Abstract: This study analyzed the resistome, virulome and mobilome of an MCR-9-producnga Enterobacter sp. identified in a muscle sample of seabream (Sparus aurata), collected in a land from multi-trophic fish farming production. Average Nucleotide Identity analysis identified INSAq77 at the species level as an Enterobacter ludwigii INSAq77 strain 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<sub>ACT-88</sub>), chloramphenicol (catA4-type), fosfomycin (fisA2-type) and colistin (mcr-9.1), as well as several efflux pumps (e.g., oppAB-type and mar operon). Further bioinformatics analysis revealed five plasmid replicon types, including the IncH2/H12A, 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-lssgsp1-pcoS-<i>ISS903-mcr-9-wbuC</i> 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 <i>mcr</i> genes may represent a serious threat to human health.

Keywords: aquaculture; mcr-9 gene; seabream; One Health

1. 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 <i>Enterobacter</i> <i>ludwigii</i> [1]. In aquatic animal species, colistin is also used to treat bacterial
MCR-producing bacteria were mostly isolated from animals, including pigs [7]. The exception are the mcr-9 and mcr-10 that were identified in human isolates: Salmonella enterica serotype Typhimurium and Enterobacter roggenkampii, respectively [8,9].

Unlike the situation in terrestrial animals, PMCR in aquaculture has largely been ignored [10]. However, colistin-resistant bacteria have also been found in aquaculture [11,12], being considered by the 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 [13] and in E. coli recovered from grass carp fish farms [14].

In Vietnam, mcr-1 was detected in E. coli isolated from striped catfish grown in ponds [15,16] and a study from Spain reported mcr-1 in S. enterica serovar Rissen isolated from mussels [17]. Recently, mcr-1 was detected in E. coli isolated from fish guts of rainbow trout in Lebanon [18]. In Czech Republic, mcr-type genes were detected in colistin-resistant Enterobacteriales and Acinetobacter strains isolated from aquaculture products (frog legs, crab meat and pangasius meat) originating from Vietnam [19]. Shen and co-workers also demonstrated the association between aquaculture and a high incidence of mcr-1-positive E. coli carried by humans [13]. 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 [20]. 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 [10,11].

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 [8]. Along with mcr-1, among the mcr-like genes, mcr-9 is the most widely disseminated [7]. The mcr-9 gene can be found worldwide in different reservoirs (human, animal, food and environment) and in various species of Enterobacteriaceae [21], which makes this resistance mechanism a problem under the perspective of One Health.

This study aimed to analyse 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.

2. 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 [7]. 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%) [21]. 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 multitrophic 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, Marcy-l’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 [22] and that hsp60 gene sequencing showed a higher species diversity than MALDI-TOF [23]. Seven species have been grouped within the E. cloacae complex: Enterobacter cloacae, Enterobacter asburiae, Enterobacter hormacei, E. ludwigii, Enterobacter mori and Enterobacter nimipressuralis, which share at least 60% similarity in their genome with E. cloacae [24].

E. ludwigii was first described as a novel Enterobacter species in 2005 [25]. 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 [26], although a CTX-M-producing E. ludwigii was recently described in an environmental isolate collected from a wastewater treatment plant in India [27]. Recently, antibiotic-resistant E. ludwigii was also identified in India, in moribund goldfish collected from ornamental fish farms [28]. On the other hand, E. ludwigii has been suggested as a potential probiotic microorganism in agriculture and aquaculture [29,30].

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 [8,31,32]. Nevertheless, recent studies showed that mcr genes might enhance the survival ability of bacteria under clinical colistin pressure, thereby potentially leading to treatment failure [33,34].

This study also analyzed the resistome, virulome and mobilome of this MCR-9-producing E. ludwigii isolated from farmed Sparus 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 30.7 ×; 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 1).

Indeed, INSAq77 has 12% of nucleotide sequence divergence with the closest strain (NZ_VLMJ0000000), an E. ludwigii isolated from the lung of a clinical patient, in 2016, in the USA (PRJNA553678) [35]. 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 (blaACT-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 (Tables 1 and S1).
Figure 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 colour indicates cluster containing INSAq77.
Table 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 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 beta-lactamase | antibiotic efflux pump | carbapenem, cephalosporin, cephamycin, penam | antibiotic inactivation               | 98.7                       | 100.0                       |
| INSAq77p_1     | Strict       | FosA2                           | protein homolog          | fosfomycin thiol transferase | antibiotic efflux pump | antibiotic inactivation | antibiotic inactivation               | 98.6                       | 100.0                       |
| INSAq77p_4     | Strict       | Escherichia coli EF-Tu mutants (R234F) | protein variant          | elfamycin-resistant EF-Tu | antibiotic efflux pump | 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 | 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                       |
Table 1. Cont.

| Contig          | RGI Criteria | ARO Term                                | Detection Criteria Model | AMR Gene Family                        | Drug Class          | Resistance Mechanism | % Identity Matching Region | % Length Reference Sequence |
|-----------------|--------------|-----------------------------------------|--------------------------|----------------------------------------|---------------------|----------------------|--------------------------|---------------------------|
| 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 beta-lactams | fluoroquinolone antibiotic, monobactam, carbapenem, cephalosporin, glycolcycline, cephamecin, 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, glycolcycline, 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, glycolcycline, penam, tetracycline antibiotic, rifamycin antibiotic, phenicol antibiotic, triclosan | antibiotic target alteration, antibiotic efflux | 91.0          | 100.0                  |
Table 1. Cont.

| Contig       | RGI Criteria | ARO Term                          | Detection Criteria Model | AMR Gene Family                          | Drug Class | Resistance Mechanism         | % Identity Matching Region | % Length Reference Sequence |
|--------------|--------------|-----------------------------------|--------------------------|------------------------------------------|------------|------------------------------|----------------------------|----------------------------|
| INSAq77p_34  | Strict       | *Klebsiella pneumoniae* KpnF      | protein homolog          | major facilitator superfamily (MFS)      | antibiotic efflux | macrolide antibiotic,      | 89.0                       | 100.0                      |
|              |              |                                   |                          | antibiotic efflux pump                   |             | aminoglycoside antibiotic,|                           |                           |
|              |              |                                   |                          | cephalosporin, tetracycline antibiotic   |             | rifamycin antibiotic       |                           |                           |
| INSAq77p_3   | Strict       | rsmA                              | protein homolog          | resistance-nodulation-cell division (RND)| antibiotic efflux | fluoroquinolone antibiotic,| 85.3                       | 100.0                      |
|              |              |                                   |                          | antibiotic efflux pump                   |             | diaminopyrimidine antibiotic,|                           |                           |
|              |              |                                   |                          | antibiotic, phenicol antibiotic          |             |                             |                           |                           |
| INSAq77p_13  | Strict       | *Escherichia coli* ampH            | protein homolog          | *ampC*-type beta-lactamase               | antibiotic inactivation | cephalosporin, penam      | 85.2                       | 100.8                      |
|              |              |                                   |                          |                                          |             |                             |                           |                           |
| INSAq77p_34  | Strict       | *Klebsiella pneumoniae* KpnE      | protein homolog          | major facilitator superfamily (MFS)      | antibiotic efflux | macrolide antibiotic,      | 82.0                       | 83.3                       |
|              |              |                                   |                          | antibiotic efflux pump                   |             | aminoglycoside antibiotic,|                           |                           |
|              |              |                                   |                          | cephalosporin, tetracycline antibiotic   |             | rifamycin antibiotic       |                           |                           |
| INSAq77p_11  | Strict       | adeF                              | protein homolog          | resistance-nodulation-cell division (RND)| antibiotic efflux | fluoroquinolone antibiotic,| 60.9                       | 99.2                       |
|              |              |                                   |                          | antibiotic efflux pump                   |             | tetracycline antibiotic    |                           |                           |
| INSAq77p_1   | Strict       | *Haemophilus influenzae* PBP3     | protein variant          | Penicillin-binding protein mutations      | antibiotic target alteration | cephalosporin, cephemycin,penam | 53.1                       | 96.4                       |
|              |              | mutant (D350N, S357N)             |                          | conferring resistance to beta-lactam     |             |                             |                           |                           |
|              |              |                                   |                          | antibiotics                             |             |                             |                           |                           |
| INSAq77p_9   | Strict       | adeF                              | protein homolog          | resistance-nodulation-cell division (RND)| antibiotic efflux | fluoroquinolone antibiotic,| 41.2                       | 97.9                       |
|              |              |                                   |                          | antibiotic efflux pump                   |             | tetracycline antibiotic    |                           |                           |
CARD loose algorithm (match bitscore less than the curated one blastp bitscore) [36] 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 [37]. 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 [38]. Diminished susceptibility to different antibiotic classes (e.g., aminoglycosides, fluoroquinolones and tetracyclines) were bioinformatically predicted (e.g., rsmA, adeF, ramA, acrAB 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 [39].

The acquired disinfectant resistance gene formA-type, a plasmid-mediated formaldehyde resistance mechanism [40], was also identified. The ability to survive aldehyde disinfection processes is clinically significant, with possible cross-resistance to antibiotics [41]. Furthermore, the INSAq77 isolate carried the terC virulence gene, commonly associated with IncH1 plasmids and conferring resistance to tellurium, where soluble salts, especially potassium tellurite, were used clinically in humans as antimicrobial agents [42].

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 S2). Indeed, in addition to the known E. cloacae complex genes encoding pathogenic proteins and the 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 [43]. In this study, nine insertion sequences (IS) were found using MobileElementFinder tool: IS26, ISKpn28, ISSen4-type, IS30-type, ISEcl1-type, ISKpn43-type, ISKpn24-type, IS100-type and ISPpu12-type.

A total of eleven prophage regions were also identified using PHASTER tool (Figure S1), 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 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 16.7Kb to 32.6Kp with an average GC content of 52.7%. These prophages were firstly described in S. enterica subspecies salamae collected in the Czech Republic [44], E. coli isolated in Hong Kong and Shigella flexneri collected in Bangladesh [45], 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 [46,47], 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 [48].

Further bioinformatics analysis revealed the presence of five plasmid replicon types: ColE10, Col(pHAD28)-type, IncFIA(H11)-type, IncR-type, IncH12 and IncHI2A, the last two linked to the worldwide dissemination of mcr-9 gene [7,21]. The mcr-9 gene can be found in different reservoirs (human, animal, food and environment), in various species of Enterobacteriaceae strains, mostly associated with IncH12/IncH12A plasmid replicons [21]. Indeed, as observed in our study, several works demonstrated the prevalence of mcr-9-harboring IncH12/IncH12A plasmids among Enterobacteriaceae isolates: e.g., Carroll et al., in 2019, detected 59/65 assemblies where IncH12 and/or IncH12A plasmid replicon were present on the same contig as mcr-9 [8].
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 S1), 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 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 16.7Kb to 32.6Kb with an average GC content of 52.7%. These prophages were firstly described in S. enterica subspecies salamae collected in the Czech Republic [44], E. coli isolated in Hong Kong and Shigella flexneri collected in Bangladesh [45], 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 [46,47], 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 [48].

Figure 2. Schematic representation of phage-related proteins identified in the intact and questionable prophages by PHASTER prophage database (https://phaster.ca, accessed on 4 January 2022) [49]. The arrow indicates 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 Fibre), 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).

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 [7,21]. 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 [21]. Indeed, as observed in our study, several works demonstrated the prevalence of 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); the GC content was 47.6%. The analysis of mcr-9-harbouring contig using the Micrornal 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 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 [50].
Table 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 Desinfectant Resistance Genes c | GenBank Acc. No. |
|--------------|--------|-------------------------------|--------------|----------------|----------|----------------------------------------------------------|-----------------|
| INSAq77 IncHI2 (30,314)  | 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)-ld, blaSHV-12, blaTEM-1B, catA2-type, dfrA19, mcr-9, qacE, qnrA1-type, sul1, sul2, tet(D) | NZ_CP029824 |
| 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")-Ib-type, aadA5, armA, blaTEM-1B, catA2-type, dfrA1-type, mcr-9, mph(E), mbr(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)-ld, blaKPC-4, blaOXA-129, dfrA21, mcr-9, mph(E), mbr(E), qacE, sul1, sul2 | NZ_CP029410 |
| pOSUEC_D (354,256) | E. hormaechei OSUVC_MCKPC4, L | Animal/USA/2016 | 100.00% | 98.0% | DLST1 | aadA1, aph(3")-Ib-type, aph(6)-ld, blaKPC-4, blaOXA-129, dfrA21, mcr-9, mph(E), mbr(E), qacE, sul1, sul2 | NZ_CP029248 |
| pK29 (269,674) | K. pneumonae NK29 | Human/Taiwan/2001 | 100.00% | 98.0% | DLST1 | aadA2, blaCMY-8, blaCTX-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_CP0425006 |
| pE11_001 (339,433) | C. freundii E11 | Burns Unit Shower/Australia/2012 | 100.00% | 98.0% | DLST1 | formA-type, mcr-9 | NZ_CP0425235 |
| pE61_001 (357,530) | L. adecarboxylata E61 | Burns Unit Shower/Australia/2014 | 100.00% | 98.0% | DLST1 | formA-type, mcr-9 | NZ_CP0424945 |
| pS65_1 (263,189) | C. freundii 565 | Human Stool/Spain/2014 | 99.68% | 95.0% | DLST1 | aac(6')-Ib-cr, aadA1, aadA2b-type, blaCTX-M-9, blachV-12, blavIM-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%).
In INSAq77 IncHI2 plasmid and in all others studied here, except p565_1 from a C. freundii strain (NZ_CP038657, Figure 3), the mcr-9 gene was surrounded upstream by an IS903 element and downstream by a wbuC family gene, encoding a cupin fold metalloprotein, followed by IS26.

Figure 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 [51]. Indeed, this gene was proposed to have transferred together with mcr-9 as a whole fragment from Buttiauxella spp. [51]. 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 regulatory system can induce the expression of the mcr-9 gene, which results in an increase in MIC values [32,51,52]. The mcr-9 gene is being described as part of a mcr-9 cassette containing the rcnR-rcnA-pcoE-pcoS-IS903-mcr-9-wbuC core structure (Figure 3) [21].

As shown in Figures 3 and 4, the genetic background immediately upstream of mcr-9 was consistent among IncHI2 mcr-9-bearing plasmids. The exception is the presence of an ISSgsp1 element from the IS66 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 cop-
Downstream of the \textit{mcr-9} gene, the nucleotide sequences, including that of INSAq77, were genetically diverse (Figure 3). Indeed, different regions were present among the IncHI2 plasmids also analyzed in this study, namely regions involved in resistance to copper (\textit{pcoABCDRE}), silver (\textit{silESRCBAP}), arsenic (\textit{arsCBRH}) and/or mercury (\textit{merEDACPTR}).

3. Materials and Methods

3.1. Study Design and Bacterial Identification

MCR-9-producing \textit{Enterobacter} sp. INSAq77 was isolated from a seabream (\textit{S. aurata}) of commercial-size (500–1500 g), which was collected in March 2018 in a land tank from a

![Figure 4. Schematic representation of the genetic environment of INSAq77 \textit{mcr-9.1}-containing contig with others IncHI2 \textit{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 \textit{mcr-9} cassette: dark red, \textit{mcr-9} gene; cyan, mobile DNA; purple, other genes; grey, other CDSs. The scale is represented in base pairs.](image)

The abundance of \textit{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 [53]. \textit{mcr-9} gene was firstly described in the USA, in a clinical \textit{S. Typhimurium} isolate, which demonstrates the high transmission potential of this colistin resistance determinant and places this research in a One Health context [54].
3. Materials and Methods

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 multitrophic farming [55]. 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 [56]. 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 [55].

3.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by disk diffusion (amoxicillin/clavulanic acid, aztreonam, cefepime, cefotaxime, cefsulodin, cephalaxin, cefoxitin, ceftriaxone, cefotaxime, cefazolin, gentamicin, imipenem, meropenem, piperacillin/tazobactam and trimethoprimsulfamethoxazole; 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 [55].

3.3. Whole-Genome Sequencing

DNA was extracted from freshly grown overnight culture (MagnaPure 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 × 250 bp) on a MiSeq Illumina platform (Illumina Inc., San Diego, CA, USA), according to the manufacturer’s instructions.

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 4 January 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 [57]. All de novo contigs were BLAST searched against GenBank’s non-redundant nucleotide collection (nr/nt) [58]. 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.

3.5. Phylogenomic Analyses of *E. ludwigii* Genomes

All *E. ludwigii* genomes (*n* = 76, Table S3) 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 4 January 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 4 January 2022).

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 4 January 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 [36]. Issaga was used for the identification and annotation of insertion sequences [59]. PHASTER search web tool (https://phaster.ca; accessed on 4 January 2022) was applied to detect, identify and annotate prophage sequences [49]. All analyses were performed using default parameters.

### 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 4 January 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 [60].

### 4. 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 [61].

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.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11091232/s1, Figure S1: Presence of prophages in INSAq77 genome identified by PHASTER. A total of eleven prophage regions were identified, of which two regions were intact, eight regions were incomplete and one 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 S1: Loose best-hit results (≥65% of identity), by predicted gene, obtained using the Resistance Gene Identifier (RGI); Table S2: Results obtained from prediction of a bacteria’s pathogenicity towards human hosts using PathogenFinder (https://cge.cbs.dtu.dk/services/PathogenFinder/, accessed on 4 January 2022). Results highlighted in green are those not matching protein pathogenic families.; Table S3: Demographic and genomic characteristics of the *E. ludwigii* isolates used for the phylogenomic analysis.

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Data Availability Statement: The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files. The E. ludwii genome shotgun (WGS) project has the project accession JABRP000000000. The new blb\textsubscript{ACT}-type nucleotide sequence was submitted to the GenBank Database as blb\textsubscript{ACT}-86 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 4 January 2022).

Conflicts of Interest: The authors declare no conflict of interest.

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