with *L. adecarboxylata*

(Table S 15). Non-synonymous mutations G385T and C402A in *parC* gene were also detected in a *K. pneumoniae* strain isolated in seabream samples. Furthermore, eight *E. coli*, isolated in bivalve mollusks, were considered multidrug resistant.

In 2022, a report from the European Medicines Agency informs that, in 2021, penicillins was the most sold class of antibiotics for food-producing animals (including aquaculture), followed by tetracyclines and sulfonamides [71]. These classes of antibiotics are also used in human medicine. In fact, in 2018, WHO listed penicillins, tetracyclines and sulfonamides as "Critical important" and/or "Highly important antimicrobials for human medicine", as well as other classes of antibiotics, whose non-susceptibilities were detected in the work developed throughout this thesis, namely quinolones, glycopeptides, carbapenems, 3rd generation cephalosporins, polymyxins, trimethoprim ("Critical important antimicrobials for human medicine"), mupirocin and phenicols ("Highly important antimicrobials for human medicine") [104].

Noteworthy, at the time of the publication of the research papers corresponding to the chapters 3, 4 and 5, old classifications of Susceptible, Intermediate and Resistant of EUCAST were still used. Thus, in these chapters, expressions like "non-susceptibility" and "decreased susceptibility" are used to include intermediate and resistant strains. New EUCAST classifications define the older "Intermediate category" as "Susceptible, increased exposure", i.e., *"when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection"* (https://www.eucast.org/newsiandr/; accessed on Aug 30, 2022).

Staphylococcaceae was the second most prevalent bacterial family in muscles of gilthead seabream, i.e., in the edible part of this fish. In this family, *S. aureus* is one of the most successful bacteria, sometimes associated with food poisoning outbreaks (Chapter 1, section 1.3.3) and one of the top 3 pathogens responsible for the highest number of human deaths attributable to antimicrobial resistance in 2019 (surpassed only by *E. coli* and *K. pneumoniae*), being the first in the high-income super region (Western Europe, Southern Latin America, North America, Asia Pacific and Australasia) with 26.1% of deaths attributed to antimicrobial resistance [647]. Therefore, we thought it was of great interest to study this species in different reservoirs. In Chapter 5, we studied 58 *S. aureus* isolates from human samples and 24 from animals of livestock, poultry, zoo, and aquaculture. The antibiotic susceptibility analysis revealed a higher level of resistance in *S. aureus* isolated from humans, with 81% resistant to ciprofloxacin, 69% resistant to cefoxitin (only 1 strain did not have *mecA* gene), 1.7% resistant to teicoplanin and hGISA. One strain was classified as multidrug resistant. On the other hand, in animals' samples only 4 strains (isolated in rabbits) were resistant to ciprofloxacin. An

overview of the mechanism of action of β-lactams (such as cefoxitin) and quinolones (such as ciprofloxacin) antibiotics is given in Chapter 1, sections 1.5.1 and 1.5.3, respectively, and the mechanisms of resistances triggered by the expression of *mecA* gene and mutations in *parC* gene (primary target in staphylococci) are explain in Chapter 1, section 1.6.2. Genetic diversity was characterized by *agr*/*spa* typing and MLST. The group I of the regulator of virulence factors, *agr*, was the most frequent in isolates of human origin, whereas group III and group non-typable were the most frequent in samples from animal origin. Human reservoir revealed a great diversity in *spa* types, with t032 as the most frequent and t14878 and t14933 described here for the first time (Table S 15). In animal reservoir, t2383 was the most frequent *spa* type and t15307 was identified for the first time (Table S 15). Only t571 was found in both reservoirs. According to MLST study, ST22 was the most frequently found in human samples, whereas ST398 was most frequent in animals' samples (ST3254, ST3269 and ST3270 were here identified for the first time; Table S 15). ST5, ST34 and ST398 were identified in both reservoirs. The detection of *spa* and ST common to both human and animal reservoirs confirms that some lineages of *S. aureus* were able to break host species barrier and adapt to different environments. Therefore, a problematic lineage is not only a problem of a specific reservoir, but it can also become a global threat and must be addressed with a multidisciplinary approach, such as One Health. One Health concept is defined as *"an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems. It recognizes that the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent"* [648]. Due to the countless and complex interactions between humans, animals, and environment from all over the world (e.g., sewage, agriculture, aquaculture, pharmaceutical industry waste, and travelling) applying control measures for antibiotic usage only in humans or only in some countries, for example, will not be enough to control the problem of antibiotic resistance.

An example of a lineage that circulates between different hosts and that can cause infection in both animals and humans is *S. aureus* ST398 [310]. In this Ph.D. thesis, *S. aureus* ST398 was identified for the first time in gilthead seabream from aquaculture origin (Table S 15). It is thought that this lineage originated in humans, being subsequently transmitted to livestock and backwards to humans [381]. The importance of this clone as a source of zoonotic disease in several countries led us, in chapter 6, to deepen the study of the 10 MSSA ST398 found in Chapter 5: three were isolated in humans, one in a dolphin and six in gilthead seabream from aquaculture. We used WGS, to better understand how different these MSSA were concerning the presence of ARG, VF and MGE and how they were contributing to the

dissemination of antibiotic resistance in these different environments. A phylogenetic analysis using the genomes of these strains plus 40 *S. aureus* ST398 from NCBI database revealed that our human strains cluster together with one *S. aureus* isolated from a human infection in France; the dolphin strain cluster with two animal-independent MSSA isolated in humans from Dominica and United States; and gilthead seabream strains cluster together with two *S. aureus* isolated in RTE food from Russia. Recent work highlights some of the differences of ST398 associated with livestock (LA-ST398) and ST398 from human origin. These authors collected evidence that LA-ST398 can be transmitted to humans and cause infections, and that is usually associated to MRSA, tetracycline resistant and lacks ϕSa3 prophage (that encodes IEC genes); however ST398 from human origin is associated with more severe infections, can be transmitted from humans to humans, is usually MSSA, tetracycline sensitive and possess ϕSa3 prophage, compensating their lack of resistance by greater cytotoxicity and virulence [649]. In our study, all strains were similar in their ARG, VF and MGE content, with punctual differences in the environment surrounding these genes. Nine of the ten strains had an *erm(T)*-type gene, predicted to be located on plasmids, and an $iMLS_B$ phenotype, i.e., resistance to erythromycin and inducible resistance to clindamycin. Seven of the ten strains studied were resistant to benzylpenicillin and six harbor *blaZ* gene (possibly chromosomally located, except for one strain from aquaculture). The same IS were found among strains from humans and seabream from aquaculture. Except for one strain from human origin and another from gilthead seabream, all *S. aureus* ST398 harbor identical genes coding for efflux pumps and virulence factors (associated with host immune evasion, exoenzymes, adherence and toxins production). Is the ability to produce toxins that makes *S. aureus* a food poisoning agent (see Chapter 1, section 1.3.3). The severity of staphylococcal food poisoning depends on the health status of the individual and the quantity of toxins ingested. These toxins are resistant to freezing, heat, drying and the acidic environment of the digestive tract and are capable of stimulating T cells, inducing an inflammatory response by the host through the production of cytokines [64]. Therefore, all *S. aureus* ST398 were classified as pathogenic to human hosts, with mean values of 98%. Strains isolated in gilthead seabream from aquaculture showed a greater diversity of prophage regions in their genome compared to strains isolated in humans and dolphin. This is not an unusual fact since it is known that bacteriophages are abundant in aquatic environments and can be involved in the dissemination of ARG and VF through transduction process. As the association ST398-t571 is common in *S. aureus* isolated from human samples [348,374,401], we therefore consider plausible the hypothesis that the strain isolated in the dolphin had a human origin. Although *spa* type from *S. aureus* isolated in gilthead seabream was different from the human

samples, this *spa* type (t2383) was already isolated in humans and food-producing animals [350,398,399] and they exhibit some characteristics of ST398 with human origin, such as being MSSA, having low resistance, high virulence and IEC type C (for four out of six strains). Therefore, we theorize whether there was a human-to-fish transmission.

Thus, WGS has become an important tool, allowing the detection of possible transmission routes and MGE that may be involved in the spread of resistance genes. In this line of thought, we expanded the use of WGS technology to study 66 Gram-negative strains (Chapter 7) from gilthead seabream and bivalve mollusks acquired directly in aquaculture farms (strains isolated in studies described in Chapters 3 and 4) and others purchase in a market (gilthead seabream and mussels).

We detected 18 misidentified species, previously identified with MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) and/or the amplification of the 16S rRNA gene. MALDI-TOF is based on the comparison of a protein spectrum from bacteria to a database composed by several protein spectrums of reference strains. This protein spectrum is obtained by the ionization of proteins (using laser and small organic acids of the matrix) and their separation through mass-to-charge ratios [650]. This technology is very useful in clinical laboratories where it allows a fast, low-cost, and high accuracy bacterial identification. However, a correct identification depends on the diversity of species present in the database [434], which is a disadvantage when we work with samples from rich and diverse environments, such as aquatic ecosystems, where we find species that are not present in clinical settings and others not yet described. Another disadvantage of MALDI-TOF is the difficulty in distinguishing very similar protein spectrums [434], what probably happened in some of the identifications of species belonging to *Aeromonadaceae*, *Enterobacteriaceae*, *Hafniaceae*, *Shewanellaceae*, *Vibrionaceae* and *Yersiniaceae* families in studies from Chapters 3 and 4. A technique that can help in bacteria identification when MALDI-TOF fails is the amplification of the 16S rRNA gene by PCR. This gene is highly conserved and ubiquitous in bacteria, with variable regions specific to certain genus and/or species. After amplification and sequencing of the PCR product, nucleotide sequences are compared with others present in databases. However, if bacteria have high similar 16S rRNA sequences, this technique could fail in their identification (which may also have happened in our studies from Chapters 3 and 4) [651]. Therefore, tools that analyze the complete genome and not only one specific gene have evolved, such as KmerFinder. KmerFinder allows an identification to the species level based on the number of overlapping 16-mers between the unidentified genome and genomes present in a database. Once again, this method depends on the size and accuracy of the genomes present in the databases [384].

Therefore, ANI gives robustness to an identification by KmerFinder. ANI is commonly used and is considered, by some authors, as the most reliable in species identification [610]. This method gives us a similarity measurement between a pair of genomes, wherein values >95% imply that those genomes belong to the same species [652]. In conclusion, methods that use the complete genome for species identification may be more suitable for non-clinical strains. Six more species were identified for the first time: *C. portucalensis* in gilthead seabream (acquired in the market), Japanese oysters and clams (from farm 1); *K. intermedia*, *R. terrigena* and *S. liquefaciens* in gilthead seabream (from the market); and *P. terrae* and *S. chilikensis* in mussels (from market and farm 2) (Table S 15). These new species descriptions contribute to a better understanding of aquaculture microbiome. During the investigation of genetic diversity, new ST were also found among *Aeromonas* spp. (ST879, ST880, ST881, ST882, ST883, ST887 and ST888), *Shewanella* spp. (ST40, ST57, ST58, ST60, ST61 and ST62) and *Vibrio* spp. (ST205 and ST 206) (Table S 15). The additional ST identified were already described in other studies, most of them associated with human samples [447,448,451,455–457]. This proves the existence of frequent exchange of bacteria from different environments, reenforcing the need of a One Health approach to mitigate this spread that can became a hazard for human health (since 74.2% of these strains were considered pathogenic to humans), but also to animal and environmental health.

Obtaining the genomes of these 66 Gram-negative strains by WGS, allowed for faster research of genes involved in antibiotic, disinfectant, and heavy metals resistance. The resistome found was very diverse, unlike the results obtained by PCR research in Chapters 3 and 4, with antibiotic resistance genes usually associated with β-lactams, tetracyclines, aminoglycosides, quinolones, sulfonamides, trimethoprim, nitroimidazole, fosfomycin, phenicols, and macrolides resistance; disinfectant resistance genes were identified associated with quaternary ammonium compounds, peroxides, and aldehydes resistance; as well as genes related to tolerance/resistance to heavy metals, such as copper, zinc, arsenic, mercury, nickel, iron and tellurium. Nine *E. coli* were considered multidrug resistant (1 isolated in gilthead seabream acquired in market and 8 in mussels from farm 4). PCRs are used to search for the most common antibiotic resistance genes, whose presence is directly related to the phenotype of antibiotic resistance. However, this methodology does not allow the detection of antibiotic resistance associated with deletions, insertions, single nucleotide polymorphisms (SNPs), as well as derepression or hyperproduction of antibiotic genes or their regulators, making WGS based methodologies more accurate in predicting a resistance phenotype [653]. Nowadays, although WGS has already proven to be effective in the identification of β-lactams resistance in some

studies involving *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *E. cloacae* [653], it does not replace phenotypic methods since the genetic origin of some resistances are not fully understood and does not allow to assess the function of the produced protein [654]. Thus, our study detected occasional mismatches between phenotype and genotype corroborating this information. The composition of the virulome was also very diverse with 25 different genes, concentrated in *Aeromonadaceae* and *Enterobacteriaceae* families and associated with the production of toxins, transportation, red blood cell lysis, iron metabolism, increased serum survival, evasion from the immune system of the host, adherence, colonization, and resistance to acidic environments. Thirty-five percent of the strains studied had genes encoding virulence factors, some of them predicted to have a plasmid location. Indeed, mobilome analyses revealed a great number and diversity of MGE, such as plasmids, IS, transposons, composite transposons, MITEs, integron-integrases, and prophage regions. Moreover, a more detail analyses of the genetic environment of some resistance genes uncovered the possible role of MGE, such as plasmids, class 1 integrons, and Tn*As1*, in the spread of sulphonamides, aminoglycosides, trimethoprim, macrolides, phenicols, tetracyclines, quaternary ammonium compounds and mercury resistance genes.

Dissemination of resistance determinants can occur in all types of aquaculture's production systems. However, seems to exist a higher risk in integrated multi-trophic aquaculture [138], such as the system where the gilthead seabreams studied in the present thesis came from (excluding gilthead seabream acquired in the market, whose information about the production method we do not have). This production system emerged from the necessity of making aquaculture a more sustainable activity, but also more economically profitable, and consists in the co-cultivation of multiple species, namely a fed species (e.g., gilthead seabream) and an extractive species (e.g., algae and/or bivalve mollusks), that fed on organic residues left by the fed species [138,655]. Other combinations commonly used in these integrated systems are livestock-fish and poultry-fish, where the feces excreted by animals like pigs and chickens are used to fertilize the fishponds, in order to support the growth of photosynthetic organisms that are eaten by fish, thus diminishing the requirement of additional feed. Sometimes, water from the ponds is also repurposed to fertilize lands used in agriculture [656,657]. Although it stimulates a more sustainable and economical use of resources, these integrated multi-trophic systems, if not properly monitored, can be a hazard for public health by promoting an easy exchange of bacteria and resistance genes between different reservoirs [138,657]. A study by Shen et al. [607] analyzed samples from a duck-fish integrated fishery, a slaughterhouse, and a market in the same geographical area and concluded that the data collected suggests

dissemination of colistin resistance *mcr-1* gene (mostly located in IncHI2, IncI2 and IncX4 plasmids, also prevalent in humans) between ducks and fish from the integrated fishery. Together with a previous study by Shen et al. [658], this study revealed a possible transfer of *mcr* genes to humans via aquatic food chain [607]. It has been proposed that wild birds, especially migratory ones, can also play a role in the contamination of aquatic environments, manly through their feces [417,418]. Due to their feeding behavior (sometimes feeding on garbage, sewage and in agricultural areas fertilized with manure), birds can acquire resistant bacteria and/or resistance genes from livestock and human sources [659]. A meta-transcriptomics analysis performed by Marcelino et al. [417] revealed that aquatic birds can be reservoirs of functional resistance genes from several antibiotic classes, such as β-lactams, quinolones, phenicols, macrolides, aminoglycosides and tetracyclines. Migratory birds, due to their ability to travel long distances and cross several continents, may be able to disseminate resistant bacteria and/or resistance genes between different ecosystems [418]. A study by Wu et al. [660], involving egrets' feces, soil samples from the habitat of these birds and water samples from a river nearby, suggest that these birds may be responsible for the spread of ARG from the river to the soil. Among the resistance genes possibly disseminated by these egrets is *mcr-1*, found in egrets' feces and in the water from the river, and located on plasmids IncHI2, IncI2, and IncP1-type [660,661]. A subsequent study with the same *mcr-1*-positive samples and *mcr-1*-containing plasmid sequences available in GenBank database, revealed highly similar *mcr-1*-containing IncI2 plasmids from different areas across migratory routes of shorebirds, namely the East Asian-Australasian Flyway, confirming that migratory birds can play a role in ARG dispersion [661].

Likewise, our WGS analysis in the present thesis revealed an *Enterobacter* sp. harboring an *mcr-9.1* gene, that was further studied in Chapter 8. This strain was identified as *E. ludwigii* belonging to ST1342 (described in this study for the first time; Table S 15) and was isolated from a muscle of a gilthead seabream originated from the integrated multi-trophic aquaculture farm 7. The susceptibility profile analysis revealed resistances to amoxicillin/clavulanic acid, cefoxitin (intrinsic), chloramphenicol, florfenicol and fosfomycin. However, the MIC for colistin was 1 mg/L, in the susceptible category. The *mcr-9.1* gene was integrated in a cassette containing the *rcnR-rcnA-pcoE*-IS*Sgsp1-pcoS*-IS*903-mcr-9-wbuC* operon, located in an IncHI2-ST1 plasmid. This operon is associated with tolerance to copper and nickel homeostasis and is probably the reason why *mcr-9.1* remains in aquaculture environment (copper can be added to fish feed or used as anti-fouling agents in fish cages [510]), since the use of colistin is forbidden in European aquaculture settings [69,296]. Although not linked with colistin resistance

in our study, this gene was associated with several MGE (IncHI2 plasmid, IS*903*, IS*Sgsp1* and IS*26*) that can facilitate its dissemination to other strains that possessed the necessary regulators to induce the activation of *mcr-9.1* transcription. Other possible explanations for the presence of this gene in gilthead seabream can be related to contamination of aquaculture settings from other environments or a different role played by this gene in important functions of the bacterial cell (such as a defense system against natural peptides and/or bacteriophages) [640].

Throughout the studies developed during this thesis several techniques were used, complementing each other, and enabling the exploration of the microbiome and the resistome of aquaculture environments.

In conclusion, there is an exchange of resistant bacteria and/or resistance genes between farmed animals and humans, possibly with aquaculture environments acting as intermediaries. Aquaculture environments can be contaminated with antibiotic resistance genes and/or resistant bacteria originating from other environments (e.g., agriculture, hospital sewage, industry), but it can also be the source of that pollution itself. Aquaculture can contribute to the growth of antibiotic resistance through the heavy implementation of antibiotics but also for the use of disinfectants and heavy metals, responsible for co-selection of resistance genes [232]. Aquaculture can play an important role in feeding the growing world population. But for that to happen, it is necessary to guarantee its safety [662]. Therefore, it is of utmost importance to implement measures to control antibiotic resistance. The primary measure must be the reduction of antibiotic consumption. To achieve this goal, preventive measures must be adopted to avoid diseases and, consequently, the use of antibiotics. Some of those measures can be implemented directly in aquaculture settings: avoiding high density stocks, handling, high temperatures, high or low salinity, restriction of stocks movements (namely between different countries), maintenance of water quality, implementation of good nutritional programs for farmed animals, better depuration methods for bivalve mollusks, surveillance systems and fast and reliable diagnostic tools to allow an early control of diseases [32,662–664]. Registration and information sharing regarding antibiotics used in aquaculture settings, as well as regulations and supervision are indispensable to implement the most suitable measures to control emergence and spread of antibiotic resistance through this reservoir. Also, education and knowledge dissemination among farmers, veterinarians, and general consumers about the consequences of an inappropriate use of antibiotics are needed [75,665].

Likewise, alternatives to antibiotics are being developed, some of them with good results, such as vaccination in salmon farms in Norway, that registered a decrease in antibiotic consumption after the implementation of this method [662,666]. Nevertheless, this method

cannot be applied in mollusks and crustaceans, because they do not develop long-term acquired immunity, nor to young fish since their immune system is not fully developed [666]. Vaccination through injection seems to be the most effective choice, however it can be a laborious work and induce stress in fish [663]. Other alternatives are the use of probiotics, prebiotics, phytobiotics, quorum sensing interference and bacteriophage therapy. Probiotics constituted by live bacteria can compete for nutrients and space, produce antimicrobial compounds (such as bacteriocins and hydrogen peroxide), disrupt quorum sensing or inhibit the expression of VF of pathogenic bacteria, preventing their development, although they can acquire antibiotic resistance genes through HGT. These substances are also responsible for the regulation of pro- and anti-inflammatory cytokines, important for immune cells' functions. On the other hand, prebiotics consist in nondigestible food ingredients that stimulate the multiplication of favorable bacteria, like probiotics, therefore having an impact in the composition of microbiota of the gastrointestinal tract. Phytobiotics are plant extracts (such as essential oils) that can also have anti-inflammatory, antiparasitic, antimicrobial and antioxidative properties, having already shown positive results against *S. aureus*, *E. coli* and *Vibrio* spp. Both probiotics and phytobiotics can interfere with quorum sensing, a cell-to-cell communication system involved in biofilm formation and VF production. A promising alternative to the use of antibiotics is phage therapy, where lytic bacteriophages are used to lyse specific bacteria (species or strain), not affecting commensal bacteria. Nonetheless, bacteria can develop resistance to bacteriophages, and these may be involved in HGT of ARG and/or VF through transduction [136,666].

Outside aquaculture settings additional measures can be employed to avoid cross-contamination between environments, such as a more effective treatment of agricultural, industrial, livestock, hospital, and municipal wastewaters to eliminate antibiotic residues, as well as ARG and resistant bacteria, and hyperthermophilic composting of manure before use (that can eliminate not only pathogens but also antibiotics like fluoroquinolones, tetracyclines and sulfonamides) [428,665].

Food-producing animals can travel through several regions and countries before reaching the final consumer. Antibiotic resistance knows no geographic borders, thus is fundamental national and international cooperations.

In the future, new research concerning clinical breakpoints and ECOFFs (epidemiological cut-off values) for bacterial species commonly found in aquaculture, as well as for antibiotics used in these settings is needed. Nowadays, quite few clinical breakpoints and ECOFFs are available. In order to prescribe the most appropriate antibiotic (as well as dose regiment) and to understand the real status of antibiotic resistance in aquaculture farms, a correct antibiotic

susceptibility testing must be performed to the bacteria isolated in farmed animals [667]. Bacteria have inhabited this planet for millions of years, developing an extraordinary adaptability and resilience. Humanity must invest in the investigation of the bacterial mechanisms of resistance to antibiotics and in the understanding of their routes of dissemination in the different environments to be able to develop new antibiotics and effective prevention measures to control antibiotic resistance, if we want to keep up with bacteria in this war that has been going on for several decades.

BIBLIOGRAPHY

1. Food and Agricultural Organization (FAO) Aquaculture topics and activities. Aquaculture. Available online: http://www.fao.org/fishery/ (accessed on Oct 29, 2020).
2. Ottinger, M.; Clauss, K.; Kuenzer, C. Aquaculture: Relevance, distribution, impacts and spatial assessments - A review. *Ocean Coast. Manag.* **2016**, *119*, 244–266, doi:10.1016/j.ocecoaman.2015.10.015.
3. Topp, E.; Larsson, D.G.J.; Miller, D.N.; Van den Eede, C.; Virta, M.P.J. Antimicrobial resistance and the environment: Assessment of advances, gaps and recommendations for agriculture, aquaculture and pharmaceutical manufacturing. *FEMS Microbiol. Ecol.* **2018**, *94*, fix185, doi:10.1093/femsec/fix185.
4. Gaspar, P.; Pires, I.; Magalhães, A.; Lda, C. *Boas práticas em cultivo de ostra - Algarve*; APA/ARH do Algarve: Faro, Portugal, 2017; ISBN 978-972-8577-70-4.
5. Liu, X.; Steele, J.C.; Meng, X.Z. Usage, residue, and human health risk of antibiotics in Chinese aquaculture: A review. *Environ. Pollut.* **2017**, *223*, 161–169, doi:10.1016/j.envpol.2017.01.003.
6. Food and Agricultural Organization (FAO) *The State of World Fisheries and Aquaculture 2020. Sustainability in action.*; FAO: Rome, Italy, 2020; doi:10.4060/ca9229en.
7. Food and Agricultural Organization (FAO) *The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation*; FAO: Rome, Italy, 2022; doi:10.4060/cc0461en.
8. Rocha, C.P.; Cabral, H.N.; Marques, J.C.; Gonçalves, A.M.M. A Global Overview of Aquaculture Food Production with a Focus on the Activity's Development in Transitional Systems—The Case Study of a South European Country (Portugal). *J. Mar. Sci. Eng.* **2022**, *10*, 417, doi:10.3390/jmse10030417.
9. Araújo, J.; Soares, F.; Pousão-Ferreira, P. Offshore Production of Mediterranean Mussels in Southern Portugal. *World Aquac.* **2018**, *49*, 55–57. Available online: https://www.was.org/Magazine/Vol/49/2#.X5VTYIj7RPY (accessed on Oct 25, 2020).
10. Direção-Geral de Recursos Naturais Segurança e Serviços Marítimos *Plano Estratégico para a Aquicultura Portuguesa 2014-2020*; Direção-Geral de Recursos Naturais Segurança e Serviços Marítimos: Lisbon, Portugal, 2013.
11. Instituto Nacional de Estatística *Estatísticas da Pesca: 2021*; Instituto Nacional de Estatística: Lisbon, Portugal, 2022; ISBN: 9789892506029.
12. Martins, R.; Carneiro, M. *Manual de identificação de peixes ósseos da costa continental portuguesa - Principais característica Diagnosticantes*; Portuguese Institute for the Sea and Atmosphere: Lisbon, Portugal, 2018; ISBN 9789729083198.

13. Colloca, F.; Cerasi, S. Cultured Aquatic Species Information Programme. *Sparus aurata*. Available online: http://www.fao.org/fishery/culturedspecies/Sparus_aurata/en (accessed on Jan 6, 2021).
14. Portuguese Institute for Sea and Atmosphere Dourada - *Sparus aurata*. Available online: http://www.ipma.pt/pt/pescas/eppo/dourada/index.jsp (accessed on Jan 6, 2021).
15. Portuguese Institute for Sea and Atmosphere Estação Piloto de Piscicultura de Olhão. Available online: http://www.ipma.pt/pt/pescas/eppo/ (accessed on Feb 12, 2021).
16. Müller, J. *Sparus aurata* escapes from offshore fish farms around Madeira, Universidade do Algarve, 2019. Available online: http://hdl.handle.net/10400.1/14892 (accessed on Sep 10, 2021).
17. Elbashir, S.; Parveen, S.; Schwarz, J.; Rippen, T.; Jahncke, M.; DePaola, A. Seafood pathogens and information on antimicrobial resistance: A review. *Food Microbiol.* **2018**, *70*, 85–93, doi:10.1016/j.fm.2017.09.011.
18. Anacleto, P.; Maulvault, A.L.; Barbosa, V.; Nunes, M.L.; Marques, A. Shellfish: Characteristics of Crustaceans and Mollusks. In *Encyclopedia of Food and Health*; Caballero, B., Finglas, P.M., Toldrá, F., Eds.; Academic Press, 2016; pp. 764–771 ISBN 9780123849533.
19. Goulletquer, P. Cultured Aquatic Species Information Programme. *Mytilus edulis*. Available online: http://www.fao.org/fishery/culturedspecies/Mytilus_edulis/en (accessed on Aug 13, 2020).
20. Figueras, A. Cultured Aquatic Species Information Programme. *Mytilus galloprovincialis*. Available online: http://www.fao.org/fishery/culturedspecies/Mytilus_galloprovincialis/en (accessed on Feb 8, 2021).
21. Chiesa, L.M.; Nobile, M.; Malandra, R.; Panseri, S.; Arioli, F. Occurrence of antibiotics in mussels and clams from various FAO areas. *Food Chem.* **2018**, *240*, 16–23, doi:10.1016/j.foodchem.2017.07.072.
22. Shukla, P. Will Mussel Aquaculture Be Viable Along The California Coast? Available online: https://marinescience.ucdavis.edu/blog/will-mussel-aquaculture-be-viable-along-california-coast (accessed on Feb 12, 2021).
23. Craeymeersch, J.A.; Jansen, H.M. Bivalve Assemblages as Hotspots for Biodiversity. In *Goods and Services of Marine Bivalves*; Smaal, A.C., Ferreira, J.G., Grant, J., Petersen, J.K., Strand, Ø., Eds.; Springer: Halifax, 2019; pp. 275–294 ISBN 9783319967769.
24. Kamermans, P.; Capelle, J.J. Provisioning of Mussel Seed and Its Efficient Use in Culture. In *Goods and Services of Marine Bivalves*; Smaal, A.C., Ferreira, J.G., Grant, J., Petersen, J.K., Strand, Ø., Eds.; Springer: Halifax, 2019; pp. 27–49 ISBN 978-3-319-96775-2.
25. Noor, N.M.; Nursyam, H.; Widodo, M.S.; Risjani, Y. Biological aspects of green mussels *Perna viridis* cultivated on raft culture in Pasaran coastal waters, Indonesia. *AACL Bioflux* **2019**, *12*, 448–456. Available online: http://www.bioflux.com.ro/docs/2019.448-456.pdf (accessed on Sep 10, 2021).
26. Helm, M.M. Cultured Aquatic Species Information Programme. *Crassostrea gigas*. Available online: http://www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en (accessed on Feb 10, 2021).
27. Lu, C. The Different Methods of Growing Oysters. Available online: https://www.pangeashellfish.com/blog/the-different-methods-of-growing-oysters

(accessed on Feb 15, 2021).
28. Figueras, A. Cultured Aquatic Species Information Programme. *Ruditapes decussatus*. Available online: http://www.fao.org/fishery/culturedspecies/Ruditapes_decussatus/en (accessed on Aug 13, 2020).
29. FAO Fisheries and Aquaculture Department Species Fact Sheets. *Ruditapes decussatus* (Linnaeus, 1758). Available online: http://www.fao.org/fishery/species/3542/en (accessed on Feb 11, 2021).
30. Pękala-Safińska, A. Contemporary threats of bacterial infections in freshwater fish. *J. Vet. Res.* **2018**, *62*, 261–267, doi:10.2478/jvetres-2018-0037.
31. Lafferty, K.D.; Harvell, C.D.; Conrad, J.M.; Friedman, C.S.; Kent, M.L.; Kuris, A.M.; Powell, E.N.; Rondeau, D.; Saksida, S.M. Infectious diseases affect marine fisheries and aquaculture economics. *Ann. Rev. Mar. Sci.* **2015**, *7*, 471–496, doi:10.1146/annurev-marine-010814-015646.
32. Zannella, C.; Mosca, F.; Mariani, F.; Franci, G.; Folliero, V.; Galdiero, M.; Tiscar, P.G.; Galdiero, M. Microbial Diseases of Bivalve Mollusks: Infections, Immunology and Antimicrobial Defense. *Mar. Drugs* **2017**, *15*, 182, doi:10.3390/md15060182.
33. Kousar, R.; Shafi, N.; Andleeb, S.; Ali, N.M.; Akhtar, T.; Khalid, S. Assessment and incidence of fish associated bacterial pathogens at hatcheries of Azad Kashmir, Pakistan. *Brazilian J. Biol.* **2020**, *80*, 607–614, doi:10.1590/1519-6984.217435.
34. Defoirdt, T.; Sorgeloos, P.; Bossier, P. Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Curr. Opin. Microbiol.* **2011**, *14*, 251–258, doi:10.1016/j.mib.2011.03.004.
35. de Bruijn, I.; Liu, Y.; Wiegertjes, G.F.; Raaijmakers, J.M. Exploring fish microbial communities to mitigate emerging diseases in aquaculture. *FEMS Microbiol. Ecol.* **2018**, *94*, fix161, doi:10.1093/femsec/fix161.
36. Gauthier, D.T. Bacterial zoonoses of fishes: A review and appraisal of evidence for linkages between fish and human infections. *Vet. J.* **2015**, *203*, 27–35, doi:10.1016/j.tvjl.2014.10.028.
37. Matanza, X.M.; Osorio, C.R. Exposure of the Opportunistic Marine Pathogen *Photobacterium damselae* subsp. *damselae* to Human Body Temperature Is a Stressful Condition That Shapes the Transcriptome, Viability, Cell Morphology, and Virulence. *Front. Microbiol.* **2020**, *11*, 1771, doi:10.3389/fmicb.2020.01771.
38. Chiu, T.H.; Kao, L.Y.; Chen, M.L. Antibiotic resistance and molecular typing of *Photobacterium damselae* subsp. *damselae*, isolated from seafood. *J. Appl. Microbiol.* **2012**, *114*, 1184–1192, doi:10.1111/jam.12104.
39. Andreoni, F.; Magnani, M. Photobacteriosis: Prevention and diagnosis. *J. Immunol. Res.* **2014**, *2014*, 793817, doi:10.1155/2014/793817.
40. Osorio, C.R.; Vences, A.; Matanza, X.M.; Terceti, M.S. *Photobacterium damselae* subsp. *damselae*, a Generalist Pathogen with Unique Virulence Factors and High Genetic Diversity. *J. Bacteriol.* **2018**, *200*, e00002-18, doi:10.1128/JB.00002-18.
41. Fernández-Bravo, A.; Figueras, M.J. An update on the genus *Aeromonas*: Taxonomy, epidemiology, and pathogenicity. *Microorganisms* **2020**, *8*, 129, doi:10.3390/microorganisms8010129.
42. McCoy, E.; Morrison, J.; Cook, V.; Johnston, J.; Eblen, D.; Guo, C. Foodborne Agents Associated with the Consumption of Aquaculture Catfish. *J. Food Prot.* **2011**, *74*, 500–

516, doi:10.4315/0362-028X.JFP-10-341.
43. Walczak, N.; Puk, K.; Guz, L. Bacterial flora associated with diseased freshwater ornamental fish. *J. Vet. Res.* **2017**, *61*, 445–449, doi:10.1515/jvetres-2017-0070.
44. Silby, M.W.; Winstanley, C.; Godfrey, S.A.C.; Levy, S.B.; Jackson, R.W. *Pseudomonas* genomes: Diverse and adaptable. *FEMS Microbiol. Rev.* **2011**, *35*, 652–680, doi:10.1111/j.1574-6976.2011.00269.x.
45. Wong, M.H.Y.; Chan, E.W.C.; Chen, S. Isolation of carbapenem-resistant *Pseudomonas* spp. from food. *J. Glob. Antimicrob. Resist.* **2015**, *3*, 109–114, doi:10.1016/j.jgar.2015.03.006.
46. Leung, K.Y.; Wang, Q.; Yang, Z.; Siame, B.A. *Edwardsiella piscicida*: A versatile emerging pathogen of fish. *Virulence* **2019**, *10*, 555–567, doi:10.1080/21505594.2019.1621648.
47. Buján, N.; Toranzo, A.E.; Magariños, B. *Edwardsiella piscicida*: A significant bacterial pathogen of cultured fish. *Dis. Aquat. Organ.* **2018**, *131*, 59–71, doi:10.3354/dao03281.
48. Rösch, R.M.; Buschmann, K.; Brendel, L.; Schwanz, T.; Vahl, C.-F. *Lactococcus garvieae* Endocarditis in a Prosthetic Aortic Valve: A Case Report and Literature Review. *J. Investig. Med. High Impact Case Reports* **2019**, *7*, 2324709619832052, doi:10.1177/2324709619832052.
49. Ortega, C.; Irgang, R.; Valladares-Carranza, B.; Collarte, C.; Avendaño-Herrera, R. First identification and characterization of *Lactococcus garvieae* isolated from rainbow trout (*Oncorhynchus mykiss*) cultured in Mexico. *Animals* **2020**, *10*, 1609, doi:10.3390/ani10091609.
50. Bayliss, S.C.; Verner-Jeffreys, D.W.; Bartie, K.L.; Aanensen, D.M.; Sheppard, S.K.; Adams, A.; Feil, E.J. The promise of whole genome pathogen sequencing for the molecular epidemiology of emerging aquaculture pathogens. *Front. Microbiol.* **2017**, *8*, 121, doi:10.3389/fmicb.2017.00121.
51. López-Campos, G.; Aguado-Urda, M.; Blanco, M.M.; Gibello, A.; Cutuli, M.T.; López-Alonso, V.; Martín-Sánchez, F.; Fernández-Garayzábal, J.F. *Lactococcus garvieae*: a small bacteria and a big data world. *Heal. Inf. Sci. Syst.* **2015**, *3*, S5, doi:10.1186/2047-2501-3-s1-s5.
52. World Health Organization (WHO) *WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015.*; WHO: Geneva, Switzerland, 2015; ISBN: 9789241565165.
53. European Food Safety Authority (EFSA); European Centre for Disease Prevention and Control (ECDC) The European Union One Health 2021 Zoonoses Report. *EFSA J.* **2022**, *20*, 7666, doi:10.2903/j.efsa.2022.7666.
54. Chlebicz, A.; Śliżewska, K. Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. *Int. J. Environ. Res. Public Health* **2018**, *15*, 863, doi:10.3390/ijerph15050863.
55. Novotny, L.; Dvorska, L.; Lorencova, A.; Beran, V.; Pavlik, I. Fish: A potential source of bacterial pathogens for human beings. *Vet. Med. (Praha).* **2004**, *49*, 343–358, doi:10.17221/5715-VETMED.
56. Uddin, G.M.N.; Larsen, M.H.; Barco, L.; Phu, T.M.; Dalsgaard, A. Clonal occurrence of *Salmonella* Weltevreden in cultured shrimp in the mekong delta, Vietnam. *PLoS One* **2015**, *10*, e0134252, doi:10.1371/journal.pone.0134252.
57. Naser, A.A.H. Special biochemical profiles of *Escherichia coli* strains isolated from

humans and camels by the VITEK 2 automated system in Al-Ahsa, Saudi Arabia. *African J. Microbiol. Res.* **2016**, *10*, 783–790, doi:10.5897/ajmr2016.8047.
58. Jang, J.; Hur, H.G.; Sadowsky, M.J.; Byappanahalli, M.N.; Yan, T.; Ishii, S. Environmental *Escherichia coli*: ecology and public health implications—a review. *J. Appl. Microbiol.* **2017**, *123*, 570–581, doi:10.1111/jam.13468.
59. Hassan, R.; Seelman, S.; Peralta, V.; Booth, H.; Tewell, M.; Melius, B.; Whitney, B.; Sexton, R.; Dwarka, A.; Vugia, D.; et al. A Multistate Outbreak of *E. coli* O157:H7 Infections Linked to Soy Nut Butter. *Pediatrics* **2019**, *144*, e20183978, doi:10.1542/peds.2018-3978.
60. Lagerqvist, N.; Löf, E.; Enkirch, T.; Nilsson, P.; Roth, A.; Jernberg, C. Outbreak of gastroenteritis highlighting the diagnostic and epidemiological challenges of enteroinvasive *Escherichia coli*, County of Halland, Sweden, November 2017. *Eurosurveillance* **2020**, *25*, 1900466, doi:10.2807/1560-7917.ES.2020.25.9.1900466.
61. Ribeiro, L.F.; Barbosa, M.M.C.; de Rezende Pinto, F.; Guariz, C.S.L.; Maluta, R.P.; Rossi, J.R.; Rossi, G.A.M.; Lemos, M.V.F.; do Amaral, L.A. Shiga toxigenic and enteropathogenic *Escherichia coli* in water and fish from pay-to-fish ponds. *Lett. Appl. Microbiol.* **2015**, *62*, 216–220, doi:10.1111/lam.12536.
62. Yang, S.C.; Lin, C.H.; Aljuffali, I.A.; Fang, J.Y. Current pathogenic *Escherichia coli* foodborne outbreak cases and therapy development. *Arch. Microbiol.* **2017**, *199*, 811–825, doi:10.1007/s00203-017-1393-y.
63. Silva, V.; Capelo, J.L.; Igrejas, G.; Poeta, P. Molecular epidemiology of *Staphylococcus aureus* lineages in wild animals in europe: A review. *Antibiotics* **2020**, *9*, 122, doi:10.3390/antibiotics9030122.
64. Sergelidis, D.; Angelidis, A.S. Methicillin-resistant *Staphylococcus aureus*: a controversial food-borne pathogen. *Lett. Appl. Microbiol.* **2017**, *64*, 409–418, doi:10.1111/lam.12735.
65. Vaiyapuri, M.; Joseph, T.C.; Rao, B.M.; Lalitha, K.V.; Prasad, M.M. Methicillin-Resistant *Staphylococcus aureus* in Seafood: Prevalence, Laboratory Detection, Clonal Nature, and Control in Seafood Chain. *J. Food Sci.* **2019**, *84*, 3341–3351, doi:10.1111/1750-3841.14915.
66. Gastalho, S.; Silva, G.J.; Ramos, F. Antibiotics in aquaculture and bacterial resistance: Health care impact. *Acta Farm. Port.* **2014**, *3*, 29–45, doi:10.13140/2.1.4697.0560.
67. Done, H.Y.; Venkatesan, A.K.; Halden, R.U. Does the Recent Growth of Aquaculture Create Antibiotic Resistance Threats Different from those Associated with Land Animal Production in Agriculture? *AAPS J.* **2015**, *17*, 513–524, doi:10.1208/s12248-015-9722-z.
68. Rodgers, C.J.; Furones, M.D. Antimicrobial agents in aquaculture: practice, needs and issues. *Options Méditerranéennes Série A. Séminaires Méditerranéens* **2009**, 41–59, doi:10.13140/2.1.4697.0560.
69. Santos, L.; Ramos, F. Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. *Int. J. Antimicrob. Agents* **2018**, *52*, 135–143, doi:10.1016/j.ijantimicag.2018.03.010.
70. Federation of Veterinarians of Europe *Antimicrobial Use in Food Producing Animals*; Federation of Veterinarians of Europe: Brussels, Belgium, 2016. Available online: 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 on Sep 10, 2021).
71. European Medicines Agency (EMA) *Sales of veterinary antimicrobial agents in 31*

European countries in 2021; Publications Office of the European Union: Amsterdam, Netherlands, 2022; doi:10.2809/39517.
72. Watts, J.E.M.; Schreier, H.J.; Lanska, L.; Hale, M.S. The rising tide of antimicrobial resistance in aquaculture: Sources, sinks and solutions. *Mar. Drugs* **2017**, *15*, 158, doi:10.3390/md15060158.
73. Cabello, F.C.; Godfrey, H.P.; Buschmann, A.H.; Dölz, H.J. Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance. *Lancet Infect. Dis.* **2016**, *16*, e127–e133, doi:10.1016/S1473-3099(16)00100-6.
74. Henriksson, P.J.G.; Rico, A.; Troell, M.; Klinger, D.H.; Buschmann, A.H.; Saksida, S.; Chadag, M. V.; Zhang, W. Unpacking factors influencing antimicrobial use in global aquaculture and their implication for management: a review from a systems perspective. *Sustain. Sci.* **2018**, *13*, 1105–1120, doi:10.1007/s11625-017-0511-8.
75. Carvalho, I.T.; Santos, L. Antibiotics in the aquatic environments: A review of the European scenario. *Environ. Int.* **2016**, *94*, 736–757, doi:10.1016/j.envint.2016.06.025.
76. Cabello, F.C.; Godfrey, H.P.; Tomova, A.; Ivanova, L.; Dölz, H.; Millanao, A.; Buschmann, A.H. Antimicrobial use in aquaculture re-examined: Its relevance to antimicrobial resistance and to animal and human health. *Environ. Microbiol.* **2013**, *15*, 1917–1942, doi:10.1111/1462-2920.12134.
77. Han, Q.F.; Zhao, S.; Zhang, X.R.; Wang, X.L.; Song, C.; Wang, S.G. Distribution, combined pollution and risk assessment of antibiotics in typical marine aquaculture farms surrounding the Yellow Sea, North China. *Environ. Int.* **2020**, *138*, 105551, doi:10.1016/j.envint.2020.105551.
78. O'Neill, J. *Review on Antimicrobial Resistance. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations*; London, United Kingdom, 2014; Available online: https://amr-review.org/Publications.html (accessed on 12 April 2021).
79. Hutchings, M.; Truman, A.; Wilkinson, B. Antibiotics: past, present and future. *Curr. Opin. Microbiol.* **2019**, *51*, 72–80, doi:10.1016/j.mib.2019.10.008.
80. World Health Organization (WHO) *WHO guidelines on use of medically important antimicrobials in food-producing animals*; WHO: Geneva, Switzerland, 2017; ISBN 9241550139.
81. Crofts, T.S.; Gasparrini, A.J.; Dantas, G. Next-generation approaches to understand and combat the antibiotic resistome. *Nat. Rev. Microbiol.* **2017**, *15*, 422–434, doi:10.1038/nrmicro.2017.28.
82. Bonomo, R.A. β-Lactamases: A Focus on Current Challenges. *Cold Spring Harb. Perspect. Med.* **2017**, *7*, a025239, doi:10.1101/cshperspect.a025239.
83. Drawz, S.M.; Bonomo, R.A. Three Decades of β-Lactamase Inhibitors. *Clin. Microbiol. Rev.* **2010**, *23*, 160–201, doi:10.1128/CMR.00037-09.
84. Díez-aguilar, M.; Cantón, R. New microbiological aspects of fosfomycin. *Spanish J. Chemother.* **2019**, *32 Suppl 1*, 8–18. PMID: 31131587.
85. Falagas, M.E.; Athanasaki, F.; Voulgaris, G.L.; Triarides, N.A.; Vardakas, K.Z. Resistance to fosfomycin: Mechanisms, Frequency and Clinical Consequences. *Int. J. Antimicrob. Agents* **2019**, *53*, 22–28, doi:10.1016/j.ijantimicag.2018.09.013.
86. Zeng, D.; Debabov, D.; Hartsell, T.L.; Cano, R.J.; Adams, S.; Schuyler, J.A.; McMillan, R.; Pace, J.L. Approved glycopeptide antibacterial drugs: Mechanism of action and

resistance. *Cold Spring Harb. Perspect. Med.* **2016**, *6*, a026989, doi:10.1101/cshperspect.a026989.
87. Allison, D.G.; Lambert, P.A. *Modes of Action of Antibacterial Agents*; Elsevier Ltd, 2015; ISBN 9780123971692.
88. Miller, W.R.; Bayer, A.S.; Arias, C.A. Mechanism of action and resistance to daptomycin in *Staphylococcus aureus* and *Enterococci*. *Cold Spring Harb. Perspect. Med.* **2016**, *6*, a026997, doi:10.1101/cshperspect.a026997.
89. Aghapour, Z.; Gholizadeh, P.; Ganbarov, K.; Bialvaei, A.Z.; Mahmood, S.S.; Tanomand, A.; Yousefi, M.; Asgharzadeh, M.; Yousefi, B.; Kafil, H.S. Molecular mechanisms related to colistin resistance in *Enterobacteriaceae*. *Infect. Drug Resist.* **2019**, *12*, 965–975, doi:10.2147/IDR.S199844.
90. El-Sayed Ahmed, M.A.E.G.; Zhong, L.L.; Shen, C.; Yang, Y.; Doi, Y.; Tian, G.B. Colistin and its role in the Era of antibiotic resistance: an extended review (2000–2019). *Emerg. Microbes Infect.* **2020**, *9*, 868–885, doi:10.1080/22221751.2020.1754133.
91. Correia, S.; Poeta, P.; Hébraud, M.; Capelo, J.L.; Igrejas, G. Mechanisms of quinolone action and resistance: where do we stand? *J. Med. Microbiol.* **2017**, *66*, 551–559, doi:10.1099/jmm.0.000475.
92. Yanat, B.; Rodríguez-Martínez, J.M.; Touati, A. Plasmid-mediated quinolone resistance in *Enterobacteriaceae*: a systematic review with a focus on Mediterranean countries. *Eur. J. Clin. Microbiol. Infect. Dis.* **2017**, *36*, 421–435, doi:10.1007/s10096-016-2847-x.
93. Bush, N.G.; Diez-Santos, I.; Abbott, L.R.; Maxwell, A. Quinolones: Mechanism, Lethality and Their Contributions to Antibiotic Resistance. *Molecules* **2020**, *25*, 5662, doi:10.3390/molecules25235662.
94. Cai, X.C.; Xi, H.; Liang, L.; Liu, J.D.; Liu, C.H.; Xue, Y.R.; Yu, X.Y. Rifampicin-resistance mutations in the *rpoB* gene in *Bacillus velezensis* CC09 have pleiotropic effects. *Front. Microbiol.* **2017**, *8*, 178, doi:10.3389/fmicb.2017.00178.
95. Tupin, A.; Gualtieri, M.; Roquet-Banères, F.; Morichaud, Z.; Brodolin, K.; Leonetti, J.P. Resistance to rifampicin: At the crossroads between ecological, genomic and medical concerns. *Int. J. Antimicrob. Agents* **2010**, *35*, 519–523, doi:10.1016/j.ijantimicag.2009.12.017.
96. Krause, K.M.; Serio, A.W.; Kane, T.R.; Connolly, L.E. Aminoglycosides: An Overview. *Cold Spring Harb. Perspect. Med.* **2016**, *6*, a027029, doi:10.1101/cshperspect.a027029.
97. Schwarz, S.; Shen, J.; Kadlec, K.; Wang, Y.; Michael, G.B.; Feßler, A.T.; Vester, B. Lincosamides, streptogramins, phenicols, and pleuromutilins: Mode of action and mechanisms of resistance. *Cold Spring Harb. Perspect. Med.* **2016**, *6*, a027037, doi:10.1101/cshperspect.a027037.
98. Ero, R.; Kumar, V.; Su, W.; Gao, Y.G. Ribosome protection by ABC-F proteins—Molecular mechanism and potential drug design. *Protein Sci.* **2019**, *28*, 684–693, doi:10.1002/pro.3589.
99. Hashemian, S.M.R.; Farhadi, T.; Ganjparvar, M. Linezolid: A review of its properties, function, and use in critical care. *Drug Des. Devel. Ther.* **2018**, *12*, 1759–1767, doi:10.2147/DDDT.S164515.
100. Grossman, T.H. Tetracycline antibiotics and resistance. *Cold Spring Harb. Perspect. Med.* **2016**, *6*, a025387, doi:10.1101/cshperspect.a025387.
101. Khoshnood, S.; Heidary, M.; Asadi, A.; Soleimani, S.; Motahar, M.; Savari, M.; Saki, M.;

Abdi, M. A review on mechanism of action, resistance, synergism, and clinical implications of mupirocin against *Staphylococcus aureus*. *Biomed. Pharmacother.* **2019**, *109*, 1809–1818, doi:10.1016/j.biopha.2018.10.131.
102. Williamson, D.A.; Carter, G.P.; Howden, B.P. Current and Emerging Topical Antibacterials and Antiseptics: Agents, Action, and Resistance Patterns. *Clin. Microbiol. Rev.* **2017**, *30*, 827–860, doi:10.1128/CMR.00112-16.
103. Sierra, Y.; Tubau, F.; González-Díaz, A.; Carrera-Salinas, A.; Moleres, J.; Bajanca-Lavado, P.; Garmendia, J.; Domínguez, M.Á.; Ardanuy, C.; Martí, S. Assessment of trimethoprim-sulfamethoxazole susceptibility testing methods for fastidious *Haemophilus* spp. *Clin. Microbiol. Infect.* **2020**, *26*, 944.e1-944.e7, doi:10.1016/j.cmi.2019.11.022.
104. World Health Organization (WHO) *Critically Important Antimicrobials for Human Medicine*; 6th ed.; WHO: Geneva, Switzerland, 2018; ISBN 978-92-4-151552-8.
105. Reygaert, W.C. An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiol.* **2018**, *4*, 482–501, doi:10.3934/microbiol.2018.3.482.
106. Bayer, A.S.; Schneider, T.; Sahl, H. Mechanisms of daptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall. *Ann. N. Y. Acad. Sci.* **2013**, *1277*, 139–158, doi:10.1111/j.1749-6632.2012.06819.x.
107. Gómez Casanova, N.; Siller Ruiz, M.; Muñoz Bellido, J.L. Mechanisms of resistance to daptomycin in *Staphylococcus aureus*. *Rev. Española Quimioter.* **2017**, *30*, 391–396.
108. Ramirez, M.S.; Tolmasky, M.E. Aminoglycoside modifying enzymes. *Drug Resist. Updat.* **2010**, *13*, 151–171, doi:10.1016/j.drup.2010.08.003.
109. Fernández, M.; Conde, S.; de la Torre, J.; Molina-Santiago, C.; Ramos, J.-L.; Duque, E. Mechanisms of resistance to chloramphenicol in *Pseudomonas putida* KT2440. *Antimicrob. Agents Chemother.* **2012**, *56*, 1001–1009, doi:10.1128/AAC.05398-11.
110. Wróbel, A.; Arciszewska, K.; Maliszewski, D.; Drozdowska, D. Trimethoprim and other nonclassical antifolates an excellent template for searching modifications of dihydrofolate reductase enzyme inhibitors. *J. Antibiot. (Tokyo).* **2020**, *73*, 5–27, doi:10.1038/s41429-019-0240-6.
111. Azmi, K.; Qrei, W.; Abdeen, Z. Screening of genes encoding adhesion factors and biofilm production in methicillin resistant strains of *Staphylococcus aureus* isolated from Palestinian patients. *BMC Genomics* **2019**, *20*, 578, doi:10.1186/s12864-019-5929-1.
112. Anderson, A.C.; Jonas, D.; Huber, I.; Karygianni, L.; Wölber, J.; Hellwig, E.; Arweiler, N.; Vach, K.; Wittmer, A.; Al-ahmad, A. *Enterococcus faecalis* from Food, Clinical Specimens, and Oral Sites: Prevalence of Virulence Factors in Association with Biofilm Formation. *Front. Microbiol.* **2016**, *6*, 1534, doi:10.3389/fmicb.2015.01534.
113. Pang, Z.; Raudonis, R.; Glick, B.R.; Lin, T.J.; Cheng, Z. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnol. Adv.* **2019**, *37*, 177–192, doi:10.1016/j.biotechadv.2018.11.013.
114. Wang, G.; Zhao, G.; Chao, X.; Xie, L.; Wang, H. The characteristic of virulence, biofilm and antibiotic resistance of *Klebsiella pneumoniae*. *Int. J. Environ. Res. Public Health* **2020**, *17*, 6278, doi:10.3390/ijerph17176278.
115. Sharma, G.; Sharma, S.; Sharma, P.; Chandola, D.; Dang, S.; Gupta, S.; Gabrani, R. *Escherichia coli* biofilm: development and therapeutic strategies. *J. Appl. Microbiol.* **2016**, *121*, 309–319, doi:10.1111/jam.13078.
116. Guérillot, R.; da Silva, A.G.; Monk, I.; Giulieri, S.; Tomita, T.; Alison, E.; Porter, J.; Pidot, S.;

Gao, W.; Peleg, A.Y.; et al. Convergent Evolution Driven by Rifampin Exacerbates the Global Burden of Drug-Resistant *Staphylococcus aureus*. *mSphere* **2018**, *3*, e00550-17, doi:10.1128/mSphere.00550-17.
117. Mendes, R.E.; Deshpande, L.M.; Jones, R.N. Linezolid update: Stable in vitro activity following more than a decade of clinical use and summary of associated resistance mechanisms. *Drug Resist. Updat.* **2014**, *17*, 1–12, doi:10.1016/j.drup.2014.04.002.
118. Mąka, Ł.; Maćkiw, E.; Ścieżyńska, H.; Modzelewska, M.; Popowska, M. Resistance to Sulfonamides and Dissemination of sul Genes Among *Salmonella* spp. Isolated from Food in Poland. *Foodborne Pathog. Dis.* **2015**, *12*, 383–389, doi:10.1089/fpd.2014.1825.
119. Shirshikova, T. V.; Sierra-Bakhshi, C.G.; Kamaletdinova, L.K.; Matrosova, L.E.; Khabipova, N.N.; Evtugyn, V.G.; Khilyas, I. V.; Danilova, I. V.; Mardanova, A.M.; Sharipova, M.R.; et al. The ABC-Type Efflux Pump MacAB Is Involved in Protection of *Serratia marcescens* against Aminoglycoside Antibiotics, Polymyxins, and Oxidative Stress. *mSphere* **2021**, *6*, e00033-21, doi:10.1128/msphere.00033-21.
120. Beceiro, A.; Tomás, M.; Bou, G. Antimicrobial Resistance and Virulence: a Successful or Deleterious Association in the Bacterial World? *Clin. Microbiol. Rev.* **2013**, *26*, 185–230, doi:10.1128/CMR.00059-12.
121. Partridge, S.R.; Kwong, S.M.; Firth, N.; Jensen, S.O. Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.* **2018**, *31*, e00088-17, doi:10.1128/CMR.00088-17.
122. Alibayov, B.; Baba-Moussa, L.; Sina, H.; Zdeňková, K.; Demnerová, K. *Staphylococcus aureus* mobile genetic elements. *Mol. Biol. Rep.* **2014**, *41*, 5005–5018, doi:10.1007/s11033-014-3367-3.
123. Schleinitz, K.M.; Vallaeys, T.; Kleinsteuber, S. Structural characterization of IS*CR8*, IS*CR22*, and IS*CR23*, subgroups of IS*91*-like insertion elements. *Antimicrob. Agents Chemother.* **2010**, *54*, 4321–4328, doi:10.1128/AAC.00006-10.
124. Escudero, J.A.; Loot, C.; Nivina, A.; Mazel, D. The integron: Adaptation on demand. *Microbiol. Spectr.* **2015**, *3*, MDNA3-0019–2014, doi:10.1128/microbiolspec.MDNA3-0019-2014.
125. Stalder, T.; Barraud, O.; Casellas, M.; Dagot, C.; Ploy, M.-C. Integron involvement in environmental spread of antibiotic resistance. *Front. Microbiol.* **2012**, *3*, 119, doi:10.3389/fmicb.2012.00119.
126. Smillie, C.; Garcillan-Barcia, M.P.; Francia, M. V.; Rocha, E.P.C.; de la Cruz, F. Mobility of Plasmids. *Microbiol. Mol. Biol. Rev.* **2010**, *74*, 434–452, doi:10.1128/MMBR.00020-10.
127. Elena, A.; Quinteros, M.; Di Conza, J.; Gutkind, G.; Cejas, D.; Radice, M.A. Full characterization of an IncR plasmid harboring *qnrS1* recovered from a VIM-11-producing *Pseudomonas aeruginosa*. *Rev. Argent. Microbiol.* **2020**, *52*, 298–304, doi:10.1016/j.ram.2019.12.001.
128. Modi, S.R.; Collins, J.J.; Relman, D.A. Antibiotics and the gut microbiota. *J. Clin. Invest.* **2014**, *124*, 4212–4218, doi:10.1172/JCI72333.themselves.
129. Lerminiaux, N.A.; Cameron, A.D.S. Horizontal transfer of antibiotic resistance genes in clinical environments. *Can. J. Microbiol.* **2019**, *65*, 34–44, doi:10.1139/cjm-2018-0275.
130. Quainoo, S.; Coolen, J.P.M.; van Hijum, S.A.F.T.; Huynen, M.A.; Melchers, W.J.G.; van Schaik, W.; Wertheim, H.F.L. Whole-Genome Sequencing of Bacterial Pathogens: the Future of Nosocomial Outbreak Analysis. *Clin. Microbiol. Rev.* **2017**, *30*, 1015–1063,

doi:10.1128/CMR.00016-17.
131. Sabat, A.J.; Budimir, A.; Nashev, D.; Sá-Leão, R.; van Dijl, J.M.; Laurent, F.; Grundmann, H.; Friedrich, A.W. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Eurosurveillance* **2013**, *18*, 20380, doi:10.2807/ese.18.04.20380-en.
132. Zago, V.; Veschetti, L.; Patuzzo, C.; Malerba, G.; Lleo, M.M. Resistome, mobilome and virulome analysis of *Shewanella algae* and *Vibrio* spp. Strains isolated in Italian aquaculture centers. *Microorganisms* **2020**, *8*, 572, doi:10.3390/microorganisms8040572.
133. Vaughn, E.L.; Vo, Q.T.; Vostok, J.; Stiles, T.; Lang, A.; Brown, C.M.; Klevens, R.M.; Madoff, L. Linking Epidemiology and Whole-Genome Sequencing to Investigate *Salmonella* Outbreak, Massachusetts, USA, 2018. *Emerg. Infect. Dis.* **2020**, *26*, 1538–1541, doi:10.3201/eid2607.200048.
134. Benulič, K.; Pirš, M.; Couto, N.; Chlebowicz, M.; Rossen, J.W.A.; Zorec, T.M.; Seme, K.; Poljak, M.; Zupanc, T.L.; Ružić-Sabljić, E.; et al. Whole genome sequencing characterization of Slovenian carbapenem-resistant *Klebsiella pneumoniae*, including OXA-48 and NDM-1 producing outbreak isolates. *PLoS One* **2020**, *15*, e0231503, doi:10.1371/journal.pone.0231503.
135. Sukhum, K. V.; Diorio-Toth, L.; Dantas, G. Genomic and Metagenomic Approaches for Predictive Surveillance of Emerging Pathogens and Antibiotic Resistance. *Clin. Pharmacol. Ther.* **2019**, *106*, 512–524, doi:10.1002/cpt.1535.
136. Pollock, J.; Low, A.S.; McHugh, R.E.; Muwonge, A.; Stevens, M.P.; Corbishley, A.; Gally, D.L. Alternatives to antibiotics in a One Health context and the role genomics can play in reducing antimicrobial use. *Clin. Microbiol. Infect.* **2020**, *26*, 1617–1621, doi:10.1016/j.cmi.2020.02.028.
137. Li, Y.M.; Cao, W.W.; Liang, S.L.; Yamasaki, S.; Chen, X.; Shi, L.; Ye, L. Metagenomic characterization of bacterial community and antibiotic resistance genes in representative ready-to-eat food in southern China. *Sci. Rep.* **2020**, *10*, 15175, doi:10.1038/s41598-020-72620-4.
138. Xu, C.; Lv, Z.; Shen, Y.; Liu, D.; Fu, Y.; Zhou, L.; Liu, W.; Chen, K.; Ye, H.; Xia, X.; et al. Metagenomic insights into differences in environmental resistome profiles between integrated and monoculture aquaculture farms in China. *Environ. Int.* **2020**, *144*, 106005, doi:10.1016/j.envint.2020.106005.
139. Wang, W.L.; Xu, S.Y.; Ren, Z.G.; Tao, L.; Jiang, J.W.; Zheng, S. Sen Application of metagenomics in the human gut microbiome. *World J. Gastroenterol.* **2015**, *21*, 803–814, doi:10.3748/wjg.v21.i3.803.
140. Pierce, M.L.; Ward, J.E. Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) and the Blue Mussel (*Mytilus edulis*): Temporal Variation and the Influence of Marine Aggregate-Associated Microbial Communities. *mSphere* **2019**, *4*, e00730-19, doi:10.1128/mSphere.00730-19.
141. Novais, C.; Campos, J.; Freitas, A.R.; Barros, M.; Silveira, E.; Coque, T.M.; Antunes, P.; Peixe, L. Water supply and feed as sources of antimicrobial-resistant *Enterococcus* spp. in aquacultures of rainbow trout (*Oncorhyncus mykiss*), Portugal. *Sci. Total Environ.* **2018**, *625*, 1102–1112, doi:10.1016/j.scitotenv.2017.12.265.
142. Pereira, A.M.P.T.; Silva, L.J.G.; Meisel, L.M.; Pena, A. Fluoroquinolones and Tetracycline

Antibiotics in a Portuguese Aquaculture System and Aquatic Surroundings: Occurrence and Environmental Impact. *J. Toxicol. Environ. Heal. - Part A Curr. Issues* **2015**, *78*, 959–975, doi:10.1080/15287394.2015.1036185.

143. Antunes, P.; Campos, J.; Mourão, J.; Pereira, J.; Novais, C.; Peixe, L. Inflow water is a major source of trout farming contamination with *Salmonella* and multidrug resistant bacteria. *Sci. Total Environ.* **2018**, *642*, 1163–1171, doi:10.1016/j.scitotenv.2018.06.143.
144. Pereira, C.; Salvador, S.; Arrojado, C.; Silva, Y.; Santos, A.L.; Cunha, A.; Gomes, N.C.M.; Almeida, A. Evaluating seasonal dynamics of bacterial communities in marine fish aquaculture: a preliminary study before applying phage therapy. *J. Environ. Monit.* **2011**, *13*, 1053–1058, doi:10.1039/c0em00434k.
145. FAO Fisheries and Aquaculture Departement Fishery and Aquaculture Country Profiles. Portugal (2005). Country Profile Fact Sheets. Available online: http://www.fao.org/fishery/ (accessed on Jul 20, 2020).
146. Heather, F.J.; Childs, D.Z.; Darnaude, A.M.; Blanchard, J.L. Using an integral projection model to assess the effect of temperature on the growth of gilthead seabream *Sparus aurata*. *PLoS One* **2018**, *13*, e0196092, doi:10.5061/dryad.fb128m1.
147. Adeogun, A.O.; Ibor, O.R.; Onoja, A.B.; Arukwe, A. Fish condition factor, peroxisome proliferator activated receptors and biotransformation responses in *Sarotherodon melanotheron* from a contaminated freshwater dam (Awba Dam) in Ibadan, Nigeria. *Mar. Environ. Res.* **2016**, *121*, 74–86, doi:10.1016/j.marenvres.2016.02.002.
148. International Standard *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*; 2nd ed.; ISO copyright office: Geneva, Switzerland, 2017.
149. International Standard *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*; 2nd ed.; ISO copyright office: Geneva, 2017.
150. Jones-Dias, D.; Manageiro, V.; Caniça, M. 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.* **2016**, *18*, 260–272, doi:10.1111/1462-2920.13021.
151. EFSA Panel on Biological Hazards (BIOHAZ) Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals. *EFSA J.* **2011**, *9*, 2322, doi:10.2903/j.efsa.2011.2322.
152. EFSA Panel on Biological Hazards (BIOHAZ) Scientific Opinion on Carbapenem resistance in food animal ecosystems. *EFSA J.* **2013**, *11*, 3501, doi:10.2903/j.efsa.2013.3501.
153. Schwarz, S.; Silley, P.; Simjee, S.; Woodford, N.; van Duijkeren, E.; Johnson, A.P.; Gaastra, W. Assessing the antimicrobial susceptibility of bacteria obtained from animals. *Vet. Microbiol.* **2010**, *141*, 1–4, doi:10.1016/j.vetmic.2009.12.013.
154. Dean, A.G.; Sullivan, K.M.; Soe, M.M. OpenEpi: Open Source Epidemiologic Statistics for Public Health. Available online: www.OpenEpi.com (accessed on Jul 29, 2020).
155. Dallenne, C.; Da Costa, A.; Decre, D.; Favier, C.; Arlet, G. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in

Enterobacteriaceae. *J. Antimicrob. Chemother.* **2010**, *65*, 490–495, doi:10.1093/jac/dkp498.
156. Mendonça, N.; Leitão, J.; Manageiro, V.; Ferreira, E.; Caniça, M. Spread of extended-spectrum β-lactamase CTX-M-producing *Escherichia coli* clinical isolates in community and nosocomial environments in Portugal. *Antimicrob. Agents Chemother.* **2007**, *51*, 1946–1955, doi:10.1128/AAC.01412-06.
157. Fallah, F.; Borhan, R.S.; Hashemi, A. Detection of *bla*(IMP) and *bla*(VIM) metallo-β-lactamases genes among *Pseudomonas aeruginosa* strains. *Int. J. Burns Trauma* **2013**, *3*, 122–124. PMID: 23638331.
158. Khudhair, A.M.; Saadallah, S.; Al-faham, M. Isolation of Multi Antibiotic Resistance *Serratia marcescens* and the Detection of AmpC & GESβL Genes by Polymerase Chain Reaction Technique. *Int. Assoc. Jungian Stud.* **2011**, *22*, 329–346. Available online: https://www.iasj.net/iasj/download/8283187028133a34 (accessed on Jul 20, 2020).
159. Manageiro, V.; Ferreira, E.; Caniça, M.; Manaia, C. GES-5 among the β-lactamases detected in ubiquitous bacteria isolated from aquatic environment samples. *FEMS Microbiol. Lett.* **2014**, *351*, 64–69, doi:10.1111/1574-6968.12340.
160. Rodríguez-Martínez, J.-M.; Poirel, L.; Nordmann, P. Molecular Epidemiology and Mechanisms of Carbapenem Resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **2009**, *53*, 4783–4788, doi:10.1128/AAC.00574-09.
161. Shoja, S.; Moosavian, M.; Peymani, A.; Tabatabaiefar, M.A. Genotyping of carbapenem resistant *Acinetobacter baumannii* isolated from tracheal tube discharge of hospitalized patients in intensive care. *Iran. J. Microbiol.* **2013**, *5*, 315–322. PMID: 25848498.
162. Chen, X.; Zhang, W.; Pan, W.; Yin, J.; Pan, Z.; Gao, S.; Jiao, X. Prevalence of *qnr*, *aac(6′)-Ib-cr*, *qepA*, and *oqxAB* in *Escherichia coli* Isolates from Humans, Animals, and the Environment. *Antimicrob. Agents Chemother.* **2012**, *56*, 3423–3427, doi:10.1128/AAC.06191-11.
163. Ellington, M.J.; Hope, R.; Turton, J.F.; Warner, M.; Woodford, N.; Livermore, D.M. Detection of *qnrA* among *Enterobacteriaceae* from South-East England with extended-spectrum and high-level AmpC beta-lactamases. *J. Antimicrob. Chemother.* **2007**, *60*, 1176–1178, doi:10.1093/jac/dkm308.
164. Jones-Dias, D.; Manageiro, V.; Francisco, A.P.; Martins, A.P.; Domingues, G.; Louro, D.; Ferreira, E.; Caniça, M. Assessing the molecular basis of transferable quinolone resistance in *Escherichia coli* and *Salmonella* spp. from food-producing animals and food products. *Vet. Microbiol.* **2013**, *167*, 523–531, doi:10.1016/j.vetmic.2013.08.010.
165. Robicsek, A.; Strahilevitz, J.; Sahm, D.F.; Jacoby, G.A.; Hooper, D.C. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. *Antimicrob. Agents Chemother.* **2006**, *50*, 2872–2874, doi:10.1128/AAC.01647-05.
166. Wu, J.J.; Ko, W.C.; Tsai, S.H.; Yan, J.J. Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of *Enterobacter cloacae* in a Taiwanese hospital. *Antimicrob. Agents Chemother.* **2007**, *51*, 1223–1227, doi:10.1128/AAC.01195-06.
167. Ahmed, A.M.; Miyoshi, S.; Shinoda, S.; Shimamoto, T. Molecular characterization of a multidrug-resistant strain of enteroinvasive *Escherichia coli* O164 isolated in Japan. *J. Med. Microbiol.* **2005**, *54*, 273–278, doi:10.1099/jmm.0.45908-0.
168. Everett, M.J.; Jin, Y.U.F.; Ricci, V.; Piddock, L.J. V Contributions of Individual Mechanisms

to Fluoroquinolone Resistance in 36 *Escherichia coli* Strains Isolated from Humans and Animals. *Antimicrob. Agents Chemother.* **1996**, *40*, 2380–2386, doi:10.1128/AAC.40.10.2380.

169. Mammeri, H.; Loo, M. Van De; Poirel, L.; Martinez-martinez, L.; Nordmann, P. Emergence of Plasmid-Mediated Quinolone Resistance in *Escherichia coli* in Europe. *Antimicrob. Agents Chemother.* **2005**, *49*, 71–76, doi:10.1128/AAC.49.1.71.
170. Rebelo, A.R.; Bortolaia, V.; Kjeldgaard, J.S.; Pedersen, S.K.; Leekitcharoenphon, P.; Hansen, I.M.; Guerra, B.; Malorny, B.; Borowiak, M.; Hammerl, J.A. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance purposes. *Eurosurveillance* **2018**, *23*, 17–00672, doi:10.2807/1560-7917.ES.2018.23.6.17-00672.
171. Pajić, M.J.; Rašić, Z.B.; Velebit, B.M.; Boboš, S.F.; Mihajlović-Ukropina, M.M.; Radinović, M.Ž.; Galfi, A.L.; Petković, J.M.; Trojačanec, S.I. The prevalence of methicillin resistance and Panton-Valentine leukocidin synthesis genes in *Staphylococcus aureus* isolates of bovine and human origin. *Vet. Arch.* **2014**, *84*, 205–214. Available online: https://hrcak.srce.hr/file/180385 (accessed on Mar 25, 2023).
172. Kang, C.; Shin, Y.; Kim, W.; Kim, Y.; Song, K.; Oh, E.; Kim, S.; Yu, H.; So, J. Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from oysters in Korea. *Environ. Sci. Pollut. Res.* **2016**, *23*, 918–926, doi:10.1007/s11356-015-5650-9.
173. Ng, C.; Chen, H.; Goh, S.G.; Haller, L.; Wu, Z.; Charles, F.R.; Trottet, A.; Gin, K. Microbial water quality and the detection of multidrug resistant *E. coli* and antibiotic resistance genes in aquaculture sites of Singapore. *Mar. Pollut. Bull.* **2018**, *135*, 475–480, doi:10.1016/j.marpolbul.2018.07.055.
174. Scarano, C.; Piras, F.; Virdis, S.; Ziino, G.; Nuvoloni, R.; Dalmasso, A.; De Santis, E.P.L.; Spanu, C.; Santis, E.P.L. De; Spanu, C. Antibiotic resistance of *Aeromonas* ssp. strains isolated from *Sparus aurata* reared in Italian mariculture farms. *Int. J. Food Microbiol.* **2018**, *284*, 91–97, doi:10.1016/j.ijfoodmicro.2018.07.033.
175. Tajbakhsh, E.; Khamesipour, F.; Ranjbar, R.; Ugwu, I.C. Prevalence of class 1 and 2 integrons in multi-drug resistant *Escherichia coli* isolated from aquaculture water in Chaharmahal Va Bakhtiari province, Iran. *Ann. Clin. Microbiol. Antimicrob.* **2015**, *14*, 37, doi:10.1186/s12941-015-0096-y.
176. Pujalte, M.J.; Sitjà-Bobadilla, A.; Álvarez-Pellitero, P.; Garay, E. Carriage of potentially fish-pathogenic bacteria in *Sparus aurata* cultured in Mediterranean fish farms. *Dis. Aquat. Organ.* **2003**, *54*, 119–126, doi:10.3354/dao054119.
177. Lalucat, J.; Bennasar, A.; Bosch, R.; Garcia-Valdes, E.; Palleroni, N.J. Biology of *Pseudomonas stutzeri*. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 510–547, doi:10.1128/mmbr.00047-05.
178. Cho, C.H.; Lee, S.B. Comparison of clinical characteristics and antibiotic susceptibility between *Pseudomonas aeruginosa* and *P. putida* keratitis at a tertiary referral center: A retrospective study. *BMC Ophthalmol.* **2018**, *18*, 204, doi:10.1186/s12886-018-0882-3.
179. Halabi, Z.; Mocadie, M.; El Zein, S.; Kanj, S.S. *Pseudomonas stutzeri* prosthetic valve endocarditis: A case report and review of the literature. *J. Infect. Public Health* **2019**, *12*, 434–437, doi:10.1016/j.jiph.2018.07.004.
180. Ouchenir, L.; Renaud, C.; Khan, S.; Bitnun, A.; Boisvert, A.A.; McDonald, J.; Bowes, J.; Brophy, J.; Barton, M.; Ting, J.; et al. The epidemiology, management, and outcomes of

bacterial meningitis in infants. *Pediatrics* **2017**, *140*, e20170476, doi:10.1542/peds.2017-0476.
181. Raphael, E.; Riley, L.W. Infections caused by antimicrobial drug-resistant saprophytic Gram-negative bacteria in the environment. *Front. Med.* **2017**, *4*, 183, doi:10.3389/fmed.2017.00183.
182. Yoshino, Y.; Kitazawa, T.; Kamimura, M.; Tatsuno, K.; Ota, Y.; Yotsuyanagi, H. *Pseudomonas putida* bacteremia in adult patients: Five case reports and a review of the literature. *J. Infect. Chemother.* **2011**, *17*, 278–282, doi:10.1007/s10156-010-0114-0.
183. Adrian, T.-G.-S.; Tan, P.-W.; Chen, J.-W.; Yin, W.-F.; Chan, K.-G. Draft Genome Sequence of *Kocuria rhizophila* strain TPW45, an Actinobacterium Isolated from Freshwater. *J. Genomics* **2016**, *4*, 16–18, doi:10.7150/jgen.15063.
184. Purty, S.; Saranathan, R.; Prashanth, K.; Narayanan, K.; Asir, J.; Devi, C.S.; Amarnath, S.K. The expanding spectrum of human infections caused by *Kocuria* species: a case report and literature review. *Emerg. Microbes Infect.* **2013**, *2*, e71, doi:10.1038/emi.2013.71.
185. Yuan, M.; Yu, Y.; Li, H.R.; Dong, N.; Zhang, X.H. Phylogenetic diversity and biological activity of actinobacteria isolated from the Chukchi Shelf marine sediments in the arctic ocean. *Mar. Drugs* **2014**, *12*, 1281–1297, doi:10.3390/md12031281.
186. Yun, J.H.; Roh, S.W.; Jung, M.J.; Kim, M.S.; Park, E.J.; Shin, K.S.; Nam, Y. Do; Bae, J.W. *Kocuria salsicia* sp. nov., isolated from salt-fermented seafood. *Int. J. Syst. Evol. Microbiol.* **2011**, *61*, 286–289, doi:10.1099/ijs.0.021469-0.
187. Mehrabadi, J.F.; Mirzaie, A.; Ahangar, N.; Rahimi, A.; Rokni-zadeh, H. Draft Genome Sequence of *Kocuria rhizophila* RF, a Radiation-Resistant Soil Isolate. *Genome* **2016**, *4*, e00095-16, doi:10.1128/genomeA.00095-16.
188. Moissenet, D.; Becker, K.; Mérens, A.; Ferroni, A.; Dubern, B.; Vu-Thien, H. Persistent bloodstream infection with *Kocuria rhizophila* related to a damaged central catheter. *J. Clin. Microbiol.* **2012**, *50*, 1495–1498, doi:10.1128/JCM.06038-11.
189. Das, A.; Behera, B.K.; Acharya, S.; Paria, P.; Chakraborty, H.J.; Parida, P.K.; Das, B.K. Genetic diversity and multiple antibiotic resistance index study of bacterial pathogen, *Klebsiella pneumoniae* strains isolated from diseased Indian major carps. *Folia Microbiol. (Praha).* **2019**, *64*, 875–887, doi:10.1007/s12223-019-00701-7.
190. Rahman, M.; Rahman, M.; Deb, S.C.; Alam, S. Molecular Identification of Multiple Antibiotic Resistant Fish Pathogenic *Enterococcus faecalis* and their Control by Medicinal Herbs. *Sci. Rep.* **2017**, *7*, 3747, doi:10.1038/s41598-017-03673-1.
191. Regecová, I.; Pipová, M.; Jevinová, P.; Marušková, K.; Kmeť, V.; Popelka, P. Species identification and antimicrobial resistance of coagulase-negative staphylococci isolated from the meat of sea fish. *J. Food Sci.* **2014**, *79*, M898-902, doi:10.1111/1750-3841.12429.
192. Thillai Sekar, V.; Santiago, T.C.; Vijayan, K.K.; Alavandi, S. V.; Stalin Raj, V.; Rajan, J.J.S.; Sanjuktha, M.; Kalaimani, N. Involvement of *Enterobacter cloacae* in the mortality of the fish, *Mugil cephalus*. *Lett. Appl. Microbiol.* **2008**, *46*, 667–672, doi:10.1111/j.1472-765X.2008.02365.x.
193. Akbari, M.; Bakhshi, B.; Peerayeh, S.N. Particular Distribution of *Enterobacter cloacae* Strains Isolated from Urinary Tract Infection within Clonal Complexes. *Iran. Biomed. J.* **2016**, *20*, 49–55, doi:10.7508/ibj.2016.01.007.
194. Savini, V.; Catavitello, C.; Bianco, A.; Balbinot, A.; Antonio, D.D. Epidemiology,

Pathogenicity and Emerging Resistances in *Staphylococcus pasteuri*: From Mammals and Lampreys, to Man. *Recent Pat. Antiinfect. Drug Discov.* **2009**, *4*, 123–129, doi:10.2174/157489109788490352.

195. Delshad, S.T.; Soltanian, S.; Sharifiyazdi, H.; Haghkhah, M.; Bossier, P. Identification of *N*-acyl homoserine lactone-degrading bacteria isolated from rainbow trout (*Oncorhynchus mykiss*). *J. Appl. Microbiol.* **2018**, *125*, 356–369, doi:10.1111/jam.13891.
196. Sousa, M.; Torres, C.; Barros, J.; Somalo, S.; Igrejas, G.; Poeta, P. Gilthead seabream (*Sparus aurata*) as carriers of SHV-12 and TEM-52 extended-spectrum beta-lactamases-containing *Escherichia coli* isolates. *Foodborne Pathog. Dis.* **2011**, *8*, 1139–41, doi:10.1089/fpd.2011.0866.
197. Srisapoome, P.; Areechon, N. Efficacy of viable *Bacillus pumilus* isolated from farmed fish on immune responses and increased disease resistance in Nile tilapia (*Oreochromis niloticus*): Laboratory and on-farm trials. *Fish Shellfish Immunol.* **2017**, *67*, 199–210, doi:10.1016/j.fsi.2017.06.018.
198. Bottone, E.J. *Bacillus cereus*, a Volatile Human Pathogen. *Clin. Microbiol. Rev.* **2010**, *23*, 382–398, doi:10.1128/CMR.00073-09.
199. Clements, A.; Young, J.C.; Constantinou, N.; Frankel, G. Infection strategies of enteric pathogenic *Escherichia coli*. *Gut Microbes* **2012**, *3*, 71–87, doi:10.4161/gmic.19182.
200. Dyabi, F.Z.; Bennaoui, F.; Slitine, N.E.I.; Soraa, N.; Maoulainine, F.M.. *Enterobacter hormaechei*: New Neonatal Infection in Morocco. *Open Infect. Dis. J.* **2018**, *10*, 147–150, doi:10.2174/1874279301810010147.
201. Interaminense, J.A.; Nascimento, D.C.O.; Ventura, R.F.; Batista, J.E.C.; Souza, M.M.C.; Hazin, F.H. V; Pontes-Filho, N.T.; Lima-Filho, J. V Recovery and screening for antibiotic susceptibility of potential bacterial pathogens from the oral cavity of shark species involved in attacks on humans in Recife, Brazil. *J. Med. Microbiol.* **2010**, *59*, 941–947, doi:10.1099/jmm.0.020453-0.
202. Keren, Y.; Keshet, D.; Eidelman, M.; Geffen, Y.; Raz-pasteur, A.; Hussein, K. Is *Leclercia adecarboxylata* a New and Unfamiliar Marine Pathogen? *J. Clin. Microbiol.* **2014**, *52*, 1775–1776, doi:10.1128/JCM.03239-13.
203. Tam, V.; Nayak, S. Isolation of *Leclercia adecarboxylata* from a wound infection after exposure to hurricane-related floodwater. *BMJ Case Rep.* **2012**, *2012*, bcr-2012-007298, doi:10.1136/bcr-2012-007298.
204. Lodovico, S. Di; Cataldi, V.; Campli, E. Di; Ancarani, E.; Cellini, L.; Giulio, M. Di. *Enterococcus hirae* biofilm formation on hospital material surfaces and effect of new biocides. *Environ. Health Prev. Med.* **2017**, *22*, 63, doi:10.1186/s12199-017-0670-3.
205. Ccorahua-Santo, R.; Cervantes, M.; Duran, Y.; Aguirre, M.; Marin, C.; Ramírez, P. Draft genome sequence of *Klebsiella michiganensis* 3T412C, harboring an arsenic resistance genomic island, isolated from mine tailings in Peru. *Genome Announc.* **2017**, *5*, e00611-17, doi:10.1128/genomeA.00611-17.
206. Heinle, C.E.; Junqueira, A.C.M.; Uchida, A.; Purbojati, R.W.; Houghton, J.N.I.; Chénard, C.; Drautz-Moses, D.I.; Wong, A.; Kolundžija, S.; Clare, M.E.; et al. Complete genome sequence of *Lelliottia nimipressuralis* type strain SGAir0187, isolated from tropical air collected in Singapore. *Genome Announc.* **2018**, *6*, e00231-18, doi:10.1128/genomeA.00231-18.
207. Kämpfer, P.; Glaeser, S.P.; Packroff, G.; Behringer, K.; Exner, M.; Chakraborty, T.;

Schmithausen, R.M.; Doijad, S. *Lelliottia aquatilis* sp. Nov., isolated from drinking water. *Int. J. Syst. Evol. Microbiol.* **2018**, *68*, 2454–2461, doi:10.1099/ijsem.0.002854.
208. Mitra, S.; Pramanik, K.; Ghosh, P.K.; Soren, T.; Sarkar, A.; Dey, R.S.; Pandey, S.; Maiti, T.K. Characterization of Cd-resistant *Klebsiella michiganensis* MCC3089 and its potential for rice seedling growth promotion under Cd stress. *Microbiol. Res.* **2018**, *210*, 12–25, doi:10.1016/j.micres.2018.03.003.
209. Palmer, M.; de Maayer, P.; Poulsen, M.; Steenkamp, E.T.; van Zyl, E.; Coutinho, T.A.; Venter, S.N. Draft genome sequences of *Pantoea agglomerans* and *Pantoea vagans* isolates associated with termites. *Stand. Genomic Sci.* **2016**, *11*, 23, doi:10.1186/s40793-016-0144-z.
210. Wang, S.S.; Liu, J.M.; Sun, J.; Sun, Y.F.; Liu, J.N.; Jia, N.; Fan, B.; Dai, X.F. Diversity of culture-independent bacteria and antimicrobial activity of culturable endophytic bacteria isolated from different *Dendrobium* stems. *Sci. Rep.* **2019**, *9*, 10389, doi:10.1038/s41598-019-46863-9.
211. Qiao, Y.; Peng, Q.; Yan, J.; Wang, H.; Ding, H.; Shi, B. Gene cloning and enzymatic characterization of alkali- tolerant type I pullulanase from *Exiguobacterium acetylicum*. *Lett. Appl. Microbiol.* **2014**, *60*, 52–59, doi:10.1111/lam.12333.
212. Keynan, Y.; Weber, G.; Sprecher, H. Molecular identification of *Exiguobacterium acetylicum* as the aetiological agent of bacteraemia. *J. Med. Microbiol.* **2007**, *56*, 563–564, doi:10.1099/jmm.0.46866-0.
213. Seiffert, S.N.; Wüthrich, D.; Gerth, Y.; Egli, A. First clinical case of KPC-3–producing *Klebsiella michiganensis* in Europe. *New Microbes New Infect.* **2019**, *29*, 100516, doi:10.1016/j.nmni.2019.100516.
214. Sekyere, J.O.; Amoako, D.G. Genomic and phenotypic characterisation of fluoroquinolone resistance mechanisms in *Enterobacteriaceae* in Durban , South Africa. *PLoS One* **2017**, *12*, e0178888, doi:10.1371/ journal.pone.0178888.
215. Zheng, B.; Xu, H.; Yu, X.; Lv, T.; Jiang, X.; Cheng, H.; Zhang, J.; Chen, Y.; Huang, C.; Xiao, Y. Identification and genomic characterization of a KPC-2-, NDM-1- and NDM-5-producing *Klebsiella michiganensis* isolate. *J. Antimicrob. Chemother.* **2018**, *73*, 536–538, doi:10.1093/jac/dkx415.
216. Leal-Negredo, Á.; Castelló-Abietar, C.; Leiva, P.S.; Fernández, J. Infección urinaria por *Lelliottia amnigena* (*Enterobacter amnigenus*): un patógeno infrecuente. *Rev. Española Quimioter.* **2017**, *30*, 483–484. PMID: 29199415.
217. Xu, L.; Yin, M.; Zhu, T.; Liu, Y.; Ying, Y.; Lu, J.; Lin, C.; Ying, J.; Xu, T.; Ni, L.; et al. Comparative Genomics Analysis of Plasmid pPV989-94 from a Clinical Isolate of *Pantoea vagans* PV989. *Int. J. Genomics* **2018**, *2018*, 1242819, doi:10.1155/2018/1242819.
218. Barros, J.; Andrade, M.; Radhouani, H.; López, M.; Igrejas, G.; Poeta, P.; Torres, C. Detection of *vanA*-containing *Enterococcus* species in faecal microbiota of gilthead seabream (*Sparus aurata*). *Microbes Environ.* **2012**, *27*, 509–511, doi:10.1264/jsme2.ME11346.
219. Cardozo, M.V.; Borges, C.A.; Beraldo, L.G.; Maluta, R.P.; Pollo, A.S.; Borzi, M.M.; dos Santos, L.F.; Kariyawasam, S.; de Ávila, F.A. Shigatoxigenic and atypical enteropathogenic *Escherichia coli* in fish for human consumption. *Brazilian J. Microbiol.* **2018**, *49*, 936–941, doi:10.1016/j.bjm.2018.02.013.
220. Adnan, M.; Patel, M.; Hadi, S. Functional and health promoting inherent attributes of

Enterococcus hirae F2 as a novel probiotic isolated from the digestive tract of the freshwater fish *Catla catla*. *PeerJ* **2017**, *5*, e3085, doi:10.7717/peerj.3085.
221. Jinendiran, S.; Boopathi, S.; Sivakumar, N.; Selvakumar, G. Functional Characterization of Probiotic Potential of Novel Pigmented Bacterial Strains for Aquaculture Applications. *Probiotics Antimicrob. Proteins* **2019**, *11*, 186–197, doi:10.1007/s12602-017-9353-z.
222. Adelowo, O.O.; Caucci, S.; Banjo, O.A.; Nnanna, O.C.; Awotipe, E.O.; Peters, F.B.; Fagade, O.E.; Berendonk, T.U. Extended Spectrum Beta-Lactamase (ESBL)-producing bacteria isolated from hospital wastewaters, rivers and aquaculture sources in Nigeria. *Env. Sci Pollut Res* **2018**, *25*, 2744–2755, doi:10.1007/s11356-017-0686-7.
223. Almeida, M.V.A.; Cangussú, Í.M.; Carvalho, A.L.S.; Brito, I.L.P.; Costa, R.A. Drug resistance, AmpC-β-lactamase and ESBL producing *Enterobacteriaceae* isolated from fish and shrimp. *Rev. Inst. Med. Trop. Sao Paulo* **2017**, *59*, e70, doi:10.1590/S1678-9946201759070.
224. Cheng, H.; Jiang, H.; Fang, J.; Zhu, C. Antibiotic Resistance and Characteristics of Integrons in *Escherichia coli* Isolated from *Penaeus vannamei* at a Freshwater Shrimp Farm in Zhejiang Province, China. *J. Food Prot.* **2019**, *82*, 470–478, doi:10.4315/0362-028X.JFP-18-444.
225. Resende, J.A.; Silva, V.L.; Fontes, C.O.; Souza-Filho, J.A.; Oliveira, T.L.R.; Coelho, C.M.; César, D.E.; Diniz, C.G. Multidrug-Resistance and Toxic Metal Tolerance of Medically Important Bacteria Isolated from an Aquaculture System. *Microbes Environ.* **2012**, *27*, 449–455, doi:10.1264/jsme2.ME12049.
226. Codjoe, F.; Donkor, E. Carbapenem Resistance: A Review. *Med. Sci.* **2017**, *6*, 1, doi:10.3390/medsci6010001.
227. Jacoby, G.A. AmpC β-Lactamases. *Clin. Microbiol. Rev.* **2009**, *22*, 161–182, doi:10.1128/CMR.00036-08.
228. Papp-Wallace, K.M.; Endimiani, A.; Taracila, M.A.; Bonomo, R.A. Carbapenems: Past, present, and future. *Antimicrob. Agents Chemother.* **2011**, *55*, 4943–4960, doi:10.1128/AAC.00296-11.
229. Nordkvist, E.; Zuidema, T.; Herbes, R.G.; Berendsen, B.J.A. Occurrence of chloramphenicol in cereal straw in north-western Europe. *Food Addit. Contam. - Part A Chem. Anal. Control. Expo. Risk Assess.* **2016**, *33*, 798–803, doi:10.1080/19440049.2016.1176450.
230. Nguyen, H.N.K.; Van, T.T.H.; Nguyen, H.T.; Smooker, P.M.; Shimeta, J.; Coloe, P.J. Molecular characterization of antibiotic resistance in *Pseudomonas* and *Aeromonas* isolates from catfish of the Mekong Delta, Vietnam. *Vet. Microbiol.* **2014**, *171*, 397–405, doi:10.1016/j.vetmic.2014.01.028.
231. Adewoye, L.; Topp, E.; Li, X.-Z. Antimicrobial Drug Efflux Genes and Pumps in Bacteria of Animal and Environmental Origin. In *Efflux-Mediated Antimicrobial Resistance in Bacteria*; Li, X., Elkins, C., Zgurskaya, H., Eds.; Adis, Cham, 2016; pp. 561–593 ISBN 978-3-319-39658-3.
232. Muziasari, W.I.; Pärnänen, K.; Johnson, T.A.; Lyra, C.; Karkman, A.; Stedtfeld, R.D.; Tamminen, M.; Tiedje, J.M.; Virta, M. Aquaculture changes the profile of antibiotic resistance and mobile genetic element associated genes in Baltic Sea sediments. *FEMS Microbiol. Ecol.* **2016**, *92*, fiw052, doi:10.1093/femsec/fiw052.
233. Miranda, C.D.; Godoy, F.A.; Lee, M.R. Current status of the use of antibiotics and the antimicrobial resistance in the chilean salmon farms. *Front. Microbiol.* **2018**, *9*, 1284,

doi:10.3389/fmicb.2018.01284.
234. Higuera-Llantén, S.; Vásquez-Ponce, F.; Barrientos-Espinoza, B.; Mardones, F.O.; Marshall, S.H.; Olivares-Pacheco, J. Extended antibiotic treatment in salmon farms select multiresistant gut bacteria with a high prevalence of antibiotic resistance genes. *PLoS One* **2018**, *13*, e0203641, doi:10.1371/journal.pone.0203641.
235. Syrova, E.; Kohoutova, L.; Dolejska, M.; Papezikova, I.; Kutilova, I.; Cizek, A.; Navratil, S.; Minarova, H.; Palikova, M. Antibiotic resistance and virulence factors in mesophilic *Aeromonas* spp. from Czech carp fisheries. *J. Appl. Microbiol.* **2018**, *125*, 1702–1713, doi:10.1111/jam.14075.
236. Mata, W.; Putita, C.; Dong, H.T.; Kayansamruaj, P.; Senapin, S.; Rodkhum, C. Quinolone-resistant phenotype of *Flavobacterium columnare* isolates harbouring point mutations both in *gyrA* and *parC* but not in *gyrB* or *parE*. *J. Glob. Antimicrob. Resist.* **2018**, *15*, 55–60, doi:10.1016/j.jgar.2018.05.014.
237. Hossain, S.; De Silva, B.C.J.; Wickramanayake, M.V.K.S.; Dahanayake, P.S.; Wimalasena, S.H.M.P.; Heo, G.J. Incidence of antimicrobial resistance genes and class 1 integron gene cassettes in multidrug-resistant motile *Aeromonas* sp. isolated from ornamental guppy (*Poecilia reticulata*). *Lett. Appl. Microbiol.* **2019**, *69*, 2–10, doi:10.1111/lam.13162.
238. Jiang, H.X.; Tang, D.; Liu, Y.H.; Zhang, X.H.; Zeng, Z.L.; Xu, L.; Hawkey, P.M. Prevalence and characteristics of β-lactamase and plasmid-mediated quinolone resistance genes in *Escherichia coli* isolated from farmed fish in China. *J. Antimicrob. Chemother.* **2012**, *67*, 2350–2353, doi:10.1093/jac/dks250.
239. Chenia, H.Y. Prevalence and characterization of plasmid-mediated quinolone resistance genes in *Aeromonas* spp. isolated from South African freshwater fish. *Int. J. Food Microbiol.* **2016**, *231*, 26–32, doi:10.1016/j.ijfoodmicro.2016.04.030.
240. Hordijk, J.; Bosman, A.B.; van Essen-Zandbergen, A.; Veldman, K.; Dierikx, C.; Wagenaar, J.A.; Mevius, D. *qnrB19* Gene Bracketed by IS*26* on a 40-Kilobase IncR Plasmid from an *Escherichia coli* Isolate from a Veal Calf. *Antimicrob. Agents Chemother.* **2011**, *55*, 453–454, doi:10.1128/AAC.00866-10.
241. Martins, W.M.B.S.; Almeida, A.C.S.; Nicoletti, A.G.; Cayô, R.; Gales, A.C.; Alves, L.C.; Brayner, F.B.; Vilela, M.A.; Morais, M.M.C. Coproduction of KPC-2 and QnrB19 in *Klebsiella pneumoniae* ST340 isolate in Brazil. *Diagn. Microbiol. Infect. Dis.* **2015**, *83*, 375–376, doi:10.1016/j.diagmicrobio.2015.09.003.
242. Richter, S.N.; Frasson, I.; Bergo, C.; Manganelli, R.; Cavallaro, A.; Palù, G. Characterisation of *qnr* plasmid-mediated quinolone resistance in *Enterobacteriaceae* from Italy: association of the *qnrB19* allele with the integron element IS*CR1* in *Escherichia coli*. *Int. J. Antimicrob. Agents* **2012**, *35*, 578–583, doi:10.1016/j.ijantimicag.2010.02.015.
243. Moreno-Switt, A.I.; Pezoa, D.; Sepúlveda, V.; González, I.; Rivera, D.; Retamal, P.; Navarrete, P.; Reyes-Jara, A.; Toro, M. Transduction as a Potential Dissemination Mechanism of a Clonal *qnrB19*-Carrying Plasmid Isolated From *Salmonella* of Multiple Serotypes and Isolation Sources. *Front. Microbiol.* **2019**, *10*, 2503, doi:10.3389/fmicb.2019.02503.
244. Araújo, B.F.; De Campos, P.A.; Royer, S.; Ferreira, M.L.; Gonçalves, I.R.; Resende, D.S.; De Brito, C.S.; Gontijo-Filho, P.P.; Ribas, R.M. High frequency of the combined presence of QRDR mutations and PMQR determinants in multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli* isolates from nosocomial and community-acquired infections. *J.*

Med. Microbiol. **2017**, *66*, 1144–1150, doi:10.1099/jmm.0.000551.
245. Minarini, L.A.R.; Darini, A.L.C. Mutations in the quinolone resistance-determining regions of *gyrA* and *parC* in *Enterobacteriaceae* isolates from Brazil. *Brazilian J. Microbiol.* **2012**, *43*, 1309–1314, doi:10.1590/S1517-838220120004000010.
246. Bisicchia, P.; Bui, N.K.; Aldridge, C.; Vollmer, W.; Devine, K.M. Acquisition of VanB-type vancomycin resistance by *Bacillus subtilis*: the impact on gene expression, cell wall composition and morphology. *Mol. Microbiol.* **2011**, *81*, 157–178, doi:10.1111/j.1365-2958.2011.07684.x.
247. Patel, J.B.; Gorwitz, R.J.; Jernigan, J.A. Mupirocin Resistance. *Clin. Infect. Dis.* **2009**, *49*, 935–941, doi:10.1086/605495.
248. Trabal Fernández, N.; Mazón-Suástegui, J.M.; Vázquez-Juárez, R.; Ascencio-Valle, F.; Romero, J. Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. *FEMS Microbiol. Ecol.* **2014**, *88*, 69–83, doi:10.1111/1574-6941.12270.
249. Tseng, S.Y.; Liu, P.Y.; Lee, Y.H.; Wu, Z.Y.; Huang, C.C.; Cheng, C.C.; Tung, K.C. The pathogenicity of *Shewanella algae* and ability to tolerate a wide range of temperatures and salinities. *Can. J. Infect. Dis. Med. Microbiol.* **2018**, *2018*, 6976897, doi:10.1155/2018/6976897.
250. Grevskott, D.H.; Svanevik, C.S.; Sunde, M.; Wester, A.L.; Lunestad, B.T. Marine bivalve mollusks as possible indicators of multidrug-resistant *Escherichia coli* and other species of the *Enterobacteriaceae* family. *Front. Microbiol.* **2017**, *8*, 24, doi:10.3389/fmicb.2017.00024.
251. Lopatek, M.; Wieczorek, K.; Osek, J. Prevalence and antimicrobial resistance of *Vibrio parahaemolyticus* isolated from raw shellfish in Poland. *J. Food Prot.* **2015**, *78*, 1029–1033, doi:10.4315/0362-028X.JFP-14-437.
252. Furushita, M.; Shiba, T.; Maeda, T.; Yahata, M.; Kaneoka, A.; Takahashi, Y.; Torii, K.; Hasegawa, T.; Ohta, M. Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl. Environ. Microbiol.* **2003**, *69*, 5336–5342, doi:10.1128/AEM.69.9.5336-5342.2003.
253. Akinbowale, O.L.; Peng, H.; Barton, M.D. Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *J. Appl. Microbiol.* **2007**, *103*, 2016–2025, doi:10.1111/j.1365-2672.2007.03445.x.
254. Rivas, A.J.; Lemos, M.L.; Osorio, C.R. *Photobacterium damselae* subsp. *damselae*, a bacterium pathogenic for marine animals and humans. *Front. Microbiol.* **2013**, *4*, 283, doi:10.3389/fmicb.2013.00283.
255. Froelich, B.A.; Noble, R.T. Vibrio bacteria in raw oysters: Managing risks to human health. *Philos. Trans. R. Soc. B Biol. Sci.* **2016**, *371*, 20150209, doi:10.1098/rstb.2015.0209.
256. Tewari, A.; Abdullah, S. *Bacillus cereus* food poisoning: international and Indian perspective. *J. Food Sci. Technol.* **2015**, *52*, 2500–2511, doi:10.1007/s13197-014-1344-4.
257. Salgueiro, V.; Manageiro, V.; Bandarra, N.M.; Reis, L.; Caniça, M. Bacterial Diversity and Antibiotic Susceptibility of *Sparus aurata* from Aquaculture. *Microorganisms* **2020**, *8*, 1343, doi:10.3390/microorganisms8091343.
258. McAuliffe, G.N.; Hennessy, J.; Baird, R.W. Relative frequency, characteristics, and antimicrobial susceptibility patterns of *Vibrio* spp., *Aeromonas* spp., *Chromobacterium*

violaceum, and *Shewanella* spp. in the Northern Territory of Australia, 2000-2013. *Am. J. Trop. Med. Hyg.* **2015**, *92*, 605–610, doi:10.4269/ajtmh.14-0715.
259. Lasa, A.; Mira, A.; Camelo-Castillo, A.; Belda-Ferre, P.; Romalde, J.L. Characterization of the microbiota associated to *Pecten maximus* gonads using 454-pyrosequencing. *Int. Microbiol.* **2016**, *19*, 93–99, doi:10.2436/20.1501.01.267.
260. Kacar, A. Some microbial characteristics of mussels (*Mytilus galloprovincialis*) in coastal city area. *Environ. Sci. Pollut. Res.* **2011**, *18*, 1384–1389, doi:10.1007/s11356-011-0487-3.
261. Radó, J.; Kaszab, E.; Benedek, T.; Kriszt, B.; Szoboszlay, S. First isolation of carbapenem-resistant *Acinetobacter beijerinckii* from an environmental sample. *Acta Microbiol. Immunol. Hung.* **2019**, *66*, 113–130, doi:10.1556/030.66.2019.004.
262. Richards, G.P.; Watson, M.A.; Crane, E.J.; Burt, I.G.; Bushek, D. *Shewanella* and *Photobacterium* spp. in oysters and seawater from the Delaware bay. *Appl. Environ. Microbiol.* **2008**, *74*, 3323–3327, doi:10.1128/AEM.00060-08.
263. Hernández-Robles, M.F.; Álvarez-Contreras, A.K.; Juárez-García, P.; Natividad-Bonifacio, I.; Curiel-Quesada, E.; Vázquez-Salinas, C.; Quiñones-Ramírez, E.I. Virulence factors and antimicrobial resistance in environmental strains of *Vibrio alginolyticus*. *Int. Microbiol.* **2016**, *19*, 191–198, doi:10.2436/20.1501.01.277.
264. Khan, A.; Aung, T.T.; Chaudhuri, D. The First Case of Native Mitral Valve Endocarditis due to *Micrococcus luteus* and Review of the Literature. *Case Reports Cardiol.* **2019**, *2019*, 5907319, doi:10.1155/2019/5907319.
265. Kim, H.W.; Hong, Y.J.; Jo, J.I.; Ha, S.D.; Kim, S.H.; Lee, H.J.; Rhee, M.S. Raw ready-to-eat seafood safety: microbiological quality of the various seafood species available in fishery, hyper and online markets. *Lett. Appl. Microbiol.* **2017**, *64*, 27–34, doi:10.1111/lam.12688.
266. Bozcal, E.; Dagdeviren, M. Bacterial metagenome analysis of *Mytilus galloprovincialis* collected from Istanbul and Izmir coastal stations of Turkey. *Environ. Monit. Assess.* **2020**, *192*, 186, doi:10.1007/s10661-020-8129-1.
267. Fernández-Delgado, M.; Contreras, M.; García-Amado, M.A.; Gueneau, P.; Suárez, P. Occurrence of *Proteus mirabilis* associated with two species of Venezuelan oysters. *Rev. Inst. Med. Trop. Sao Paulo* **2007**, *49*, 355–359, doi:10.1590/s0036-46652007000600004.
268. Sacramento, A.G.; Fernandes, M.R.; Sellera, F.P.; Dolabella, S.S.; Zanella, R.C.; Cerdeira, L.; Lincopan, N. VanA-type vancomycin-resistant *Enterococcus faecium* ST1336 isolated from mussels in an anthropogenically impacted ecosystem. *Mar. Pollut. Bull.* **2019**, *142*, 533–536, doi:10.1016/j.marpolbul.2019.04.014.
269. Roslev, P.; Iversen, L.; Sønderbo, H.L.; Iversen, N.; Bastholm, S. Uptake and persistence of human associated *Enterococcus* in the mussel *Mytilus edulis*: relevance for faecal pollution source tracking. *J. Appl. Microbiol.* **2009**, *107*, 944–953, doi:10.1111/j.1365-2672.2009.04272.x.
270. Chapman, C. Investigation into the microbiological causes of epizootics of Pacific oyster larvae (*Crassostrea gigas*) in commercial production, University of Tasmania, 2012. Available online: https://eprints.utas.edu.au/15901/1/front-chapman-2012.pdf (accessed on Oct 25, 2020).
271. Drzewiecka, D. Significance and Roles of *Proteus* spp. Bacteria in Natural Environments. *Microb. Ecol.* **2016**, *72*, 741–758, doi:10.1007/s00248-015-0720-6.

272. de Sousa, O.V.; dos Fernandes Vieira, R.H.S.; de Menezes, F.G.R.; dos Reis, C.M.F.; Hofer, E. Detection of *Vibrio parahaemolyticus* and *Vibrio cholerae* in oyster, *Crassostrea rhizophorae*, collected from a natural nursery in the Cocó river estuary, Fortaleza, Ceará, Brazil. *Rev. Inst. Med. Trop. Sao Paulo* **2004**, *46*, 59–62, doi:10.1590/s0036-46652004000200001.
273. Baldi, F.; Gallo, M.; Marchetto, D.; Faleri, C.; Maida, I.; Fani, R. Manila clams from Hg polluted sediments of Marano and Grado lagoons (Italy) harbor detoxifying Hg resistant bacteria in soft tissues. *Environ. Res.* **2013**, *125*, 188–196, doi:10.1016/j.envres.2012.11.008.
274. Santoiemma, P.P.; Kalainov, D.M.; Mehta, M.P.; Bolon, M.K. An unusual case of *Staphylococcus pasteuri* osteomyelitis. *Infect. Dis. Rep.* **2020**, *12*, 8523, doi:10.4081/idr.2020.8523.
275. Jadhav, K.P.; Pai, P.G. A rare infective endocarditis caused by *Vagococcus fluvialis*. *J. Cardiol. Cases* **2019**, *20*, 129–131, doi:10.1016/j.jccase.2019.07.001.
276. Matsuo, T.; Mori, N.; Kawai, F.; Sakurai, A.; Toyoda, M.; Mikami, Y.; Uehara, Y.; Furukawa, K. *Vagococcus fluvialis* as a causative pathogen of bloodstream and decubitus ulcer infection: Case report and systematic review of the literature. *J. Infect. Chemother.* **2021**, *27*, 359–363, doi:10.1016/j.jiac.2020.09.019.
277. Liu, H.; Zhu, J.; Hu, Q.; Rao, X. *Morganella morganii*, a non-negligent opportunistic pathogen. *Int. J. Infect. Dis.* **2016**, *50*, 10–17, doi:10.1016/j.ijid.2016.07.006.
278. Sharma, D.; Sharma, P.; Soni, P. First case report of *Providencia Rettgeri* neonatal sepsis. *BMC Res. Notes* **2017**, *10*, 536, doi:10.1186/s13104-017-2866-4.
279. Kanuparthy, A.; Challa, T.; Meegada, S.; Siddamreddy, S.; Muppidi, V. *Staphylococcus warneri*: Skin Commensal and a Rare Cause of Urinary Tract Infection. *Cureus* **2020**, *12*, e8337, doi:10.7759/cureus.8337.
280. Zhou, Y.H.; Xu, C.G.; Yang, Y.B.; Xing, X.X.; Liu, X.; Qu, Q.W.; Ding, W.Y.; Bello-Onaghise, G.; Li, Y.H. Histidine metabolism and IGPD play a key role in cefquinome inhibiting biofilm formation of *Staphylococcus xylosus*. *Front. Microbiol.* **2018**, *9*, 665, doi:10.3389/fmicb.2018.00665.
281. Al-Kharousi, Z.S.; Guizani, N.; Al-Sadi, A.M.; Al-Bulushi, I.M.; Shaharoona, B. Hiding in Fresh Fruits and Vegetables: Opportunistic Pathogens May Cross Geographical Barriers. *Int. J. Microbiol.* **2016**, *2016*, 4292417, doi:10.1155/2016/4292417.
282. Herridge, W.P.; Shibu, P.; O'Shea, J.; Brook, T.C.; Hoyles, L. Bacteriophages of *Klebsiella* spp., their diversity and potential therapeutic uses. *J. Med. Microbiol.* **2020**, *69*, 176–194, doi:10.1099/jmm.0.001141.
283. Pérez-Vazquez, M.; Oteo-Iglesias, J.; Sola-Campoy, P.J.; Carrizo-Manzoni, H.; Bautista, V.; Lara, N.; Aracil, B.; Alhambra, A.; Martínez-Martínez, L.; Campos, J.; et al. Characterization of Carbapenemase-Producing *Klebsiella oxytoca* in Spain, 2016–2017. *Antimicrob. Agents Chemother.* **2019**, *63*, e02529-18, doi:10.1128/AAC.02529-18.
284. Chen, Z.; Li, H.; Feng, J.; Li, Y.; Chen, X.; Guo, X.; Chen, W.; Wang, L.; Lin, L.; Yang, H.; et al. NDM-1 encoded by a pNDM-BJ01-like plasmid p3SP-NDM in clinical *Enterobacter aerogenes*. *Front. Microbiol.* **2015**, *6*, 294, doi:10.3389/fmicb.2015.00294.
285. Abriouel, H.; Omar, N. Ben; Molinos, A.C.; López, R.L.; Grande, M.J.; Martínez-Viedma, P.; Ortega, E.; Cañamero, M.M.; Galvez, A. Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations

from raw fruit and vegetable foods, water and soil, and clinical samples. *Int. J. Food Microbiol.* **2008**, *123*, 38–49, doi:10.1016/j.ijfoodmicro.2007.11.067.
286. Kubota, H.; Mitani, A.; Niwano, Y.; Takeuchi, K.; Tanaka, A.; Yamaguchi, N.; Kawamura, Y.; Hitomi, J. *Moraxella* species are primarily responsible for generating malodor in laundry. *Appl. Environ. Microbiol.* **2012**, *78*, 3317–3324, doi:10.1128/AEM.07816-11.
287. Alkhatib, N.J.; Younis, M.H.; Alobaidi, A.S.; Shaath, N.M. An unusual osteomyelitis caused by *Moraxella osloensis*: A case report. *Int. J. Surg. Case Rep.* **2017**, *41*, 146–149, doi:10.1016/j.ijscr.2017.10.022.
288. Kaeuffer, C.; Schramm, F.; Meyer, A.; Hansmann, Y.; Guffroy, A.; Argemi, X. First case of *Comamonas aquatica* bacteremia complicated by septic shock. *Med. Mal. Infect.* **2018**, *48*, 540–542, doi:10.1016/j.medmal.2018.08.004.
289. Parthasarathy, A.; Gan, H.M.; Wong, N.H.; Savka, M.A.; Steiner, K.K.; Henry, K.R.; Hudson, A.O. Isolation and genomic characterization of six endophytic bacteria isolated from *Saccharum* sp (sugarcane): Insights into antibiotic, secondary metabolite and quorum sensing metabolism. *J. Genomics* **2018**, *6*, 117–121, doi:10.7150/jgen.28335.
290. Gaastra, W.; Kusters, J.G.; van Duijkeren, E.; Lipman, L.J.A. *Escherichia fergusonii*. *Vet. Microbiol.* **2014**, *172*, 7–12, doi:10.1016/j.vetmic.2014.04.016.
291. Hajjar, R.; Ambaraghassi, G.; Sebajang, H.; Schwenter, F.; Su, S.H. *Raoultella ornithinolytica*: Emergence and resistance. *Infect. Drug Resist.* **2020**, *13*, 1091–1104, doi:10.2147/IDR.S191387.
292. Iguchi, A.; Nagaya, Y.; Pradel, E.; Ooka, T.; Ogura, Y.; Katsura, K.; Kurokawa, K.; Oshima, K.; Hattori, M.; Parkhill, J.; et al. Genome evolution and plasticity of *Serratia marcescens*, an important multidrug-resistant nosocomial pathogen. *Genome Biol. Evol.* **2014**, *6*, 2096–2110, doi:10.1093/gbe/evu160.
293. Baek, S.D.; Chun, C.; Hong, K.S. Hemolytic uremic syndrome caused by *Escherichia fergusonii* infection. *Kidney Res. Clin. Pract.* **2019**, *38*, 253–255, doi:10.23876/j.krcp.19.012.
294. Wang, R.X.; Wang, A.; Wang, J.Y. Antibiotic resistance monitoring in heterotrophic bacteria from anthropogenic-polluted seawater and the intestines of oyster *Crassostrea hongkongensis*. *Ecotoxicol. Environ. Saf.* **2014**, *109*, 27–31, doi:10.1016/j.ecoenv.2014.07.028.
295. Catry, B.; Cavaleri, M.; Baptiste, K.; Grave, K.; Grein, K.; Holm, A.; Jukes, H.; Liebana, E.; Navas, A.L.; Mackay, D.; et al. Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): development of resistance in animals and possible impact on human and animal health. *Int. J. Antimicrob. Agents* **2015**, *46*, 297–306, doi:10.1016/j.ijantimicag.2015.06.005.
296. Shen, Y.; Zhang, R.; Schwarz, S.; Wu, C.; Shen, J.; Walsh, T.R.; Wang, Y. Farm animals and aquaculture: significant reservoirs of mobile colistin resistance genes. *Environ. Microbiol.* **2020**, *22*, 2469–2484, doi:10.1111/1462-2920.14961.
297. Dahanayake, P.S.; Hossain, S.; Wickramanayake, M.V.K.S.; Wimalasena, S.H.M.P.; Heo, G.-J.. Manila clam (*Ruditapes philippinarum*) marketed in Korea as a source of vibrios harbouring virulence and β-lactam resistance genes. *Lett. Appl. Microbiol.* **2020**, *71*, 46–53, doi:10.1111/lam.13229.
298. Su, H.; Hu, X.; Wang, L.; Xu, W.; Xu, Y.; Wen, G.; Li, Z.; Cao, Y. Contamination of antibiotic resistance genes (ARGs) in a typical marine aquaculture farm: source tracking of ARGs

in reared aquatic organisms. *J. Environ. Sci. Heal. Part B.* **2020**, *55*, 220–229, doi:10.1080/03601234.2019.1684747.
299. De Silva, B.C.J.; Hossain, S.; Dahanayake, P.S.; Heo, G.-J. *Aeromonas* spp. from marketed Yesso scallop (*Patinopecten yessoensis*): molecular characterization, phylogenetic analysis, virulence properties and antimicrobial susceptibility. *J. Appl. Microbiol.* **2019**, *126*, 288–299, doi:10.1111/jam.14106.
300. Giedraitiene, A.; Vitkauskiene, A.; Naginiene, R.; Pavilonis, A. Antibiotic resistance mechanisms of clinically important bacteria. *Medicina (B. Aires).* **2011**, *47*, 137–146, doi:10.3390/medicina47030019.
301. Foster, T.J.; Geoghegan, J.A. *Staphylococcus aureus*. In *Molecular Medical Microbiology*, Tang, Y.W., Sussman, M., Liu, D., Poxton, I., Schwartzman, J., Eds.; Elsevier Ltd: Dublin, 2015; pp. 655–674 ISBN 3522738284.
302. Lakhundi, S.; Zhang, K. Methicillin-Resistant *Staphylococcus aureus*: Molecular Characterization, Evolution, and Epidemiology. *Clin. Microbiol. Rev.* **2018**, *31*, e00020-18, doi:10.1128/CMR.00020-18.
303. Piecková, E. Indoor Microbial Aerosol and Its Health Effects: Microbial Exposure in Public Buildings - Viruses, Bacteria, and Fungi. In *Exposure to Microbiological Agents in Indoor and Occupational Environments*; Viegas, C., Viegas, S., Gomes, A., Täubel, M., Sabino, R., Eds.; Springer: Cham, Switzerland, 2017; pp. 237–252 ISBN 978-3-319-61686-5.
304. Conceição, T.; Coelho, C.; Santos-Silva, I.; de Lencastre, H.; Aires-de-Sousa, M. Epidemiology of Methicillin-Resistant and -Susceptible *Staphylococcus aureus* in Luanda, Angola: First Description of the Spread of the MRSA ST5-IVa Clone in the African Continent. *Microb. drug Resist.* **2014**, *20*, 441–449, doi:10.1089/mdr.2014.0007.
305. Cuny, C.; Layer, F.; Werner, G.; Harmsen, D.; Jurke, A.; Mellmann, A.; Witte, W.; Köck, R. State-wide surveillance of antibiotic resistance patterns and spa types of methicillin-resistant *Staphylococcus aureus* from blood cultures in North. *Clin. Microbiol. Infect.* **2015**, *21*, 750–757, doi:10.1016/j.cmi.2015.02.013.
306. Cong, Y.; Yang, S.; Rao, X. Vancomycin resistant *Staphylococcus aureus* infections: A review of case updating and clinical features. *J. Adv. Res.* **2020**, *21*, 169–176, doi:10.1016/j.jare.2019.10.005.
307. Howden, B.P.; Peleg, A.Y.; Stinear, T.P. The evolution of vancomycin intermediate *Staphylococcus aureus* (VISA) and heterogenous-VISA. *Infect. Genet. Evol.* **2014**, *21*, 575–582, doi:10.1016/j.meegid.2013.03.047.
308. Lee, A.S.; de Lencastre, H.; Garau, J.; Kluytmans, J.; Malhotra-Kumar, S.; Peschel, A.; Harbarth, S. Methicillin-resistant *Staphylococcus aureus*. *Nat. Rev. Dis. Prim.* **2018**, *4*, doi:10.1038/nrdp.2018.33.
309. Aires-de-Sousa, M. Methicillin-resistant *Staphylococcus aureus* among animals: current overview. *Clin. Microbiol. Infect.* **2017**, *23*, 373–380, doi:10.1016/j.cmi.2016.11.002.
310. Heaton, C.J.; Gerbig, G.R.; Sensius, L.D.; Patel, V.; Smith, T.C. *Staphylococcus aureus* epidemiology in wildlife: A systematic review. *Antibiotics* **2020**, *9*, 89, doi:10.3390/antibiotics9020089.
311. Arfatahery, N.; Davoodabadi, A.; Abedimohtasab, T. Characterization of Toxin Genes and Antimicrobial Susceptibility of *Staphylococcus aureus* Isolates in Fishery Products in Iran. *Sci. Rep.* **2016**, *6*, 34216, doi:10.1038/srep34216.
312. van Duijkeren, E.; Hengeveld, P.; Zomer, T.P.; Landman, F.; Bosch, T.; Haenen, A.; van de

Giessen, A. Transmission of MRSA between humans and animals on duck and turkey farms. *J. Antimicrob. Chemother.* **2016**, *71*, 58–62, doi:10.1093/jac/dkv313.
313. Pirolo, M.; Visaggio, D.; Gioffrè, A.; Artuso, I.; Gherardi, M.; Pavia, G.; Samele, P.; Ciambrone, L.; Di Natale, R.; Spatari, G.; et al. Unidirectional animal-to-human transmission of methicillin-resistant *Staphylococcus aureus* ST398 in pig farming; Evidence from a surveillance study in southern Italy. *Antimicrob. Resist. Infect. Control* **2019**, *8*, 187, doi:10.1186/s13756-019-0650-z.
314. Unnerstad, H.E.; Mieziewska, K.; Börjesson, S.; Hedbäck, H.; Strand, K.; Hallgren, T.; Landin, H.; Skarin, J.; Bengtsson, B. Suspected transmission and subsequent spread of MRSA from farmer to dairy cows. *Vet. Microbiol.* **2018**, *225*, 114–119, doi:10.1016/j.vetmic.2018.09.017.
315. Sousa, M.; Silva, N.; Manageiro, V.; Ramos, S.; Coelho, A.; Gonçalves, D.; Caniça, M.; Torres, C.; Igrejas, G.; Poeta, P. First report on MRSA CC398 recovered from wild boars in the north of Portugal. Are we facing a problem? *Sci. Total Environ.* **2017**, *596–597*, 26–31, doi:10.1016/j.scitotenv.2017.04.054.
316. Larsen, A.R.; Stegger, M.; Sørum, M. *spa* typing directly from a *mecA*, *spa* and *pvl* multiplex PCR assay - a cost-effective improvement for methicillin-resistant *Staphylococcus aureus* surveillance. *Clin. Microbiol. Infect.* **2008**, *14*, 611–614, doi:10.1111/j.1469-0691.2008.01995.x.
317. Sousa, M.; Silva, N.; Igrejas, G.; Silva, F.; Sargo, R.; Alegria, N.; Benito, D.; Gómez, P.; Lozano, C.; Gómez-Sanz, E.; et al. Antimicrobial resistance determinants in *Staphylococcus* spp. recovered from birds of prey in Portugal. *Vet. Microbiol.* **2014**, *171*, 436–440, doi:10.1016/j.vetmic.2014.02.034.
318. Gilot, P.; Lina, G.; Cochard, T.; Poutrel, B. Analysis of the genetic variability of genes encoding the RNA III-activating components Agr and TRAP in a population of *Staphylococcus aureus* strains isolated from cows with mastitis. *J. Clin. Microbiol.* **2002**, *40*, 4060–4067, doi:10.1128/JCM.40.11.4060-4067.2002.
319. Feil, E.J.; Li, B.C.; Aanensen, D.M.; Hanage, W.P.; Spratt, B.G. eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. *J. Bacteriol.* **2004**, *186*, 1518–1530, doi:10.1128/JB.186.5.1518-1530.2004.
320. Espadinha, D.; Faria, N.A.; Miragaia, M.; Lito, L.M.; Melo-Cristino, J.; de Lencastre, H. Extensive Dissemination of Methicillin-Resistant *Staphylococcus aureus* (MRSA) between the Hospital and the Community in a Country with a High Prevalence of Nosocomial MRSA. *PLoS One* **2013**, *8*, e59960, doi:10.1371/journal.pone.0059960.
321. Bhattacharya, S.; Bir, R.; Majumdar, T. Evaluation of multidrug resistant *Staphylococcus aureus* and their association with biofilm production in a tertiary care hospital, Tripura, Northeast India. *J. Clin. Diagnostic Res.* **2015**, *9*, DC01-4, doi:10.7860/JCDR/2015/13965.6417.
322. Medina, G.; Egea, A.L.; Otth, C.; Otth, L.; Fernández, H.; Bocco, J.L.; Wilson, M.; Sola, C. Molecular epidemiology of hospital-onset methicillin-resistant *Staphylococcus aureus* infections in Southern Chile. *Eur J Clin Microbiol Dis* **2013**, *32*, 1533–1540, doi:10.1007/s10096-013-1907-8.
323. Darboe, S.; Dobreniecki, S.; Jarju, S.; Jallow, M.; Mohammed, N.I.; Wathuo, M.; Ceesay, B.; Tweed, S.; Roy, R.B.; Okomo, U.; et al. Prevalence of Panton-Valentine Leukocidin (PVL)

and Antimicrobial Resistance in Community-Acquired Clinical *Staphylococcus aureus* in an Urban Gambian Hospital: A 11-year period retrospective pilot study. *Front. Cell. Infect. Microbiol.* **2019**, *9*, 170, doi:10.3389/fcimb.2019.00170.

324. Velasco, V.; Buyukcangaz, E.; Sherwood, J.S.; Stepan, R.M.; Koslofsky, R.J.; Logue, C.M. Characterization of *Staphylococcus aureus* from Humans and a Comparison with İsolates of Animal Origin, in North Dakota, United States. *PLoS One* **2015**, *10*, e0140497, doi:10.1371/journal.pone.0140497.
325. Pomba, C.; Baptista, F.M.; Couto, N.; Loução, F.; Hasman, H. Methicillin-resistant *Staphylococcus aureus* CC398 isolates with indistinguishable ApaI restriction patterns in colonized and infected pigs and humans. *J. Antimicrob. Chemother.* **2010**, *65*, 2479–2481, doi:10.1093/jac/dkq330.
326. Couto, N.; Belas, A.; Centeno, M.; van Duijkeren, E.; Pomba, C. First description of *fexA*-positive meticillin-resistant *Staphylococcus aureus* ST398 from calves in Portugal. *J. Glob. Antimicrob. Resist.* **2014**, *2*, 342–343, doi:10.1016/j.jgar.2014.06.003.
327. Porrero, M.C.; Mentaberre, G.; Sánchez, S.; Fernández-Llario, P.; Gómez-Barrero, S.; Navarro-Gonzalez, N.; Serrano, E.; Casas-Díaz, E.; Marco, I.; Fernández-Garayzabal, J.-F.; et al. Methicillin resistant *Staphylococcus aureus* (MRSA) carriage in different free-living wild animal species in Spain. *Vet. J.* **2013**, *198*, 127–130, doi:10.1016/j.tvjl.2013.06.004.
328. Omoshaba, E.O.; Ojo, O.E.; Oyekunle, M.A.; Sonibare, A.O.; Adebayo, A.O. Methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from raw milk and nasal swabs of small ruminants in Abeokuta, Nigeria. *Trop. Anim. Health Prod.* **2020**, *52*, 2599–2608, doi:10.1007/s11250-020-02301-x.
329. European Centre for Disease Prevention and Control (ECDC) European Surveillance of Antimicrobial Comsuption Available online: https://www.ecdc.europa.eu/en/antimicrobial-consumption/database/distribution-by-antimicrobial-group (accessed on Jul 25, 2020).
330. Hashem, R.A.; Yassin, A.S.; Zedan, H.H.; Amin, M.A. Fluoroquinolone resistant mechanisms in methicillin-resistant *Staphylococcus aureus* clinical isolates in Cairo, Egypt. *J. Infect. Dev. Ctries.* **2013**, *7*, 796–803, doi:10.3855/jidc.3105.
331. Bakthavatchalam, Y.D.; Babu, P.; Munusamy, E.; Dwarakanathan, T.; Rupali, P.; Zervos, M.; Victor, P.J.; Veeraraghavan, B. Genomic insights on heterogeneous resistance to vancomycin and teicoplanin in Methicillin-resistant *Staphylococcus aureus*: A first report from South India. *PLoS One* **2019**, *14*, e0227009, doi:10.1371/journal.pone.0227009.
332. Simões, R.R.; Aires-de-Sousa, M.; Conceição, T.; Antunes, F.; da Costa, P.M.; de Lencastre, H. High prevalence of EMRSA-15 in Portuguese public buses: a worrisome finding. *PLoS One* **2011**, *6*, e17630, doi:10.1371/journal.pone.0017630.
333. Tavares, A.; Faria, N.A.; de Lencastre, H.; Miragaia, M. Population structure of methicillin-susceptible *Staphylococcus aureus* (MSSA) in Portugal over a 19-year period (1992-2011). *Eur. J. Clin. Microbiol. Infect. Dis.* **2014**, *33*, 423–432, doi:10.1007/s10096-013-1972-z.
334. Plano, L.R.W.; Shibata, T.; Garza, A.C.; Kish, J.; Fleisher, J.M.; Sinigalliano, C.D.; Gidley, M.L.; Withum, K.; Elmir, S.M.; Hower, S.; et al. Human-Associated Methicillin-Resistant *Staphylococcus aureus* from a Subtropical Recreational Marine Beach. *Microb. Ecol.* **2013**, *65*, 1039–1051, doi:10.1007/s00248-013-0216-1.
335. Pereira-Franchi, E.P.L.; Barreira, M.R.N.; da Costa, N. de S.L.M.; Riboli, D.; Abraão, L.;

Martins, K.; Victória, C.; da Cunha, M. de L. Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in the Brazilian primary health care system. *Trop. Med. Int. Heal.* **2019**, *24*, 339–347, doi:10.1111/tmi.13192.

336. Silva, V.; Sousa, T. De; Gómez, P.; Sabença, C.; Vieira-Pinto, M.; Capita, R.; Alonso-Calleja, C.; Torres, C.; Capelo, J.L.; Igrejas, G.; et al. Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Purulent Subcutaneous Lesions of Farm Rabbits. *Foods* **2020**, *9*, 439, doi:10.3390/foods9040439.
337. Sunde, M.; Tharaldsen, H.; Marstein, L.; Haugum, M.; Norström, M.; Jacobsen, T.; Lium, B. Detection of methicillin-resistant *Staphylococcus aureus* sequence type 8 in pigs, production environment, and human beings. *J. Vet. Diagnostic Investig.* **2011**, *23*, 348–350, doi:10.1177/104063871102300226.
338. Chen, Y.; Liu, Z.; Duo, L.; Xiong, J.; Gong, Y.; Yang, J.; Wang, Z.; Wu, X.; Lu, Z.; Meng, X.; et al. Characterization of *Staphylococcus aureus* from distinct geographic locations in China: an increasing prevalence of *spa*-t030 and SCC*mec* type III. *PLoS One* **2014**, *9*, e96255, doi:10.1371/journal.pone.0096255.
339. Conceição, T.; Diamantino, F.; Coelho, C.; de Lencastre, H.; Aires-de-Sousa, M. Contamination of public buses with MRSA in Lisbon, Portugal: a possible transmission route of major MRSA clones within the community. *PLoS One* **2013**, *8*, e77812, doi:10.1371/journal.pone.0077812.
340. Ghaznavi-Rad, E.; Shamsudin, M.N.; Sekawi, Z.; Khoon, L.Y.; Aziz, M.N.; Hamat, R.A.; Othman, N.; Chong, P.P.; Van Belkum, A.; Ghasemzadeh-Moghaddam, H.; et al. Predominance and emergence of clones of hospital-acquired methicillin-resistant *Staphylococcus aureus* in Malaysia. *J. Clin. Microbiol.* **2010**, *48*, 867–872, doi:10.1128/JCM.01112-09.
341. Goudarzi, M.; Eslami, G.; Rezaee, R.; Heidary, M.; Khoshnood, S.; Nia, S. Clonal dissemination of *Staphylococcus aureus* isolates causing nosocomial infections, Tehran, Iran. *Iran. J. Basic Med. Sci.* **2019**, *22*, 238–245, doi:10.22038/ijbms.2018.30067.7245.
342. Harastani, H.H.; Araj, G.F.; Tokajian, S.T. Molecular characteristics of *Staphylococcus aureus* isolated from a major hospital in Lebanon. *Int. J. Infect. Dis.* **2014**, *19*, 33–38, doi:10.1016/j.ijid.2013.10.007.
343. Nagel, M.; Dischinger, J.; Türck, M.; Verrier, D.; Oedenkoven, M.; Ngoubangoye, B.; Le Flohic, G.; Drexler, J.F.; Bierbaum, G.; Gonzalez, J.-P. Human-associated *Staphylococcus aureus* strains within great ape populations in Central Africa (Gabon). *Clin. Microbiol. Infect.* **2013**, *19*, 1072–1077, doi:10.1111/1469-0691.12119.
344. Tavares, A.; Miragaia, M.; Rolo, J.; Coelho, C.; de Lencastre, H. High prevalence of hospital-associated methicillin-resistant *Staphylococcus aureus* in the community in Portugal: Evidence for the blurring of community-hospital boundaries. *Eur. J. Clin. Microbiol. Infect. Dis.* **2013**, *32*, 1269–1283, doi:10.1007/s10096-013-1872-2.
345. Lin, Y.; Barker, E.; Kislow, J.; Kaldhone, P.; Stemper, M.E.; Pantrangi, M.; Moore, F.M.; Hall, M.; Fritsche, T.R.; Novicki, T.; et al. Evidence of multiple virulence subtypes in nosocomial and community-associated MRSA genotypes in companion animals from the upper midwestern and northeastern United States. *Clin. Med. Res.* **2011**, *9*, 7–16, doi:10.3121/cmr.2010.944.
346. Vestergaard, M.; Cavaco, L.M.; Sirichote, P.; Unahalekhaka, A.; Dangsakul, W.; Svendsen, C.A.; Aarestrup, F.M.; Hendriksen, R.S. SCC*mec* type IX element in methicillin resistant

Staphylococcus aureus spa type t337 (CC9) isolated from pigs and pork in Thailand. *Front. Microbiol.* **2012**, *3*, 103, doi:10.3389/fmicb.2012.00103.
347. David, M.Z.; Siegel, J.; Lowy, F.D.; Zychowski, D.; Taylor, A.; Lee, C.J.; Boyle-Vavra, S.; Daum, R.S. Asymptomatic carriage of sequence type 398, *spa* type t571 methicillin-susceptible *Staphylococcus aureus* in an urban jail: A newly emerging, transmissible pathogenic strain. *J. Clin. Microbiol.* **2013**, *51*, 2443–2447, doi:10.1128/JCM.01057-13.
348. Moon, D.C.; Jeong, S.K.; Hyun, B.-H.; Lim, S.-K. Prevalence and Characteristics of Methicillin-Resistant *Staphylococcus aureus* Isolates in Pigs and Pig Farmers in Korea. *Foodborne Pathog. Dis.* **2019**, *16*, 256–261, doi:10.1089/fpd.2018.2509.
349. Bosch, T.; de Neeling, A.J.; Schouls, L.M.; van der Zwaluw, K.W.; Kluytmans, J.A.J.W.; Grundmann, H.; Huijsdens, X.W. PFGE diversity within the methicillin-resistant *Staphylococcus aureus* clonal lineage ST398. *BMC Microbiol.* **2010**, *10*, 40, doi:10.1186/1471-2180-10-40.
350. European Food Safety Authority (EFSA); European Centre for Disease Prevention and Control (ECDC) The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018. *EFSA J.* **2020**, *18*, 6007, doi:10.2903/j.efsa.2020.6007.
351. Paterson, G.K.; Harrison, E.M.; Holmes, M.A. The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*. *Trends Microbiol.* **2014**, *22*, 42–47, doi:10.1016/j.tim.2013.11.003.
352. Gharsa, H.; Ben Slama, K.; Gómez-Sanz, E.; Lozano, C.; Zarazaga, M.; Messadi, L.; Boudabous, A.; Torres, C. Molecular characterization of *Staphylococcus aureus* from nasal samples of healthy farm animals and pets in Tunisia. *Vector borne zoonotic Dis.* **2015**, *15*, 109–115, doi:10.1089/vbz.2014.1655.
353. Silva, V.; Pereira, J.E.; Maltez, L.; Ferreira, E.; Manageiro, V.; Caniça, M.; Capelo, J.L.; Igrejas, G.; Poeta, P. Diversity of methicillin-resistant staphylococci among wild *Lepus granatensis*: first detection of *mecA*-MRSA in hares. *FEMS Microbiol. Ecol.* **2020**, *96*, fiz204, doi:10.1093/femsec/fiz204.
354. Couto, N.; Monchique, C.; Belas, A.; Marques, C.; Gama, L.T.; Pomba, C. Trends and molecular mechanisms of antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16 year period. *J. Antimicrob. Chemother.* **2016**, *71*, 1479–1487, doi:10.1093/jac/dkw029.
355. Lopes, E.; Conceição, T.; Poirel, L.; de Lencastre, H.; Aires-de-Sousa, M. Epidemiology and antimicrobial resistance of methicillin-resistant *Staphylococcus aureus* isolates colonizing pigs with different exposure to antibiotics. *PLoS One* **2019**, *14*, e0225497, doi:10.1371/journal.pone.0225497.
356. Mediavilla, J.R.; Chen, L.; Uhlemann, A.-C.; Hanson, B.M.; Rosenthal, M.; Stanak, K.; Koll, B.; Fries, B.C.; Armellino, D.; Schilling, M.E.; et al. Methicillin-susceptible *Staphylococcus aureus* ST398, New York and New Jersey, USA. *Emerg. Infect. Dis.* **2012**, *18*, 700–702, doi:10.3201/eid1804.111419.
357. Kashif, A.; McClure, J.A.; Lakhundi, S.; Pham, M.; Chen, S.; Conly, J.M.; Zhang, K. *Staphylococcus aureus* ST398 Virulence Is Associated With Factors Carried on Prophage ϕSa3. *Front. Microbiol.* **2019**, *10*, 2219, doi:10.3389/fmicb.2019.02219.
358. Sakoulas, G.; Eliopoulos, G.M.; Moellering, Jr., R.C.; Novick, R.P.; Venkataraman, L.; Wennersten, C.; DeGirolami, P.C.; Schwaber, M.J.; Gold, H.S. *Staphylococcus aureus*

Accessory Gene Regulator (*agr*) Group II: Is There a Relationship to the Development of Intermediate-Level Glycopeptide Resistance? *J. Infect. Dis.* **2003**, *187*, 929–938, doi:10.1086/368128.
359. Moise-Broder, P.A.; Sakoulas, G.; Eliopoulos, G.M.; Schentag, J.J.; Forrest, A.; Moellering, R.C. Accessory gene regulator group II polymorphism in methicillin-resistant *Staphylococcus aureus* is predictive of failure of vancomycin therapy. *Clin. Infect. Dis.* **2004**, *38*, 1700–1705, doi:10.1086/421092.
360. Purrello, S.M.; Daum, R.S.; Edwards, G.F.S.; Lina, G.; Lindsay, J.; Peters, G.; Stefani, S. Meticillin-resistant *Staphylococcus aureus* (MRSA) update: New insights into bacterial adaptation and therapeutic targets. *J. Glob. Antimicrob. Resist.* **2014**, *2*, 61–69, doi:10.1016/j.jgar.2014.02.003.
361. Smyth, D.S.; Feil, E.J.; Meaney, W.J.; Hartigan, P.J.; Tollersrud, T.; Fitzgerald, J.R.; Enright, M.C.; Smyth, C.J. Molecular genetic typing reveals further insights into the diversity of animal-associated *Staphylococcus aureus*. *J. Med. Microbiol.* **2009**, *58*, 1343–1353, doi:10.1099/jmm.0.009837-0.
362. Gómez, P.; Lozano, C.; González-Barrio, D.; Zarazaga, M.; Ruiz-Fons, F.; Torres, C. High prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) carrying the *mecC* gene in a semi-extensive red deer (*Cervus elaphus hispanicus*) farm in Southern Spain. *Vet. Microbiol.* **2015**, *177*, 326–331, doi:10.1016/j.vetmic.2015.03.029.
363. Gharsa, H.; Ben Slama, K.; Lozano, C.; Gómez-Sanz, E.; Klibi, N.; Ben Sallem, R.; Gómez, P.; Zarazaga, M.; Boudabous, A.; Torres, C. Prevalence, antibiotic resistance, virulence traits and genetic lineages of *Staphylococcus aureus* in healthy sheep in Tunisia. *Vet. Microbiol.* **2012**, *156*, 367–373, doi:10.1016/j.vetmic.2011.11.009.
364. Hau, S.J.; Sun, J.; Davies, P.R.; Frana, T.S.; Nicholson, T.L. Comparative prevalence of immune evasion complex genes associated with β-hemolysin converting bacteriophages in MRSA ST5 isolates from swine, swine facilities, humans with swine contact, and humans with no swine contact. *PLoS One* **2015**, *10*, e0142832, doi:10.1371/journal.pone.0142832.
365. Lowder, B. V.; Guinane, C.M.; Zakour, N.L.B.; Weinert, L.A.; Conway-Morris, A.; Cartwright, R.A.; Simpson, A.J.; Rambaut, A.; Nübel, U.; Fitzgerald, J.R. Recent human-to-poultry host jump, adaptation, and pandemic spread of *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 19545–19550, doi:10.1073/pnas.0909285106.
366. European Centre for Disease Prevention and Control (ECDC) *Surveillance of antimicrobial resistance in Europe 2018*; ECDC: Stockholm, Sweden, 2018; doi: 10.2900/22212.
367. Melo-Cristino, J.; Resina, C.; Manuel, V.; Lito, L.; Ramirez, M. First case of infection with vancomycin-resistant *Staphylococcus aureus* in Europe. *Lancet* **2013**, *382*, 205, doi:10.1016/S0140-6736(13)61219-2.
368. Sieber, R.N.; Larsen, A.R.; Urth, T.R.; Iversen, S.; Møller, C.H.; Skov, R.L.; Larsen, J.; Stegger, M. Genome investigations show host adaptation and transmission of LA-MRSA CC398 from pigs into Danish healthcare institutions. *Sci. Rep.* **2019**, *9*, 18655, doi:10.1038/s41598-019-55086-x.
369. Salgueiro, V.; Manageiro, V.; Bandarra, N.M.; Clemente, L.; Caniça, M. Genetic Relatedness and Diversity of *Staphylococcus aureus* from Different Reservoirs: Humans and Animals of Livestock, Poultry, Zoo, and Aquaculture. *Microorganisms* **2020**, *8*, 1345,

doi:10.3390/microorganisms8091345.
370. Uhlemann, A.C.; McAdam, P.R.; Sullivan, S.B.; Knox, J.R.; Khiabanian, H.; Rabadan, R.; Davies, P.R.; Fitzgerald, J.R.; Lowy, F.D. Evolutionary dynamics of pandemic methicillin-sensitive *Staphylococcus aureus* ST398 and its international spread via routes of human migration. *MBio* **2017**, *8*, e01375-16, doi:10.1128/mBio.01375-16.
371. Mostofsky, E.; Lipsitch, M.; Regev-yochay, G. Is methicillin-resistant *Staphylococcus aureus* replacing methicillin-susceptible S. aureus? *J. Antimicrob. Chemother.* **2011**, *66*, 2199–2214, doi:10.1093/jac/dkr278.
372. Monaco, M.; de Araujo, F.P.; Cruciani, M.; Coccia, E.M.; Pantosti, A. Worldwide Epidemiology and Antibiotic Resistance of *Staphylococcus aureus*. *Curr. Top. Microbiol. Immunol.* **2017**, *409*, 21–56, doi:10.1007/82_2016_3.
373. Mama, O.M.; Aspiroz, C.; Ruiz-Ripa, L.; Ceballos, S.; Iñiguez-Barrio, M.; Cercenado, E.; Azcona, J.M.; López-Cerero, L.; Seral, C.; López-Calleja, A.I.; et al. Prevalence and Genetic Characteristics of *Staphylococcus aureus* CC398 Isolates From Invasive Infections in Spanish Hospitals, Focusing on the Livestock-Independent CC398-MSSA Clade. *Front. Microbiol.* **2021**, *12*, 623108, doi:10.3389/fmicb.2021.623108.
374. Li, G.; Wu, C.; Wang, X.; Meng, J. Prevalence and characterization of methicillin susceptible *Staphylococcus aureus* ST398 isolates from retail foods. *Int. J. Food Microbiol.* **2015**, *196*, 94–97, doi:10.1016/j.ijfoodmicro.2014.12.002.
375. Eom, H.S.; Back, S.H.; Lee, H.H.; Lee, G.Y. Prevalence and characteristics of livestock-associated methicillin-susceptible *Staphylococcus aureus* in the pork production chain in Korea. *J. Vet. Sci.* **2019**, *20*, e69, doi:10.4142/jvs.2019.20.e69.
376. Austin, B.; Austin, D.A. *Bacterial Fish Pathogens: Disease of Farmed and Wild Fish*; 6th ed.; Springer: Cham, Switzerland, 2016; ISBN 978-3-319-32673-3.
377. Oh, W.T.; Jun, J.W.; Giri, S.S.; Yun, S.; Kim, H.J.; Kim, S.G.; Kim, S.W.; Han, S.J.; Kwon, J.; Park, S.C. *Staphylococcus xylosus* infection in rainbow trout (*Oncorhynchus mykiss*) as a primary pathogenic cause of eye protrusion and mortality. *Microorganisms* **2019**, *7*, 330, doi:10.3390/microorganisms7090330.
378. Algammal, A.M.; Hetta, H.F.; Elkelish, A.; Alkhalifah, D.H.H.; Hozzein, W.N.; Batiha, G.E.S.; Nahhas, N. El; Mabrok, M.A. Methicillin-resistant *Staphylococcus aureus* (MRSA): One health perspective approach to the bacterium epidemiology, virulence factors, antibiotic-resistance, and zoonotic impact. *Infect. Drug Resist.* **2020**, *13*, 3255–3265, doi:10.2147/IDR.S272733.
379. Much, P.; Pichler, J.; Kasper, S.S.; Allerberger, F. Foodborne outbreaks, Austria 2007. *Wien. Klin. Wochenschr.* **2009**, *121*, 77–85, doi:10.1007/s00508-008-1125-z.
380. Macori, G.; Bellio, A.; Bianchi, D.; Gallina, S.; Adriano, D.; Zuccon, F.; Chiesa, F.; Acutis, P.; Casalinuovo, F.; Decastelli, L. Molecular typing of *Staphylococcus aureus* isolate responsible for staphylococcal poisoning incident in homemade food. *Ital. J. Food Saf.* **2016**, *5*, 5736, doi:10.4081/ijfs.2016.5736.
381. Price, L.B.; Stegger, M.; Hasman, H.; Aziz, M.; Larsen, J.; Andersen, P.S.; Pearson, T.; Waters, A.E.; Foster, J.T.; Schupp, J.; et al. *Staphylococcus aureus* CC398: Host adaptation and emergence of methicillin resistance in livestock. *MBio* **2012**, *3*, e00305-11, doi:10.1128/mBio.00305-11.
382. Lienen, T.; Schnitt, A.; Cuny, C.; Maurischat, S.; Tenhagen, B.A. Phylogenetic tracking of LA-MRSA ST398 intra-farm transmission among animals, humans and the environment

on german dairy farms. *Microorganisms* **2021**, *9*, 1119, doi:10.3390/microorganisms9061119.
383. Jones-Dias, D.; Clemente, L.; Moura, I.B.; Sampaio, D.A.; Albuquerque, T.; Vieira, L.; Manageiro, V.; Caniça, M. Draft genomic analysis of an avian multidrug resistant *Morganella morganii* isolate carrying *qnrD1*. *Front. Microbiol.* **2016**, *7*, 1660, doi:10.3389/fmicb.2016.01660.
384. Larsen, M. V.; Cosentino, S.; Lukjancenko, O.; Saputra, D.; Rasmussen, S.; Hasman, H.; Sicheritz-Pontén, T.; Aarestrup, F.M.; Ussery, D.W.; Lund, O. Benchmarking of methods for genomic taxonomy. *J. Clin. Microbiol.* **2014**, *52*, 1529–1539, doi:10.1128/JCM.02981-13.
385. 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.; et al. Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* **2012**, *50*, 1355–1361, doi:10.1128/JCM.06094-11.
386. Bartels, M.D.; Petersen, A.; Worning, P.; Nielsen, J.B.; Larner-Svensson, H.; Johansen, H.K.; Andersen, L.P.; Jarløv, J.O.; Boye, K.; Larsen, A.R.; et al. Comparing whole-genome sequencing with sanger sequencing for spa typing of methicillin-resistant *Staphylococcus aureus*. *J. Clin. Microbiol.* **2014**, *52*, 4305–4308, doi:10.1128/JCM.01979-14.
387. 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.; et al. ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* **2020**, *75*, 3491–3500, doi:10.1093/jac/dkaa345.
388. Tetzschner, A.M.M.; Johnson, J.R.; Johnston, B.D.; Lund, O.; Scheutz, F. *In Silico* Genotyping of *Escherichia coli* Isolates for Extraintestinal Virulence Genes by Use of Whole-Genome Sequencing Data. *J. Clin. Microbiol.* **2020**, *58*, e01269-20, doi:10.1128/JCM.01269-20.
389. Carattoli, A.; Zankari, E.; Garciá-Fernández, A.; Larsen, M.V.; Lund, O.; Villa, L.; Aarestrup, F.M.; Hasman, H. *In Silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* **2014**, *58*, 3895–3903, doi:10.1128/AAC.02412-14.
390. Johansson, M.H.K.; Bortolaia, V.; Tansirichaiya, S.; Aarestrup, F.M.; Roberts, A.P.; Petersen, T.N. Detection of mobile genetic elements associated with antibiotic resistance in *Salmonella enterica* using a newly developed web tool: MobileElementFinder. *J. Antimicrob. Chemother.* **2021**, *76*, 101–109, doi:10.1093/jac/dkaa390.
391. Ito, T.; Hiramatsu, K.; Oliveira, D.C.; de Lencastre, H.; Zhang, K.; Westh, H.; O'Brien, F.; Giffard, P.M.; Coleman, D.; Tenover, F.C.; et al. Classification of Staphylococcal cassette chromosome *mec* (SCC*mec*): Guidelines for reporting novel SCC*mec* elements. *Antimicrob. Agents Chemother.* **2009**, *53*, 4961–4967, doi:10.1128/AAC.00579-09.
392. Cosentino, S.; Larsen, M.V.; Aarestrup, F.M.; Lund, O. PathogenFinder - Distinguishing Friend from Foe Using Bacterial Whole Genome Sequence Data. *PLoS One* **2013**, *8*, e77302, doi:10.1371/journal.pone.0077302.
393. 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.; et al. CARD 2020: Antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleic Acids Res.*

2020, *48*, D517–D525, doi:10.1093/nar/gkz935.
394. Varani, A.M.; Siguier, P.; Gourbeyre, E.; Charneau, V.; Chandler, M. ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. *Genome Biol.* **2011**, *12*, R30, doi:10.1186/gb-2011-12-3-r30.
395. Arndt, D.; Grant, J.R.; Marcu, A.; Sajed, T.; Pon, A.; Liang, Y.; Wishart, D.S. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* **2016**, *44*, W16–W21, doi:10.1093/nar/gkw387.
396. Kaas, R.S.; Leekitcharoenphon, P.; Aarestrup, F.M.; Lund, O. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. *PLoS One* **2014**, *9*, e104984, doi:10.1371/journal.pone.0104984.
397. van der Graaf-Van Bloois, L.; Wagenaar, J.A.; Zomer, A.L. RFPlasmid: predicting plasmid sequences from short-read assembly data using machine learning. *Microb. Genomics* **2021**, *7*, 000683, doi:10.1099/mgen.0.000683.
398. Fanoy, E.; Helmhout, L.C.; van der Vaart, W.L.; Weijdema, K.; van Santen-Verheuvel, M.G.; Thijsen, S.F.; de Neeling, A.J.; van Wamel, W.J.; Maňásková, S.H.; Kingma-Thijssen, J.L. An outbreak of non-typeable MRSA within a residential care facility. *Euro Surveill.* **2009**, *14*, 19080, doi:10.2807/ese.14.01.19080-en.
399. Graveland, H.; Wagenaar, J.A.; Heesterbeek, H.; Mevius, D.; van Duijkeren, E.; Heederik, D. Methicillin resistant *Staphylococcus aureus* ST398 in veal calf farming: Human MRSA carriage related with animal antimicrobial usage and farm hygiene. *PLoS One* **2010**, *5*, e10990, doi:10.1371/journal.pone.0010990.
400. European Centre for Disease Prevention and Control (ECDC); European Food Safety Authority (EFSA); European Medicines Agency (EMA) *Antimicrobial consumption and resistance in bacteria from humans and animals: Third joint inter-agency report on integrated analysis of consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals in the EU/EEA, JIACRA III. 2016-2018*; ECDC, EFSA, EMA: Stockholm, Parma, Amsterdam, 2021; doi:10.2900/056892.
401. Chen, L.; Tang, Z.Y.; Cui, S.Y.; Ma, Z.B.; Deng, H.; Kong, W.L.; Yang, L.W.; Lin, C.; Xiong, W.G.; Zeng, Z.L. Biofilm production ability, virulence and antimicrobial resistance genes in *Staphylococcus aureus* from various veterinary hospitals. *Pathogens* **2020**, *9*, 264, doi:10.3390/pathogens9040264.
402. Bouchami, O.; Fraqueza, M.J.; Faria, N.A.; Alves, V.; Lawal, O.U.; de Lencastre, H.; Miragaia, M. Evidence for the dissemination to humans of methicillin-resistant *Staphylococcus aureus* ST398 through the pork production chain: A study in a portuguese slaughterhouse. *Microorganisms* **2020**, *8*, 1892, doi:10.3390/microorganisms8121892.
403. Schürch, A.C.; Arredondo-Alonso, S.; Willems, R.J.L.; Goering, R. V. 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.* **2018**, *24*, 350–354, doi:10.1016/j.cmi.2017.12.016.
404. Long, S.W.; Beres, S.B.; Olsen, R.J.; Musser, J.M. Absence of Patient-to-Patient intrahospital transmission of *Staphylococcus aureus* as determined by Whole-Genome sequencing. *MBio* **2014**, *5*, e01692-14, doi:10.1128/mBio.01692-14.
405. Price, J.R.; Golubchik, T.; Cole, K.; Wilson, D.J.; Crook, D.W.; Thwaites, G.E.; Bowden, R.;

Walker, A.S.; Peto, T.E.A.; Paul, J.; et al. Whole-genome sequencing shows that patient-to-patient transmission rarely accounts for acquisition of *Staphylococcus aureus* in an intensive care unit. *Clin. Infect. Dis.* **2014**, *58*, 609–618, doi:10.1093/cid/cit807.

406. Busenlehner, L.S.; Pennella, M.A.; Giedroc, D.P. The SmtB/ArsR family of metalloregulatory transcriptional repressors: Structural insights into prokaryotic metal resistance. *FEMS Microbiol. Rev.* **2003**, *27*, 131–143, doi:10.1016/S0168-6445(03)00054-8.
407. Koch, M.; Willi, J.; Pradere, U.; Hall, J.; Polacek, N. Critical 23S rRNA interactions for macrolide-dependent ribosome stalling on the ErmCL nascent peptide chain. *Nucleic Acids Res.* **2017**, *45*, 6717–6728, doi:10.1093/nar/gkx195.
408. Kwong, S.M.; Ramsay, J.P.; Jensen, S.O.; Firth, N. Replication of Staphylococcal resistance plasmids. *Front. Microbiol.* **2017**, *8*, 2279, doi:10.3389/fmicb.2017.02279.
409. Takayama, Y.; Tanaka, T.; Oikawa, K.; Fukano, N.; Goto, M.; Takahashi, T. Prevalence of *blaZ* gene and performance of phenotypic tests to detect penicillinase in *Staphylococcus aureus* isolates from Japan. *Ann. Lab. Med.* **2018**, *38*, 155–159, doi:10.3343/alm.2018.38.2.155.
410. Falagas, M.E.; Roussos, N.; Gkegkes, I.D.; Rafailidis, P.I.; Karageorgopoulos, D.E. Fosfomycin for the treatment of infections caused by Gram-positive cocci with advanced antimicrobial drug resistance: A review of microbiological, animal and clinical studies. *Expert Opin. Investig. Drugs* **2009**, *18*, 921–944, doi:10.1517/13543780902967624.
411. Fu, Z.; Ma, Y.; Chen, C.; Guo, Y.; Hu, F.; Liu, Y.; Xu, X.; Wang, M. Prevalence of fosfomycin resistance and mutations in *murA*, *glpT*, and *uhpT* in methicillin-resistant *Staphylococcus aureus* strains isolated from blood and cerebrospinal fluid samples. *Front. Microbiol.* **2016**, *6*, 1544, doi:10.3389/fmicb.2015.01544.
412. Xu, S.; Fu, Z.; Zhou, Y.; Liu, Y.; Xu, X.; Wang, M. Mutations of the transporter proteins GlpT and UhpT confer fosfomycin resistance in *Staphylococcus aureus*. *Front. Microbiol.* **2017**, *8*, 914, doi:10.3389/fmicb.2017.00914.
413. van Wamel, W.J.B.; Rooijakkers, S.H.M.; Ruyken, M.; van Kessel, K.P.M.; van Strijp, J.A.G. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on β-hemolysin-converting bacteriophages. *J. Bacteriol.* **2006**, *188*, 1310–1315, doi:10.1128/JB.188.4.1310-1315.2006.
414. Verkaik, N.J.; Benard, M.; Boelens, H.A.; de Vogel, C.P.; Nouwen, J.L.; Verbrugh, H.A.; Melles, D.C.; van Belkum, A.; van Wamel, W.J.B. Immune evasion cluster-positive bacteriophages are highly prevalent among human *Staphylococcus aureus* strains, but they are not essential in the first stages of nasal colonization. *Clin. Microbiol. Infect.* **2011**, *17*, 343–348, doi:10.1111/j.1469-0691.2010.03227.x.
415. Baquero, F.; Martínez, J.L.; Cantón, R. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* **2008**, *19*, 260–265, doi:10.1016/j.copbio.2008.05.006.
416. Hatosy, S.M.; Martiny, A.C. The ocean as a global reservoir of antibiotic resistance genes. *Appl. Environ. Microbiol.* **2015**, *81*, 7593–7599, doi:10.1128/AEM.00736-15.
417. Marcelino, V.R.; Wille, M.; Hurt, A.C.; González-Acuña, D.; Klaassen, M.; Schlub, T.E.; Eden, J.S.; Shi, M.; Iredell, J.R.; Sorrell, T.C.; et al. Meta-transcriptomics reveals a diverse antibiotic resistance gene pool in avian microbiomes. *BMC Biol.* **2019**, *17*, 31,

doi:10.1186/s12915-019-0649-1.
418. Zeballos-Gross, D.; Rojas-Sereno, Z.; Salgado-Caxito, M.; Poeta, P.; Torres, C.; Benavides, J.A. The Role of Gulls as Reservoirs of Antibiotic Resistance in Aquatic Environments: A Scoping Review. *Front. Microbiol.* **2021**, *12*, 703886, doi:10.3389/fmicb.2021.703886.
419. Diene, S.M.; Corvaglia, A.R.; François, P.; van der Mee-Marquet, N.; Amirault, P.; Lehiani, O.; Archambault, M.; Prevost-Oussar, M.; Bachelier, M.N.; Guinard, F.; et al. Prophages and adaptation of *Staphylococcus aureus* ST398 to the human clinic. *BMC Genomics* **2017**, *18*, 133, doi:10.1186/s12864-017-3516-x.
420. Avberšek, J.; Golob, M.; Papić, B.; Dermota, U.; Grmek Košnik, I.; Kušar, D.; Ocepek, M.; Zdovc, I. Livestock-associated methicillin-resistant *Staphylococcus aureus*: Establishing links between animals and humans on livestock holdings. *Transbound. Emerg. Dis.* **2021**, *68*, 789–801, doi:10.1111/tbed.13745.
421. van der Mee-Marquet, N.; Corvaglia, A.R.; Valentin, A.S.; Hernandez, D.; Bertrand, X.; Girard, M.; Kluytmans, J.; Donnio, P.Y.; Quentin, R.; François, P. Analysis of prophages harbored by the human-adapted subpopulation of *Staphylococcus aureus* CC398. *Infect. Genet. Evol.* **2013**, *18*, 299–308, doi:10.1016/j.meegid.2013.06.009.
422. Salgueiro, V.; Reis, L.; Botelho, M.J.; Manageiro, V.; Caniça, M. Assessing the Bacterial Community Composition of Bivalve Mollusks Collected in Aquaculture Farms and Respective Susceptibility to Antibiotics. *Antibiotics* **2021**, *10*, 1135, doi:10.3390/antibiotics10091135.
423. Salgueiro, V.; Manageiro, V.; Bandarra, N.M.; Ferreira, E.; Clemente, L.; Caniça, M. First comparative genomic characterization of the MSSA ST398 lineage detected in aquaculture and other reservoirs. *Front. Microbiol.* **2023**, *14*, 1035547, doi:10.3389/fmicb.2023.1035547.
424. 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. The scourge of antibiotic resistance: the important role of the environment. *Clin. Infect. Dis.* **2013**, *57*, 704–10, doi:10.1093/cid/cit355.
425. Lupo, A.; Coyne, S.; Berendonk, T.U. Origin and evolution of antibiotic resistance: The common mechanisms of emergence and spread in water bodies. *Front. Microbiol.* **2012**, *3*, 18, doi:10.3389/fmicb.2012.00018.
426. Poirel, L.; Rodriguez-Martinez, J.M.; Mammeri, H.; Liard, A.; Nordmann, P. Origin of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob. Agents Chemother.* **2005**, *49*, 3523–3525, doi:10.1128/AAC.49.8.3523-3525.2005.
427. Poirel, L.; Potron, A.; Nordmann, P. OXA-48-like carbapenemases: the phantom menace. *J. Antimicrob. Chemother.* **2012**, *67*, 1597–1606, doi:10.1093/jac/dks121.
428. 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. Antimicrobial resistance: more than 70 years of war between humans and bacteria. *Crit. Rev. Microbiol.* **2020**, *46*, 578–599, doi:10.1080/1040841X.2020.1813687.
429. Breitbart, M. Marine viruses: Truth or dare. *Ann. Rev. Mar. Sci.* **2012**, *4*, 425–448, doi:10.1146/annurev-marine-120709-142805.
430. Wanyan, R.; Pan, M.; Mai, Z.; Xiong, X.; Su, W.; Yang, J.; Yu, Q.; Wang, X.; Han, Q.; Li, H.; et al. Distribution and influencing factors of antibiotic resistance genes of crayfish (*Procambarus clarkii*) intestine in main crayfish breeding provinces in China. *Sci. Total*

Environ. **2023**, *857*, 159611, doi:10.1016/j.scitotenv.2022.159611.
431. Alikhan, N.F.; Petty, N.K.; Ben Zakour, N.L.; Beatson, S.A. BLAST Ring Image Generator (BRIG): Simple prokaryote genome comparisons. *BMC Genomics* **2011**, *12*, 402, doi:10.1186/1471-2164-12-402.
432. Pal, C.; Bengtsson-Palme, J.; Rensing, C.; Kristiansson, E.; Larsson, D.G.J. BacMet: Antibacterial biocide and metal resistance genes database. *Nucleic Acids Res.* **2014**, *42*, D737-743, doi:10.1093/nar/gkt1252.
433. Cao, X.; Xie, H.; Huang, D.; Zhou, W.; Liu, Y.; Shen, H.; Zhou, K. Detection of a clinical carbapenem-resistant *Citrobacter portucalensis* strain and the dissemination of *C. portucalensis* in clinical settings. *J. Glob. Antimicrob. Resist.* **2021**, *27*, 79–81, doi:10.1016/j.jgar.2021.04.027.
434. Yu, K.; Huang, Z.; Li, Y.; Fu, Q.; Lin, L.; Wu, S.; Dai, H.; Cai, H.; Xiao, Y.; Lan, R.; et al. Establishment and Application of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for Detection of *Shewanella* Genus. *Front. Microbiol.* **2021**, *12*, 625821, doi:10.3389/fmicb.2021.625821.
435. Wu, W.; Feng, Y.; Zong, Z. Precise Species Identification for *Enterobacter*: a Genome Sequence-Based Study with Reporting of Two Novel Species, *Enterobacter quasiroggenkampii* sp. nov. and *Enterobacter quasimori* sp. nov. *mSystems* **2020**, *5*, e00527-20, doi:10.1128/msystems.00527-20.
436. 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.; et al. 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.* **2013**, *32*, 1571–1578, doi:10.1007/s10096-013-1912-y.
437. Turner, J.W.; Tallman, J.J.; Macias, A.; Pinnell, L.J.; Elledge, N.C.; Azadani, D.N.; Nilsson, W.B.; Paranjpye, R.N.; Armbrust, E. V.; Strom, M.S. Comparative genomic analysis of *Vibrio diabolicus* and six taxonomic synonyms: A first look at the distribution and diversity of the expanded species. *Front. Microbiol.* **2018**, *9*, 1893, doi:10.3389/fmicb.2018.01893.
438. Harmon, D.E.; Miranda, O.A.; McCarley, A.; Eshaghian, M.; Carlson, N.; Ruiz, C. Prevalence and characterization of carbapenem-resistant bacteria in water bodies in the Los Angeles–Southern California area. *Microbiologyopen* **2019**, *8*, e00692, doi:10.1002/mbo3.692.
439. Singh, A.K.; Das, S.; Kumar, S.; Gajamer, V.R.; Najar, I.N.; Lepcha, Y.D.; Tiwari, H.K.; Singh, S. Distribution of Antibiotic-Resistant *Enterobacteriaceae* Pathogens in Potable Spring Water of Eastern Indian Himalayas: Emphasis on Virulence Gene and Antibiotic Resistance Genes in *Escherichia coli*. *Front. Microbiol.* **2020**, *11*, 581072, doi:10.3389/fmicb.2020.581072.
440. Bjornsdottir-Butler, K.; Bowers, J.C.; Benner, R.A. Prevalence and characterization of high histamine-producing bacteria in Gulf of Mexico fish species. *J. Food Prot.* **2015**, *78*, 1335–1342, doi:10.4315/0362-028X.JFP-15-012.
441. Navarrete, P.; Magne, F.; Mardones, P.; Riveros, M.; Opazo, R.; Suau, A.; Pochart, P.; Romero, J. Molecular analysis of intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*). *FEMS Microbiol. Ecol.* **2010**, *71*, 148–156, doi:10.1111/j.1574-6941.2009.00769.x.
442. Thakur, P.; Gauba, P. Genomic characterization of *Lelliottia amnigena* PTJIIT1005, a

nitrate tolerant strain isolated from water sample of Yamuna River, Delhi, India. *Microbiol. Resour. Annoucements* **2022**, *11*, e0022922, doi:10.1128/mra.00229-22.
443. Fazal, M.; Alexander, S.; Grayson, N.E.; Deheer-graham, A.; Oliver, K.; Holroyd, N.; Parkhill, J.; Russell, J.E. Complete Whole-Genome Sequences of Two *Raoultella terrigena* Strains, NCTC 13097 and NCTC 13098, Isolated from Human Cases. *Microbiol. Resour. Annoucements* **2019**, *8*, e00239-19, doi:10.1128/MRA.00239-19.
444. Begrem, S.; Jérôme, M.; Leroi, F.; Delbarre-Ladrat, C.; Grovel, O.; Passerini, D. Genomic diversity of *Serratia proteamaculans* and *Serratia liquefaciens* predominant in seafood products and spoilage potential analyses. *Int. J. Food Microbiol.* **2021**, *354*, 109326, doi:10.1016/j.ijfoodmicro.2021.109326.
445. Li, J.; McLaughlin, R.W.; Chen, M.; Liu, Y.L.; Xie, H.X.; Wan, X.L.; Zhou, J.Y.; Zheng, J.S. First case of *Shewanella indica* isolated from a Bryde's whale (*Balaenoptera edeni*) stranded in the northern Beibu Gulf, China. *Antonie Van Leeuwenhoek* **2020**, *113*, 1385–1391, doi:10.1007/s10482-020-01444-z.
446. Sucharita, K.; Sasikala, C.; Park, S.C.; Baik, K.S.; Seong, C.N.; Ramana, C. V. *Shewanella chilikensis* sp. nov., a moderately alkaliphilic gammaproteobacterium isolated from a lagoon. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 3111–3115, doi:10.1099/ijs.0.010918-0.
447. Villa, J.; Arana, D.M.; Viedma, E.; Perez-Montarelo, D.; Chaves, F. Characterization of mobile genetic elements carrying VIM-1 and KPC-2 carbapenemases in *Citrobacter freundii* isolates in Madrid. *Int. J. Med. Microbiol.* **2017**, *307*, 340–345, doi:10.1016/j.ijmm.2017.07.001.
448. Lalaoui, R.; Djukovic, A.; Bakour, S.; Hadjadj, L.; Sanz, J.; Salavert, M.; López-Hontangas, J.L.; Sanz, M.A.; Ubeda, C.; Rolain, J.M. Genomic characterization of *Citrobacter freundii* strains coproducing OXA-48 and VIM-1 carbapenemase enzymes isolated in leukemic patient in Spain. *Antimicrob. Resist. Infect. Control* **2019**, *8*, 167, doi:10.1186/s13756-019-0630-3.
449. Lin, M.; Yang, Y.; Yang, Y.; Chen, G.; He, R.; Wu, Y.; Zhong, L.L.; Ahmed, M.A.E.G.E.S.; Feng, S.; Shen, C.; et al. Co-occurrence of *mcr-9* and bla_{NDM-1} in *Enterobacter cloacae* isolated from a patient with bloodstream infection. *Infect. Drug Resist.* **2020**, *13*, 1397–1402, doi:10.2147/IDR.S248342.
450. Liu, S.; Huang, N.; Zhou, C.; Lin, Y.; Zhang, Y.; Wang, L.; Zheng, X.; Zhou, T.; Wang, Z. Molecular mechanisms and epidemiology of carbapenem-resistant *Enterobacter cloacae* complex isolated from Chinese patients during 2004–2018. *Infect. Drug Resist.* **2021**, *14*, 3647–3658, doi:10.2147/IDR.S327595.
451. Manges, A.R.; Geum, H.M.; Guo, A.; Edens, T.J.; Fibke, C.D.; Pitout, J.D.D. Global Extraintestinal Pathogenic *Escherichia coli* (ExPEC) Lineages. *Clin. Microbiol. Rev.* **2019**, *32*, e00135-18, doi:10.1128/CMR.00135-18.
452. Roschanski, N.; Guenther, S.; Vu, T.T.T.; Fischer, J.; Semmler, T.; Huehn, S.; Alter, T.; Roesler, U. VIM-1 carbapenemase-producing *Escherichia coli* isolated from retail seafood, Germany 2016. *Eurosurveillance* **2017**, *22*, 17–00032, doi:10.2807/1560-7917.ES.2017.22.43.17-00032.
453. Müller, A.; Stephan, R.; Nüesch-Inderbinen, M. Distribution of virulence factors in ESBL-producing *Escherichia coli* isolated from the environment, livestock, food and humans. *Sci. Total Environ.* **2016**, *541*, 667–672, doi:10.1016/j.scitotenv.2015.09.135.
454. Vignaroli, C.; Di Sante, L.; Leoni, F.; Chierichetti, S.; Ottaviani, D.; Citterio, B.; Biavasco, F.

Multidrug-resistant and epidemic clones of *Escherichia coli* from natural beds of Venus clam. *Food Microbiol.* **2016**, *59*, 1–6, doi:10.1016/j.fm.2016.05.003.
455. 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.; et al. A role for CoIV plasmids in the evolution of pathogenic *Escherichia coli* ST58. *Nat. Commun.* **2022**, *13*, 683, doi:10.1038/s41467-022-28342-4.
456. Mori, N.; Kagawa, N.; Aoki, K.; Ishi, Y.; Tateda, K.; Aoki, Y. Clinical and molecular analyses of bloodstream infections caused by IMP metallo-β-lactamase-producing *Enterobacteriaceae* in a tertiary hospital in Japan. *J. Infect. Chemother.* **2020**, *26*, 144–147, doi:10.1016/j.jiac.2019.07.017.
457. Liu, Y.; Wan, L.G.; Deng, Q.; Cao, X.W.; Yu, Y.; Xu, Q.F. First description of NDM-1-, KPC-2-, VIM-2- and IMP-4-producing *Klebsiella pneumoniae* strains in a single Chinese teaching hospital. *Epidemiol. Infect.* **2015**, *143*, 376–384, doi:10.1017/S0950268814000995.
458. Maravić, A.; Skočibušić, M.; Šamanić, I.; Fredotović, Ž.; Cvjetan, S.; Jutronić, M.; Puizina, J. *Aeromonas* spp. simultaneously harbouring $bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}12}$, $bla_{PER\text{-}1}$ and $bla_{FOX\text{-}2}$, in wild-growing Mediterranean mussel (*Mytilus galloprovincialis*) from Adriatic Sea, Croatia. *Int. J. Food Microbiol.* **2013**, *166*, 301–308, doi:10.1016/j.ijfoodmicro.2013.07.010.
459. Huang, L.; Xu, Y. Bin; Xu, J.X.; Ling, J.Y.; Chen, J.L.; Zhou, J. Le; Zheng, L.; Du, Q.P. Antibiotic resistance genes (ARGs) in duck and fish production ponds with integrated or non-integrated mode. *Chemosphere* **2017**, *168*, 1107–1114, doi:10.1016/j.chemosphere.2016.10.096.
460. Juan, C.; Torrens, G.; González-Nicolau, M.; Oliver, A. Diversity and regulation of intrinsic β-lactamases from non-fermenting and other Gram-negative opportunistic pathogens. *FEMS Microbiol. Rev.* **2017**, *41*, 781–815, doi:10.1093/femsre/fux043.
461. Jacobs, L.; Chenia, H.Y. Characterization of integrons and tetracycline resistance determinants in *Aeromonas* spp. isolated from South African aquaculture systems. *Int. J. Food Microbiol.* **2007**, *114*, 295–306, doi:10.1016/j.ijfoodmicro.2006.09.030.
462. Igbinosa, I.H.; Beshiru, A.; Odjadjare, E.E.; Ateba, C.N.; Igbinosa, E.O. Pathogenic potentials of *Aeromonas* species isolated from aquaculture and abattoir environments. *Microb. Pathog.* **2017**, *107*, 185–192, doi:10.1016/j.micpath.2017.03.037.
463. Vásquez-Ponce, F.; Higuera-Llantén, S.; Parás-Silva, J.; Gamboa-Acuña, N.; Cortés, J.; Opazo-Capurro, A.; Ugalde, J.A.; Alcalde-Rico, M.; Olivares-Pacheco, J. Genetic characterization of clinically relevant class 1 integrons carried by multidrug resistant bacteria (MDRB) isolated from the gut microbiota of highly antibiotic treated *Salmo salar*. *J. Glob. Antimicrob. Resist.* **2022**, *29*, 55–62, doi:10.1016/j.jgar.2022.02.003.
464. 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. Analysis of antibiotic multi-resistant bacteria and resistance genes in the effluent of an intensive shrimp farm (Long An, Vietnam). *J. Environ. Manage.* **2018**, *214*, 149–156, doi:10.1016/j.jenvman.2018.02.089.
465. Walckenaer, E.; Delmas, J.; Leflon-Guibout, V.; Bonnet, R.; Nicolas-Chanoine, M.H. Genetic, biochemical characterization and mutagenesis of the chromosomal class A β-lactamase of *Raoultella* (formerly *Klebsiella*) *terrigena*. *Pathol. Biol.* **2015**, *63*, 158–163, doi:10.1016/j.patbio.2015.05.002.

466. Sivaraman, G.K.; Rajan, V.; Vijayan, A.; Elangovan, R.; Prendiville, A.; Bachmann, T.T. 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.* **2021**, *12*, 622891, doi:10.3389/fmicb.2021.622891.
467. Said, L. Ben; Hamdaoui, M.; Jouini, A.; Boudabous, A.; Slama, K. Ben; Torres, C.; Klibi, N. First detection of CTX-M-1 in extended-spectrum β-lactamase-producing *Escherichia coli* in seafood from Tunisia. *J. Food Prot.* **2017**, *80*, 1877–1881, doi:10.4315/0362-028X.JFP-16-324.
468. Ahmed, A.M.; Maruyama, A.; Khalifa, H.O.; Shimamoto, T.; Shimamoto, T. Seafood as a Reservoir of Gram-negative Bacteria Carrying Integrons and Antimicrobial Resistance Genes in Japan. *Biomed. Environ. Sci.* **2015**, *28*, 924–926, doi:10.3967/bes2015.128.
469. Ye, L.; Zhang, L.; Li, X.; Shi, L.; Huang, Y.; Wang, H.H. Antibiotic-resistant bacteria associated with retail aquaculture products from Guangzhou, China. *J. Food Prot.* **2013**, *76*, 295–301, doi:10.4315/0362-028X.JFP-12-288.
470. Cantón, R.; González-Alba, J.M.; Galán, J.C. CTX-M enzymes: Origin and diffusion. *Front. Microbiol.* **2012**, *3*, 110, doi:10.3389/fmicb.2012.00110.
471. González-López, J.J.; Coelho, A.; Larrosa, M.N.; Lavilla, S.; Bartolomé, R.; Prats, G. First detection of plasmid-encoded bla_{OXY} β-Lactamase. *Antimicrob. Agents Chemother.* **2009**, *53*, 3143–3146, doi:10.1128/AAC.01473-08.
472. Walckenaer, E.; Poirel, L.; Leflon-Guibout, V.; Nordmann, P.; Nicolas-Chanoine, M.H. Genetic and Biochemical Characterization of the Chromosomal Class A β-Lactamases of *Raoultella* (formerly *Klebsiella*) *planticola* and *Raoultella ornithinolytica*. *Antimicrob. Agents Chemother.* **2004**, *48*, 305–312, doi:10.1128/AAC.48.1.305-312.2004.
473. Lin, M.; Wu, X.; Yan, Q.; Ma, Y.; Huang, L.; Qin, Y.; Xu, X. Incidence of antimicrobial-resistance genes and integrons in antibiotic-resistant bacteria isolated from eels and aquaculture ponds. *Dis. Aquat. Organ.* **2016**, *120*, 115–123, doi:10.3354/dao03013.
474. Nonaka, L.; Maruyama, F.; Suzuki, S.; Masuda, M. Novel macrolide-resistance genes, *mef(C)* and *mph(G)*, carried by plasmids from *Vibrio* and *Photobacterium* isolated from sediment and seawater of a coastal aquaculture site. *Lett. Appl. Microbiol.* **2015**, *61*, 1–6, doi:10.1111/lam.12414.
475. Gao, Q.; Li, Y.; Qi, Z.; Yue, Y.; Min, M.; Peng, S.; Shi, Z.; Gao, Y. Diverse and abundant antibiotic resistance genes from mariculture sites of China's coastline. *Sci. Total Environ.* **2018**, *630*, 117–125, doi:10.1016/j.scitotenv.2018.02.122.
476. Ye, M.; Hu, X.; Lü, A.; Sun, J.; Chen, C. Isolation and genomic characterization of a pathogenic *Providencia rettgeri* strain G0519 in turtle *Trachemys scripta*. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* **2020**, *113*, 1633–1662, doi:10.1007/s10482-020-01469-4.
477. Chenia, H.Y.; Jacobs, A. Antimicrobial resistance, heavy metal resistance and integron content in bacteria isolated from a South African tilapia aquaculture system. *Dis. Aquat. Organ.* **2017**, *126*, 199–209, doi:10.3354/dao03173.
478. Indugu, N.; Sharma, L.; Jackson, C.R.; Singh, P. Whole-Genome Sequence Analysis of Multidrug-Resistant *Enterobacter hormaechei* Isolated from Imported Retail Shrimp. *Microbiol. Resour. Announc.* **2020**, *9*, e01103-20, doi:10.1128/mra.01103-20.
479. Zhou, Q.; Wang, M.; Zhong, X.; Liu, P.; Xie, X.; Wangxiao, J.; Sun, Y. 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.* **2019**, *26*, 8182–8193, doi:10.1007/s11356-018-04065-2.

480. Manageiro, V.; Salgueiro, V.; Rosado, T.; Bandarra, N.M.; Smith, T.; Dias, E.; Caniça, M. Genomic Analysis of a *mcr-9.1*-Harbouring IncHI2-ST1 Plasmid from *Enterobacter ludwigii* Isolated in Fish Farming. *Antibiotics* **2022**, *11*, 1232, doi:10.3390/antibiotics11091232.
481. Libisch, B.; Abdulkadir, S.; Keresztény, T.; Papp, P.P.; Olasz, F.; Fébel, H.; Sándor, Z.J.; Rasschaert, G.; Lambrecht, E.; Heyndrickx, M.; et al. Detection of Acquired Antibiotic Resistance Genes in Domestic Pig (*Sus scrofa*) and Common Carp (*Cyprinus carpio*) Intestinal Samples by Metagenomics Analyses in Hungary. *Antibiotics* **2022**, *11*, 1441, doi:10.3390/antibiotics11101441.
482. Shah, S.Q.A.; Cabello, F.C.; L'Abée-Lund, T.M.; Tomova, A.; Godfrey, H.P.; Buschmann, A.H.; Sørum, H. Antimicrobial resistance and antimicrobial resistance genes in marine bacteria from salmon aquaculture and non-aquaculture sites. *Environ. Microbiol.* **2014**, *16*, 1310–1320, doi:10.1111/1462-2920.12421.
483. Jayol, A.; Saly, M.; Nordmann, P.; Ménard, A.; Poirel, L.; Dubois, V. Hafnia, an enterobacterial genus naturally resistant to colistin revealed by three susceptibility testing methods. *J. Antimicrob. Chemother.* **2017**, *72*, 2507–2511, doi:10.1093/jac/dkx154.
484. Janda, J.M.; Abbott, S.L. The genus *Hafnia*: From soup to nuts. *Clin. Microbiol. Rev.* **2006**, *19*, 12–28, doi:10.1128/CMR.19.1.12-28.2006.
485. Koivula, T.T.; Juvonen, R.; Haikara, A.; Suihko, M.L. Characterization of the brewery spoilage bacterium *Obesumbacterium proteus* by automated ribotyping and development of PCR methods for its biotype 1. *J. Appl. Microbiol.* **2006**, *100*, 398–406, doi:10.1111/j.1365-2672.2005.02794.x.
486. Vu, T.T.T.; Alter, T.; Roesler, U.; Roschanski, N.; Huehn, S. Investigation of Extended-Spectrum and AmpC β-Lactamase-Producing *Enterobacteriaceae* from retail seafood in Berlin, Germany. *J. Food Prot.* **2018**, *81*, 1079–1086, doi:10.4315/0362-028X.JFP-18-029.
487. Briales, A.; Rodriguez-Martinez, J.M.; Velasco, C.; Machuca, J.; Díaz de Alba, P.; Blazquez, J.; Pascual, A. Exposure to diverse antimicrobials induces the expression of *qnrB1*, *qnrD* and *smaqnr* genes by SOS-dependent regulation. *J. Antimicrob. Chemother.* **2012**, *67*, 2854–2859, doi:10.1093/jac/dks326.
488. Kraychete, G.B.; Campana, E.H.; Picão, R.C.; Bonelli, R.R. *qnrD*-harboring plasmids in *Providencia* spp. recovered from food and environmental Brazilian sources. *Sci. Total Environ.* **2019**, *646*, 1290–1292, doi:10.1016/j.scitotenv.2018.07.378.
489. Su, H.; Liu, S.; Hu, X.; Xu, X.; Xu, W.; Xu, Y.; Li, Z.; Wen, G.; Liu, Y.; Cao, Y. Occurrence and temporal variation of antibiotic resistance genes (ARGs) in shrimp aquaculture: ARGs dissemination from farming source to reared organisms. *Sci. Total Environ.* **2017**, *607–608*, 357–366, doi:10.1016/j.scitotenv.2017.07.040.
490. Jang, H.M.; Kim, Y.B.; Choi, S.; Lee, Y.; Shin, S.G.; Unno, T.; Kim, Y.M. Prevalence of antibiotic resistance genes from effluent of coastal aquaculture, South Korea. *Environ. Pollut.* **2018**, *233*, 1049–1057, doi:10.1016/j.envpol.2017.10.006.
491. Abgottspon, H.; Zurfluh, K.; Nüesch-Inderbinen, M.; Hächler, H.; Stephan, R. 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.* **2014**, *58*, 3560–3563, doi:10.1128/AAC.02404-14.

492. Tamminen, M.; Karkman, A.; Lõhmus, A.; Muziasari, W.I.; Takasu, H.; Wada, S.; Suzuki, S.; Virta, M. Tetracycline resistance genes persist at aquaculture farms in the absence of selection pressure. *Environ. Sci. Technol.* **2011**, *45*, 386–391, doi:10.1021/es102725n.
493. Herrera, F.C.; Santos, J.A.; Otero, A.; García-López, M.L. Occurrence of *Plesiomonas shigelloides* in displayed portions of saltwater fish determined by a PCR assay based on the *hugA* gene. *Int. J. Food Microbiol.* **2006**, *108*, 233–238, doi:10.1016/j.ijfoodmicro.2005.12.008.
494. Zheng, X.; Ma, J.; Lu, Y.; Sun, D.; Yang, H.; Xia, F.; Tang, B. Detection of *tet(X6)* variant–producing *Proteus terrae* subsp. *cibarius* from animal cecum in Zhejiang, China. *J. Glob. Antimicrob. Resist.* **2022**, *29*, 124–130, doi:10.1016/j.jgar.2022.02.011.
495. Tomova, A.; Ivanova, L.; Buschmann, A.H.; Rioseco, M.L.; Kalsi, R.K.; Godfrey, H.P.; Cabello, F.C. Antimicrobial resistance genes in marine bacteria and human uropathogenic *Escherichia coli* from a region of intensive aquaculture. *Environ. Microbiol. Rep.* **2015**, *7*, 803–809, doi:10.1111/1758-2229.12327.
496. Zhang, R.Q.; Ying, G.G.; Su, H.C.; Zhou, L.J.; Liu, Y.S. Antibiotic resistance and genetic diversity of *Escherichia coli* isolates from traditional and integrated aquaculture in South China. *J. Environ. Sci. Heal. - Part B Pestic. Food Contam. Agric. Wastes* **2013**, *48*, 999–1013, doi:10.1080/03601234.2013.816611.
497. 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.; et al. Diagnosis, genetic variations, virulence, and toxicity of AHPND-positive *Vibrio parahaemolyticus* in *Penaeus monodon*. *Aquac. Int.* **2020**, *28*, 2531–2546, doi:10.1007/s10499-020-00607-z.
498. Michaud, L.; Lo Giudice, A.; Troussellier, M.; Smedile, F.; Bruni, V.; Blancheton, J.P. Phylogenetic characterization of the heterotrophic bacterial communities inhabiting a marine recirculating aquaculture system. *J. Appl. Microbiol.* **2009**, *107*, 1935–1946, doi:10.1111/j.1365-2672.2009.04378.x.
499. Miranda, C.D.; Kehrenberg, C.; Ulep, C.; Schwarz, S.; Roberts, M.C. Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob. Agents Chemother.* **2003**, *47*, 883–888, doi:10.1128/AAC.47.3.883-888.2003.
500. Mon-On, N.; Surachetpong, W.; Mongkolsuk, S.; Sirikanchana, K. 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.* **2018**, *41*, 783–789, doi:10.1111/jfd.12776.
501. Hu, J.; Le, Q.; Wang, Y.; Yu, N.; Cao, X.; Kuang, S.; Zhang, M.; Gu, W.; Sun, Y.; Yang, Y.; et al. Effects of formaldehyde on detoxification and immune responses in silver pomfret (*Pampus argenteus*). *Fish Shellfish Immunol.* **2019**, *88*, 449–457, doi:10.1016/j.fsi.2019.03.019.
502. Jia, R.; Du, J.; Cao, L.; Feng, W.; He, Q.; Xu, P.; Yin, G. Application of transcriptome analysis to understand the adverse effects of hydrogen peroxide exposure on brain function in common carp (*Cyprinus carpio*). *Environ. Pollut.* **2021**, *286*, 117240, doi:10.1016/j.envpol.2021.117240.
503. Výrostková, J.; Pipová, M.; Semjon, B.; Jevinová, P.; Regecová, I.; Malová, J. Antibacterial

effects of hydrogen peroxide and caprylic acid on selected foodborne bacteria. *Pol. J. Vet. Sci.* **2020**, *23*, 439–446, doi:10.24425/pjvs.2020.134689.
504. Sabri, M.; Léveillé, S.; Dozois, C.M. A SitABCD homologue from an avian pathogenic *Escherichia coli* strain mediates transport of iron and manganese and resistance to hydrogen peroxide. *Microbiology* **2006**, *152*, 745–758, doi:10.1099/mic.0.28682-0.
505. Galardini, M.; Clermont, O.; Baron, A.; Busby, B.; Dion, S.; Schubert, S.; Beltrao, P.; Denamur, E. 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.* **2020**, *16*, e1009065, doi:10.1371/journal.pgen.1009065.
506. Boyer, E.; Bergevin, I.; Malo, D.; Gros, P.; Cellier, M.F.M. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* **2002**, *70*, 6032–6042, doi:10.1128/IAI.70.11.6032.
507. Green, R.T.; Todd, J.D.; Johnston, A.W.B. Manganese uptake in marine bacteria; The novel MntX transporter is widespread in Roseobacters, Vibrios, Alteromonadales and the SAR11 and SAR116 clades. *ISME J.* **2013**, *7*, 581–591, doi:10.1038/ismej.2012.140.
508. Lv, J.; Hu, R.; Wang, N.; Zhu, L.; Zhang, X.; Yuan, X.; Liu, B. 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* **2021**, *278*, 130352, doi:10.1016/j.chemosphere.2021.130352.
509. Razak, M.R.; Aris, A.Z.; Zakaria, N.A.C.; Wee, S.Y.; Ismail, N.A.H. Accumulation and risk assessment of heavy metals employing species sensitivity distributions in Linggi River, Negeri Sembilan, Malaysia. *Ecotoxicol. Environ. Saf.* **2021**, *211*, 111905, doi:10.1016/j.ecoenv.2021.111905.
510. Farmaki, E.G.; Thomaidis, N.S.; Pasias, I.N.; Baulard, C.; Papaharisis, L.; Efstathiou, C.E. Environmental impact of intensive aquaculture: Investigation on the accumulation of metals and nutrients in marine sediments of Greece. *Sci. Total Environ.* **2014**, *485–486*, 554–562, doi:10.1016/j.scitotenv.2014.03.125.
511. Zagui, G.S.; Moreira, N.C.; Santos, D.V.; Darini, A.L.C.; Domingo, J.L.; Segura-Muñoz, S.I.; Andrade, L.N. High occurrence of heavy metal tolerance genes in bacteria isolated from wastewater: A new concern? *Environ. Res.* **2021**, *196*, 110352, doi:10.1016/j.envres.2020.110352.
512. Sinha, R.K.; Krishnan, K.P.; Kurian, P.J. 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* **2021**, *55*, 100804, doi:10.1016/j.margen.2020.100804.
513. Wang, D.; Fierke, C.A. The BaeSR regulon is involved in defense against zinc toxicity in *E. coli*. *Met. Integr. biometal Sci.* **2013**, *5*, 372–83, doi:10.1039/c3mt20217h.
514. Boyd, E.S.; Barkay, T. The mercury resistance operon: from an origin in a geothermal environment to an efficient detoxification machine. *Front. Microbiol.* **2012**, *3*, 349, doi:10.3389/fmicb.2012.00349.
515. Koler, M.; Frank, V.; Amartely, H.; Friedler, A.; Vaknin, A. Dynamic clustering of the bacterial sensory kinase BaeS. *PLoS One* **2016**, *11*, e0150349, doi:10.1371/journal.pone.0150349.
516. Higgins, K.A.; Carr, C.E.; Maroney, M.J. Specific metal recognition in nickel trafficking. *Biochemistry* **2012**, *51*, 7816–7832, doi:10.1021/bi300981m.

517. Ouyang, A.; Gasner, K.M.; Neville, S.L.; McDevitt, C.A.; Frawley, E.R. MntP and YiiP Contribute to Manganese Efflux in *Salmonella enterica* Serovar Typhimurium under Conditions of Manganese Overload and Nitrosative Stress. *Microbiol. Spectr.* **2022**, *10*, e0131621, doi:10.1128/spectrum.01316-21.
518. Sherpa, M.T.; Najar, I.N.; Das, S.; Thakur, N. Distribution of antibiotic and metal resistance genes in two glaciers of North Sikkim, India. *Ecotoxicol. Environ. Saf.* **2020**, *203*, 111037, doi:10.1016/j.ecoenv.2020.111037.
519. Kajfasz, J.K.; Katrak, C.; Ganguly, T.; Vargas, J.; Wright, L.; Peters, Z.T.; Spatafora, G.A.; Abranches, J.; Lemos, J.A. Manganese Uptake, Mediated by SloABC and MntH, Is Essential for the Fitness of *Streptococcus mutans*. *mSphere* **2020**, *5*, e00764-19, doi:10.1128/mSphere.00764-19.
520. Jie, S.; Li, M.; Gan, M.; Zhu, J.; Yin, H.; Liu, X. Microbial functional genes enriched in the Xiangjiang River sediments with heavy metal contamination. *BMC Microbiol.* **2016**, *16*, 179, doi:10.1186/s12866-016-0800-x.
521. Ju, Y.R.; Chen, C.W.; Chen, C.F.; Chuang, X.Y.; Dong, C. Di Assessment of heavy metals in aquaculture fishes collected from southwest coast of Taiwan and human consumption risk. *Int. Biodeterior. Biodegrad.* **2017**, *124*, 314–325, doi:10.1016/j.ibiod.2017.04.003.
522. Wang, C.; Wang, Y.; Liu, P.; Sun, Y.; Song, Z.; Hu, X. Characteristics of bacterial community structure and function associated with nutrients and heavy metals in coastal aquaculture area. *Environ. Pollut.* **2021**, *275*, 116639, doi:10.1016/j.envpol.2021.116639.
523. Jiang, H.; Yu, T.; Yang, Y.; Yu, S.; Wu, J.; Lin, R.; Li, Y.; Fang, J.; Zhu, C. 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.* **2020**, *11*, 1294, doi:10.3389/fmicb.2020.01294.
524. He, Y.; Wang, S.; Zhang, J.; Zhang, X.; Sun, F.; He, B.; Liu, X. Integrative and conjugative elements-positive *Vibrio parahaemolyticus* isolated from aquaculture shrimp in Jiangsu, China. *Front. Microbiol.* **2019**, *10*, 1574, doi:10.3389/fmicb.2019.01574.
525. Nabinger, D.D.; Altenhofen, S.; Bitencourt, P.E.R.; Nery, L.R.; Leite, C.E.; Vianna, M.R.M.R.; Bonan, C.D. Nickel exposure alters behavioral parameters in larval and adult zebrafish. *Sci. Total Environ.* **2018**, *624*, 1623–1633, doi:10.1016/j.scitotenv.2017.10.057.
526. Rabsch, W.; Methner, U.; Voigt, W.; Tschäpe, H.; Reissbrodt, R.; Williams, P.H. Role of Receptor Proteins for Enterobactin and 2,3-Dihydroxybenzoylserine in Virulence of *Salmonella enterica*. *Infect. Immun.* **2003**, *71*, 6953–6961, doi:10.1128/IAI.71.12.6953-6961.2003.
527. Greig, S.L.; Radjainia, M.; Mitra, A.K. Oligomeric structure of Colicin Ia channel in lipid bilayer membranes. *J. Biol. Chem.* **2009**, *284*, 16126–16134, doi:10.1074/jbc.M900292200.
528. Baquero, F.; Lanza, V.F.; Baquero, M.R.; del Campo, R.; Bravo-Vázquez, D.A. Microcins in *Enterobacteriaceae*: Peptide Antimicrobials in the Eco-Active Intestinal Chemosphere. *Front. Microbiol.* **2019**, *10*, 2261, doi:10.3389/fmicb.2019.02261.
529. Nicholson, B.A.; West, A.C.; Mangiamele, P.; Barbieri, N.; Wannemuehler, Y.; Nolan, L.K.; Logue, C.M.; Li, G. Genetic characterization of ExPEC-like virulence plasmids among a subset of NMEC. *PLoS One* **2016**, *11*, e0147757, doi:10.1371/journal.pone.0147757.
530. Massip, C.; Oswald, E. Siderophore-Microcins in *Escherichia coli*: Determinants of

Digestive Colonization, the First Step Toward Virulence. *Front. Cell. Infect. Microbiol.* **2020**, *10*, 381, doi:10.3389/fcimb.2020.00381.
531. Murase, K.; Martin, P.; Porcheron, G.; Houle, S.; Helloin, E.; Pénary, M.; Nougayrède, J.P.; Dozois, C.M.; Hayashi, T.; Oswald, E. HlyF produced by extraintestinal pathogenic *Escherichia coli* is a virulence factor that regulates outer membrane vesicle biogenesis. *J. Infect. Dis.* **2015**, *213*, 856–865, doi:10.1093/infdis/jiv506.
532. Ling, J.; Pan, H.; Gao, Q.; Xiong, L.; Zhou, Y.; Zhang, D.; Gao, S.; Liu, X. Aerobactin Synthesis Genes *iucA* and *iucC* Contribute to the Pathogenicity of Avian Pathogenic *Escherichia coli* O2 Strain E058. *PLoS One* **2013**, *8*, e57794, doi:10.1371/journal.pone.0057794.
533. Messaili, C.; Messai, Y.; Bakour, R. Virulence gene profiles, antimicrobial resistance and phylogenetic groups of fecal *Escherichia coli* strains isolated from broiler chickens in Algeria. *Vet. Ital.* **2019**, *55*, 35–46, doi:10.12834/VetIt.799.3865.2.
534. 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. *Escherichia coli* Bloodstream Infections in Patients at a University Hospital: Virulence factors and clinical characteristics. *Front. Cell. Infect. Microbiol.* **2019**, *9*, 191, doi:10.3389/fcimb.2019.00191.
535. Desloges, I.; Taylor, J.A.; Leclerc, J.M.; Brannon, J.R.; Portt, A.; Spencer, J.D.; Dewar, K.; Marczynski, G.T.; Manges, A.; Gruenheid, S.; et al. Identification and characterization of OmpT-like proteases in uropathogenic *Escherichia coli* clinical isolates. *Microbiologyopen* **2019**, *8*, e915, doi:10.1002/mbo3.915.
536. Massella, E.; Reid, C.J.; Cummins, M.L.; Anantanawat, K.; Zingali, T.; Serraino, A.; Piva, S.; Giacometti, F.; Djordjevic, S.P. Snapshot study of whole genome sequences of *Escherichia coli* from healthy companion animals, livestock, wildlife, humans and food in Italy. *Antibiotics* **2020**, *9*, 782, doi:10.3390/antibiotics9110782.
537. Zhang, Y.; Zhao, L.; Chen, W.; Huang, Y.; Yang, L.; Sarathbabu, V.; Wu, Z.; Li, J.; Nie, P.; Lin, L. Complete genome sequence analysis of the fish pathogen *Flavobacterium columnare* provides insights into antibiotic resistance and pathogenicity related genes. *Microb. Pathog.* **2017**, *111*, 203–211, doi:10.1016/j.micpath.2017.08.035.
538. Medina-Pizzali, M.L.; Venkatesh, A.; Riveros, M.; Cuicapuza, D.; Salmon-Mulanovich, G.; Mäusezahl, D.; Hartinger, S.M. Whole-Genome Characterisation of ESBL-Producing *E. coli* Isolated from Drinking Water and Dog Faeces from Rural Andean Households in Peru. *Antibiotics* **2022**, *11*, 692, doi:10.3390/antibiotics11050692.
539. Vingino, A.; Roberts, M.C.; Wainstein, M.; West, J.; Norman, S.A.; Lambourn, D.; Lahti, J.; Ruiz, R.; D'angeli, M.; Weissman, S.J.; et al. Surveillance for Antibiotic-Resistant *E. coli* in the Salish Sea Ecosystem. *Antibiotics* **2021**, *10*, 1201, doi:10.3390/antibiotics10101201.
540. 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.; et al. World Health Organization critical priority *Escherichia coli* clone ST648 in magnificent frigatebird (*Fregata magnificens*) of an uninhabited insular environment. *Front. Microbiol.* **2022**, *13*, 940600, doi:10.3389/fmicb.2022.940600.
541. McDougall, F.; Boardman, W.; Power, M. High Prevalence of Beta-Lactam-Resistant *Escherichia coli* in South Australian Grey-Headed Flying Fox Pups (*Pteropus poliocephalus*). *Microorganisms* **2022**, *10*, 1589, doi:10.3390/microorganisms10081589.
542. Elabd, H.; Abd El-latif, A.; Shaheen, A.; Matter, A. Identification of emerging *Acinetobacter johnsonii* virulence and antibiotic resistance genes associated with high

mortality in cultured *Oreochromis niloticus*. *Egypt. J. Aquac.* **2020**, *10*, 19–33, doi:10.21608/eja.2020.29010.1021.
543. Tang, X.; Wang, H.; Liu, F.; Sheng, X.; Xing, J.; Zhan, W. Recombinant outer membrane protein T (OmpT) of *Vibrio ichthyoenteri*, a potential vaccine candidate for flounder (*Paralichthys olivaceus*). *Microb. Pathog.* **2019**, *126*, 185–192, doi:10.1016/j.micpath.2018.11.001.
544. 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. Molecular characterization and phylogeny of Shiga toxin–producing *Escherichia coli* isolates obtained from two Dutch regions using whole genome sequencing. *Clin. Microbiol. Infect.* **2016**, *22*, 642.e1-642.e9, doi:10.1016/j.cmi.2016.03.028.
545. Javadi, K.; Mohebi, S.; Motamedifar, M.; Hadi, N. Characterization and antibiotic resistance pattern of diffusely adherent *Escherichia coli* (DAEC), isolated from paediatric diarrhoea in Shiraz, southern Iran. *New Microbes New Infect.* **2020**, *38*, 100780, doi:10.1016/j.nmni.2020.100780.
546. Umpiérrez, A.; Ernst, D.; Fernández, M.; Oliver, M.; Casaux, M.L.; Caffarena, R.D.; Schild, C.; Giannitti, F.; Fraga, M.; Zunino, P. Virulence genes of *Escherichia coli* in diarrheic and healthy calves. *Rev. Argent. Microbiol.* **2021**, *53*, 34–38, doi:10.1016/j.ram.2020.04.004.
547. Karama, M.; Mainga, A.O.; Cenci-Goga, B.T.; Malahlela, M.; El-Ashram, S.; Kalake, A. Molecular profiling and antimicrobial resistance of Shiga toxin-producing *Escherichia coli* O26, O45, O103, O121, O145 and O157 isolates from cattle on cow-calf operations in South Africa. *Sci. Rep.* **2019**, *9*, 11930, doi:10.1038/s41598-019-47948-1.
548. Srinivasan, U.; Foxman, B.; Marrs, C.F. Identification of a gene encoding heat-resistant agglutinin in *Escherichia coli* as a putative virulence factor in urinary tract infection. *J. Clin. Microbiol.* **2003**, *41*, 285–289, doi:10.1128/JCM.41.1.285-289.2003.
549. Li, D.; Shen, M.; Xu, Y.; Liu, C.; Wang, W.; Wu, J.; Luo, X.; Jia, X.; Ma, Y. Virulence gene profiles and molecular genetic characteristics of diarrheagenic *Escherichia coli* from a hospital in western China. *Gut Pathog.* **2018**, *10*, 35, doi:10.1186/s13099-018-0262-9.
550. 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. Phylogeny, resistome, and virulome of *Escherichia coli* causing biliary tract infections. *J. Clin. Med.* **2019**, *8*, 2118, doi:10.3390/jcm8122118.
551. Lima, I.F.N.; Boisen, N.; da Silva Quetz, J.; Havt, A.; de Carvalho, E.B.; Soares, A.M.; Lima, N.L.; Mota, R.M.S.; Nataro, J.P.; Guerrant, R.L.; et al. Prevalence of enteroaggregative *Escherichia coli* and its virulence-related genes in a case-control study among children from north-eastern Brazil. *J. Med. Microbiol.* **2013**, *62*, 683–693, doi:10.1099/jmm.0.054262-0.
552. Ngo, T.P.H.; Vu, H.T.T.; Le, T.T.T.; Bui, H.C.N.; Liles, M.R.; Rodkhum, C. Comparative genomic analysis of hypervirulent *Aeromonas hydrophila* strains from striped catfish (*Pangasianodon hypophthalmus*) in Vietnam. *Aquaculture* **2022**, *558*, 738364, doi:10.1016/j.aquaculture.2022.738364.
553. Bueris, V.; Sellera, F.P.; Fuga, B.; Sano, E.; Carvalho, M.P.N.; Couto, S.C.F.; Moura, Q.; Lincopan, N. Convergence of virulence and resistance in international clones of WHO critical priority enterobacterales isolated from Marine Bivalves. *Sci. Rep.* **2022**, *12*, 5707, doi:10.1038/s41598-022-09598-8.
554. Nadya, S.; Delaquis, P.; Chen, J.; Allen, K.; Johnson, R.P.; Ziebell, K.; Laing, C.; Gannon, V.;

Bach, S.; Topp, E. 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.* **2016**, *6*, 36, doi:10.3389/fcimb.2016.00036.

555. 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. Microbiome patterns reveal the transmission of pathogenic bacteria in hilsa fish (*Tenualosa ilisha*) marketed for human consumption in Bangladesh. *J. Appl. Microbiol.* **2019**, *126*, 1879–1890, doi:10.1111/jam.14257.
556. Magaña-Lizárraga, J.A.; Gómez-Gil, B.; Rendón-Maldonado, J.G.; Delgado-Vargas, F.; Vega-López, I.F.; Báez-Flores, M.E. 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* **2022**, *10*, 662, doi:10.3390/microorganisms10030662.
557. Vignaroli, C.; Luna, G.M.; Rinaldi, C.; Di Cesare, A.; Danovaro, R.; Biavasco, F. New Sequence Types and Multidrug Resistance among Pathogenic *Escherichia coli* Isolates from Coastal Marine Sediments. *Appl. Environ. Microbiol.* **2012**, *78*, 3916–3922, doi:10.1128/AEM.07820-11.
558. Deng, Y.; Xu, L.; Chen, H.; Liu, S.; Guo, Z.; Cheng, C.; Ma, H.; Feng, J. Prevalence, virulence genes, and antimicrobial resistance of *Vibrio* species isolated from diseased marine fish in South China. *Sci. Rep.* **2020**, *10*, 14329, doi:10.1038/s41598-020-71288-0.
559. Nhinh, D.T.; Le, D.V.; Van, K. Van; Giang, N.T.H.; Dang, L.T.; Hoai, T.D. Prevalence, virulence gene distribution and alarming the multidrug resistance of *Aeromonas hydrophila* associated with disease outbreaks in freshwater aquaculture. *Antibiotics* **2021**, *10*, 532, doi:10.3390/antibiotics10050532.
560. Ryu, A.R.; Mok, J.S.; Lee, D.E.; Kwon, J.Y.; Park, K. Occurrence, virulence, and antimicrobial resistance of *Vibrio parahaemolyticus* isolated from bivalve shellfish farms along the southern coast of Korea. *Environ. Sci. Pollut. Res.* **2019**, *26*, 21034–21043, doi:10.1007/s11356-019-05426-1.
561. Ceccarelli, D.; Hasan, N.A.; Huq, A.; Colwell, R.R. Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. *Front. Cell. Infect. Microbiol.* **2013**, *3*, 97, doi:10.3389/fcimb.2013.00097.
562. Çam, S.; Brinkmeyer, R. 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.* **2020**, *113*, 1437–1446, doi:10.1007/s10482-020-01452-z.
563. Rubio-Portillo, E.; Martin-Cuadrado, A.B.; Caraballo-Rodríguez, A.M.; Rohwer, F.; Dorrestein, P.C.; Antón, J. Virulence as a side effect of interspecies interaction in *Vibrio* coral pathogens. *MBio* **2020**, *11*, e00201-20, doi:10.1128/mBio.00201-20.
564. Pattanayak, S.; Priyadarsini, S.; Paul, A.; Kumar, P.R.; Sahoo, P.K. Diversity of virulence-associated genes in pathogenic *Aeromonas hydrophila* isolates and their in vivo modulation at varied water temperatures. *Microb. Pathog.* **2020**, *147*, 104424, doi:10.1016/j.micpath.2020.104424.
565. Ranquet, C.; Toussaint, A.; De Jong, H.; Maenhaut-Michel, G.; Geiselmann, J. Control of bacteriophage Mu lysogenic repression. *J. Mol. Biol.* **2005**, *353*, 186–195, doi:10.1016/j.jmb.2005.08.015.

566. Ramsamy, Y.; Mlisana, K.P.; Amoako, D.G.; Allam, M.; Ismail, A.; Singh, R.; Abia, A.L.K.; Essack, S.Y. Pathogenomic analysis of a novel extensively drug-resistant *Citrobacter freundii* isolate carrying a *bla*$_{NDM-1}$ carbapenemase in South Africa. *Pathogens* **2020**, *9*, 89, doi:10.3390/pathogens9020089.
567. Enany, S.; Zakeer, S.; Diab, A.A.; Bakry, U.; Sayed, A.A. Whole genome sequencing of *Klebsiella pneumoniae* clinical isolates sequence type 627 isolated from Egyptian patients. *PLoS One* **2022**, *17*, e0265884, doi:10.1371/journal.pone.0265884.
568. Ragab, W.; Kawato, S.; Nozaki, R.; Kondo, H.; Hirono, I. Comparative genome analyses of five *Vibrio penaeicida* strains provide insights into their virulence-related factors. *Microb. Genomics* **2022**, *8*, 000766, doi:10.1099/mgen.0.000766.
569. Wendling, C.C.; Refardt, D.; Hall, A.R. Fitness benefits to bacteria of carrying prophages and prophage-encoded antibiotic-resistance genes peak in different environments. *Evolution (N. Y).* **2021**, *75*, 515–528, doi:10.1111/evo.14153.
570. Olszak, T.; Latka, A.; Roszniowski, B.; Valvano, M.A.; Drulis-Kawa, Z. Phage Life Cycles Behind Bacterial Biodiversity. *Curr. Med. Chem.* **2017**, *24*, 3987–4001, doi:10.2174/0929867324666170413100136.
571. Sultan, I.; Ali, A.; Gogry, F.A.; Rather, I.A.; Sabir, J.S.M.; Haq, Q.M.R. Bacterial isolates harboring antibiotics and heavy-metal resistance genes co-existing with mobile genetic elements in natural aquatic water bodies. *Saudi J. Biol. Sci.* **2020**, *27*, 2660–2668, doi:10.1016/j.sjbs.2020.06.002.
572. Jones-Dias, D.; Manageiro, V.; Graça, R.; Sampaio, D.A.; Albuquerque, T.; Themudo, P.; Vieira, L.; Ferreira, E.; Clemente, L.; Caniça, M. QnrS1- and Aac(6')-Ib-cr-producing *Escherichia coli* among isolates from animals of different sources: Susceptibility and genomic characterization. *Front. Microbiol.* **2016**, *7*, 671, doi:10.3389/fmicb.2016.00671.
573. Nicolas, E.; Lambin, M.; Dandoy, D.; Galloy, C.; Nguyen, N.; Oger, C.A.; Hallet, B. The Tn*3*-family of replicative transposons. *Microbiol. Spectr.* **2015**, *3*, doi:10.1128/microbiolspec.MDNA3-0060-2014.
574. Zhao, H.; Chen, W.; Xu, X.; Zhou, X.; Shi, C. Transmissible ST3-IncHI2 plasmids are predominant carriers of diverse complex IS*26*-class 1 integron arrangements in multidrug-resistant *Salmonella*. *Front. Microbiol.* **2018**, *9*, 2492, doi:10.3389/fmicb.2018.02492.
575. Kubomura, A.; Sekizuka, T.; Onozuka, D.; Murakami, K.; Kimura, H.; Sakaguchi, M.; Oishi, K.; Hirai, S.; Kuroda, M.; Okabe, N. Truncated Class 1 Integron Gene Cassette Arrays Contribute to Antimicrobial Resistance of Diarrheagenic *Escherichia coli*. *Biomed Res. Int.* **2020**, *2020*, 4908189, doi:10.1155/2020/4908189.
576. Gomes-Neves, E.; Manageiro, V.; Ferreira, E.; Correia da Costa, J.M.; Caniça, M. First description of food-borne *Salmonella enterica* resistance regions R1 and R3 associated with IS*26* elements. *Res. Microbiol.* **2015**, *166*, 570–573, doi:10.1016/j.resmic.2015.03.007.
577. Betteridge, T.; Yang, J.; Pittard, A.J.; Praszkier, J. Role of RepA and DnaA proteins in the opening of the origin of DNA replication of an IncB plasmid. *J. Bacteriol.* **2004**, *186*, 3785–3793, doi:10.1128/JB.186.12.3785-3793.2004.
578. Jiang, H.; Cheng, H.; Liang, Y.; Yu, S.; Yu, T.; Fang, J.; Zhu, C. 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.* **2019**, *10*, 1787, doi:10.3389/fmicb.2019.01787.
579. Suzuki, S.; Kadoya, A.; Masuda, N.; Sugimoto, Y.; Takada, H.; Mizukawa, K.; Takei, A.; Chou, H.Y.; Wu, J.H. Macrolide resistance genes and mobile genetic elements in waterways from pig farms to the sea in Taiwan. *J. Glob. Antimicrob. Resist.* **2022**, *29*, 360–370, doi:10.1016/j.jgar.2022.04.024.
580. Andersson, D.I.; Hughes, D. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev. Microbiol.* **2014**, *12*, 465–478, doi:10.1038/nrmicro3270.
581. Piotrowska, M.; Przygodzinska, D.; Matyjewicz, K.; Popowska, M. Occurrence and variety of ß-lactamase genes among *Aeromonas* spp. isolated from Urban Wastewater treatment plant. *Front. Microbiol.* **2017**, *8*, 863, doi:10.3389/fmicb.2017.00863.
582. Ross, K.; Varani, A.M.; Snesrud, E.; Huang, H.; Alvarenga, D.O.; Zhang, J.; Wu, C.; McGann, P.; Chandlere, M. TnCentral: a Prokaryotic Transposable Element Database and Web Portal for Transposon Analysis. *MBio* **2021**, *12*, e0206021, doi:10.1128/mBio.02060-21.
583. Mbelle, N.M.; Feldman, C.; Osei Sekyere, J.; Maningi, N.E.; Modipane, L.; Essack, S.Y. The Resistome, Mobilome, Virulome and Phylogenomics of Multidrug-Resistant *Escherichia coli* Clinical Isolates from Pretoria, South Africa. *Sci. Rep.* **2019**, *9*, 16457, doi:10.1038/s41598-019-52859-2.
584. Møller, T.S.B.; Overgaard, M.; Nielsen, S.S.; Bortolaia, V.; Sommer, M.O.A.; Guardabassi, L.; Olsen, J.E. Relation between *tetR* and *tetA* expression in tetracycline resistant *Escherichia coli*. *BMC Microbiol.* **2016**, *16*, 39, doi:10.1186/s12866-016-0649-z.
585. Li, Y.; Zhang, Y.; Chen, M.; Hu, J.; Zhang, H.; Xiang, Y.; Yang, H.; Qiu, S.; Song, H. 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.* **2021**, *103*, 13–18, doi:10.1016/j.ijid.2020.11.150.
586. Hong, H.; Jung, J.; Park, W. Plasmid-encoded tetracycline efflux pump protein alters bacterial stress responses and ecological fitness of *Acinetobacter oleivorans*. *PLoS One* **2014**, *9*, e107716, doi:10.1371/journal.pone.0107716.
587. Tran, T.; Andres, P.; Petroni, A.; Soler-Bistué, A.; Albornoz, E.; Zorreguieta, A.; Reyes-Lamothe, R.; Sherratt, D.J.; Corso, A.; Tolmaskya, M.E. Small plasmids harboring *qnrB19*: A model for plasmid evolution mediated by site-specific recombination at *oriT* and Xer sites. *Antimicrob. Agents Chemother.* **2012**, *56*, 1821–1827, doi:10.1128/AAC.06036-11.
588. Pallecchi, L.; Riccobono, E.; Sennati, S.; Mantella, A.; Bartalesi, F.; Trigoso, C.; Gotuzzo, E.; Bartoloni, A.; Rossolini, G.M. Characterization of small ColE-like plasmids mediating widespread dissemination of the *qnrB19* gene in commensal enterobacteria. *Antimicrob. Agents Chemother.* **2010**, *54*, 678–682, doi:10.1128/AAC.01160-09.
589. Caniça, M.; Manageiro, V.; Jones-Dias, D.; Clemente, L.; Gomes-Neves, E.; Poeta, P.; Dias, E.; Ferreira, E. Current perspectives on the dynamics of antibiotic resistance in different reservoirs. *Res. Microbiol.* **2015**, *166*, 594–600, doi:10.1016/j.resmic.2015.07.009.
590. Kempf, I.; Jouy, E.; Chauvin, C. Colistin use and colistin resistance in bacteria from animals. *Int. J. Antimicrob. Agents* **2016**, *48*, 598–606, doi:10.1016/j.ijantimicag.2016.09.016.
591. Poirel, L.; Jayol, A.; Nordmann, P. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. *Clin. Microbiol. Rev.* **2017**, *30*, 557–596, doi:10.1128/CMR.00064-16.
592. Andrade, F.F.; Silva, D.; Rodrigues, A.; Pina-Vaz, C. Colistin update on its mechanism of

action and resistance, present and future challenges. *Microorganisms* **2020**, *8*, 1716, doi:10.3390/microorganisms8111716.
593. Sun, J.; Zhang, H.; Liu, Y.H.; Feng, Y. Towards Understanding MCR-like Colistin Resistance. *Trends Microbiol.* **2018**, *26*, 794–808, doi:10.1016/j.tim.2018.02.006.
594. Zhang, S.; Abbas, M.; Rehman, M.U.; Wang, M.; Jia, R.; Chen, S.; Liu, M.; Zhu, D.; Zhao, X.; Gao, Q.; et al. Updates on the global dissemination of colistin-resistant *Escherichia coli*: An emerging threat to public health. *Sci. Total Environ.* **2021**, *799*, 149280, doi:10.1016/j.scitotenv.2021.149280.
595. Ling, Z.; Yin, W.; Shen, Z.; Wang, Y.; Shen, J.; Walsh, T.R. Epidemiology of mobile colistin resistance genes *mcr-1* to *mcr-9*. *J. Antimicrob. Chemother.* **2020**, *75*, 3087–3095, doi:10.1093/jac/dkaa205.
596. Carroll, L.M.; Gaballa, A.; Guldimann, C.; Sullivan, G.; Henderson, L.O.; Wiedmanna, M. Identification of Novel Mobilized Colistin Resistance Gene *mcr-9* in a Multidrug-Resistant, Colistin-Susceptible *Salmonella enterica* Serotype Typhimurium Isolate. *MBio* **2019**, *10*, e00853-19, doi:10.1128/mBio.00853-19.
597. Wang, C.; Feng, Y.; Liu, L.; Wei, L.; Kang, M.; Zong, Z. Identification of novel mobile colistin resistance gene *mcr-10*. *Emerg. Microbes Infect.* **2020**, *9*, 508–516, doi:10.1080/22221751.2020.1732231.
598. Cabello, F.C.; Godfrey, H.P. Aquaculture, exaptation, and the origin of *mcr*-positive colistin resistance. *Antimicrob. Agents Chemother.* **2018**, *62*, e01903-18, doi:10.1128/AAC.01903-18.
599. Cabello, F.C.; Tomova, A.; Ivanova, L.; Godfrey, H.P. Aquaculture and *mcr* colistin resistance determinants. *MBio* **2017**, *8*, e01229-17, doi:10.1128/mBio.01229-17.
600. Lei, T.; Zhang, J.; Jiang, F.; He, M.; Zeng, H.; Chen, M.; Wu, S.; Wang, J.; Ding, Y.; Wu, Q. First detection of the plasmid-mediated colistin resistance gene *mcr-1* in virulent *Vibrio parahaemolyticus*. *Int. J. Food Microbiol.* **2019**, *308*, 108290, doi:10.1016/j.ijfoodmicro.2019.108290.
601. Lv, L.; Cao, Y.; Yu, P.; Huang, R.; Wang, J.; Wen, Q.; Zhi, C.; Zhang, Q.; Liu, J.-H. Detection of *mcr-1* Gene among *Escherichia coli* Isolates from Farmed Fish and Characterization of *mcr-1*-Bearing IncP Plasmids. *Antimicrob. Agents Chemother.* **2018**, *62*, e02378-17, doi:10.1128/AAC.02378-17.
602. Hoa, T.T.T.; Nakayama, T.; Huyen, H.M.; Harada, K.; Hinenoya, A.; Phuong, N.T.; Yamamoto, Y. Extended-spectrum beta-lactamase-producing *Escherichia coli* harbouring *sul* and *mcr-1* genes isolates from fish gut contents in the Mekong Delta, Vietnam. *Lett. Appl. Microbiol.* **2020**, *71*, 78–85, doi:10.1111/lam.13222.
603. Yamaguchi, T.; Kawahara, R.; Harada, K.; Teruya, S.; Nakayama, T.; Motooka, D.; Nakamura, S.; Do Nguyen, P.; Kumeda, Y.; Dang, C. Van; et al. The presence of colistin resistance gene *mcr-1* and *-3* in ESBL producing *Escherichia coli* isolated from food in Ho Chi Minh City, Vietnam. *FEMS Microbiol. Lett.* **2018**, *365*, doi:10.1093/femsle/fny100.
604. Lozano-Leon, A.; Garcia-Omil, C.; Dalama, J.; Rodriguez-Souto, R.; Martinez-Urtaza, J.; Gonzalez-Escalona, N. Detection of colistin resistance *mcr-1* gene in *Salmonella enterica* serovar Rissen isolated from mussels, Spain, 2012- to 2016. *Eurosurveillance* **2019**, *24*, 1900200, doi:10.2807/1560-7917.es.2019.24.16.1900200.
605. Hassan, J.; Eddine, R.Z.; Mann, D.; Li, S.; Deng, X.; Saoud, I.P.; Kassem, I.I. The mobile colistin resistance gene, *mcr-1.1*, is carried on Incx4 plasmids in multidrug resistant *E.*

coli isolated from rainbow trout aquaculture. *Microorganisms* **2020**, *8*, 1636, doi:10.3390/microorganisms8111636.
606. Kalová, A.; Gelbíčová, T.; Overballe-Petersen, S.; Litrup, E.; Karpíšková, R. Characterisation of colistin-resistant *Enterobacterales* and *Acinetobacter* strains carrying *mcr* genes from asian aquaculture products. *Antibiotics* **2021**, *10*, 838, doi:10.3390/antibiotics10070838.
607. Shen, Y.; Lv, Z.; Yang, L.; Liu, D.; Ou, Y.; Xu, C.; Liu, W.; Yuan, D.; Hao, Y.; He, J.; et al. Integrated aquaculture contributes to the transfer of *mcr-1* between animals and humans via the aquaculture supply chain. *Environ. Int.* **2019**, *130*, 104708, doi:10.1016/j.envint.2019.03.056.
608. Li, Y.; Dai, X.; Zeng, J.; Gao, Y.; Zhang, Z.; Zhang, L. Characterization of the global distribution and diversified plasmid reservoirs of the colistin resistance gene *mcr-9*. *Sci. Rep.* **2020**, *10*, 8113, doi:10.1038/s41598-020-65106-w.
609. European Comimission; Directorate-General for Health and Food Safety; Braak, K.; Schrijver, R.; Bergevoet, R.; et al. *Welfare of farmed fish: Common practices during transport and at slaughter - Final Report*; Publications Office of European Union: Luxembourg, Luxembourg, 2017; doi:10.2875/172078.
610. Ciufo, S.; Kannan, S.; Sharma, S.; Badretdin, A.; Clark, K.; Turner, S.; Brover, S.; Schoch, C.L.; Kimchi, A.; DiCuccio, M. Using average nucleotide identity to improve taxonomic assignments in prokaryotic genomes at the NCBI. *Int. J. Syst. Evol. Microbiol.* **2018**, *68*, 2386–2392, doi:10.1099/ijsem.0.002809.
611. Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; Madden, T.L. BLAST+: Architecture and applications. *BMC Bioinformatics* **2009**, *10*, 421, doi:10.1186/1471-2105-10-421.
612. Sullivan, M.J.; Petty, N.K.; Beatson, S.A. Easyfig: A genome comparison visualizer. *Bioinformatics* **2011**, *27*, 1009–1010, doi:10.1093/bioinformatics/btr039.
613. Mateos, M.; Hernández-García, M.; Del Campo, R.; Martínez-García, L.; Gijón, D.; Morosini, M.I.; Ruiz-Garbajosa, P.; Cantón, R. Emergence and Persistence over Time of Carbapenemase-Producing *Enterobacter* Isolates in a Spanish University Hospital in Madrid, Spain (2005-2018). *Microb. Drug Resist.* **2021**, *27*, 895–903, doi:10.1089/mdr.2020.0265.
614. Mezzatesta, M.L.; Gona, F.; Stefani, S. *Enterobacter cloacae* complex: clinical impact and emerging antibiotic resistance. *Future Microbiol.* **2012**, *7*, 887–902, doi:10.2217/fmb.12.61.
615. Hoffmann, H.; Stindl, S.; Stumpf, A.; Mehlen, A.; Monget, D.; Heesemann, J.; Schleifer, K.H.; Roggenkamp, A. Description of *Enterobacter ludwigii* sp. nov., a novel *Enterobacter* species of clinical relevance. *Syst. Appl. Microbiol.* **2005**, *28*, 206–212, doi:10.1016/j.syapm.2004.12.009.
616. Davin-Regli, A.; Lavigne, J.P.; Pagès, J.M. *Enterobacter* spp.: update on taxonomy, clinical aspects, and emerging antimicrobial resistance. *Clin. Microbiol. Rev.* **2019**, *32*, e00002-19, doi:10.1128/CMR.00002-19.
617. Ali, A.; Sultan, I.; Mondal, A.H.; Siddiqui, M.T.; Gogry, F.A.; Haq, Q.M.R. Lentic and effluent water of Delhi-NCR: A reservoir of multidrug-resistant bacteria harbouring bla_{CTX-M}, bla_{TEM} and bla_{SHV} type ESBL genes. *J. Water Health* **2021**, *19*, 592–603, doi:10.2166/WH.2021.085.
618. Preena, P.G.; Dharmaratnam, A.; Raj, N.S.; Raja, S.A.; Nair, R.R.; Swaminathan, T.R.

Antibiotic-resistant *Enterobacteriaceae* from diseased freshwater goldfish. *Arch. Microbiol.* **2021**, *203*, 219–231, doi:10.1007/s00203-020-02021-8.
619. Lee, K.E.; Adhikari, A.; Kang, S.M.; You, Y.H.; Joo, G.J.; Kim, J.H.; Kim, S.J.; Lee, I.J. Isolation and characterization of the high silicate and phosphate solubilizing novel strain *Enterobacter ludwigii* GAK2 that promotes growth in rice plants. *Agronomy* **2019**, *9*, 144, doi:10.3390/agronomy9030144.
620. Porto de Souza Vandenberghe, L.; Marcela Blandon Garcia, L.; Rodrigues, C.; Cândido Camara, M.; Vinícius de Melo Pereira, G.; de Oliveira, J.; Ricardo Soccol, C. Potential applications of plant probiotic microorganisms in agriculture and forestry. *AIMS Microbiol.* **2017**, *3*, 629–648, doi:10.3934/microbiol.2017.3.629.
621. Börjesson, S.; Greko, C.; Myrenås, M.; Landén, A.; Nilsson, O.; Pedersen, K. A link between the newly described colistin resistance gene *mcr-9* and clinical *Enterobacteriaceae* isolates carrying bla_{SHV-12} from horses in Sweden. *J. Glob. Antimicrob. Resist.* **2020**, *20*, 285–289, doi:10.1016/j.jgar.2019.08.007.
622. Tyson, G.H.; Li, C.; Hsu, C.H.; Ayers, S.; Borenstein, S.; Mukherjee, S.; Tran, T.T.; McDermot, P.F.; Zhao, S. The *mcr-9* gene of *Salmonella* and *Escherichia coli* is not associated with colistin resistance in the United States. *Antimicrob. Agents Chemother.* **2020**, *64*, e00573-20, doi:10.1128/AAC.00573-20.
623. Zhang, H.; Zhao, D.; Quan, J.; Hua, X.; Yu, Y. *mcr-1* facilitated selection of high-level colistin-resistant mutants in *Escherichia coli*. *Clin. Microbiol. Infect.* **2019**, *25*, 517.e1-517.e4, doi:10.1016/j.cmi.2018.12.014.
624. Zhu, X.Q.; Liu, Y.Y.; Wu, R.; Xun, H.; Sun, J.; Li, J.; Feng, Y.; Liu, J.H. Impact of *mcr-1* on the Development of High Level Colistin Resistance in *Klebsiella pneumoniae* and *Escherichia coli*. *Front. Microbiol.* **2021**, *12*, 666782, doi:10.3389/fmicb.2021.666782.
625. Ferreira, I.; Beisken, S.; Lueftinger, L.; Weinmaier, T.; Klein, M.; Bacher, J.; Patel, R.; von Haeseler, A.; Posch, A.E. Species identification and antibiotic resistance prediction by analysis of whole-genome sequence data by use of ARESdb: An analysis of isolates from the unyvero lower respiratory tract infection trial. *J. Clin. Microbiol.* **2020**, *58*, e00273-20, doi:10.1128/JCM.00273-20.
626. Schwarz, S.; Kehrenberg, C.; Doublet, B.; Cloeckaert, A. Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol. Rev.* **2004**, *28*, 519–542, doi:10.1016/j.femsre.2004.04.001.
627. Li, J.; Zhang, H.; Ning, J.; Sajid, A.; Cheng, G.; Yuan, Z.; Hao, H. The nature and epidemiology of OqxAB, a multidrug efflux pump. *Antimicrob. Resist. Infect. Control* **2019**, *8*, 44, doi:10.1186/s13756-019-0489-3.
628. Fernández, L.; Hancock, R.E.W. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin. Microbiol. Rev.* **2012**, *25*, 661–681, doi:10.1128/CMR.00043-12.
629. Kümmerle, N.; Feucht, H.H.; Kaulfers, P.M. Plasmid-mediated formaldehyde resistance in *Escherichia coli*: Characterization of resistance gene. *Antimicrob. Agents Chemother.* **1996**, *40*, 2276–2279, doi:10.1128/aac.40.10.2276.
630. Cloete, T.E. Resistance mechanisms of bacteria to antimicrobial compounds. *Int. Biodeterior. Biodegrad.* **2003**, *51*, 277–282, doi:10.1016/S0964-8305(03)00042-8.
631. Taylor, D.E. Bacterial tellurite resistance. *Trends Microbiol.* **1999**, *7*, 111–115, doi:10.1016/S0966-842X(99)01454-7.

632. Mikalová, L.; Bosák, J.; Hříbková, H.; Dědičová, D.; Benada, O.; Šmarda, J.; Šmajs, D. Novel temperate phages of *Salmonella enterica* subsp. *salamae* and subsp. *diarizonae* and their activity against pathogenic *S. enterica* subsp. *enterica* isolates. *PLoS One* **2017**, *12*, e0170734, doi:10.1371/journal.pone.0170734.
633. Jakhetia, R.; Talukder, K.A.; Verma, N.K. Isolation, characterization and comparative genomics of bacteriophage SfIV: A novel serotype converting phage from *Shigella flexneri*. *BMC Genomics* **2013**, *14*, 677, doi:10.1186/1471-2164-14-677.
634. Pan, Y.; Fang, Y.; Feng, Y.; Lyu, N.; Chen, L.; Li, J.; Xu, X.; Zhu, B.; Hu, Y. Discovery of *mcr-3.1* gene carried by a prophage located in a conjugative IncA/C2 plasmid from a *Salmonella* Choleraesuis clinical isolate. *J. Infect.* **2021**, *82*, 414–451, doi:10.1016/j.jinf.2020.09.036.
635. Zhang, C.; Feng, Y.; Liu, F.; Jiang, H.; Qu, Z.; Lei, M.; Wang, J.; Zhang, B.; Hu, Y.; Ding, J.; et al. A Phage-Like IncY Plasmid Carrying the *mcr-1* Gene in *Escherichia coli* from a Pig Farm in China. *Antimicrob. Agents Chemother.* **2017**, *61*, e02035-16, doi:10.1128/AAC.02035-16.
636. Wang, X.; Wood, T.K. Cryptic prophages as targets for drug development. *Drug Resist. Updat.* **2016**, *27*, 30–38, doi:10.1016/j.drup.2016.06.001.
637. Kizny Gordon, A.; Phan, H.T.T.; Lipworth, S.I.; Cheong, E.; Gottlieb, T.; George, S.; Peto, T.E.A.; Mathers, A.J.; Walker, A.S.; Crook, D.W.; et al. Genomic dynamics of species and mobile genetic elements in a prolonged bla_{IMP-4}-associated carbapenemase outbreak in an Australian hospital. *J. Antimicrob. Chemother.* **2020**, *75*, 873–882, doi:10.1093/jac/dkz526.
638. Kieffer, N.; Royer, G.; Decousser, J.-W.; Bourrel, A.-S.; Palmieri, M.; Ortiz De La Rosa, J.-M.; Jacquier, H.; Denamur, E.; Nordmann, P.; Poirel, L. *mcr-9*, an Inducible Gene Encoding an Acquired Phosphoethanolamine Transferase in *Escherichia coli*, and Its Origin. *Antimicrob. Agents Chemother.* **2019**, *63*, e00965-19, doi:10.1128/AAC.00965-19.
639. Bitar, I.; Papagiannitsis, C.C.; Kraftova, L.; Chudejova, K.; Mattioni Marchetti, V.; Hrabak, J. Detection of Five *mcr-9*-Carrying *Enterobacterales* Isolates in Four Czech Hospitals. *mSphere* **2020**, *5*, e01008-20, doi:10.1128/msphere.01008-20.
640. Khedher, M. Ben; Baron, S.A.; Riziki, T.; Ruimy, R.; Raoult, D.; Diene, S.M.; Rolain, J.M. Massive analysis of 64,628 bacterial genomes to decipher water reservoir and origin of mobile colistin resistance genes: is there another role for these enzymes? *Sci. Rep.* **2020**, *10*, 5970, doi:10.1038/s41598-020-63167-5.
641. Xiaomin, S.; Yiming, L.; Yuying, Y.; Zhangqi, S.; Yongning, W.; Shaolin, W. Global impact of *mcr-1*-positive *Enterobacteriaceae* bacteria on "one health." *Crit. Rev. Microbiol.* **2020**, *46*, 565–577, doi:10.1080/1040841X.2020.1812510.
642. Cherak, Z.; Loucif, L.; Moussi, A.; Rolain, J.M. Epidemiology of mobile colistin resistance (*mcr*) genes in aquatic environments. *J. Glob. Antimicrob. Resist.* **2021**, *27*, 51–62, doi:10.1016/j.jgar.2021.07.021.
643. Averhoff, B.; Kirchner, L.; Pfefferle, K.; Yaman, D. Natural transformation in Gram-negative bacteria thriving in extreme environments: from genes and genomes to proteins, structures and regulation. *Extremophiles* **2021**, *25*, 425–436, doi:10.1007/s00792-021-01242-z.
644. Fraune, S.; Bosch, T.C.G. Why bacteria matter in animal development and evolution. *BioEssays* **2010**, *32*, 571–580, doi:10.1002/bies.200900192.

645. World Health Organization (WHO) Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Available online: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed on Aug 30, 2022).
646. Bereded, N.K.; Abebe, G.B.; Fanta, S.W.; Curto, M.; Waidbacher, H.; Meimberg, H.; Domig, K.J. The impact of sampling season and catching site (wild and aquaculture) on gut microbiota composition and diversity of Nile tilapia (*Oreochromis niloticus*). *Biology (Basel).* **2021**, *10*, 180, doi:10.3390/biology10030180.
647. Murray, C.J.; Ikuta, K.S.; Sharara, F.; Swetschinski, L.; Robles Aguilar, G.; Gray, A.; Han, C.; Bisignano, C.; Rao, P.; Wool, E.; et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet* **2022**, *399*, 629–655, doi:10.1016/S0140-6736(21)02724-0.
648. One Health High-Level Expert Panel (OHHLEP); Adisasmito, W.B.; Almuhairi, S.; Behravesh, C.B.; Bilivogui, P.; Bukachi, S.A.; Casas, N.; Cediel Becerra, N.; Charron, D.F.; Chaudhary, A.; et al. One Health: A new definition for a sustainable and healthy future. *PLOS Pathog.* **2022**, *18*, e1010537, doi:10.1371/journal.ppat.1010537.
649. Zhao, X.; Chlebowicz-Flissikowska, M.A.; Wang, M.; Vera Murguia, E.; de Jong, A.; Becher, D.; Maaß, S.; Buist, G.; van Dijl, J.M. Exoproteomic profiling uncovers critical determinants for virulence of livestock-associated and human-originated *Staphylococcus aureus* ST398 strains. *Virulence* **2020**, *11*, 947–963, doi:10.1080/21505594.2020.1793525.
650. Dubois, D.; Grare, M.; Prere, M.F.; Segonds, C.; Marty, N.; Oswald, E. Performances of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid identification of bacteria in routine clinical microbiology. *J. Clin. Microbiol.* **2012**, *50*, 2568–2576, doi:10.1128/JCM.00343-12.
651. Jenkins, C.; Ling, C.L.; Ciesielczuk, H.L.; Lockwood, J.; Hopkins, S.; McHugh, T.D.; Gillespie, S.H.; Kibbler, C.C. Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: Comparison of two different approaches in clinical practice. *J. Med. Microbiol.* **2012**, *61*, 483–488, doi:10.1099/jmm.0.030387-0.
652. Figueras, M.J.; Beaz-Hidalgo, R.; Hossain, M.J.; Liles, M.R. Taxonomic affiliation of new genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis. *Genome Announc.* **2014**, *2*, e00927-14, doi:10.1128/genomeA.00927-14.
653. Shelburne, S.A.; Kim, J.; Munita, J.M.; Sahasrabhojane, P.; Shields, R.K.; Press, E.G.; Li, X.; Arias, C.A.; Cantarel, B.; Jiang, Y.; et al. Whole-genome sequencing accurately identifies resistance to extended-spectrum β-lactams for major gram-negative bacterial pathogens. *Clin. Infect. Dis.* **2017**, *65*, 738–745, doi:10.1093/cid/cix417.
654. Ruppé, E.; Cherkaoui, A.; Charretier, Y.; Girard, M.; Schicklin, S.; Lazarevic, V.; Schrenzel, J. From genotype to antibiotic susceptibility phenotype in the order *Enterobacterales*: a clinical perspective. *Clin. Microbiol. Infect.* **2020**, *26*, 643.e1-643.e7, doi:10.1016/j.cmi.2019.09.018.
655. Alexander, K.A.; Angel, D.; Freeman, S.; Israel, D.; Johansen, J.; Kletou, D.; Meland, M.; Pecorino, D.; Rebours, C.; Rousou, M.; et al. Improving sustainability of aquaculture in Europe: Stakeholder dialogues on Integrated Multi-trophic Aquaculture (IMTA). *Environ. Sci. Policy* **2016**, *55*, 96–106, doi:10.1016/j.envsci.2015.09.006.
656. Dang, S.T.T.; Dalsgaard, A. *Escherichia coli* contamination of fish raised in integrated pig-

fish aquaculture systems in Vietnam. *J. Food Prot.* **2012**, *75*, 1317–1319, doi:10.4315/0362-028X.JFP-11-501.
657. Gibson, J.S.; Wai, H.; Oo, S.S.M.L.; Hmwe, E.M.M.; Wai, S.S.; Htun, L.L.; Lim, H.P.; Latt, Z.M.; Henning, J. Antimicrobials use and resistance on integrated poultry-fish farming systems in the Ayeyarwady Delta of Myanmar. *Sci. Rep.* **2020**, *10*, 16149, doi:10.1038/s41598-020-73076-2.
658. Shen, Y.; Zhou, H.; Xu, J.; Wang, Y.; Zhang, Q.; Walsh, T.R.; Shao, B.; Wu, C.; Hu, Y.; Yang, L.; et al. Anthropogenic and environmental factors associated with high incidence of *mcr-1* carriage in humans across China. *Nat. Microbiol.* **2018**, *3*, 1054–1062, doi:10.1038/s41564-018-0205-8.
659. Alcalá, L.; Alonso, C.A.; Simón, C.; González-Esteban, C.; Orós, J.; Rezusta, A.; Ortega, C.; Torres, C. Wild Birds, Frequent Carriers of Extended-Spectrum β-Lactamase (ESBL) Producing *Escherichia coli* of CTX-M and SHV-12 Types. *Microb. Ecol.* **2016**, *72*, 861–869, doi:10.1007/s00248-015-0718-0.
660. Wu, J.; Huang, Y.; Rao, D.; Zhang, Y.; Yang, K. Evidence for environmental dissemination of antibiotic resistance mediated by wild birds. *Front. Microbiol.* **2018**, *9*, 745, doi:10.3389/fmicb.2018.00745.
661. Lin, Y.; Dong, X.; Wu, J.; Rao, D.; Zhang, L.; Faraj, Y.; Yang, K. Metadata Analysis of *mcr-1*-Bearing Plasmids Inspired by the Sequencing Evidence for Horizontal Transfer of Antibiotic Resistance Genes Between Polluted River and Wild Birds. *Front. Microbiol.* **2020**, *11*, 352, doi:10.3389/fmicb.2020.00352.
662. Reverter, M.; Sarter, S.; Caruso, D.; Avarre, J.C.; Combe, M.; Pepey, E.; Pouyaud, L.; Vega-Heredía, S.; de Verdal, H.; Gozlan, R.E. Aquaculture at the crossroads of global warming and antimicrobial resistance. *Nat. Commun.* **2020**, *11*, 1870, doi:10.1038/s41467-020-15735-6.
663. Assefa, A.; Abunna, F. Maintenance of Fish Health in Aquaculture: Review of Epidemiological Approaches for Prevention and Control of Infectious Disease of Fish. *Vet. Med. Int.* **2018**, *2018*, 5432497, doi:10.1155/2018/5432497.
664. Martinez-Albores, A.; Lopez-Santamarina, A.; Rodriguez, J.A.; Ibarra, I.S.; Del Carmen Mondragón, A.; Miranda, J.M.; Lamas, A.; Cepeda, A. Complementary methods to improve the depuration of Bivalves: A review. *Foods* **2020**, *9*, 129, doi:10.3390/foods9020129.
665. Zhao, Y.; Yang, Q.E.; Zhou, X.; Wang, F.H.; Muurinen, J.; Virta, M.P.; Brandt, K.K.; Zhu, Y.G. Antibiotic resistome in the livestock and aquaculture industries: Status and solutions. *Crit. Rev. Environ. Sci. Technol.* **2021**, *51*, 2159–2196, doi:10.1080/10643389.2020.1777815.
666. Pérez-Sánchez, T.; Mora-Sánchez, B.; Balcázar, J.L. Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. *Trends Microbiol.* **2018**, *26*, 896–903, doi:10.1016/j.tim.2018.05.002.
667. Miller, R.A.; Harbottle, H. Antimicrobial Drug Resistance in Fish Pathogens. *Microbiol. Spectr.* **2018**, *6*, ARBA-0017-2017, doi:10.1128/microbiolspec.arba-0017-2017.

A

SUPPLEMENTARY MATERIAL

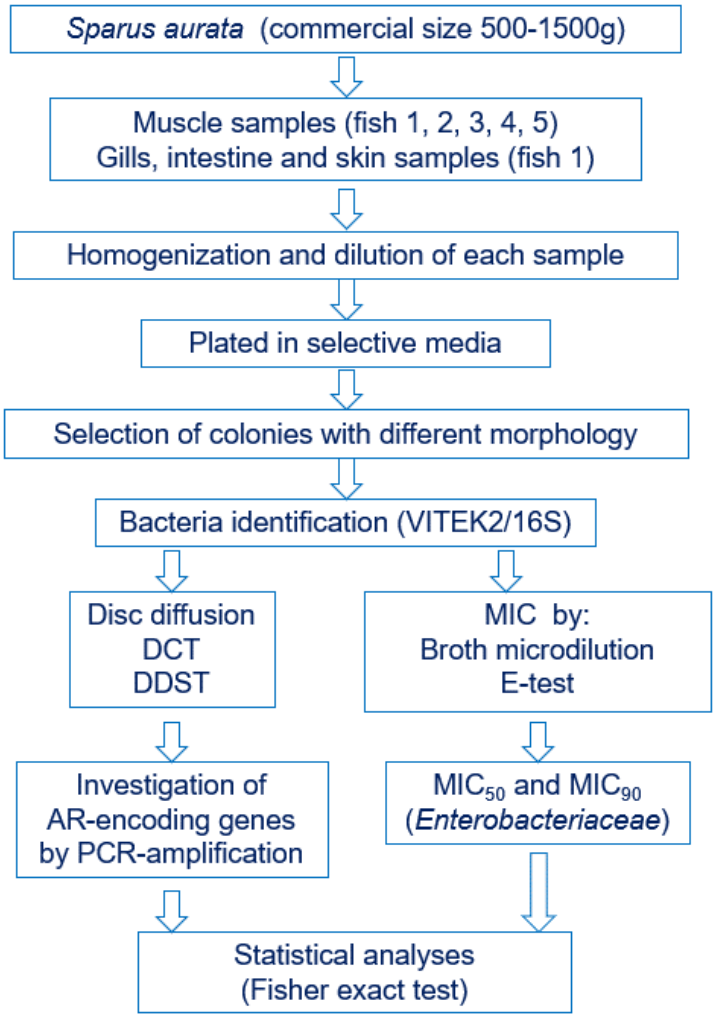

Figure S 1 — Diagram summarizing the experimental design. DCT: disc combination test. DDST: double disc synergy test.

Table S 1 — Odds ratio (OR) and 95% confidence intervals (CI) ($p \leq 0.05$) from the analysis of negative and positive correlations between fish samples (muscle vs. gills, intestine, and skin) and each bacterial species and non-susceptibility to different antibiotic's class (detailed results). Numbers in blue represent significant associations.

Fish sample	Bacterial family	Bacterial species	Antibiotic's class	Odds Ratio (OR)	(95% CI) lower	(95% CI) upper	*p* value (one-tail)	*p* value (two-tail)	P
Muscle	*Bacillaceae*	ALL	-	0.5294	0.1615	1.823	0.1831	0.3662	P
Muscle	*Bacillales* Family XII. *Incertae Sedis*	ALL	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	*Comamonadaceae*	ALL	-	undefined	0.08359	undefined	0.4761	0.9523	
Muscle	*Enterobacteriaceae*	ALL	-	0.8419	0.3739	1.871	0.3949	0.7898	P
Muscle	*Enterococcaceae*	ALL	-	0.8889	0.1218	10.21	0.6030	>0.9999999	P
Muscle	*Micrococcaceae*	ALL	-	undefined	0.2952	undefined	0.2237	0.4473	
Muscle	*Pseudomonadaceae*	ALL	-	3.299	0.3994	152.2	0.2300	0.4600	
Muscle	*Staphylococcaceae*	ALL	-	1.139	0.3778	3.883	0.5123	>0.9999999	
Muscle	ALL	ALL	Glycopeptides	undefined	0.1841	undefined	0.3269	0.6538	
Muscle	ALL	ALL	Mupirocin	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	ALL	ALL	Phenicols	0.3921	0.1701	0.912	0.01406	0.02812	
Non-Muscle	ALL	ALL	Phenicols	2.55	1.096	5.879	0.01406	0.02812	
Muscle	ALL	ALL	Quinolones	1.609	0.2879	16.5	0.4348	0.8696	
Muscle	ALL	ALL	β-lactams	1.39	0.3869	6.289	0.4108	0.8216	
Muscle	-	*Bacillus cereus*	-	0.6593	0.07282	8.204	0.4917	0.9833	P
Muscle	-	*Bacillus pumilus*	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	-	*Bacillus* sp.	-	0.5778	0.09334	4.148	0.3717	0.7434	P
Muscle	-	*Bacillus thuringiensis*	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	-	*Citrobacter freundii*	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	-	*Citrobacter freundii* complex	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	-	*Comamonas aquatica*	-	undefined	0.08359	undefined	0.4761	0.9523	
Muscle	-	*Enterobacter cloacae*	-	3.299	0.3994	152.2	0.2300	0.4600	
Muscle	-	*Enterobacter hormaechei*	-	0.7317	0.2416	2.393	0.3596	0.7191	P

Muscle	-	*Enterobacter* sp.	-	0.1648	0.02645	0.7834	0.009823	0.01965	P
Non-Muscle	-	*Enterobacter* sp.	-	6.067	1.277	37.8	0.009823	0.01965	
Muscle	-	*Enterococcus hirae*	-	1.822	0.1725	91.9	0.5083	>0.9999999	
Muscle	-	*Exiguobacterium acetylicum*	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	-	*Klebsiella michiganensis*	-	undefined	0.08359	undefined	0.4761	0.9523	
Muscle	-	*Klebsiella pneumoniae*	-	2.303	0.2451	111.6	0.3970	0.7939	
Muscle	-	*Kocuria rhizophila*	-	undefined	0.2952	undefined	0.2237	0.4473	
Muscle	-	*Leclercia adecarboxylata*	-	2.407	0.7236	10.36	0.09561	0.1912	
Muscle	-	*Lelliottia* sp.	-	0.4348	0.03073	6.235	0.3634	0.7268	P
Muscle	-	*Pseudomonas putida*	-	undefined	0.2952	undefined	0.2237	0.4473	
Muscle	-	*Pseudomonas stutzeri*	-	1.352	0.1047	72.66	0.6366	>0.9999999	
Muscle	-	*Staphylococcus aureus*	-	0.5778	0.09334	4.148	0.3717	0.7434	P
Muscle	-	*Staphylococcus capitis*	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	-	*Staphylococcus epidermidis*	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	-	*Staphylococcus haemolyticus*	-	0.6593	0.07282	8.204	0.4917	0.9833	P
Muscle	-	*Staphylococcus pasteuri*	-	0.8913	0.04525	53.85	0.6731	>0.9999999	P
Muscle	-	*Staphylococcus petrasii* subsp. *pragensis*	-	undefined	0.08359	undefined	0.4761	0.9523	
Muscle	-	*Staphylococcus saprophyticus*	-	undefined	0.01146	undefined	0.6912	>0.9999999	
Muscle	-	*Staphylococcus* sp.	-	undefined	0.01146	undefined	0.6912	>0.9999999	

P: indicates an OR value for a protective or negative association; otherwise, values should be interpreted as a positive association.

Table S 2 — Distribution of bacterial families among the six aquaculture farms in summer and autumn.

Family	Clams (*n*=42)		Mussels (*n*=102)						Japanese oysters (*n*=78)				Total
	Farm 1		Farm 2		Farm 3		Farm 4		Farm 1		Farm 5	Farm 6	
	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn	Summer	Autumn	
Aeromonadaceae	9.5%	0%	5.6%	0%	0%	0%	0%	0%	0%	0%	0%	0%	1.4%
Bacillaceae	0%	9.5%	11.1%	6.7%	20.0%	0%	0%	0%	0%	0%	6.3%	0%	3.2%
Comamonadaceae	4.8%	0%	0.0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0.5%
Enterobacteriaceae	28.6%	52.4%	5.6%	13.3%	60.0%	44.4%	23.5%	93.1%	64.7%	25.0%	6.3%	41.4%	40.5%
Enterococcaceae	9.5%	0%	0.0%	6.7%	0%	11.1%	0%	3.4%	11.8%	6.3%	6.3%	6.9%	5.4%
Micrococcaceae	0%	0%	0.0%	13.3%	0%	5.6%	5.9%	0%	0%	0%	0%	0%	1.8%
Moraxellaceae	0%	0%	0.0%	0%	0%	0%	0%	0.0%	5.9%	12.5%	6.3%	6.9%	2.7%
Morganellaceae	38.1%	14.3%	5.6%	13.3%	0%	27.8%	17.6%	0%	5.9%	37.5%	0%	27.6%	16.7%
Pseudomonadaceae	0%	4.8%	5.6%	13.3%	0%	0%	11.8%	0%	0%	0%	0%	13.8%	4.5%
Shewanellaceae	0%	0%	33.3%	0%	0%	5.6%	0%	0%	0%	0%	18.8%	0%	4.5%
Staphylococcaceae	0%	9.5%	5.6%	0%	0%	0%	11.8%	3.4%	0%	0%	0%	0%	2.7%
Streptococcaceae	0%	4.8%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0.5%
Vibrionaceae	9.5%	4.8%	27.8%	33.3%	20.0%	5.6%	29.4%	0%	11.8%	18.8%	43.8%	3.4%	14.9%
Yersiniaceae	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	12.5%	0%	0.9%
Total	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%

Table S 3 — Percentage of strains with decreased susceptibility to antibiotics used in the initial screening.

Bivalve species	Aquaculture farm	Season	Decreased susceptibility					
			AMX	CHL	COL	CTX	NAL	OTC
Clams	1	Summer	15.4%	15.4%	0%	7.7%	15.4%	46.2%
		Autumn	7.7%	0%	0%	7.7%	15.4%	69.2%
Mussels	2	Summer	25.0%	0%	16.7%	0%	0%	58.3%
		Autumn	30.0%	0%	0%	0%	10.0%	60.0%
	3	Summer	33.3%	0%	0%	0%	0%	66.7%
		Autumn	8.3%	16.7%	0%	0%	0%	75.0%
	4	Summer	23.1%	0%	0%	0%	7.7%	69.2%
		Autumn	7.4%	18.5%	0%	0%	29.6%	44.4%
Japanese oysters	5	Summer	7.7%	0%	38.5%	0%	0%	53.8%
	6	Autumn	25.0%	6.3%	0%	0%	31.3%	37.5%
	1	Summer	0%	8.3%	0%	8.3%	0%	83.3%
		Autumn	0%	0%	0%	20.0%	10.0%	70.0%
Total			13.6%	7.1%	4.5%	3.2%	13.0%	58.4%

These percentages do not include known intrinsic resistances. AMX: amoxicillin; CHL: chloramphenicol; COL: colistin; CTX: cefotaxime; NAL: nalidixic acid; OTC: oxytetracycline.

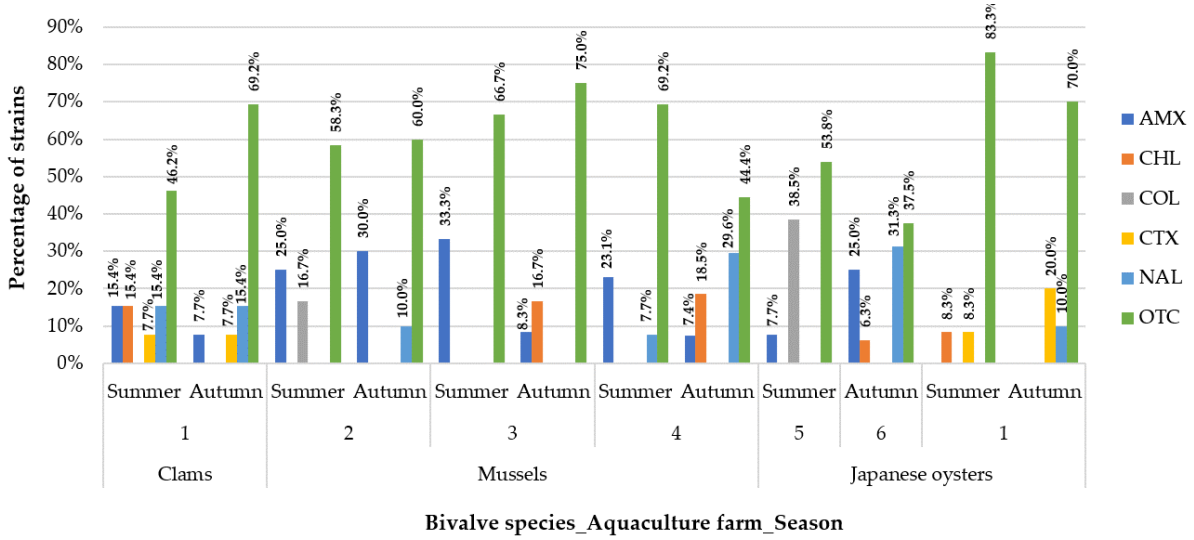

Figure S 2 — Decreased susceptibilities found in bivalves' samples. These results were obtained through the initial screening with selective media containing antibiotics and do not include known intrinsic resistances. AMX: amoxicillin; CHL: chloramphenicol; COL: colistin; CTX: cefotaxime; NAL: nalidixic acid; OTC: oxytetracycline.

Table S 4 — Information about the 50 *S. aureus* ST398 used in the construction of SNPs-based tree and heat map. Strains from this study are highlighted according to the origin of samples (orange: humans; green: dolphin; seabream: blue).

Accession number	Host	Country	Observations
AIDT01	Human (abscess)	Dominica	Animal-independent MSSA
AM990992	Human (with endocarditis)	Netherlands	LA-MRSA
CP003808	Human (with soft tissue infection)	Canada	LA-MRSA
CP013218	Chicken meat	Canada	LA-MRSA
CP017091	Pig	United States of America (USA)	-
CP021178	Human	China	CA-MRSA without livestock contact
CP077738	Human (sinus pus)	Netherlands	-
JABWSZ01	Swine (nasal swab)	Brazil	Borderline oxacillin resistant
JADRJJ01	Frozen Tangyuan product	China	LA-MRSA
JAEUEC01	Pig	China	MRSA
JAHVAQ01	Milk	China	MSSA
JAKFAR01 (INSa934)	Human	Portugal	This study. MSSA
JAKFAS01 (INSa910)	Human	Portugal	This study. MSSA
JAKFAT01 (INSa869)	Human	Portugal	This study. MSSA
JAKFAU01 (LV31741/11)	Dolphin (zoo)	Portugal	This study. MSSA
JAKFAV01 (INSaAq156)	Gilthead seabream (aquaculture)	Portugal	This study. MSSA
JAKFAW01 (INSaAq134)	Gilthead seabream (aquaculture)	Portugal	This study. MSSA
JAKFAX01 (INSaAq83)	Gilthead seabream (aquaculture)	Portugal	This study. MSSA
JAKFAY01 (INSaAq69)	Gilthead seabream (aquaculture)	Portugal	This study. MSSA
JAKFAZ01 (INSaAq61)	Gilthead seabream (aquaculture)	Portugal	This study. MSSA
JAKFBA01 (INSaAq36)	Gilthead seabream (aquaculture)	Portugal	This study. MSSA
JAKKUY01	Pork rinsed after slaughter	China	LA-MRSA
JAKKUZ01	Swine	China	LA-MRSA
JAKKVB01	Human (abattoir employee)	China	LA-MRSA
JAKKVN01	Carrier vehicle from a swine slaughterhouse	China	LA-MRSA

JAKKVO01	Fishpond water	China	LA-MRSA
JAKKWG01	Airborne dust from a swine slaughterhouse	China	LA-MRSA
JALJBR01	Ready-to-eat food (minced pork cutlet)	Russia	-
JALJBZ01	Ready-to-eat food (salad "Vesna" with radish and dressing)	Russia	-
JALJCA01	Ready-to-eat food (salad with tomato, cucumber, and dressing)	Russia	-
LAWV01	Human (nose)	Denmark	MRSA
LKYR01	Pig	USA	-
LNJF01	Human (infection)	France	-
LNTF01	Swine (skin swab)	Brazil	LA-MRSA
LXGP01	Human (bloodstream infection)	France	-
LZQL01	Human (sputum of patient with cystic fibrosis)	Brazil	MRSA
NAIC01	Human (veterinarian)	Australia	MRSA
NC_017333	Human (with endocarditis)	Netherlands	LA-MRSA
NC_017673	Human	USA	Animal-independent MSSA
NC_018608	Human (soft tissue infection)	Canada	MRSA
NZ_CP029172	Pig	Australia	-
NZ_CP065194	Pig	China	MRSA
QXEI01	Human	Greece	MLS_B-resistant
QYAT01	Poultry meat	Germany	LA-MRSA
QYAV01	Poultry meat	Poland	LA-MRSA
QYAX01	Human (nasal swab)	Czech Republic	LA-MRSA
RKRI01	Human (from swine farm)	South Korea	MRSA
RQIW01	Swine (nasal swab)	South Korea	MRSA
VRRB01	Human (farm worker)	Australia	LA-MRSA
WNWI01	Raw milk products (*Bos taurus*)	China	LA-MRSA

CA-MRSA: community-acquired MRSA. LA-MRSA: livestock-associated MRSA. MLS_B: Macrolide-lincosamide-streptogramin B. MSSA: methicillin-sensitive *Staphylococcus aureus*. MRSA: methicillin-resistant *S. aureus*.

Table S 5 — SNPs (Single-nucleotide polymorphisms) matrix generated by CSI Phylogeny 1.4 [396] including 50 strains of *S. aureus* ST398 (10 from our study and 40 from NCBI database). Strains from our study are highlighted (strains from humans in orange, from dolphin in green and from gilthead seabream originated in aquaculture in blue). SNPs highlighted from dark (less than 15 SNPs) to light purple (between 40 and 49 SNPs) represent the most closely related strains. Available at: https://docs.google.com/spreadsheets/d/1JEQPdF4DlaoozxHIx7Q7m0de-2hHnpxx/edit?usp=sharing&ouid=113583921972656433491&rtpof=true&sd=true

Table S 6 — Information of number of reads (before and after trim and used in the assembly), bases, contigs, consensus length, average coverage and contigs N50 for each strain investigated in this study.

Strain	BioProject	Accession number	Number of reads	Number of reads after trim	Number of reads used in the assembly	Number of bases	Number of contigs	Consensus length	Average coverage	Contigs N50
Sa869	PRJNA795413	JAKFAT000000000	2401320	2401016	2393737	308862461	45	2706000	230,3	136630
Sa910	PRJNA795413	JAKFAS000000000	2685662	2685336	2678094	345145220	41	2685117	185,7	201488
Sa934	PRJNA795413	JAKFAR000000000	2792566	2792035	2785140	356271991	49	2667024	189,3	134483
LV31741/11	PRJNA795413	JAKFAU000000000	2039258	2036373	2022123	246241966	56	2682326	130,0	85832
SaAq36	PRJNA795413	JAKFBA000000000	1621196	1621131	1615589	210023099	49	2741025	107,4	134239
SaAq61	PRJNA795413	JAKFAZ000000000	2501324	2501010	2441067	314672160	41	2685117	293,8	200413
SaAq69	PRJNA795413	JAKFAY000000000	2304032	2303570	2297097	294979786	41	2742049	178,2	136727
SaAq83	PRJNA795413	JAKFAX000000000	1895848	1891829	1873869	217724627	227	2744508	78,1	30491
SaAq134	PRJNA795413	JAKFAW000000000	2289240	2289087	2281605	295652293	34	2739934	161,3	136580
SaAq156	PRJNA795413	JAKFAV000000000	1885256	1881410	1870433	220335479	182	2736317	83,6	38501

Table S 7 — Distribution of strains included in this study among bacterial families, aquaculture species and origin (aquaculture farm/market).

Bacterial family	Aquaculture species	Origin	Portuguese Region	No. of strains
Aeromonadaceae	*Sparus aurata*	Market	central	6
	Mytilus spp.	Market	central	2
Enterobacteriaceae	*Sparus aurata*	Market	central	5
		Aquaculture farm 7	southern	13
	Crassostrea gigas	Aquaculture farm 1	southern	1
	Mytilus spp.	Market	central	2
		Aquaculture farm 4	southern	11
	Ruditapes decussatus	Aquaculture farm 1	southern	1
Hafniaceae	*Sparus aurata*	Market	central	10
	Mytilus spp.	Market	central	1
Morganellaceae	*Mytilus* spp.	Market	central	1
Pseudomonadaceae	*Sparus aurata*	Aquaculture farm 7	southern	2
Shewanellaceae	*Crassostrea gigas*	Aquaculture farm 5	central	1
	Mytilus spp.	Market	central	1
		Aquaculture farm 2/3	southern	5
Vibrionaceae	*Mytilus* spp.	Market	central	3
Yersiniaceae	*Sparus aurata*	Market	central	1
Total				66

Table S 8 — Antibiotics, respective concentrations and breakpoints (by bacterial family) used to perform antibiotic susceptibility testing.

Bacterial family	Method	Antibiotics tested (concentration)	Breakpoints
Aeromonadaceae	Disk diffusion	AZT (30 µg), CAZ (10 µg), CIP (5 µg), FEP (30 µg), LEV (5 µg), SXT (25 µg)	EUCAST
		AN (30 µg), CAZ (30 µg), CTX (30 µg), DOR (10 µg), ERT (10 µg), FOX (30 µg), GEN (10 µg), IMP (10 µg), MEM (10 µg)	CLSI M45
	MIC	CHL, FLO, FMQ, OTC	
Enterobacteriaceae *Hafniaceae* *Morganellaceae* *Yersiniaceae*	Disk diffusion	AMC (20+10 µg), AZT (30 µg), CAZ (10 µg), CIP (5 µg), CTX (5 µg), ERT (10 µg), FEP (30 µg), FOX (30 µg), GEN (10 µg), IMP (10 µg), MEM (10 µg), PTZ (36 µg), SXT (25 µg)	EUCAST
	MIC	CHL, FLO, OTC	CLSI VET08
		FMQ	CASFM VET 2019
		CIP	EUCAST
Pseudomonadaceae	Disk diffusion	AN (30 µg), AZT (30 µg), CAZ (10 µg), CIP (5 µg), DOR (10 µg), ERT (10 µg), FEP (30 µg), GEN (10 µg), IMP (10 µg), LEV (5 µg), MEM (10 µg), NET (10 µg), PTZ (36 µg), TMN (10 µg)	EUCAST
	MIC	CHL, FLO, FMQ, OTC	CLSI M100[1]
Shewanellaceae	Disk diffusion	AN (30 µg), AZT (30 µg), CAZ (10 µg), CIP (5 µg), FEP (30 µg), GEN (10 µg), IMP (10 µg), LEV (5 µg), MEM (10 µg), NET (10 µg), PTZ (36 µg), TMN (10 µg)	EUCAST[2]
	MIC	CHL, CIP, FLO, FMQ, OTC,	CLSI M100[1,2]
Vibrionaceae	Disk diffusion	AN (30 µg), AMC (20+10 µg), AMP (10 µg), CHL (30 µg), CIP (5 µg), FEP (30 µg), FOX (30 µg), GEN (10 µg), IMP (10 µg), LEV (5 µg), MEM (10 µg), SXT (25 µg), TET (30 µg)	CLSI M45[1]
	MIC	CHL, FLO, FMQ, OTC	

AMC: amoxicillin/clavulanic acid; AMP: ampicillin; AN: amikacin; AZT: aztreonam; CAZ: ceftazidime; CHL: chloramphenicol; CIP: ciprofloxacin; ; CTX: cefotaxime; DOR: doripenem; ERT: ertapenem; FEP: cefepime; FLO: florfenicol; FMQ: flumequine; FOX: cefoxitin; GEN: gentamicin; IMP: imipenem; LEV: levofloxacin; MEM: meropenem; NET: netilmicin; OTC: oxytetracycline; PTZ: piperacillin/tazobactam; SXT: trimethoprim/sulfamethoxazole; TET: tetracycline; TMN: tobramycin; EUCAST: European Committee on Antimicrobial Susceptibility Testing; CLSI: Clinical and Laboratory Standards Institute; CASFM VET: Comité de l'antibiogramme de la Société Française de Microbiologie Recommandations Vétérinaires.

[1]Breakpoints for CHL were used for FLO as well; breakpoints for CIP were used for FMQ; and breakpoints for TET were used for OTC.

[2]Breakpoints for *Shewanella* spp. were not available, therefore breakpoints from EUCAST and CLSI M100 for *Pseudomonas* spp. were used, as reported elsewhere [258].

Table S 9 — Information of BioProject and Accession numbers, reads, paired-end, bases, contigs, consensus length, average coverage and contigs N50 for each strain investigated in this study.

Strain	BioProject	Accession number	Number of reads	Paired-end (bp)	Number of bases	Number of contigs	Consensus length	Average coverage	Contigs N50
INSAq21	PRJNA762299	JAKCMK000000000	2924434	150	364462806	183	4942503	132,41	63999
INSAq40	PRJNA762299	JAKCML000000000	3762020	150	472393120	230	5479214	71,17	94289
INSAq43	PRJNA762299	JAKCMM000000000	4369515	150	547242250	151	5298171	248,06	105786
INSAq73	PRJNA762299	JAKCMN000000000	3588330	150	450199068	252	6260195	119,29	73198
INSAq87	PRJNA762299	JAKCMO000000000	348425	250	71768617	338	4695662	14,53	41690
INSAq93	PRJNA762299	JAKCMP000000000	520763	250	107594093	104	4699132	30,25	147692
INSAq99	PRJNA762299	JAKCMQ000000000	1625754	250	334881280	121	4942454	98,16	182015
INSAq107	PRJNA762299	JAKCMR000000000	2298047	150	285652996	172	4994183	92,42	51331
INSAq121	PRJNA762299	JAKCMS000000000	590721	250	113755344	265	5446657	22,33	61623
INSAq140	PRJNA762299	JAKCMT000000000	2294955	150	284817084	242	4915559	66,34	46516
INSAq143	PRJNA762299	JAKCMU000000000	1929636	150	240569459	216	4858654	66,87	47598
INSAq146	PRJNA762299	JAKCMV000000000	581750	250	114449218	153	4531490	52,57	90525
INSAq159	PRJNA762299	JAKCMW000000000	484597	250	94449253	337	5135645	20,43	51447
INSAq160	PRJNA762299	JAKCMX000000000	580307	250	114763455	210	5142371	35,14	79365
INSAq163	PRJNA762299	JAKCMY000000000	3798688	150	459191275	333	5114485	122,48	57511
INSAq169	PRJNA762299	JAKCMZ000000000	302225	250	60147329	761	5060107	11,07	16471
INSAq172	PRJNA762299	JAKMAH000000000	284909	250	55550557	840	4793476	8,67	15621
INSAq176	PRJNA762299	JAKCNA000000000	423804	250	83129516	351	4589461	22,55	51297
INSAq177	PRJNA762299	JAKCNB000000000	768241	250	148128890	279	4584357	42,53	43316
INSAq178	PRJNA762299	JAKCNC000000000	496359	250	108869130	6664	6161540	3,88	33608
INSAq180	PRJNA762299	JAKCND000000000	736222	250	144283505	535	4728366	16,33	50888
INSAq183	PRJNA762299	JAKCNE000000000	2311768	150	286749802	340	4874182	65,09	33039
INSAq191	PRJNA762299	JAKCNF000000000	610244	250	118172544	202	4712960	30,68	46221
INSAq192	PRJNA762299	JAKCNG000000000	541115	250	106963135	145	4795526	21,18	74829
INSAq193	PRJNA762299	JAKCNH000000000	610364	250	118011877	2379	9486800	9,61	10567
INSAq195	PRJNA762299	JAKCNI000000000	642976	250	125509188	298	5258180	34,39	53277

INSAq197	PRJNA762299	JAKCNJ000000000	793076	250	150548966	90	4803171	34,53	87181
INSAq204	PRJNA762299	JAKCNK000000000	768386	250	160879711	8135	7054845	5,65	17049
INSAq207	PRJNA762299	JAKCNL000000000	566717	250	109026285	333	5521348	24,31	79279
INSAq215	PRJNA762299	JAKCNM000000000	559968	250	107198609	620	4680519	26,61	16163
INSAq216	PRJNA762299	JAKCNN000000000	523389	250	101060146	208	4854731	19,68	50278
INSAq217	PRJNA762299	JAKCNO000000000	634884	250	121151921	258	4718040	56,42	34932
INSAq219	PRJNA762299	JAKCNP000000000	555472	250	105306466	589	4991695	24,59	19358
INSAq225	PRJNA762299	JAKCNQ000000000	488756	250	91921963	560	4723471	23,07	19014
INSAq228	PRJNA762299	JAKCNR000000000	3812662	150	470681706	150	5734461	116,35	111364
INSAq229	PRJNA762299	JAKCNS000000000	2839351	150	353162067	832	4973586	23,28	58224
INSAq234	PRJNA762299	JAKCNT000000000	4473695	150	552737385	131	5384082	193,71	132025
INSAq237	PRJNA762299	JAKCNU000000000	3932978	150	487226469	109	4747973	185,64	118870
INSAq239	PRJNA762299	JAKCNV000000000	456937	250	87550013	458	4415111	19,35	24954
INSAq240	PRJNA762299	JAKCNW000000000	4253188	150	527400479	237	6059267	106,24	75343
INSAq241	PRJNA762299	JAKCNX000000000	659608	250	128631417	176	4596490	26,56	119677
INSAq243	PRJNA762299	JAKCNY000000000	394216	250	75920271	308	4541062	17,88	42038
INSAq246	PRJNA762299	JAKCNZ000000000	671703	250	124534991	112	4140369	28,84	133871
INSAq249	PRJNA762299	JAKCOA000000000	1312623	250	247827275	140	5017759	69,61	127032
INSAq250	PRJNA762299	JAKCOB000000000	470999	250	89720847	209	5068449	17,03	78039
INSAq251	PRJNA762299	JAKCOC000000000	671450	250	129387437	132	5199631	27,10	194849
INSAq252	PRJNA762299	JAKCOD000000000	1149368	250	222239546	133	5219752	66,47	216055
INSAq258	PRJNA762299	JAKCOE000000000	3674220	150	454693322	126	4829915	120,83	64729
INSAq311	PRJNA762299	JAKCOF000000000	5504710	150	675680672	259	6011481	199,09	84652
INSAq315	PRJNA762299	JAKCOG000000000	2855191	150	355001784	307	6006541	89,54	62463
INSAq316	PRJNA762299	JAKCOH000000000	4210179	150	512449385	279	5145536	252,04	56571
INSAq317	PRJNA762299	JAKCOI000000000	9830213	150	1152208808	284	5151190	401,00	61041
INSAq319	PRJNA762299	JAKCOJ000000000	6924672	150	829056777	296	5152954	308,77	59946
INSAq320	PRJNA762299	JAKCOK000000000	5817476	150	684445258	303	5149470	237,99	59916
INSAq321	PRJNA762299	JAKCOL000000000	5609949	150	685727385	321	5155931	232,04	54665
INSAq322	PRJNA762299	JAKCOM000000000	3272831	150	402740727	272	5145315	155,34	61112
INSAq324	PRJNA762299	JAKCON000000000	3490950	150	433070578	303	5149610	151,03	52536

INSAq326	PRJNA762299	JAKCOO000000000	3386581	150	416778657	233	5986194	138,26	82579
INSAq334	PRJNA762299	JAKCOP000000000	3173735	150	394632899	115	4510319	109,10	117304
INSAq347	PRJNA762299	JAKCOQ000000000	4522182	150	567040778	122	4424751	230,38	101116
INSAq354	PRJNA762299	JAKCOR000000000	3209408	150	403436819	317	5149571	139,90	40818
INSAq424	PRJNA762299	JAKCOS000000000	6275793	150	787979361	81	4915595	530,34	140008
INSAq485	PRJNA762299	JAKCOT000000000	4990008	150	624685396	72	5079653	212,30	166720
INSAq494	PRJNA762299	JAKCOU000000000	3567675	150	448463348	123	4866345	125,13	84758
INSAq495	PRJNA762299	JAKCOV000000000	4254733	150	535069406	144	4824694	125,36	59695
INSAq497	PRJNA762299	JAKCOW000000000	1243076	150	156512572	437	4792727	46,58	23805

Table S 10 — Results of the 18 misidentified bacterial strains and the 7 strains identified to the species level in this study by MALDI-TOF, 16S rRNA, KmerFinder 3.2 and Average Nucleotide Identity (ANI).

Strain	MALDI-TOF	16S rRNA	KmerFinder 3.2	Average Nucleotide Identity (ANI)
INSaAq21	*Enterobacter cloacae* complex	*Enterobacter cloacae*	*Enterobacter hormaechei*	*Enterobacter hormaechei*
INSaAq107	-	*Enterobacter cloacae*	*Enterobacter hormaechei*	*Enterobacter hormaechei*
INSaAq140	-	*Enterobacter* sp.	*Enterobacter hormaechei*	*Enterobacter hormaechei*
INSaAq146	-	*Lelliottia* sp.	*Enterobacter asburiae*	*Enterobacter asburiae*
INSaAq172	*Hafnia alvei*	*Hafnia alvei*	*Obesumbacterium proteus*	*Obesumbacterium proteus*
INSaAq177	*Aeromonas media*	*Aeromonas* sp.	*Aeromonas rivipollensis*	*Aeromonas rivipollensis*
INSaAq178	*Aeromonas veronii*	*Aeromonas* sp.	*Aeromonas veronii*	*Aeromonas allosaccharophila*
INSaAq180	*Aeromonas media*	*Aeromonas* sp.	*Aeromonas rivipollensis*	*Aeromonas rivipollensis*
INSaAq191	*Hafnia alvei*	*Hafnia alvei*	*Hafnia paralvei*	*Hafnia paralvei*
INSaAq192	*Hafnia alvei*	*Hafnia alvei*	*Obesumbacterium proteus*	*Obesumbacterium proteus*
INSaAq193	*Aeromonas* sp.	*Aeromonas* sp.	*Aeromonas media*	*Aeromonas media*
INSaAq195	*Aeromonas* sp.	*Aeromonas* sp.	*Aeromonas salmonicida*	*Aeromonas salmonicida*
INSaAq197	*Hafnia alvei*	*Hafnia alvei*	*Obesumbacterium proteus*	*Obesumbacterium proteus*
INSaAq204	-	*Hafnia* sp.	*Hafnia paralvei*	*Hafnia paralvei*
INSaAq207	-	*Serratia* sp.	*Serratia liquefaciens*	*Serratia liquefaciens*
INSaAq216	-	*Hafnia alvei*	*Obesumbacterium proteus*	*Obesumbacterium proteus*
INSaAq217	-	*Hafnia alvei*	*Hafnia paralvei*	*Hafnia paralvei*
INSaAq225	-	*Hafnia alvei*	*Hafnia paralvei*	*Hafnia paralvei*
INSaAq228	-	*Raoultella ornithinolytica*	*Raoultella terrigena*	*Raoultella terrigena*
INSaAq229	-	*Kluyvera cryocrescens*	*Kluyvera intermedia*	*Kluyvera intermedia*
INSaAq234	-	*Citrobacter freundii*	*Citrobacter freundii*	*Citrobacter portucalensis*
INSaAq241	*Aeromonas veronii*	*Aeromonas* sp.	*Aeromonas allosaccharophila*	*Aeromonas allosaccharophila*
INSaAq246	-	*Proteus terrae*	*Proteus vulgaris*	*Proteus terrae*
INSaAq250	-	*Vibrio* sp.	*Vibrio diabolicus*	*Vibrio diabolicus*
INSaAq251	-	*Vibrio* sp.	*Vibrio antiquarius*	*Vibrio antiquarius*
INSaAq334	-	*Shewanella algae*	*Shewanella chilikensis*	*Shewanella chilikensis*
INSaAq347	-	*Shewanella algae*	*Shewanella chilikensis*	*Shewanella indica*
INSaAq424	*Citrobacter freundii*	*Citrobacter* sp.	*Citrobacter freundii*	*Citrobacter portucalensis*
INSaAq485	*Citrobacter braakii*	-	*Citrobacter freundii* complex	*Citrobacter portucalensis*

"-": method not used.

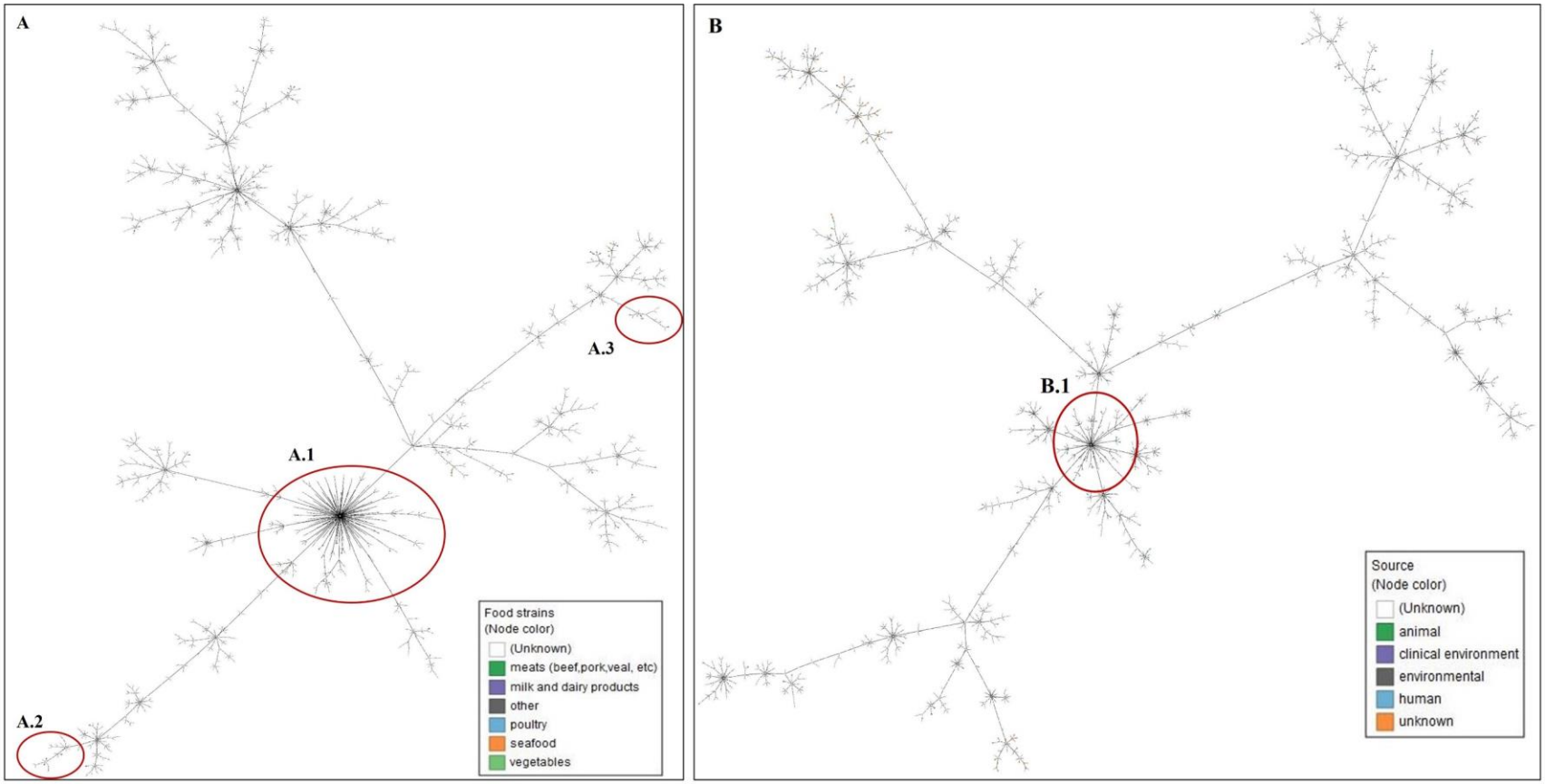

Figure S 3 — Minimum spanning trees (MST) constructed with CLC Genomics Workbench version 21.0.3, using MLST schemes to understand possible evolutionary relationships among the new ST detected in: **(A)** *Aeromonas* spp. (zooms of **A.1**, **A.2** and **A.3** are represented in Figure 7.1); **(B)** *Enterobacter cloacae* complex (zoom of **B.1** is represented in Figure 7.1).

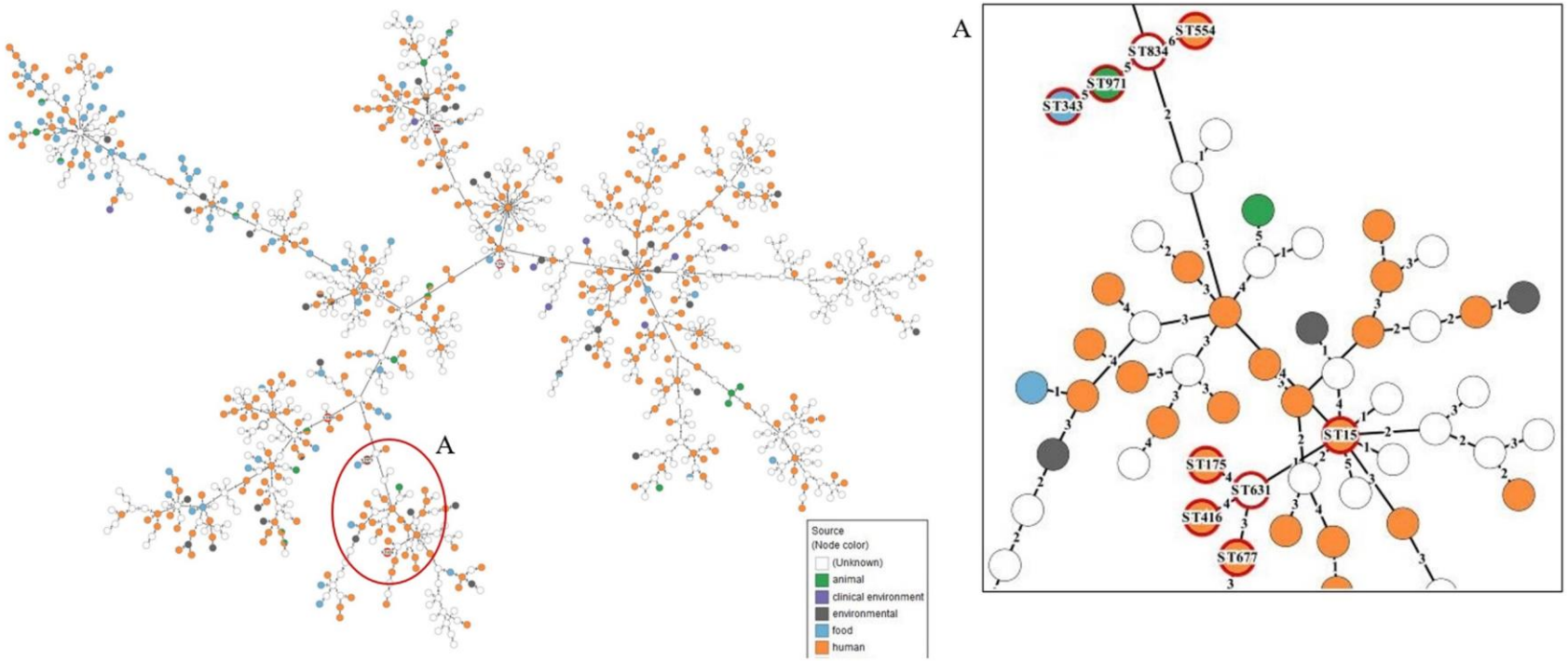

Figure S 4 — Minimum spanning tree (MST) constructed with CLC Genomics Workbench version 21.0.3, using MLST schemes to understand possible evolutionary relationships among the new ST detected in *Citrobacter portucalensis*. **(A)** Zoom of the MST, highlighting the new ST971. ST found in this study and their closest neighbors are highlighted with a red circle.

Figure S 5 — Single nucleotide polymorphism (SNPs)-based tree, constructed with CSI Phylogeny 1.4 [396], showing the relationship between all *Escherichia coli* strains isolated in our study. All *E. coli* from farm 4 are closely related and very distinct from *E. coli* found in farm 7 and market.

Table S 11 — SNPs (Single-nucleotide polymorphisms) matrix generated by CSI Phylogeny 1.4 [396] including all *Escherichia coli* strains identified in our study.

	INSAq159	INSAq163	INSAq183	INSAq316	INSAq317	INSAq319	INSAq320	INSAq321	INSAq322	INSAq324	INSAq354	Reference/ CP053284
INSAq159	0	120	15253	26686	26679	26686	26678	26677	26687	26679	26681	26443
INSAq163	120	0	15256	26716	26707	26718	26710	26709	26713	26705	26713	26467
INSAq183	15253	15256	0	27073	27068	27073	27069	27062	27076	27068	27064	26466
INSAq316	26686	26716	27073	0	9	6	18	11	11	15	12	3625
INSAq317	26679	26707	27068	9	0	11	13	12	14	6	15	3618
INSAq319	26686	26718	27073	6	11	0	14	11	11	17	14	3625
INSAq320	26678	26710	27069	18	13	14	0	13	19	11	18	3621
INSAq321	26677	26709	27062	11	12	11	13	0	14	12	7	3614
INSAq322	26687	26713	27076	11	14	11	19	14	0	18	15	3628
INSAq324	26679	26705	27068	15	6	17	11	12	18	0	15	3618
INSAq354	26681	26713	27064	12	15	14	18	7	15	15	0	3613
Reference/ CP053284	26443	26467	26466	3625	3618	3625	3621	3614	3628	3618	3613	0

min: 6, max: 27076

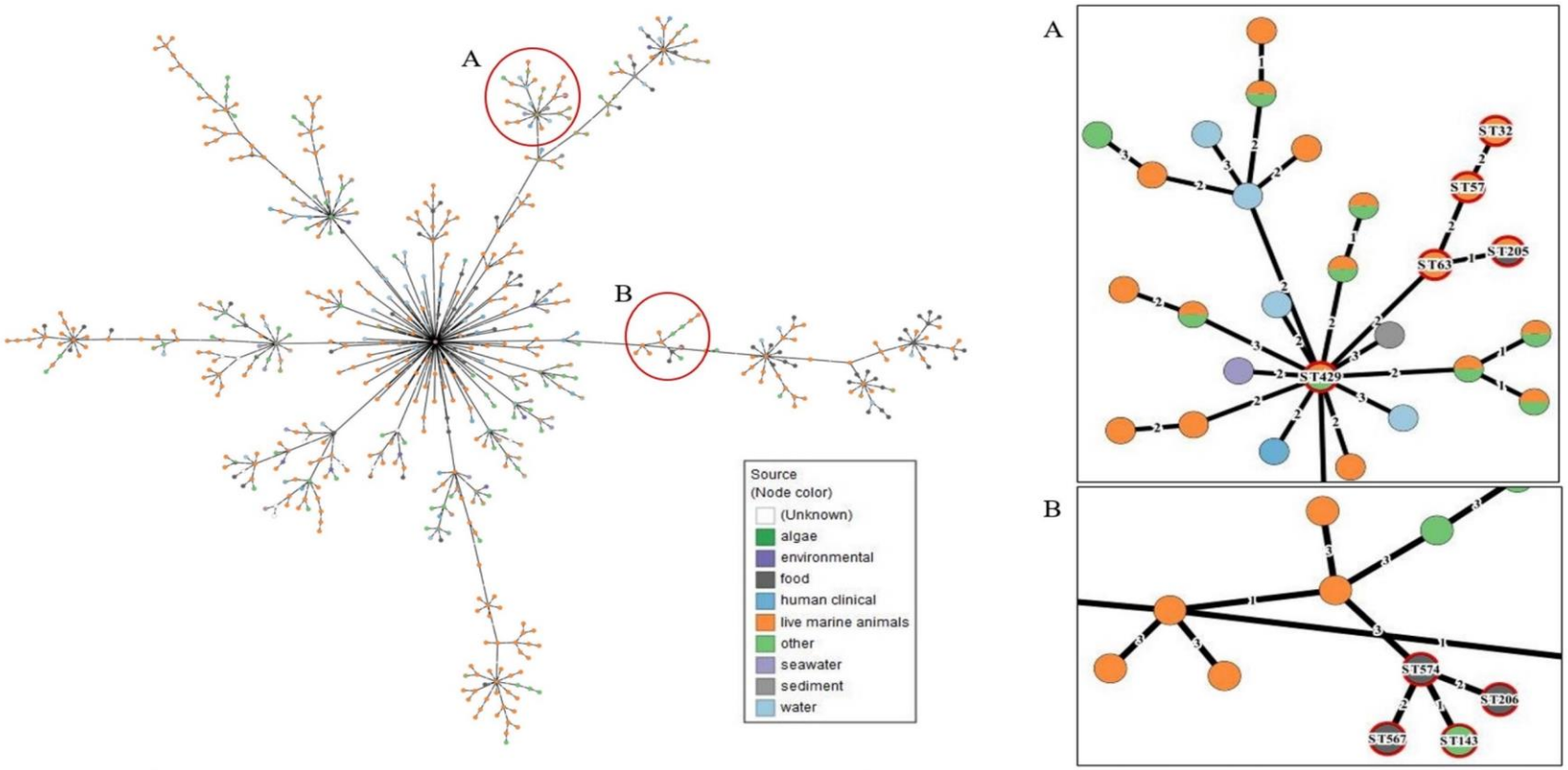

Figure S 6 — Minimum spanning tree (MST) constructed with CLC Genomics Workbench version 21.0.3, using MLST schemes to understand possible evolutionary relationships among the new ST detected in *Vibrio* spp. **(A)** Zoom of the MST, highlighting the new ST205. **(B)** Zoom of the MST, highlighting the new ST206. ST found in this study and their closest neighbors are highlighted with a red circle.

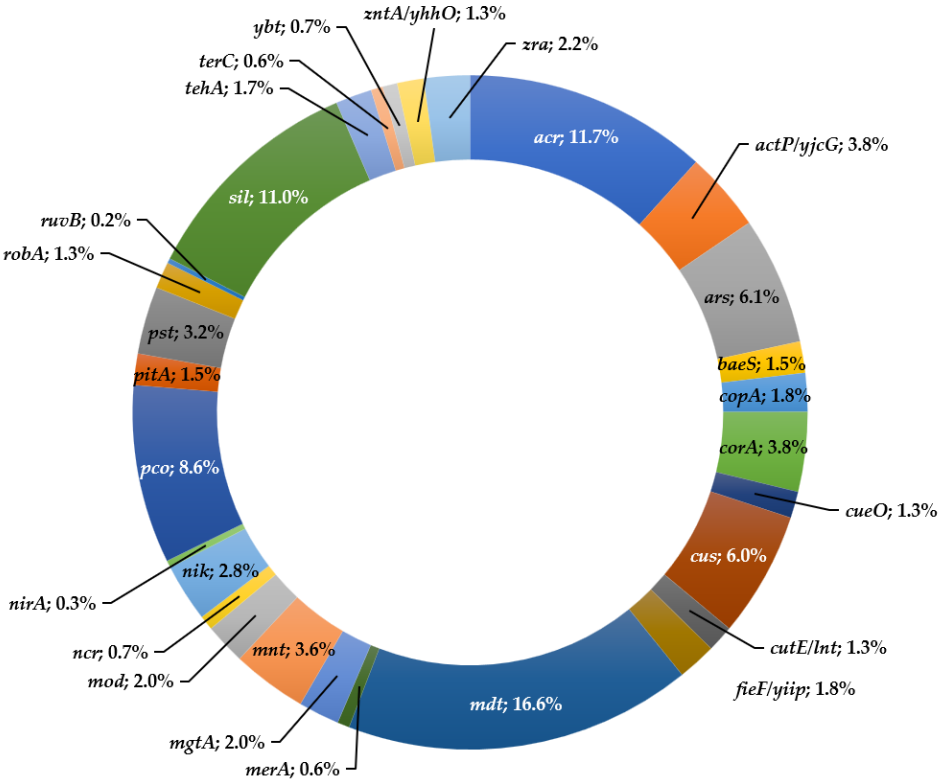

Figure S 7 — Total of genes associated with tolerance to heavy metals (%) found in this study. We can observe a great variety of heavy metals resistance/tolerance genes, with the group of *mdt* genes being the most frequent (16.6%), followed by *acr* genes group (11.7%) and *sil* genes (11.0%).

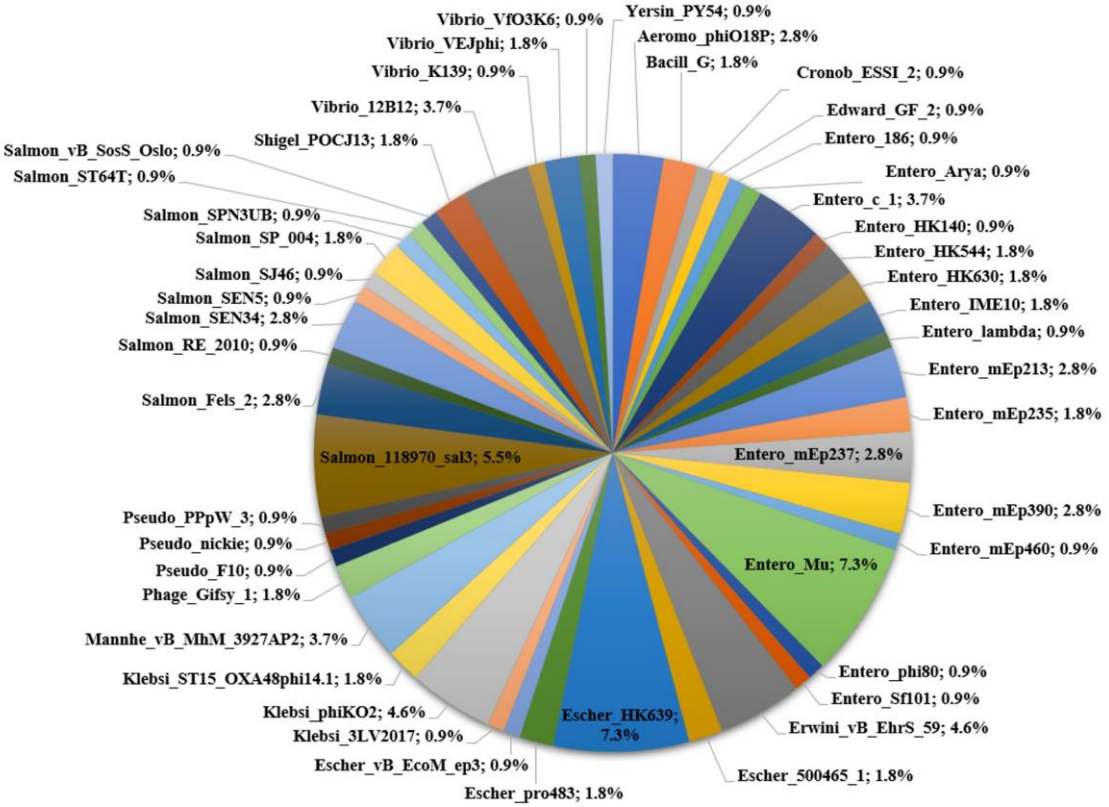

Figure S 8 — Total of phages (%) found in this study by PHASTER search web tool [395]. The most frequent prophage are Entero_Mu and Escher_HK639 (7.3% each).

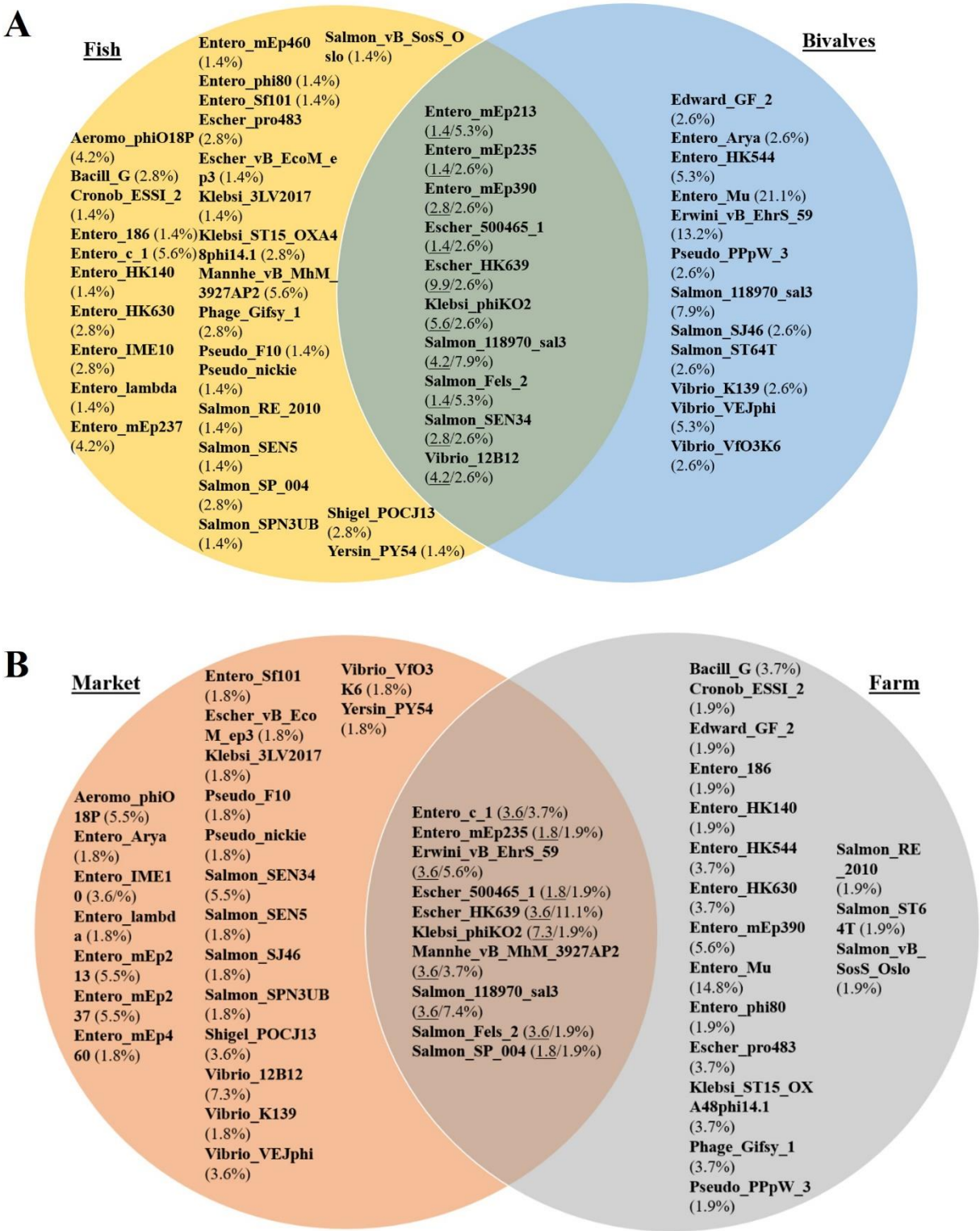

Figure S 9 — Venn diagrams representing prophages (and their prevalence in parentheses) found in this study in: **(A)** Fish vs. bivalve samples. **(B)** Market vs. farm samples. In the group of prophages found in both reservoirs, the underlined percentages correspond to the prevalence of that prophage in fish/market (A/B) samples and the non-underlined ones correspond to bivalve/farm samples (A/B).

Table S 12. — Demographic and genomic characteristics of the *E. ludwigii* isolates used for the phylogenomic analysis.

Strain	Collection Year	Location	Isolation Source	Isolation type	AMR genotypes	BioSample	NCBI Accession Number	Contigs/ Scaffolds	N50 (bp)	Length (bp)
JP6	2011	China	Rhizosphere soil	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN11415570	NZ_CP040256	1	4681598	4681598
JP9	2011	China	Soil	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN11415807	NZ_CP040527	1	4681542	4681542
I42	2015	China	*Lycium barba-rum* rhizosphere soil	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN11415809	NZ_CP040606	1	4719369	4719369
120152	2019	China	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN19316194	JAHEUQ000000000	27	685439	4722419
WCHEL090041	2016	China	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN09845216	RXRU00000000	38	486168	4783603
A3203	n.a.	USA	Rhizosphere	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN05589724	MSDM00000000	17	792170	4812349
WCHEL090017	2017	China	Human	Clinical	*aph(3'')-Ib*-type, *aph(6)-Id*, *bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN09845195	RXRA00000000	87	250730	5167660
JGM43	2015	USA	Kitchen B	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA2*	SAMN17146117	JAERJF000000000	97	190941	5302774
DSM16688T	n.a.	Germany	n.a.	n.a.	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMEA104113918	FYBD00000000	26	544330	4908609
LecVs2	n.a.	Italy	n.a.	n.a.	*ampC**, *oqxB*, *vat**	SAMEA3213012	CEFR00000000	197	62320	5285925
EnVs2	n.a.	Italy	n.a.	n.a.	*ampC**, *oqxB*	SAMEA3213011	CEFQ00000000	108	393305	5079786
GN02454	2005	USA	Human bodily fluid	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN03732707	LEDW00000000	22	586033	4715527
AA4	2012	USA	*Zea mays* root	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN06130962	CP018785	1	4799256	4799256
GN02730	2008	USA	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN03732724	LEER00000000	75	322082	5204500
GN02226	2003	USA	Human bodily fluid	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN03732688	LEEO00000000	36	276882	4836923
EnVs6	n.a.	Italy	n.a.	n.a.	*ampC**, *oqxB*, *vat**	SAMEA3213010	CEFO00000000	64	208468	5220112
MGYG-HGUT-02503	n.a.	Australia	Human gut	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMEA5852008	CABMNI000000000	26	332883	4697885
BIDMC121	n.a.	USA	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN08148284	JACRRX000000000	30	284260	5224129
EcWSU1	2007/2008	USA	Onion bulbs	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN02604307	CP002886	2	4734438	4798091
OLC-1682	2014	Canada	n.a.	Food	*bla*$_{ACT}$, *catA**, *oqxB*	SAMN03292329	JXWK00000000	1341	5354	4910606
e1617	2007	United King-dom	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMEA2273503	FJZU00000000	37	229677	4784689

e864	2004	United Kingdom	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMEA2273255	FKHG00000000	36	234939	4786532
PDC34	n.a.	USA	n.a.	n.a.	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN05428986	FRCI00000000	19	697266	4683223
Hanford	2008	USA	Water sample	Environmental/ other	*bla*$_{ACT}$, *bla*$_{TEM-116}$, *catA1*, *catA**, *fosA*, *oqxB*	SAMN02212350	ATCK00000000	62	640760	4832153
MGH160	2015	USA	Human	Clinical	*bla*$_{ACT}$, *bla*$_{FONA}$, *catA**, *fosA*, *oqxB*	SAMN04521909	NGRN00000000	59	233137	4925300
DLL7524	2015	Australia	Human feces	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN08374129	PQCT00000000	26	332883	4697885
P101	n.a.	USA	Switchgrass	Environmental/ other	*bla*$_{ACT-12}$, *catA**, *fosA2*, *oqxB*	SAMN02641647	NZ_CP006580	1	5369929	5369929
e1026	2005	United Kingdom	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMEA2273485	FJWD00000000	27	494790	4725159
e2350	2010	United Kingdom	Human blood	Clinical	*bla*$_{ACT}$, *bla*$_{NMC-A}$, *catA**, *fosA*, *oqxB*	SAMEA2273452	FKCG00000000	40	491260	4963845
EN-119	n.a.	China	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN05787341	NZ_CP017279	2	4857439	4952770
E8	2015	Germany	Cucumber	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*, *tet(A)*-type	SAMN12560200	VTUA00000000	140	496749	4848102
NR1491	2013	Japan	n.a.	n.a.	*bla*$_{ACT-54}$, *bla*$_{NMC-A}$, *catA**, *fosA*, *oqxB*	SAMD00184384	BKZO00000000	32	456530	4785407
ES-1	2015	USA	Soil	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN11281800	SZVD00000000	95	129134	4858644
AS012248	2015	USA	Human lung	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN12250567	VLNN00000000	739	71890	5204705
AS012244	2015	USA	Human lung	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN12250563	VLNO00000000	548	209857	4972320
D42-sc-1712201	n.a.	Switzerland	n.a.	n.a.	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN15300311	NZ_CP056119	1	4875486	4875486
FDAARGOS 1436	2004	Germany	Human mid-stream urine	Clinical	*bla*$_{ACT}$, *fosA*, *oqxA*, *oqxB*	SAMN16357578	CP077223	1	4943437	4943437
K37	2020	China	Human feces	Clinical	*bla*$_{ACT}$, *fosA2*, *oqxA*, *oqxB*	SAMN14389561	JAASIR000000000	17	667499	4902033
K32	2020	China	Human feces	Clinical	*bla*$_{ACT}$, *fosA2*, *oqxA*, *oqxB*	SAMN14389555	JAASIL000000000	18	721734	4736013
K31_2	2020	China	Human feces	Clinical	*bla*$_{ACT}$, *fosA2*, *oqxA*, *oqxB*	SAMN14389554	JAASIK000000000	15	721734	4736350
FDAAR-GOS_1475	2002	Greece	Soil from a land farm for treatment of refinery waste sludge	Environmental/ other	*bla*$_{ACT}$, *fosA2*, *oqxA*, *oqxB*	SAMN20888892	CP082860	1	4730716	4730716
FDAAR-GOS_1498	2002	Greece	Soil from a land farm for treatment of refinery waste sludge	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN21218854	CP083640	1	4730711	4730711
UW5	1994	Canada	Soil	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN03743787	NZ_CP011798	1	4904981	4904981

2485STDY5438318	n.a.	United Kingdom	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMEA1964557	UNVN00000000	35	539931	4810044
2485STDY5438320	n.a.	United Kingdom	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMEA2053720	UNVX00000000	23	540025	4761804
EC57	2018	Japan	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN16911616	JADRHX000000000	32	314090	4721280
MML25	2018	Japan	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN16911600	JADRIN000000000	22	697873	4737023
OLC-1683	2014	Canada	n.a.	Food	*fosA2*, *oqxB*-type	SAMN03292330	JXWL00000000	955	8888	5136359
AOUC-8/14	2014	Italy	Human rectal swab from female	Clinical	*bla*$_{ACT}$, *bla*$_{NMC-A}$, *catA**, *fosA2*, *oqxB*	SAMN03861996	LGIV00000000	15	695071	4766022
GN04920	2012	USA	n.a.	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN04572595	LVTW00000000	92	82088	4924730
NCR3	2014	Australia	*Carpobrotus rossii* rhizosphere	Environmental/ other	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN05462048	MCGF00000000	23	771694	4779415
4928STDY7071139	2018	United Kingdom	Human fecal	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMEA104567250	CABGVX000000000	48	285987	5138127
4928STDY7071138	2018	United Kingdom	Human fecal	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMEA104567249	CABGVW000000000	47	209911	5136800
CEB04	2003	Sweden	Human urinary tract catheters	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN11357407	NZ_CP039741	1	4892375	4892475
2021EL-00127	2020	USA	Human urine	Clinical	*bla*$_{ACT}$, *bla*$_{NMC-A}$, *catA**, *fosA2*, *oqxB*	SAMN18511098	JAGKLL000000000	20	585339	4892341
2020EL-00108	2020	USA	Human peritoneum	Clinical	*bla*$_{ACT}$, *bla*$_{NMC-A}$, *catA**, *fosA2*, *oqxB*	SAMN18511080	JAGKMD000000000	38	336354	4793318
4928STDY7071136	2018	United Kingdom	Human fecal	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMEA104567247	CABGVO000000000	47	286026	5137156
4928STDY7071140	2018	United Kingdom	Human fecal	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMEA104567251	CABGVZ000000000	59	209917	5061284
I140	2017	USA	Human hospital patient	Clinical	*bla*$_{ACT-12}$, *catA**, *fosA7*, *fosA*, *oqxB*	SAMN15689507	JACJHJ000000000	25	542092	5039619
Res13-Abat-PEB19-P1-02-A	2017	Canada	Swab	Environmental/ other	*bla*$_{ACT-12}$, *catA**, *fosA2*, *oqxB*	SAMN16304044	JADAJZ000000000	2	4656584	4661993
MGH216	n.a.	USA	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN08148254	JACRRJ000000000	35	252605	5225825
608_ECLO	n.a.	USA	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN03197808	JVAG00000000	222	48185	4804191
GN03638	2010	USA	Human	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*-type	SAMN04407776	LRCI00000000	167	47667	5003459
e558	2003	United Kingdom	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMEA2273189	FKEY00000000	62	158439	5091497
48	2017	Germany	Human clinical specimen	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*	SAMN12258083	VKFK00000000	200	203750	5134960

49	2017	Germany	Human clinical specimen	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*	SAMN12258084	VKFJ00000000	152	185970	5122530
AS012471	2015	USA	Human lung	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *oqxB*	SAMN12250790	VLMA00000000	754	245975	5153193
INSAq77	2018	Portugal	*Sparus aurata*	Environmental/ other	*bla*$_{ACT-88}$, *catA**, *fosA*, *mcr-9.1*, *oqxB*	SAMN15015462	JABRPH000000000	225	74544	5276953
EC56	2018	Japan	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *mcr-10.1*, *oqxB*	SAMN16911615	JADRHY000000000	80	202845	5110004
EC49	2018	Japan	Human blood	Clinical	*bla*$_{ACT}$, *catA**, *fosA2*, *oqxB*	SAMN16911589	JADRIY000000000	68	186338	4853579
11894	2011	China	Human throat swab	Clinical	*bla*$_{ACT}$, *catA**, *fosA*, *mcr-10*, *oqxB*	SAMN18435831	JAGFWU000000000	120	141921	4749540
40513	2015	France	*Corvus*	Environmental/ other	*aac(6')-Ib3*, *aadA2*, *ant(2'')-Ia*, *bla*$_{ACT}$, *bla*$_{CTX-M-9}$, *bla*$_{SHV-12}$, *catA1*, *catA**, *fosA2*, *mcr-9.1*, *oqxB*, *qnrA1*, *sul1*, *tet(A)*	SAMN15925369	JAGDFR000000000	62	529698	5031188
40508	2015	France	*Pica pica*	Environmental/ other	*aac(6')-Ib3*, *aadA2*, *ant(2'')-Ia*, *bla*$_{ACT}$, *bla*$_{CTX-M-9}$, *bla*$_{SHV-12}$, *catA1*, *catA**, *fosA2*, *mcr-9.1*, *oqxB*, *qnrA1*, *sul1*, *tet(A)*	SAMN15925368	JAGDFS000000000	66	423627	5031210
Y05	2020	China	Human feces	Clinical	*aac(6')-Ib-cr5*, *aadA16*, *aph(3'')-Ib*, *aph(6)-Id*, *arr-3*, *bla*$_{ACT}$, *dfrA27*, *fosA2*, *mph(A)*, *oqxA*, *oqxB*, *qnrB6*, *sul1*, *tet(C)*	SAMN14389599	JAASKD000000000	49	281183	5549279
Y09	2020	China	Human feces	Clinical	*aac(6')-Ib-cr5*, *aadA2*, *aph(3'')-Ib*, *aph(6)-Id*, *arr-3*, *bla*$_{ACT}$, *bla*$_{OXA-1}$, *catB3*, *dfrA12*, *dfrA14*, *fosA3*, *fosA*, *mph(A)*, *oqxA*, *oqxB*, *qnrA1*, *qnrS1*, *sul1*, *sul2*, *tet(B)*, *tet(D)*	SAMN14389603	JAASKH000000000	41	534659	5406832
AS012405	2016	Austria	Human lung	Clinical	*bla*$_{ACT-12-type}$, *fosA2*-type, *oqxA*-type, *oqxB*-type	SAMN12250724	VLMJ01000000	2375	182725	5650902

AMR: antimicrobial resistance. *(HMM): According with the NCBI Pathogen Detection (https://www.ncbi.nlm.nih.gov/pathogens; accessed on Jan 4, 2022), using hidden Markov models (HMMs) to identify protein families. n.a.: data not available.

Table S 13. — Loose best-hit results (≥65% of identity), by predicted gene, obtained using the Resistance Gene Identifier (RGI).

Contig	RGI Criteria	ARO Term	Detection Criteria Model	AMR Gene Family	Drug Class	Resistance Mechanism	% Identity of Matching Region	% Length of Reference Sequence
INSAq77p_72	Loose	ramA	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump; General Bacterial Porin with reduced permeability to β-lactams	Fluoroquinolone; monobactam; carbapenem; cephalosporin; glycylcycline; cephamycin; penam; tetracycline; rifamycin; phenicol; triclosan; penem	Antibiotic efflux; reduced permeability to antibiotic	97.4	91.1
INSAq77p_3	Loose	acrA	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone; cephalosporin; glycylcycline; penam; tetracycline; rifamycin; phenicol; triclosan	Antibiotic efflux	95.5	100.0
INSAq77p_87	Loose	cpxA	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminoglycoside; aminocoumarin	Antibiotic efflux	94.8	100.0
INSAq77p_44	Loose	bacA	Protein homolog	Undecaprenyl pyrophosphate related proteins	Peptide	Antibiotic target alteration	94.1	100.0
INSAq77p_42	Loose	soxR	Protein overexpression	ATP-binding cassette (ABC) antibiotic efflux pump; major facilitator superfamily (MFS) antibiotic efflux pump; resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone; cephalosporin; glycylcycline; penam; tetracycline; rifamycin; phenicol; triclosan	Antibiotic target alteration; antibiotic efflux	93.4	98.7
INSAq77p_2	Loose	acrB	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone; cephalosporin; glycylcycline; penam; tetracycline; rifamycin; phenicol; triclosan	Antibiotic efflux	92.9	99.9
INSAq77p_3	Loose	mdtC	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminocoumarin	Antibiotic efflux	91.4	100.0
INSAq77p_18	Loose	YojI	Protein homolog	ATP-binding cassette (ABC) antibiotic efflux pump	Peptide	Antibiotic efflux	90.1	100.0
INSAq77p_2	Loose	mdtB	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminocoumarin	Antibiotic efflux	90.0	100.0
INSAq77p_41	Loose	soxS	Protein overexpression	ATP-binding cassette (ABC) antibiotic efflux pump; major facilitator superfamily (MFS) antibiotic efflux pump; resistance-nodulation-cell division (RND) antibiotic efflux pump; General Bacterial Porin with reduced permeability to β-lactams	Fluoroquinolone; monobactam; carbapenem; cephalosporin; glycylcycline; cephamycin; penam; tetracycline; rifamycin; phenicol; triclosan; penem	Antibiotic target alteration; antibiotic efflux; reduced permeability to antibiotic	89.7	100.9
INSAq77p_6	Loose	KpnG	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Macrolide; fluoroquinolone; aminoglycoside; carbapenem; cephalosporin; penam; peptide; penem	Antibiotic efflux	87.4	100.0
INSAq77p_3	Loose	mdtG	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Fosfomycin	Antibiotic efflux	86.7	100.3

INSAq77p_83	Loose	mdfA	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Tetracycline; benzalkonium chloride; rhodamine	Antibiotic efflux	86.2	114.4
INSAq77p_5	Loose	baeS	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminoglycoside; aminocoumarin	Antibiotic efflux	86.1	100.0
INSAq77p_20	Loose	OmpA	Protein homolog	General Bacterial Porin with reduced permeability to β-lactams	Monobactam; carbapenem; cephalosporin; cephamycin; penam; penem	Reduced permeability to antibiotic	86.0	93.9
INSAq77p_6	Loose	MdtK	Protein homolog	Multidrug and toxic compound extrusion (MATE) transporter	Fluoroquinolone	Antibiotic efflux	85.8	101.3
INSAq77p_13	Loose	mdtH	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Fluoroquinolone	Antibiotic efflux	85.1	100.0
INSAq77p_32	Loose	TolC	Protein homolog	ATP-binding cassette (ABC) antibiotic efflux pump; major facilitator superfamily (MFS) antibiotic efflux pump; resistance-nodulation-cell division (RND) antibiotic efflux pump	Macrolide; fluoroquinolone; aminoglycoside; carbapenem; cephalosporin; glycylcycline; cephamycin; penam; tetracycline; peptide; aminocoumarin; rifamycin; phenicol; triclosan; penem	Antibiotic efflux	84.9	99.4
INSAq77p_188	Loose	PmrF	Protein homolog	pmr phosphoethanolamine transferase	Peptide	Antibiotic target alteration	84.8	101.6
INSAq77p_118	Loose	kdpE	Protein homolog	kdpDE	Aminoglycoside	Antibiotic efflux	84.4	100.0
INSAq77p_46	Loose	AcrF	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone; cephalosporin; cephamycin; penam	Antibiotic efflux	84.0	100.3
INSAq77p_18	Loose	ugd	Protein homolog	pmr phosphoethanolamine transferase	Peptide	Antibiotic target alteration	83.5	100.0
INSAq77p_1	Loose	mdtA	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Aminocoumarin	Antibiotic efflux	81.2	96.9
INSAq77p_21	Loose	OmpK37	Protein homolog	General Bacterial Porin with reduced permeability to β-lactams	Monobactam; carbapenem; cephalosporin; cephamycin; penam; penem	Reduced permeability to antibiotic	80.6	98.7
INSAq77p_4	Loose	acrR	Protein overexpression	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone; cephalosporin; glycylcycline; penam; tetracycline; rifamycin; phenicol; triclosan	Antibiotic target alteration; antibiotic efflux	79.8	100.9
INSAq77p_61	Loose	LptD	Protein homolog	ATP-binding cassette (ABC) antibiotic efflux pump	Carbapenem; peptide; aminocoumarin; rifamycin	Antibiotic efflux	79.6	100.1
INSAq77p_45	Loose	AcrE	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone; cephalosporin; cephamycin; penam	Antibiotic efflux	74.7	98.4
INSAq77p_17	Loose	rosB	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Peptide	Antibiotic efflux	74.0	99.1
INSAq77p_17	Loose	sdiA	Protein homolog	Resistance-nodulation-cell division (RND) antibiotic efflux pump	Fluoroquinolone; cephalosporin; glycylcycline; penam; tetracycline; rifamycin; phenicol; triclosan	Antibiotic efflux	73.3	100.0
INSAq77p_153	Loose	eptB	Protein homolog	pmr phosphoethanolamine transferase	Peptide	Antibiotic target alteration	73.2	98.3
INSAq77p_18	Loose	rosA	Protein homolog	Major facilitator superfamily (MFS) antibiotic efflux pump	Peptide	Antibiotic efflux	71.8	98.1

INSAq77p_185	Loose	ArnT	Protein homolog	pmr phosphoethanolamine transferase	Peptide	Antibiotic target alteration	70.2	100.0
INSAq77p_187	Loose	arnA	Protein homolog	pmr phosphoethanolamine transferase	Peptide	Antibiotic target alteration	69.7	99.7
INSAq77p_1	Loose	OmpK37	Protein homolog	General Bacterial Porin with reduced permeability to β-lactams	Monobactam; carbapenem; cephalosporin; cephamycin; penam; penem	Reduced permeability to antibiotic	68.1	18.7
INSAq77p_19	Loose	catA4	Protein homolog	Chloramphenicol acetyltransferase (CAT)	Phenicol	Antibiotic inactivation	65.9	106.9
INSAq77p_22	Loose	OmpK37	Protein homolog	General Bacterial Porin with reduced permeability to β-lactams	Monobactam; carbapenem; cephalosporin; cephamycin; penam; penem	Reduced permeability to antibiotic	65.7	106.2

AMR: antimicrobial resistance. ARO: antibiotic Resistance Ontology.

Table S 14. — Results obtained from prediction of a bacteria's pathogenicity towards human hosts using PathogenFinder (https://cge.cbs.dtu.dk/services/PathogenFinder/; accessed on Jan 4, 2022). Results highlighted in green are those not matching protein pathogenic families.

INSAq77 Contig	NCBI Accession number	Description	Protein Function	NCBI Protein ID	Identity (%)
INSAq771p_94_1	Unknown	*Salmonella enterica* subsp. *enterica* serovar Typhi str. CT18	Unknown	Unknown	100.0
INSAq771p_20_58	CP000880	*Salmonella enterica* subsp. *arizonae* serovar 62:z4,z23:--	Hypothetical protein	ABX24195	97.07
INSAq771p_43_25	CU928158	*Escherichia fergusonii* ATCC 35469 chromosome	Inorganic polyphosphate/ATP-NAD kinase	CAQ88017	97.6
INSAq771p_1_175	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA	4-hydroxyphenylacetate 3-hydroxylase	BAH61578	98.08
INSAq771p_3_58	CP000964	*Klebsiella pneumoniae* 342	4-hydroxybenzoate decarboxylase, subunit C	ACI07194	96.0
INSAq771p_10_38	CP000964	*Klebsiella pneumoniae* 342	Ascorbate-specific permease IIC component	ACI07087	96.78
INSAq771p_83_4	CP000948	*Escherichia coli* str. K12 substr. DH10B	CP4-6 prophage; predicted sugar transporter	ACB01437	96.73
INSAq771p_17_52	CP000822	*Citrobacter koseri* ATCC BAA-895	Hypothetical protein	ABV14881	97.03
INSAq771p_70_19	CU928163	*Escherichia coli* UMN026 chromosome	Putative pyridine nucleotide-disulfide oxidoreductase	CAR11557	99.55
INSAq771p_46_7	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R	Putative DNA methyltransferase	ABF67893	97.84
INSAq771p_4_46	CP000822	*Citrobacter koseri* ATCC BAA-895	Hypothetical protein	ABV15790	98.73
INSAq771p_91_11	CP000650	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 plasmid pKPN5	Plasmid partition protein A	ABR80603	99.5
INSAq771p_10_82	CP000822	*Citrobacter koseri* ATCC BAA-895	Hypothetical protein	ABV14677	95.78
INSAq771p_5_104	CP001138	*Salmonella enterica* subsp. *enterica* serovar Agona str. SL483	Secretion protein HlyD family protein	ACH51271	95.77
INSAq771p_46_27	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R	RepH12	ABF67907	99.45
INSAq771p_62_1	AP010960	*Escherichia coli* O111:H- str. 11128 DNA	Hypothetical protein	BAI35195	98.86
INSAq771p_8_15	CP000648	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 plasmid pKPN3	Hypothetical protein	ABR80369	97.75
INSAq771p_25_17	CP001063	*Shigella boydii* CDC 3083-94	DNA replication and repair protein RecF	ACD08678	96.36
INSAq771p_2_73	CP000468	*Escherichia coli* APEC O1	UDP-galactose-4-epimerase	ABJ00142	96.45
INSAq771p_107_4	AE014073	*Shigella flexneri* 2a str. 2457T	IS*911* orfA	AAP15762	96.31

INSAq771p_61_7	CP000886	*Salmonella enterica* subsp. *enterica* serovar Paratyphi B str. SPB7	Hypothetical protein	ABX66384	96.32
INSAq771p_22_45	CP001138	*Salmonella enterica* subsp. *enterica* serovar Agona str. SL483	Phage integrase	ACH51627	97.36
INSAq771p_93_6	AE017042	*Yersinia pestis* biovar Microtus str. 91001	Transposase for insertion sequence IS*100*	AAS60313	100.0
INSAq771p_7_29	CP000243	*Escherichia coli* UTI89	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	ABE08068	98.01
INSAq771p_91_12	CP000966	*Klebsiella pneumoniae* 342 plasmid pKP91	Plasmid partition parB protein	ACI12297	99.38
INSAq771p_15_32	CP000243	*Escherichia coli* UTI89	30S ribosomal protein S2	ABE05693	97.93
INSAq771p_193_1	CP001383	*Shigella flexneri* 2002017	IS*Ehe3*, transposase *orfB*	ADA76515	99.02
INSAq771p_91_9	CP000650	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 plasmid pKPN5	DNA replication	ABR80601	99.31
INSAq771p_6_5	CP000266	*Shigella flexneri* 5 str. 8401	Conserved hypothetical protein	ABF06037	95.75
INSAq771p_34_40	CP000822	*Citrobacter koseri* ATCC BAA-895	Hypothetical protein	ABV12545	98.0
INSAq771p_1_132	CP000822	*Citrobacter koseri* ATCC BAA-895	Hypothetical protein	ABV14493	97.3
INSAq771p_93_7	CP000244	*Escherichia coli* UTI89 plasmid pUTI89	Putative transposase	ABE10617	100.0
INSAq771p_14_9	CP000822	*Citrobacter koseri* ATCC BAA-895	Hypothetical protein	ABV15586	98.07
INSAq771p_62_3	CP001164	*Escherichia coli* O157:H7 str. EC4115	Conserved hypothetical protein	ACI36787	99.22
INSAq771p_2_77	CP000880	*Salmonella enterica* subsp. *arizonae* serovar 62:z4,z23:--	Hypothetical protein	ABX22051	96.8
INSAq771p_83_6	U00096	*Escherichia coli* str. K-12 substr. MG1655	CP4-6 prophage; predicted DNA-binding transcriptional regulator	AAC73375	95.63
INSAq771p_18_19	CP001113	*Salmonella enterica* subsp. *enterica* serovar Newport str. SL254	Ribonuclease III	ACF61892	95.82
INSAq771p_13_34	CP000822	*Citrobacter koseri* ATCC BAA-895	Hypothetical protein	ABV13829	98.55
INSAq771p_62_9	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R	TerY1	ABF67743	99.06
INSAq77_MCR-9_38	CP001125	*Salmonella enterica* subsp. *enterica* serovar Schwarzengrund str. CVM19633 plasmid pCVM1963	10, complete sequence.	ACF88522	100.0
INSAq771p_113_4	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA	Fimbrial chaperone protein mrkB precursor	BAH65060	100.0
INSAq771p_10_14	CP000247	*Escherichia coli* 536	Oligoribonuclease	ABG72350	96.13
INSAq771p_10_41	CP000964	*Klebsiella pneumoniae* 342	3-dehydro-L-gulonate-6-phosphate decarboxylase	ACI10848	96.76
INSAq771p_4_93	CP000783	*Enterobacter sakazakii* ATCC BAA-894	Hypothetical protein	ABU79530	98.27
INSAq771p_62_10	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R	TerX	ABF67742	100.0
INSAq771p_18_13	CP000243	*Escherichia coli* UTI89	RNA polymerase sigma E	ABE08355	98.95
INSAq771p_162_1	CP001063	*Shigella boydii* CDC 3083-94	IS*1* protein InsB	ACD09517	97.1
INSAq771p_15_21	CP000822	*Citrobacter koseri* ATCC BAA-895	Hypothetical protein	ABV14271	97.35
INSAq771p_3_23	CP001144	*Salmonella enterica* subsp. *enterica* serovar Dublin str. C	2021853, complete genome.	ACH75166	97.86
INSAq771p_5_36	CP001063	*Shigella boydii* CDC 3083-94	Conserved hypothetical protein	ACD09359	98.33
INSAq771p_9_48	CP000034	*Shigella dysenteriae* Sd197	Conserved hypothetical protein	ABB62686	96.45
INSAq771p_30_6	CP000243	*Escherichia coli* UTI89	50S ribosomal subunit protein L13	ABE09105	97.89
INSAq771p_54_12	XXX	*Salmonella enterica* subsp. *enterica* serovar Typhi str. CT18	XXX		98.16
INSAq771p_31_45	CP000783	*Enterobacter sakazakii* ATCC BAA-894	Hypothetical protein	ABU79120	96.88
INSAq771p_6_20	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA	Transcriptional repressor for methionine biosynthesis	BAH61007	98.1

INSAq771p_81_8	CP000857	*Salmonella enterica* subsp. *enterica* serovar Paratyphi C strain RKS4594	Co-chaperonin GroES	ACN48531	96.91
INSAq771p_62_7	DQ517526	*Escherichia coli* APEC O1 plasmid pAPEC-O1-R	TerW	ABF67744	100.0
INSAq771p_26_25	CP000948	*Escherichia coli* str. K12 substr. DH10B	CP4-6 prophage; inner membrane lipoprotein	ACB01418	100.0
INSAq771p_19_15	CP000247	*Escherichia coli* 536	Thioredoxin 1	ABG71937	98.17
INSAq771p_160_3	CP000244	*Escherichia coli* UTI89 plasmid pUTI89	Hypothetical protein	ABE10614	97.76
INSAq771p_119_5	CP000784	*Enterobacter sakazakii* ATCC BAA-894 plasmid pESA2	Hypothetical protein	ABU79601	100.0
INSAq771p_92_4	CP000036	*Shigella boydii* Sb227	Putative alpha helix protein	ABB65503	95.5
INSAq771p_29_45	AE017220	*Salmonella enterica* subsp. *enterica* serovar Choleraesuis str. SC-B67	Chemotaxis regulator, transmits chemoreceptor signals to flagellar motor components	AAX65829	95.35
INSAq771p_70_17	CP001063	*Shigella boydii* CDC 3083-94	IS*1* transposase *orfB*	ACD09692	98.86
INSAq771p_13_64	CP000243	*Escherichia coli* UTI89	Hypothetical protein YajC	ABE05930	99.09
INSAq771p_119_6	CP000784	*Enterobacter sakazakii* ATCC BAA-894 plasmid pESA2	Hypothetical protein	ABU79600	100.0
INSAq77_MCR-9_32	CP000800	*Escherichia coli* E24377A	IS*66* family element, *orf2*	ABV19481	99.13
INSAq771p_160_2	CP001063	*Shigella boydii* CDC 3083-94	IS*66* family element, *orf2*	ACD10323	100.0
INSAq771p_26_32	CP000948	*Escherichia coli* str. K12 substr. DH10B	CP4-6 prophage; toxin of the YkfI-YafW toxin-antitoxin system	ACB01411	99.12
INSAq771p_32_43	CP000880	*Salmonella enterica* subsp. *arizonae* serovar 62:z4,z23:--	Hypothetical protein	ABX21688	97.44
INSAq771p_10_39	AE014075	*Escherichia coli* CFT073	Unknown pentitol phosphotransferase enzyme II, B component	AAN83704	98.02
INSAq771p_8_5	CP000880	*Salmonella enterica* subsp. *arizonae* serovar 62:z4,z23:--	Hypothetical protein	ABX21522	97.98
INSAq771p_2_165	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA	Cold shock protein	BAH62358	100.0
INSAq771p_131_1	AE014073	*Shigella flexneri* 2a str. 2457T	IS*1 orfB*	AAP15901	98.97
INSAq771p_91_7	CU928144	*Escherichia fergusonii* str. ATCC 35469T plasmid pEFER	Hypothetical protein	CAQ86970	98.82
INSAq771p_6_137	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA	Conserved hypothetical protein	BAH60940	95.51
INSAq771p_70_18	CP000800	*Escherichia coli* E24377A	IS*1*, transposase *orfA*	ABV18481	100.0
INSAq771p_66_21	CP000799	*Escherichia coli* E24377A plasmid pETE	4, complete sequence.	ABV16448	98.9
INSAq771p_10_62	CP000026	*Salmonella enterica* subsp. *enterica* serovar Paratyphi A str. ATCC 9150	Conserved hypothetical protein	AAV79961	98.53
INSAq771p_30_19	CP000034	*Shigella dysenteriae* Sd197	Conserved hypothetical protein	ABB63408	97.01
INSAq771p_134_1	CP001113	*Salmonella enterica* subsp. *enterica* serovar Newport str. SL254	Phage transcriptional regulator, AlpA	ACF61557	96.51
INSAq771p_26_24	CP000948	*Escherichia coli* str. K12 substr. DH10B	CP4-57 prophage; predicted inner membrane protein	ACB03777	97.44
INSAq771p_22_56	CP000026	*Salmonella enterica* subsp. *enterica* serovar Paratyphi A str. ATCC 9150	Hypothetical protein	AAV78455	100.0
INSAq771p_1_137	AP006725	*Klebsiella pneumoniae* NTUH-K2044 DNA	Putative amino acid/amine transport protein	BAH61631	98.11
INSAq771p_134_3	BA000007	*Escherichia coli* O157:H7 str. Sakai DNA	Hypothetical protein	BAB33724	97.26
INSAq771p_73_3	CP000783	*Enterobacter sakazakii* ATCC BAA-894	Hypothetical protein	ABU79426	100.0

a)

Region	Region Length	Completeness	Score	# Total Proteins	Region Position	Most Common Phage	GC %
					INSAq771p_5		
1	32.7Kb	incomplete	50	14	25314-58060	PHAGE_Salmon_vB_SosS_Oslo_NC_018279(2)	53.27%
					INSAq771p_6		
2	32.6Kb	intact	150	49	47358-80029	PHAGE_Salmon_SEN5_NC_028701(27)	53.49%
					INSAq771p_7		
3	14.9Kb	incomplete	40	7	254-15224	PHAGE_Salmon_vB_SemP_Emek_NC_018275(2)	50.30%
					INSAq771p_15		
4	19.7Kb	questionable	90	26	68749-88473	PHAGE_Shigel_SfIV_NC_022749(4)	50.37%
					INSAq771p_20		
5	16Kb	incomplete	40	17	59703-75706	PHAGE_Klebsi_ST147_VIM1phi7.1_NC_049451(5)	51.33%
					INSAq771p_22		
6	35.6Kb	incomplete	30	35	37471-73143	PHAGE_Salmon_SEN8_NC_047753(20)	53.19%
					INSAq771p_35		
7	33.1Kb	incomplete	60	10	8624-41822	PHAGE_Cronob_ENT47670_NC_019927(6)	52.88%
					INSAq771p_43		
8	19Kb	incomplete	60	22	17976-37028	PHAGE_Escher_500465_1_NC_049342(18)	54.59%
					INSAq771p_74		
9	16.7Kb	intact	140	18	173-16928	PHAGE_Entero_HK542_NC_019769(8)	54.12%
					INSAq771p_84		
10	12.1Kb	incomplete	30	19	656-12792	PHAGE_Entero_c_1_NC_019706(3)	47.19%
					INSAq771p_91		
11	7.8Kb	incomplete	40	10	2658-10495	PHAGE_Entero_P1_NC_005856(3)	49.78%

b)

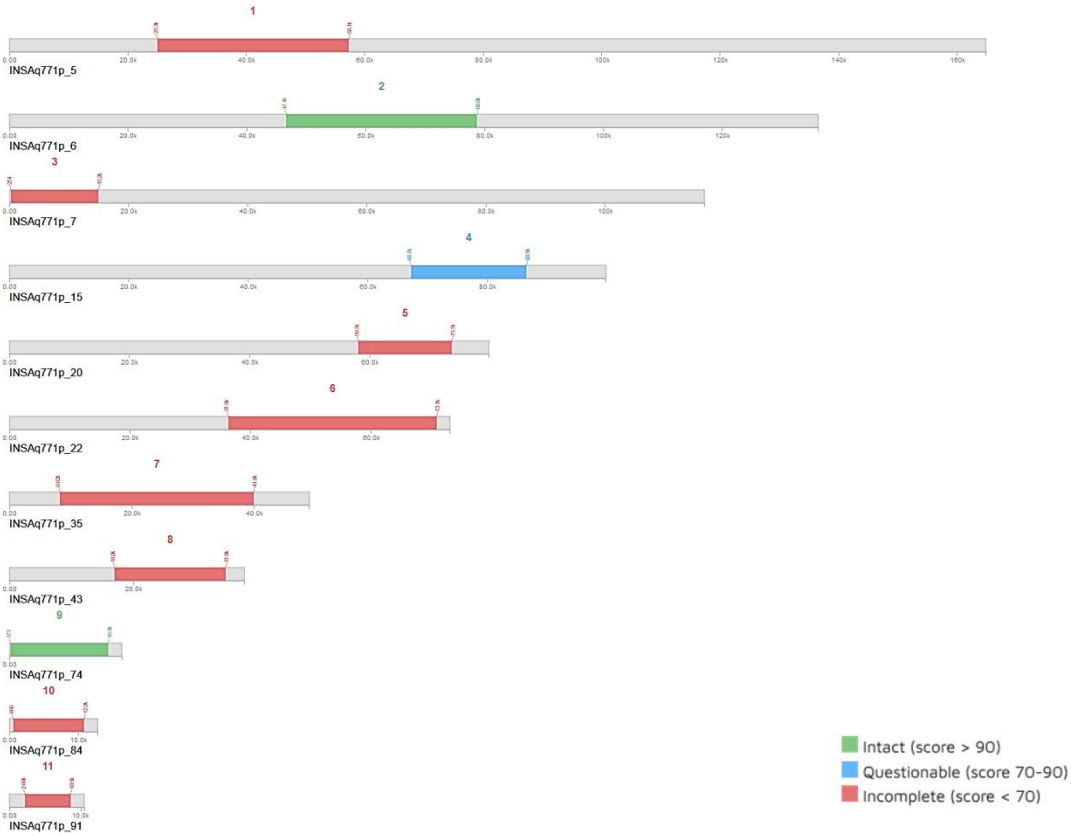

Figure S 10. — Presence of prophages in INSAq77 genome identified by PHASTER. A total of 11 prophage regions were identified, of which 2 regions were intact, 8 regions were incomplete, and 1 region was questionable. **a)** Table with prophage characteristics. **b)** Location of predicted prophages within INSAq77 contigs. Region types were marked with colors: intact (green), questionable (blue) and incomplete (red).

Table S 15 — Summary of all *spa*, ST, bacteria, antibiotic/heavy metals/disinfectants resistance genes (ARG/HMRG/DRG), and virulence factors (VF) described for the first time in this Ph.D. thesis.

Feature	New	First at our knowledge (at publication date)	Sample origin	Bacteria	Published in
spa	t14878	NA	Human	*Staphylococcus aureus*	[369]
	t14933	NA	Human		
	t15307	NA	Duck		
ST	3254	NA	Swine		
	3269	NA	Duck		
	3270	NA	Duck		
	879	NA	Gilthead seabream	*Aeromonas salmonicida*	Chapter 7*
	880	NA	Gilthead seabream	*Aeromonas rivipollensis*	
	881	NA	Gilthead seabream	*Aeromonas allosaccharophila*	
	882	NA	Gilthead seabream	*A. rivipollensis*	
	883	NA	Gilthead seabream	*Aeromonas media*	
	887	NA	Gilthead seabream	*A. salmonicida*	
	888	NA	Mussels	*A. allosaccharophila*	
	971	NA	Clams	*Citrobacter portucalensis*	
	1342	NA	Gilthead seabream	*Enterobacter ludwigii*	[480]
	1994	NA	Gilthead seabream	*Enterobacter asburiae*	Chapter 7*
	40	NA	Mussels	*Shewanella chilikensis*	
	57	NA	Mussels	*Shewanella algae*	
	58	NA	Mussels	*S. chilikensis*	
	60	NA	Mussels	*Shewanella indica*	
	61	NA	Mussels	*S. algae*	
	62	NA	Oysters	*S. algae*	
	205	NA	Mussels	*Vibrio diabolicus*	
	206	NA	Mussels	*Vibrio antiquarius*	
ST170	NA	Yes/in aquaculture	Gilthead seabream	*Enterobacter hormaechei*	
ST190					
ST664					
ST398	NA	Yes/in gilthead seabream from aquaculture	Gilthead seabream	*S. aureus*	[369]
Bacteria	NA	Yes/in gilthead seabream	Gilthead seabream	*Kluyvera intermedia*	Chapter 7*
	NA		Gilthead seabream	*Raoultella terrigena*	
	NA		Gilthead seabream	*Serratia liquefaciens*	
	NA		Gilthead seabream	*Exiguobacterium acetylicum*	[257]
	NA		Gilthead seabream	*Klebsiella michiganensis*	
	NA		Gilthead seabream	*Lelliottia* sp.	
	NA		Gilthead seabream	*Pantoea vagans*	
	NA	Yes/in *Leclercia adecarboxylata*	Gilthead seabream	*Leclercia adecarboxylata*	[257]

	NA	Yes/in mussels from aquaculture	Mussels	*Proteus terrae*	Chapter 7*
	NA		Mussels	*S. chilikensis*	
	NA	Yes/in bivalve mollusks from aquaculture	Clams	*Comamonas aquatica*	[422]
	NA		Clams	*Lactococcus garvieae*	
	NA		Clams	*Pseudocitrobacter faecalis*	
	NA		Clams and mussels	*Raoultella ornithinolytica*	
	NA		Oysters	*Escherichia fergusonii*	
	NA		Oysters	*Moraxella osloensis*	
	NA		Oysters	*Serratia marcescens*	
	NA	Yes/in gilthead seabream, oysters, and clams from aquaculture	Gilthead seabream, clams, and oysters	*C. portucalensis*	Chapter 7*
Bacteria resistant to vancomycin	NA	Yes/in gilthead seabream from aquaculture	Gilthead seabream	*Bacillus* sp.	[257]
ARG	*bla*$_{ACT-88}$	NA	Gilthead seabream	*E. ludwigii*	[480]
	bla$_{FOX-18}$	NA	Gilthead seabream	*A. allosaccharophila*	Chapter 7*
	bla$_{FOX-19}$	NA	Mussels	*A. allosaccharophila*	
	bla$_{OXA-958}$	NA	Gilthead seabream	*A. allosaccharophila*	
	bla$_{OXA-959}$	NA	Mussels	*A. allosaccharophila*	
	bla$_{OXA-960}$	NA	Mussels	*S. chilikensis*	
	bla$_{OXA-961}$	NA	Mussels	*S. chilikensis*	
	bla$_{OXA-963}$	NA	Mussels	*S. algae*	
	bla$_{OXA-965}$	NA	Oysters	*S. algae*	
	bla$_{TER-3}$	NA	Gilthead seabream	*R. terrigena*	
	bla$_{CTX-M-246}$	NA	Gilthead seabream	*K. intermedia*	
	bla$_{PLA-7}$	NA	Mussels	*Raoultella planticola*	
	bla$_{CMY-175}$	NA	Oysters	*C. portucalensis*	
ARG (*bla*$_{ACC}$-like)	NA	Yes/in *Obesumbacterium proteus*	Gilthead seabream	*O. proteus*	
ARG (*bla*$_{ORN-1}$)	NA	Yes/in aquaculture	Mussels	*R. ornithinolytica*	
ARG (*bla*$_{OXA-962}$)	NA	Yes/in mussels from aquaculture	Mussels	*S. indica*	
ARG (*bla*$_{OXA-964}$)	NA		Mussels	*S. algae*	
ARG (*bla*$_{OXY-1-3}$)	NA	Yes/in aquaculture	Gilthead seabream	*K. michiganensis*	
ARG (*eptA*)	NA		Gilthead seabream and mussels	*Escherichia coli*	
ARG (*hugA*-type)	NA		Mussels	*Proteus terrae*	
ARG (*kdpE*)	NA		Mussels	*E. coli*	
ARG (*mcr-9.1*)	NA		Gilthead seabream	*Enterobacter ludwigii*	[480]
ARG (*mdtN*)	NA		Mussels	*E. coli*	Chapter 7*
ARG (*mdtO*)	NA		Mussels	*E. coli*	
ARG (*mdtP*)	NA		Mussels	*E. coli*	
ARG (*qnrB38*)	NA		Mussels	*Citrobacter freundii*	

ARG (*qnrD2*)	NA	Yes/in aquaculture	Gilthead seabream	*Hafnia paralvei*
ARG (*yojI*)	NA		Mussels	*E. coli*
ARG/HMRG (*baeS*)	NA		Gilthead seabream and mussels	*E. coli and C. freundii*
ARG/HMRG (*emrA*)	NA		Gilthead seabream and mussels	*E. coli*
ARG/HMRG (*emrK*)	NA		Gilthead seabream and mussels	*E. coli*
ARG/HMRG (*mdtE*)	NA		Gilthead seabream and mussels	*E. coli*
ARG/VF (*gad*)	NA		Gilthead seabream and mussels	*E. coli*
VF (*astA*)	NA		Gilthead seabream	*E. coli*
VF (*cia*)	NA		Gilthead seabream	*Aeromonas media* and *E. coli*
VF (*cvaC*)	NA		Gilthead seabream	*A. media* and *E. coli*
VF (*etsC*)	NA		Gilthead seabream	*A. media* and *E. coli*
VF (*fyuA*)	NA		Gilthead seabream and mussels	*R. ornithinolytica*, *K. michiganensis* and *E. coli*
VF (*hlyF*)	NA		Gilthead seabream	*A. media* and *E. coli*
VF (*iha*)	NA		Mussels	*E. coli*
VF (*iroN*)	NA		Gilthead seabream	*A. media*, *E. coli* and *E. hormaechei*
VF (*iucC*)	NA		Gilthead seabream and mussels	*A. media* and *E. coli*
VF (*irp2*)	NA		Gilthead seabream	*E. coli*
VF (*iss*)	NA		Gilthead seabream	*A. media* and *E. coli*
VF (*lpfA*)	NA		Gilthead seabream	*E. coli*
VF (*mchC*)	NA		Mussels	*E. coli*
VF (*mchF*)	NA		Gilthead seabream and mussels	*A. media* and *E. coli*
DRG (*sitABCD*-type)	NA		Gilthead seabream and mussels	*A. media* and *E. coli*

*The manuscript corresponding to chapter 7 is not published but submitted in an international peer reviewed journal. NA: not applicable. ST: sequence type. ARG: antibiotic resistance gene. DRG: disinfectant resistance gene. HMRG: heavy metals resistance gene. VF: virulence factors.

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DECRYPTING THE DIVERSITY OF MICROBIOME IN AQUACULTURE