water

MDPI

Article

Comprehensive Profiling of *Klebsiella* in Surface Waters from Northern Portugal: Understanding Patterns in Prevalence, Antibiotic Resistance, and Biofilm Formation

Sara Araújo [1,2], Vanessa Silva [1,2,3,4], Maria de Lurdes Enes Dapkevicius [5,6,*], José Eduardo Pereira [1,7,8], Ângela Martins [7,8], Gilberto Igrejas [2,3,4] and Patricia Poeta [1,4,7,8,*]

1 Microbiology and Antibiotic Resistance Team (MicroART), Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal; saravanessaaraujo07@gmail.com (S.A.); vanessasilva@utad.pt (V.S.); jeduardo@utad.pt (J.E.P.)
2 Department of Genetics and Biotechnology, University of Trás-os-Montes and Alto Douro, 5000-801 Vila Real, Portugal; gigrejas@utad.pt
3 Functional Genomics and Proteomics Unit, University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal
4 Associated Laboratory for Green Chemistry (LAQV-REQUIMTE), University NOVA of Lisboa, 2829-516 Lisboa, Portugal
5 Faculty of Agricultural and Environmental Sciences, University of the Azores, 9500-321 Angra do Heroísmo, Portugal
6 Institute of Agricultural and Environmental Research and Technology (IITAA), University of the Azores, 9500-321 Angra do Heroísmo, Portugal
7 Veterinary and Animal Research Centre, Associate Laboratory for Animal and Veterinary Science (CECAV), University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal; angela@utad.pt
8 Associate Laboratory for Animal and Veterinary Science (AL4AnimalS), University of Trás-os-Montes and Alto Douro (UTAD), 5000-801 Vila Real, Portugal
* Correspondence: maria.ln.dapkevicius@uac.pt (M.d.L.E.D.); ppoeta@utad.pt (P.P.)

Abstract: This study investigates the prevalence of resistance and virulence genes in *Klebsiella* isolates from surface waters in Northern Portugal, within the broader context of freshwater quality challenges in Southern Europe. The aim of this research is to explain how *Klebsiella* dynamics, antibiotic resistance, and biofilm formation interact in surface waters. Antimicrobial susceptibility was examined using the Kirby–Bauer disk diffusion method against 11 antibiotics and screening for Extended-Spectrum Beta-Lactamase (ESBL) production using the double-disk synergy. PCR was employed to detect resistance and virulence genes, while biofilm production was assessed using the microplate method. Out of 77 water isolates, 33 *Klebsiella* (14 *Klebsiella* spp. and 19 *K. pneumoniae* strains) were isolated. ESBL production was observed in 36.8% of *K. pneumoniae* and 28.6% of *Klebsiella* spp. High resistance rates to $bla_{\text{CTX-U}}$ were observed in both. The *pap*C gene was prevalent, signifying potential environmental risks. Biofilm production averaged 81.3% for *K. pneumoniae* and 86.9% for *Klebsiella* spp. These findings underscore the intricate interplay between *Klebsiella*'s dynamics and freshwater quality, with ESBL's prevalence raising concerns about waterborne dissemination and public health implications. This work supports the need for vigilance of *Klebsiella* in surface waters in Southern Europe.

Keywords: *Klebsiella* spp.; *K. pneumoniae*; surface waters; aquatic environment; ESBL; biofilms

check for updates

Citation: Araújo, S.; Silva, V.; de Lurdes Enes Dapkevicius, M.; Pereira, J.E.; Martins, Â.; Igrejas, G.; Poeta, P. Comprehensive Profiling of *Klebsiella* in Surface Waters from Northern Portugal: Understanding Patterns in Prevalence, Antibiotic Resistance, and Biofilm Formation. *Water* **2024**, *16*, 1297. https://doi.org/10.3390/w16091297

Academic Editors: Daniela R. De Figueiredo, M. Teresa Condesso de Melo and Sofia Bento

Received: 15 March 2024
Revised: 26 April 2024
Accepted: 27 April 2024
Published: 2 May 2024

Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The global rise in antimicrobial-resistant bacteria (ARB) poses a formidable medical challenge, emerging as one of the most concerning issues of our era. It is estimated that by 2050, without sustained efforts, the global mortality attributed to diseases caused by ARB could potentially exceed 10 million, surpassing the mortality rate caused by cancer [1]. The decline in new antibiotic development and the increased prevalence of multidrug-resistant

bacteria, some of which are resistant to all antibiotic families, pose a major threat to global public health, potentially leading us back to a pre-antibiotic era [2]. Antimicrobial resistance (AMR) represents an ecological challenge, characterized by intricate interactions among diverse microbial populations that impact human, animal, and environmental health [3]. The assessment of the role of the environment in the development and transmission of AMR is a relatively recent approach, with actions in the environmental sector being the least implemented within the scope of public policies [4]. In recent times, there has been a growing acknowledgment of the environment as a critical source and significant pathway for the dissemination of resistance. The limited understanding of the environment's role in resistance development presents challenges in mitigating the emergence and spread of mobile resistance factors [5]. Water systems are a major focus of research as they receive high levels of ARBs and antibiotic resistance genes (ARGs) from human and animal waste. The increased concentration of antibiotic residues in wastewater fosters the development of antibiotic resistance in bacteria. Numerous studies have demonstrated that wastewater serves as a reservoir of ARGs, persisting in the effluents of wastewater treatment plants, even after filtration and disinfection [6,7]. The presence of ARB in water is becoming an increasingly pressing concern. Moreover, the presence of antibiotic-resistant bacteria serves as an indicator of antibiotic contamination in the respective aquatic environment. Overall, water quality and safety are paramount for social development and ecological sustainability [8]. The ESKAPE pathogens (*Enterococcus faecium*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and some of the *Enterobacter* species) have been detected in ecosystems influenced by anthropogenic or agricultural factors. *Klebsiella* spp. is an opportunistic pathogen found in various environments, including surface waters, plants, soil, and wastewater, among others, with its presence dependent on the phylogroup [9,10]. This bacterium demonstrates the ability to thrive in both oxygenated and non-oxygenated environments. This adaptability, coupled with its considerable resistome, poses a risk of transferring genetic determinants of antimicrobial resistance to other bacteria [11]. Numerous species and subspecies of *Klebsiella* have been identified, with *K. pneumoniae* regarded as the most clinically significant in both human and animal health, closely followed by *K. oxytoca* [12,13]. Extended-Spectrum β-Lactamase (ESBL)-producing strains of *Klebsiella* spp. are a common source of AMR in animals, humans, and the environment. These are bacterial enzymes that degrade antibiotics from the β-lactam class, such as penicillin and third- and fourth-generation cephalosporins [14]. The exploration of surface freshwater and groundwater in the context of AMR is crucial due to the global rise of AMR bacteria. Studies have identified various antibiotics and antibiotic resistance genes in surface water and groundwater, indicating that these water bodies can act as reservoirs and conduits for the spread of AMR [15,16]. Projections of decreased water levels and quality in both surface and groundwater bodies underscore the environmental, societal, political, and economic changes that have potential implications for global health [17]. In lentic water bodies, where the impact of droughts and warmer temperatures can lead to life-threatening events within local communities, the urgency of addressing freshwater quality becomes even more apparent. The potential effects on groundwater quality highlight the critical need for the preservation of freshwater quality as an urgent and crucial issue [18,19]. Thus, with the ultimate goal of contributing to a better understanding of the spread of *Klebsiella* spp. and *K. pneumoniae* in surface water, this work's aim is to investigate the presence and diversity of these pathogens in lotic (streams, rivers, fountains, irrigation ditches, and springs) and lentic (dams, water wells, water tanks, and a water mine) water bodies in Northern Portugal. Moreover, the prevalence of antibiotic-resistant phenotypes, genetic determinants of resistance and virulence, as well as biofilm formation were investigated in all isolates under study.

2. Materials and Methods

2.1. Geographical Location and Sample Collection

Seventy-seven locations across the Portuguese region of Trás-os-Montes and Alto Douro (Figure 1) were investigated. All rivers under consideration exclusively pertained to the Douro River Basin, an international hydrographic region spanning an aggregate area of approximately 97,000 km^2, of which 18,643 km^2 is situated within the confines of Portugal. This hydrological basin is demarcated by the eastern border with Spain and the western boundary adjacent to the Atlantic Ocean.

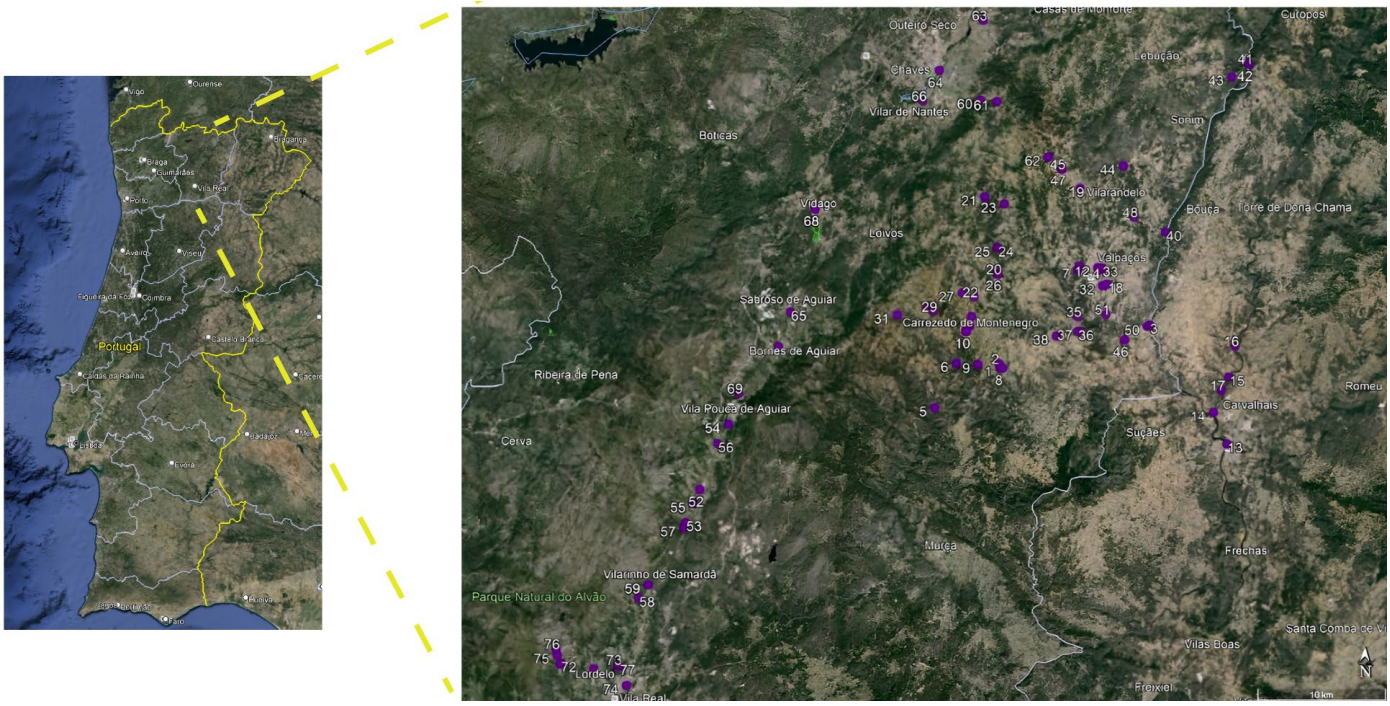

Figure 1. Geographical location of surface water sampling points in Trás-os-Montes and Alto Douro (Portugal). Each number corresponds to a specific number of water sample described in Table S1.

Samples from 18 rivers, 33 streams, 1 irrigation ditch, 1 dam, 12 fountains, 7 water wells, 2 water tanks, 1 water mine, and 2 springs were collected between October 2022 and April 2023 (Table S1). Water was sampled in 500 mL sterile plastic bottles containing sodium thiosulfate and, subsequently, preserved at 4–8 °C. The filtration of all samples occurred on the day of collection.

2.2. Bacterial Isolation

The water samples collected were filtered for *Klebsiella* spp. isolation. Approximately 100 mL of the water samples was filtered using a 0.45 µm cellulose nitrate pore membrane filter (Whatman, UK). Subsequently, the filters were immersed into tubes containing 5 mL of BHI (Brain Heart Infusion) broth and incubated at 37 °C for 24 h. After the incubation period, the samples were seeded onto Chromogenic Coliform Agar, supplemented and not supplemented with cefotaxime (CTX). The colonies that were pink in color were picked for the isolation of *Klebsiella* spp. and were subsequently sown on HiChrome *Klebsiella* Selective Agar Base. The plates were incubated for 24 h at 37 °C. All isolates that turned purple on HiChrome *Klebsiella* selective media were considered presumptive *Klebsiella* spp. The *K. pneumoniae* species identification of all isolates was carried out by polymerase chain reaction (PCR) to amplify the 428 base pairs (bps) of the *khe* gene using specific primers, as previously described [20].

2.3. Antimicrobial Susceptibility/Resistance Assessment

The Kirby–Bauer disk diffusion method was employed to evaluate antimicrobial susceptibility according to the EUCAST guidelines, with the exception of ceftazidime, tetracycline, and streptomycin, for which the CLSI guidelines were employed as standards. The following 11 antimicrobials (µg/disc) were used: amoxicillin + clavulanic acid (20 + 10), cefoxitin (30), ceftazidime (30), cefotaxime (30), meropenem (10), tetracycline (30), gentamicin (10), streptomycin (10), tobramycin (10), ciprofloxacin (5), and trimethoprim–sulfamethoxazole (1.25 + 23.75). Screening for phenotypic ESBL production was conducted through the double-disk synergy test utilizing cefotaxime, ceftazidime, and amoxicillin/clavulanic acid disks. Isolates showing resistance to three or more antibiotic classes were considered as multi-resistant (MDR).

2.4. Antimicrobial Resistance Genes and Virulence Factors

For DNA extraction, isolates were seeded on BHI agar and incubated at 37 °C for 18–24 h. After the incubation, genomic DNA from *Klebsiella* strains was extracted using the "Boiling Method" [21]. The extracted DNA was preserved at −20 °C until further analysis. All isolates were screened for the presence of antimicrobial resistance genes based on their phenotypic resistance profiles. The presence of antimicrobial-resistant genes encoding resistance to cefotaxime and β-lactams ($bla_{\text{CTX-U}}$, $bla_{\text{CTX-M3}}$, $bla_{\text{CTX-M9}}$, bla_{SHV}, bla_{TEM}, *amp*C), tetracyclines (*tet*A, and *tet*B), gentamicin (*aac*(3)-II, *aac*(3)-IV, and *ant*(2)), streptomycin (*str*A, *str*B, *aad*A1, and *aad*A5), trimethoprim–sulfamethoxazole (*sul*1, *sul*2, and *sul*3), carbapenems (bla_{OXA}, $bla_{\text{OXA-48}}$, bla_{VIM}, bla_{IMP}, and bla_{KPC}), ciprofloxacin (*par*C), and colistin (*mcr*-1) was investigated by PCR, as previously reported. The presence of virulence genes, including Pap pili (*pap*C), pilus associated with pyelonephritis G allele III (*pap*G-III), cytotoxic necrotizing factor 1 (*cnf*1), aerolysin gene (*aer*), and bundle-forming pili (*bfp*), was also tested using PCR. Positive and negative controls used in all experiments were derived from the strain collection of the University of Trás-Os-Montes and Alto Douro [22,23]. The specific primer sequences used in this study and the amplified product size are shown in Table S2.

2.5. Biofilm Production

Biofilm production was conducted using the microtiter assay, following a previously described protocol with certain modifications [24]. In short, each *Klebsiella* isolate was streaked on BHI agar plates and incubated at 37 °C for 24 h. After the incubation period, a few colonies were transferred to tubes containing 3 mL of Tryptic Soy Broth (TSB, Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C for 16 ± 1 h, with continuous shaking at 120 rpm (ES-80 Shaker-incubator, Grant Instruments, Cambridge, UK). Then, the bacterial suspension was adjusted to an optical density of 1×10^6 colony forming units, and 200 µL of bacterial suspension was added to each well of the 96-well flat-bottom microplate. To standardize the results, the biofilm formation of each isolate was given as percentage from the results obtained for the positive control strain, *Klebsiella* spp. ATCC® 13883. Isolates were characterized as strong, moderate, or weak biofilm producers when the percentages obtained were, respectively, >100%, 70–100%, or <70%. TSB without bacterial inoculum was used as a negative control. The plates were incubated at 37 °C for 24 h without shaking under aerobic conditions. All experiments were performed in duplicate and had 7 technical replicates.

To quantify biofilm biomass, the Crystal Violet (CV) staining method was used, following the procedure established by Peeters et al., with some adaptations [25]. After incubation, the medium was carefully removed from each well, and the plates were washed twice with distilled water to remove non-attached bacterial cells. The plates were allowed to dry at room temperature. To fix the biofilms, 100 µL of methanol (VWR International) was added to each well and incubated for 10 min. Methanol was then removed, the plates were air-dried at room temperature for 15 min, and 100 µL of CV at 1% (*v*/*v*) was added to each well for 15 min. Then, the CV was removed, and the plates were washed twice with

distilled water to remove the excess dye. Next, 150 µL of acetic acid 33% (*v*/*v*) was added to solubilize the CV, and the absorbance was measured at 570 nm using a microplate reader BioTek ELx808U (BioTek, Winooski, VT, USA).

2.6. Statistical Analysis

Statistical analyses were performed using GraphPad Prism Version 8.0.2. (GraphPAD Software Inc., San Diego, CA, USA) to compare the biofilm formation capacity of surface waters and MDR isolates. Results were expressed as mean values and standard deviation. The level of significance was determined using the Student *t*-test. Moreover, a principal components analysis (PCA) was carried out using the JMP®, Version 17 (SAS Institute Inc., Cary, NC, USA, 1989–2023) between source and genotype and between source and virulence genes.

3. Results

3.1. Distribution of Klebsiella in Surface Waters

Bacterial growth was observed in nearly all analyzed water samples, although only 33 (70.2%) out of the 77 samples were positive for *Klebsiella*. The distribution of *Klebsiella* spp. and *K. pneumoniae* among the different sources is shown in Table 1. *Klebsiella* was isolated from 54 lotic (streams, rivers, fountains, irrigation ditches, and springs) and 23 lentic (dams, water wells, water tanks, and water mines) water samples. *Klebsiella* spp. was detected in 14 (18.1%) of the 77 water samples, whereas *K. pneumoniae* was found in 19 (24.7%) samples.

Table 1. Prevalence of *Klebsiella* spp. and *K. pneumoniae* water samples collected from different sources.

Source	Number of Samples	*Klebsiella* spp.	*K. pneumoniae*
Rivers	18	7	8
Streams	33	3	6
Irrigation ditches	1	-	-
Dams	1	-	1
Fountains	11	2	3
Water wells	7	1	1
Water tanks	2	-	-
Water mines	1	-	-
Springs	2	1	-
Total:	77	14	19

3.2. Antimicrobial Resistance Phenotype in Klebsiella spp. and K. pneumoniae Strains

Seven (36.8%) *K. pneumoniae* isolates were ESBL producers. Most *K. pneumoniae* isolates were resistant to amoxicillin-clavulanic acid (n = 11; 57.9%), trimethoprim–sulfamethoxazole (n = 10; 52.6%), cefoxitin (n = 7; 36.8%), cefotaxime (n = 8; 42.1%), and streptomycin (n = 8; 42.1%). Moreover, the MDR phenotype was observed in seven (36.8%) of the *K. pneumoniae* isolates. However, resistance to meropenem and tetracycline was detected in only one isolate. Regarding the 14 *Klebsiella* spp. isolates, 28.6% (n = 4) were ESBL producers. A high rate of antibiotic resistance was found in these isolates for amoxicillin + clavulanic acid (n = 4; 28.6%), trimethoprim/sulfamethoxazole (n = 4; 28.6%), and cefoxitin (n = 3; 21.4%). Nevertheless, only one (7.1%) *Klebsiella* spp. isolate was categorized as MDR. Table 2 details the antimicrobial resistance profiles of the isolates under study.

Table 2. Characteristics of the isolates recovered from surface waters from Northern Portugal.

Isolate	Species	ESBL Production	Antimicrobial Resistance		Virulence Genes
			Phenotype	Genotype	
VS3296	*K. pneumoniae*	N	CN, S, TOB, CIP, SXT	*aac*(3)-IV, *aac*(3)-II, *sul*2, *str*A, *aad*A1, *aad*A5	-
VS3297	*K. pneumoniae*	N	AUG, FOX, CAZ, CTX, MRP, CN, S, SXT	*aac*(3)-IV, *aac*(3)-II, $bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, bla_{SHV}, *sul*2, *str*A, *amp*C, *aad*A1, *par*C, bla_{TEM}	-
VS3298	*K. pneumoniae*	P	AUG, CAZ, CTX, CN, S, TOB, CIP, SXT	*aac*(3)-IV, *aac*(3)-II, $bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, bla_{SHV}, *sul*2, *str*A, *amp*C, bla_{TEM}	-
VS3299	*K. pneumoniae*	N	AUG, CAZ, CTX, TE, SXT	*tet*A, bla_{SHV}, *sul*2, *amp*C, bla_{TEM}	*pap*C
VS3300	*K. pneumoniae*	N	SXT	*sul*2	*pap*G-III, *pap*C
VS3301	*K. pneumoniae*	P	AUG, CTX, S, SXT	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, bla_{SHV}, *sul*2, *strA*, *ampC*, *aadA1*	-
VS3302	*K. pneumoniae*	N	SXT	*sul*2	-
VS3303	*K. pneumoniae*	N	AUG, FOX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$	*papC*
VS3304	*K. pneumoniae*	N	AUG	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$	-
VS3305	*K. pneumoniae*	N	CIP	-	*pap*G-III
VS3306	*K. pneumoniae*	P	CAZ, CTX, S, SXT	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, bla_{SHV}, *sul*2, *str*A, *aad*A1, *aad*A5, bla_{TEM}	-
VS3307	*K. pneumoniae*	P	AUG, FOX, CAZ, CTX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, *amp*C	-
VS3308	*K. pneumoniae*	P	FOX, CAZ, CTX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, *amp*C	-
VS3309	*K. pneumoniae*	N	AUG, FOX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, *amp*C	-
VS3310	*K. pneumoniae*	N	-	-	*pap*C
VS3311	*K. pneumoniae*	N	-	-	-
VS3312	*K. pneumoniae*	P	CTX, S, SXT	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, bla_{SHV}, *sul*2, *str*A, *aad*A1, bla_{TEM}	-
VS3313	*K. pneumoniae*	P	CAZ, CTX, S, SXT	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, bla_{SHV}, *sul*2, *str*A, *aad*A1, bla_{TEM}	*pap*C, *bfp*
VS3314	*K. pneumoniae*	N	-	-	*bfp*
VS3315	*Klebsiella* spp.	N	TE, S, SXT	*sul*2, *str*A, *aad*A1	*pap*C
VS3316	*Klebsiella* spp.	P	CTX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$	-
VS3317	*Klebsiella* spp.	N	CAZ	-	-
VS3318	*Klebsiella* spp.	N	CTX, SXT	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$, *sul*2	*aer*
VS3319	*Klebsiella* spp.	N	AUG, FOX, CAZ, CTX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$	-
VS3320	*Klebsiella* spp.	N	-	-	*pap*C
VS3321	*Klebsiella* spp.	N	-	-	-
VS3322	*Klebsiella* spp.	N	AUG, FOX, CTX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$	*pap*C, *bfp*
VS3323	*Klebsiella* spp.	N	SXT	*sul*2	-
VS3324	*Klebsiella* spp.	N	AUG	-	-
VS3325	*Klebsiella* spp.	P	-	-	-
VS3326	*Klebsiella* spp.	N	S, CIP, SXT	*sul*2	*aer*
VS3327	*Klebsiella* spp.	P	AUG, CTX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$	-
VS3328	*Klebsiella* spp.	P	CTX	$bla_{\text{CTX-U}}$, $bla_{\text{CTX-M9}}$	*aer*

Notes: Trimethoprim–sulfamethoxazole (SXT), Ciprofloxacin (CIP), Tobramycin (TOB), Streptomycin (S), Gentamicin (CN), Tetracycline (TE), Meropenem (MRP), Cefotaxime (CTX), Ceftazidime (CAZ), Cefoxitin (FOX), and Amoxicillin-clavulanic acid (AUG).

3.3. Characterization of Resistance Genes and Virulence Factors

Data on the resistance and virulence phenotypes and genetic determinants are given in Table 2. A high diversity of resistance genes was detected among the 19 *K. pneumoniae* isolates, namely, bla_{CTX-U} (n = 11), bla_{CTX-M9} (n = 12), bla_{TEM} (n = 7), *aac*(3)-II (n = 3), *aac*(3)-IV (n = 3), *str*A (n = 7), *aad*A1 (n = 5), *aad*A5 (n = 2), *sul*2 (n = 10), and *par*C (n = 1). The majority of *Klebsiella* spp. isolates with phenotypic resistance to β-lactams and cefotaxime (n = 5; 100%) harbored the bla_{CTX-M9} gene. Furthermore, the diversity of other resistance genes was detected among these isolates, namely, bla_{CTX-U} (n = 6) and *str*A (n = 1). The most frequently found virulence gene in *K. pneumoniae* isolates was *pap*C (n = 6; 30%), while in *Klebsiella* spp. Isolates, a high prevalence of *pap*C (n = 3; 21.4%) and *aer* (n = 3; 21.4%) was observed. A low prevalence of *pap*G-III and *bfp* virulence genes was detected in both *K. pneumoniae* and *Klebsiella* spp.

3.4. Biofilm Production

Biofilm production was measured by a microtiter plate assay for the *Klebsiella* spp. and *K. pneumoniae* isolates from surface waters. The results were standardized using *Klebsiella* spp. ATCC® 13883 (biofilm producer) to enhance the consistency of result comparisons. Figure 2 shows the percentage of biofilm production by each of the isolates in this study.

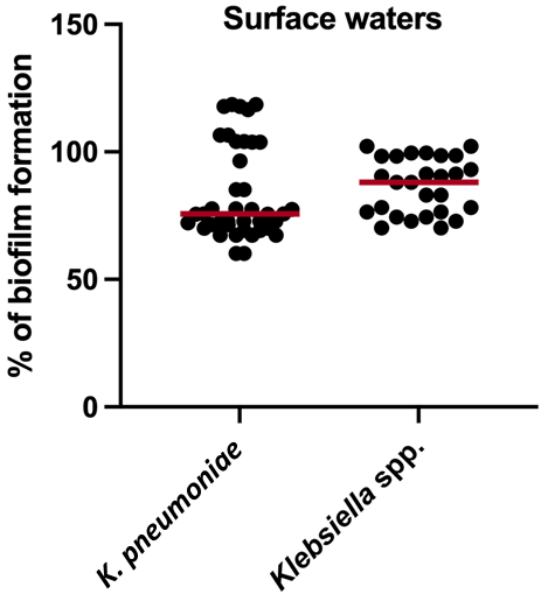

Figure 2. Ability of *K. pneumoniae* and *Klebsiella* spp. strains from surface waters to form biofilm, expressed as percentage of the positive control strain (*Klebsiella* spp. ATCC® 13883). The symbols (•) represent the average biomass of the biofilm formed in independent tests of each individual isolate tested for each surface water sample. The red lines represent the average of biofilm biomass formed by all isolates of each surface water sample. Statistical significance was determined using the Student *t*-test.

Isolates of *Klebsiella* spp. and *K. pneumoniae* produced a moderate amount of biofilm biomass, with very similar averages of biofilm formation percentages (83.1% and 86.9%, respectively). Among *K. pneumoniae* strains, 13 (65%) were confirmed as weak producers, 2 (10%) were moderate, and 5 (25%) were strong biofilm producers. Similarly, regarding *Klebsiella* spp., five isolates (35.7%) were weak producers, eight (57.1%) were moderate, and the remaining isolate (7.1%) was a strong biofilm producer. The weakest biofilm producer (60.2%) was one *K. pneumoniae* isolate from the river Rio Corgo (Vila Real), while the strongest biofilm producer (118.6%) was isolated from a stream located in Valpaços. For a more complete analysis of the prevalence of biofilm-producing species, we compared the formation of biofilms in multi-resistant and non-multi-resistant species. Among MDR

isolates, one (14.3%) was a strong biofilm producer and six (85.7%) were weak biofilm producers. Additionally, of the 26 non-MDR *Klebsiella* isolates, 5 (19.2%) had a high capacity to form biofilms, 9 (34.6%) were moderate biofilm producers, and 12 (46.1%) were weak biofilm producers. Comparing the results obtained in the MDR and non-MDR isolates under study, the difference between the means was not statistically significant, since the isolates produced approximately the same amount of biofilm (Figure 3). In addition, among 11 ESBL-producing *Klebsiella* strains, all were confirmed as biofilm producers; 8 isolates (72.7%) were weak producers, 2 (18.2%) were moderate, and 1 (9.1%) was a high biofilm producer. On the other hand, of the 21 non-ESBL-producing *Klebsiella* strains, 9 isolates (42.9%) were weak producers, 7 (33.3%) were moderate biofilm producers, and the remaining 5 (23.8%) were high biofilm producers.

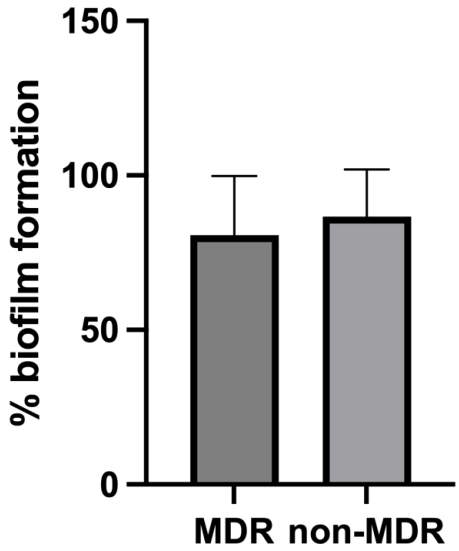

Figure 3. Ability of MDR and non-MDR *K. pneumoniae* and *Klebsiella* spp. strains from surface waters to form biofilm. Statistical significance was determined using Student *t*-test (MDR (multidrug resistance); non-MDR (non-multidrug resistance)).

3.5. Principal Component Analysis (PCA)

Regarding the relationship between source and genotypes, the first two main components do not explain most of the variability in the data (component 1—9.34%; component 2—10.2%). On the other hand, regarding the relationship between source and virulence, the first two main components explain around 37% of the total variability (component 1—19%; component 2—18%) (Figure 4).

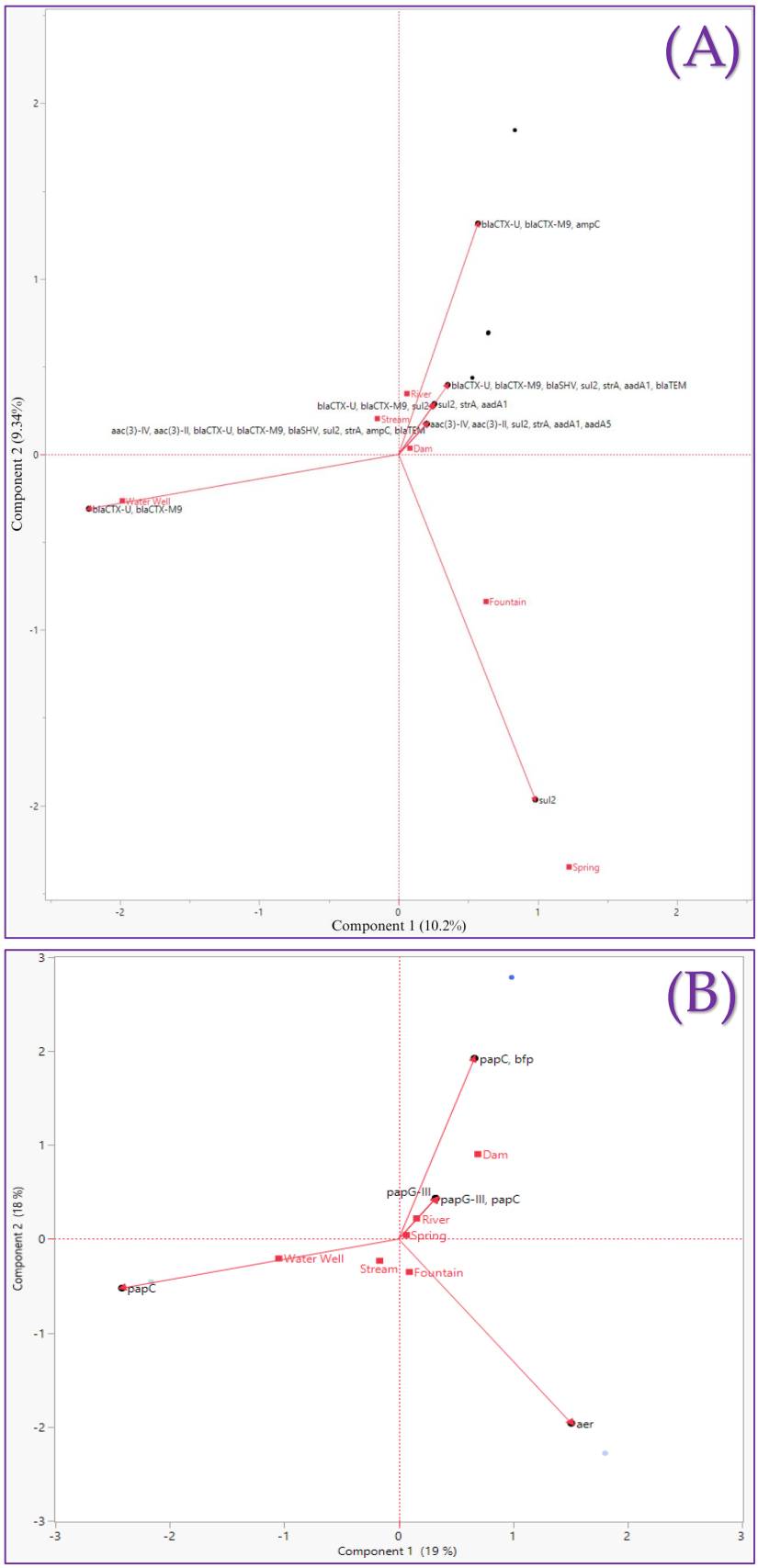

Figure 4. Principal component analysis. (**A**) Source versus genotype, biplot for surface waters PCA; (**B**) source versus virulence, biplot for surface waters PCA.

4. Discussion

The environment, particularly the aquatic environment, is recognized as a reservoir for antimicrobial resistance (AMR) and antimicrobial resistance genes (ARGs), even within highly confined habitats, like drinking water sources. Surface waters constitute one of the main sources of potable water for human and animal consumption. Therefore, when contaminated, they can become an important contributor to the dissemination of antimicrobial-resistant *Klebsiella* and its resistance and virulence factors [7]. However, data on the prevalence of antimicrobial resistance in *Klebsiella* from surface waters are scant. Therefore, studies on *Klebsiella*'s prevalence, antibiotic resistance, and virulence are of the utmost importance for evaluating the potential roles of different aquatic environments in the spread of these pathogens, as well as their impact on human and animal exposure to these pathogens.

Klebsiella spp. Is a frequent member of the environmental microbiota, including that of surface water bodies. The presence of *Klebsiella* spp. in freshwater systems, such as drinking water, rivers, lakes, and streams, as well as in seawater, has been demonstrated in several studies [26–30]. In this study, a total of 77 surface water samples were collected, and 33 *Klebsiella* strains were isolated (42.9%). When comparing different surface water sources, it became evident that rivers exhibited a higher prevalence of both *Klebsiella* spp. and *K. pneumoniae*. Specifically, among the 18 isolates from rivers, 33.3% were identified as *Klebsiella* spp., while 50% were characterized as *K. pneumoniae*. These findings are in line with several other studies, in which a higher prevalence of both *Klebsiella* spp. and *K. pneumoniae* in rivers was noted, with *K. pneumoniae* being the most prevalent [29,31].

K. pneumoniae shows resistance to a wide array of antibiotics as well as the production of β-Lactamase enzymes and the capacity to form biofilms [32]. Given that many β-Lactamase genes are plasmid-borne, or located in other mobile genetic elements, resistant strains can spread fast, leading to increased illness, death rates, and healthcare expenses. In this study, most isolates were resistant to the tested antibiotics, including amoxicillin-clavulanic acid, cefoxitin, cefotaxime, ceftazidime, streptomycin, and trimethoprim–sulfamethoxazole, belonging, respectively, to the penicillin, cephalosporin, aminoglycoside, and sulfonamide antibiotic classes. However, among these isolates, meropenem (carbapenems) was the antibiotic with the lowest resistance rate, followed by tetracycline (tetracyclines) and gentamicin (aminoglycosides). The penicillin antibiotic class exhibited the highest prevalence of resistance (44.1%) among the studied surface water isolates. Notably, isolates of *Klebsiella* spp. displayed lower resistance rates to ciprofloxacin (fluoroquinolones) and meropenem (carbapenems), with no instances of resistance observed. However, antibiotics with higher resistance rates were consistent across both taxonomic groups. Numerous reports on penicillin resistance in *Klebsiella* species from surface waters in Portugal [33] and elsewhere [30,34] align with the elevated resistance rates found in this study. Of particular concern is the alignment of these results regarding ceftazidime resistance and those of Teixeira et al. (2020), who also reported a high number of ceftazidime-resistant *Klebsiella* spp. isolates from a Portuguese river [33]. These results raise concerns, both from the environmental and public health perspectives. Ceftazidime is a third-generation cephalosporin that should be more effective against Gram-negative bacteria than both the first and second generations of antibiotics in this family. Third-generation cephalosporins are also more active against bacteria that may be resistant to previous generations of cephalosporins. These are important antibiotics, mostly used in hospital settings to treat severe infections involving multidrug-resistant Gram-negative pathogens, such as *Klebsiella* spp. and *K. pneumoniae* [35]. Moreover, third-generation oral cephalosporins, such as ceftazidime, can be combined with amoxicillin × clavulanic acid to tackle urinary tract infections involving ESBL-producing *Klebsiella* spp. [36], further enhancing the concerns these findings raise. Regarding the prevalence of *Klebsiella* spp. with the MDR phenotype, these findings differ from the results of other studies carried out in surface waters, in which high prevalences of *Klebsiella* spp. with the MDR phenotype were reported [37,38]. On the basis of antibiotic susceptibility results, it is evident that

Klebsiella spp. and *K. pneumoniae* present a significant therapeutic challenge. The slightly higher resistance rates observed in the environment raise concerns, especially regarding the potential spread of multidrug-resistant bacteria responsible for infections like pneumonia and meningitis to both humans and animals. Notably, the β-Lactam and carbapenem classes of antibiotics have garnered increased interest and concern due to the observed high rates of resistance in isolated *Klebsiella* spp. [31], which this study also shows.

Less than 50% of the isolates were ESBL producers. These results agree with the findings of Caltagirone et al., in which only 7/33 (21.2%) *K. pneumoniae* isolated from surface waters were ESBL producers [30]. However, Falgenhaeur et al. reported a higher prevalence (83.3%) of ESBL-producing *Klebsiella* spp. in surface waters, contrary to what was found in this study [39]. Several reports on the prevalence of ESBLs in surface waters report that they are frequently associated with MDR [40]; however, in this study, there was a higher prevalence of ESBL-producing *Klebsiella* in non-MDR isolates (n = 8; 24.2%) than in MDR isolates (n = 3; 9.1%). Furthermore, the most common resistance gene, both in *K. pneumoniae* and *Klebsiella* spp., was $bla_{\text{CTX-M9}}$, followed by *sul*2. However, in *K. pneumoniae*, a high prevalence of bla_{TEM}, bla_{SHV}, *amp*C, and *str*A was also observed. These findings diverge from those of several studies, where a high prevalence of $bla_{\text{CTX-U}}$, bla_{KPC}, *tet*(A), and *sul*2 genes was reported. In this study, the most prevalent gene was $bla_{\text{CTX-M9}}$, which was not reported in other studies from surface waters. However, the prevalence of *sul*2 in those studies aligns with the prevalences reported in this study [33,41,42]. In studies carried out by Caltagirone et al., Muller et al., Teixeira et al., and Hoffman et al., in Italy, Germany, and Portugal, respectively, a high prevalence of bla_{KPC} and $bla_{\text{OXA-48}}$ genes was detected [30,33,43,44]. However, in this study, none of the isolates from surface waters possessed these genes.

As previously mentioned, *pap*C—an operon that is an outer membrane protein, essential for the regulation of P fimbriae biogenesis—was the most prevalent virulence gene in both *Klebsiella* spp. and *K. pneumoniae*. This result may be related to the virulence of the isolated strains, since adhesion is the most important determinant of pathogenicity in the *Klebsiella* genus [45]. These results are quite concerning, since this genetic virulence determinant may increase the pathogenicity in *Klebsiella* strains, and we demonstrated its high prevalence in the environment. To our knowledge, the presence of these virulence genes has not been previously reported in environmental samples, including surface waters. This study represents a pioneering effort in this regard, and it highlights the importance of water as a potential reservoir of virulence genes.

The high environmental prevalence of *pap*C we found makes it important to check whether a high prevalence of this gene has also been reported in clinical samples. The results obtained in studies on hospital patients, in which the presence of these genes was investigated in isolates of *Klebsiella* spp. of diverse clinical samples, were divergent from those in the present study. In the reports of Liu et al. and Düzgün et al., *pap*C was the gene with the lowest prevalence in *Klebsiella* spp. isolates [46,47]. Nevertheless, they showed a high prevalence of *aer*, which aligns with what was observed in the *Klebsiella* spp. isolates from surface waters in this study. Moreover, in the aforementioned studies, the *bfp* and *pap*G-III genes demonstrated the lowest prevalence among hospital patients, mirroring these results. Contrastingly, Hassan et al. described a notable prevalence of the *pap*C gene in *K. pneumoniae* isolates obtained from clinical infection specimens [48], a finding that concurs with the outcomes observed in this study. The alignment of these results on the prevalence of *aer* and, in some cases, *papC* may suggest a plausible transfer of these genes between environmental sources, such as water bodies and human hosts.

Biofilm formation represents a crucial virulence trait in *Klebsiella* and serves as an adaptative response to diverse stressors, such as alterations in the physical environment and exposure to drugs (particularly antibiotics) [49]. In addition, bacteria present in surface waters can also produce biofilms, promoting an ideal environment for horizontal gene transfer that can lead to the accumulation of genetic mobile elements. The accumulation of biofilm-producing bacteria in ecosystems may pose environmental and public health

concerns [50]. Changes in water levels have been demonstrated as among the most relevant stressors affecting the structure and function of biofilms [51]. As biofilms play a crucial role in aquatic environments, studying the prevalence of biofilm production isolates is essential for preventing the spread of pathogens in the environment and ensuring the biosafety of drinking water. To the best of our knowledge, no studies have yet been conducted on environmental samples (including surface waters) on the biofilm production ability of *Klebsiella* strains. In this study, all *Klebsiella* isolates were confirmed as biofilm producers, similar to the very high (99%) rates of biofilm production reported by Türkel et al. in clinical samples. In addition, in the same study, a higher prevalence of strong biofilm producers was found in ciprofloxacin-susceptible isolates [52], similar to these results, in which 6/30 (20%) of the ciprofloxacin-susceptible *Klebsiella* had a high capacity to produce biofilms. Nevertheless, in contrast with these results, other studies have reported a correlation between multidrug resistance (MDR) and biofilm production, indicating a higher prevalence of high biofilm producers among MDR isolates [53], a concerning trend when the results on water bodies are considered.

In this study, considerable rates of weak biofilm-producing isolates were found, both among ESBL-producing (72.7%) and non-ESBL-producing *Klebsiella* isolates (43.5%). While the majority of studies suggest that β-Lactamase-producing strains are usually high biofilm producers [54], others have observed no correlation between the capacity to form biofilms and ESBL production [55].

PCA showed that the environmental variables (type of water body) under study only account for a low extent of the observed gene distribution patterns. However, in spite of the low eigenvalues observed (Table S3), the presence of *sul*2 correlated positively with "fountain" and "spring" and negatively with "water well", while $bla_{\text{CTX-U}}$ and $bla_{\text{CTX-M9}}$ correlated positively with "water well" but negatively with "fountain" and "spring". Regarding virulence determinants, *pap*C correlated positively with "water well" and "stream" but negatively with "dam". On the other hand, *pap*C and *bfp* correlated positively with "dam" but negatively with "water well" and "stream". Furthermore, *aer* correlated positively with "fountain" but negatively with "water well" and "stream". Moreover, it was observed that some virulence genes exhibited positive and negative associations with lotic and lentic waters. This was the case with *pap*C, which exhibited positive associations with "streams" and negative associations with "fountains", both lotic waters. Additionally, *aer* presented positive associations with "fountains" and negative associations with "streams", both lotic waters. Furthermore, the *pap*C and *bfp* genes also demonstrated positive associations with "dam" and negative associations with "water well", both lentic waters.

The distinctive patterns of antibiotic resistance, coupled with the incidence of virulence factors and biofilm-forming capacity found in this study, may raise concerns about the dissemination potential of pathogenic *Klebsiella* strains through surface waters. A further cause for concern is that individuals, both animal and human, engaging in recreational activities or using these waters for potable purposes, may belong to risk groups with increased susceptibility to *Klebsiella* infections or colonization. However, it must be taken into account that the available information on the connection between *Klebsiella*-contaminated waters and the onset of infections in humans is still scarce.

In the present context of the critical importance of surface freshwater, these findings underscore the urgent need for integrated research on freshwater quality. The unexpectedly high prevalence of *Klebsiella* spp. and *K. pneumoniae* in surface waters, coupled with the concerning presence of resistance and virulence genes, highlights the complex interplay between microbial dynamics and environmental factors. To address such challenges, the One Health perspective is paramount, emphasizing the interconnectedness of human, animal, and environmental health. This study supports the urgency for collaborative, multidisciplinary solutions to tackle freshwater monitoring, especially in Southern Europe, where severe water quality issues are expected to increase under extreme precipitation and droughts derived mainly from climate change. Comprehensive research on freshwater ecology, toxicity, hydrochemistry, and monitoring approaches is essential.

5. Conclusions

This work demonstrates that surface waters may act as reservoirs of *Klebsiella*, with higher prevalences of *K. pneumoniae* in river samples than in the other types of surface waters tested. In terms of phenotypic resistance to a wide range of antibiotic classes, ceftazidime (a third-generation cephalosporin), the high prevalence of $bla_{\text{CTX-M9}}$, and biofilm production are causes for concern. This study constitutes, to our knowledge, the first report on the presence of the *pap*C virulence determinant in *Klebsiella* isolates from surface waters. When compared to the findings of studies on hospital isolates, these results may suggest a plausible transfer of genetic determinants of antibiotic resistance (particularly *aer* but, also, in some cases, *pap*C) between surface waters and the human host. Thus, they highlight both the need for including these sources of pathogens under the One Health effort and the need for vigilance of *Klebsiella* in lentic and lotic water bodies to assess their distribution and dissemination in these habitats.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16091297/s1, Table S1: Location, source, and coordinates of the surface water samples in this study, and the identity of the respective isolates; Table S2: Primer pairs used for molecular typing, detection of antimicrobial resistance and virulence genes in *Klebsiella* strains; Table S3: Principal component analysis. (I) Source versus genotype, Eigenvalues for surface water PCA; (II) source versus virulence, Eigenvalues for surface water PCA. References [56–69] are cited in the Supplementary Materials.

Author Contributions: Conceptualization, S.A.; methodology, S.A., V.S., G.I. and P.P.; software, V.S. and Â.M.; validation, V.S., M.d.L.E.D., J.E.P. and P.P.; formal analysis, S.A., V.S. and Â.M.; investigation, S.A.; resources, S.A., V.S. and J.E.P.; data curation, V.S.; writing—original draft preparation, S.A.; writing—review and editing, S.A., V.S. and M.d.L.E.D.; visualization, S.A., V.S., M.d.L.E.D. and P.P; supervision, V.S., G.I. and P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the project UIDB/00772/2020 (Doi:10.54499/UIDB/00772/2020) funded by the Portuguese Foundation for Science and Technology (FCT). This work was supported by the Associate Laboratory for Green Chemistry-LAQV, which is financed by national funds from FCT/MCTES (UIDB/50006/2020, UIDP/50006/2020). MD acknowledges the funding from the Regional Government of the Azores (Portugal), reference M1.1.A/FUNC.UI&D/001/2021-2024, measure 01.1.a.Apoio UI&D.2022 (SRCCTD/DRCTD).

Data Availability Statement: All the data supporting our findings are contained within the article and Supplementary Materials.

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

References

1. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. Available online: https://iiif.wellcomecollection.org/file/b28552179_AMR%20Tackling%20a%20crisis%20for%20the%20health%20and%20wealth%20of%20nations.pdf (accessed on 19 February 2024).
2. Varaldo, P.E.; Facinelli, B.; Bagnarelli, P.; Menzo, S.; Mingoia, M.; Brenciani, A.; Giacometti, A.; Barchiesi, F.; Brescini, L.; Cirioni, O.; et al. Antimicrobial Resistance: A Challenge for the Future. In *The First Outstanding 50 Years of "Universita Politecnica delle Marche": Research Achievements in Life Sciences*; Springer: Cham, Switzerland, 2020; pp. 13–29. [CrossRef]
3. Collignon, P.J.; McEwen, S.A. One Health—Its Importance in Helping to Better Control Antimicrobial Resistance. *Trop. Med. Infect. Dis.* **2019**, *4*, 22. [CrossRef] [PubMed]
4. Haenni, M.; Dagot, C.; Chesneau, O.; Bibbal, D.; Labanowski, J.; Vialette, M.; Bouchard, D.; Martin-Laurent, F.; Calsat, L.; Nazaret, S.; et al. Environmental Contamination in a High-Income Country (France) by Antibiotics, Antibiotic-Resistant Bacteria, and Antibiotic Resistance Genes: Status and Possible Causes. *Environ. Int.* **2022**, *159*, 107047. [CrossRef] [PubMed]
5. Berendonk, T.U.; Manaia, C.M.; Merlin, C.; Fatta-Kassinos, D.; Cytryn, E.; Walsh, F.; Bürgmann, H.; Sørum, H.; Norström, M.; Pons, M.N.; et al. Tackling Antibiotic Resistance: The Environmental Framework. *Nat. Rev. Microbiol.* **2015**, *13*, 310–317. [CrossRef] [PubMed]
6. Kumar, A.; Pal, D. Antibiotic Resistance and Wastewater: Correlation, Impact and Critical Human Health Challenges. *J. Environ. Chem. Eng.* **2018**, *6*, 52–58. [CrossRef]

7. Silva, V.; Caniça, M.; Capelo, J.L.; Igrejas, G.; Poeta, P. Diversity and Genetic Lineages of Environmental Staphylococci: A Surface Water Overview. *FEMS Microbiol. Ecol.* **2020**, *96*, fiaa191. [CrossRef] [PubMed]
8. Zeng, Y.; Chang, F.; Liu, Q.; Duan, L.; Li, D.; Zhang, H. Review Article Recent Advances and Perspectives on the Sources and Detection of Antibiotics in Aquatic Environments. *J. Anal. Methods Chem.* **2022**, *2022*, 5091181. [CrossRef] [PubMed]
9. Barati, A.; Ghaderpour, A.; Chew, L.L.; Bong, C.W.; Thong, K.L.; Chong, V.C.; Chai, L.C. Isolation and Characterization of Aquatic-Borne *Klebsiella pneumoniae* from Tropical Estuaries in Malaysia. *Int. J. Environ. Res. Public Health* **2016**, *13*, 426. [CrossRef] [PubMed]
10. Hu, Y.; Anes, J.; Devineau, S.; Fanning, S. *Klebsiella pneumoniae*: Prevalence, Reservoirs, Antimicrobial Resistance, Pathogenicity, and Infection: A Hitherto Unrecognized Zoonotic Bacterium. *Foodborne Pathog. Dis.* **2021**, *18*, 63–84. [CrossRef] [PubMed]
11. Kowalczyk, J.; Czokajło, I.; Gańko, M.; Śmiałek, M.; Koncicki, A. Identification and Antimicrobial Resistance in *Klebsiella* spp. Isolates from Turkeys in Poland between 2019 and 2022. *Animals* **2022**, *12*, 3157. [CrossRef]
12. Gómez, M.; Valverde, A.; Del Campo, R.; Rodríguez, J.M.; Maldonado-Barragán, A. Phenotypic and Molecular Characterization of Commensal, Community-Acquired and Nosocomial *Klebsiella* spp. *Microorganisms* **2021**, *9*, 2344. [CrossRef] [PubMed]
13. Rajkumari, J.; Paikhomba Singha, L.; Pandey, P. Genomic Insights of Aromatic Hydrocarbon Degrading *Klebsiella pneumoniae* AWD5 with Plant Growth Promoting Attributes: A Paradigm of Soil Isolate with Elements of Biodegradation. *3 Biotech* **2018**, *8*, 118. [CrossRef] [PubMed]
14. Kimera, Z.I.; Mgaya, F.X.; Mshana, S.E.; Karimuribo, E.D.; Matee, M.I.N. Occurrence of Extended Spectrum Beta Lactamase (ESBL) Producers, Quinolone and Carbapenem Resistant Enterobacteriaceae Isolated from Environmental Samples along Msimbazi River Basin Ecosystem in Tanzania. *Int. J. Environ. Res. Public Health* **2021**, *18*, 8264. [CrossRef] [PubMed]
15. Guan, X.; He, R.; Zhang, B.; Gao, C.; Liu, F. Seasonal Variations of Microbial Community Structure, Assembly Processes, and Influencing Factors in Karst River. *Front. Microbiol.* **2023**, *14*, 1133938. [CrossRef]
16. Beal, C.; Matsubae, K.; Ylä-Mella, J.; Elagroudy, S.; Campanale, C.; Losacco, D.; Triozzi, M.; Massarelli, C.; Uricchio, V.F. An Overall Perspective for the Study of Emerging Contaminants in Karst Aquifers. *Resources* **2022**, *11*, 105. [CrossRef]
17. Cuadrado-Quesada, G.; Holley, C.; Gupta, J. Groundwater Governance in the Anthropocene: A Close Look at Costa Rica. *Water Policy* **2018**, *20*, 475–489. [CrossRef]
18. Arroita, M.; Flores, L.; Larrañaga, A.; Chauvet, E.; Elosegi, A. Hydrological Contingency: Drying History Affects Aquatic Microbial Decomposition. *Aquat. Sci.* **2018**, *80*, 31. [CrossRef]
19. Giri, S.; Mishra, A.; Zhang, Z.; Lathrop, R.G.; Alnahit, A.O. Meteorological and Hydrological Drought Analysis and Its Impact on Water Quality and Stream Integrity. *Sustainability* **2021**, *13*, 8175. [CrossRef]
20. He, Y.; Guo, X.; Xiang, S.; Li, J.; Li, X.; Xiang, H.; He, J.; Chen, D.; Chen, J. Comparative Analyses of Phenotypic Methods and 16S RRNA, Khe, RpoB Genes Sequencing for Identification of Clinical Isolates of *Klebsiella pneumoniae*. *Antonie Van Leeuwenhoek Int. J. Gen. Mol. Microbiol.* **2016**, *109*, 1029–1040. [CrossRef] [PubMed]
21. Solberg, O.D.; Ajiboye, R.M.; Riley, L.W. Origin of Class 1 and 2 Integrons and Gene Cassettes in a Population-Based Sample of Uropathogenic *Escherichia coli*. *J. Clin. Microbiol.* **2006**, *44*, 1347–1351. [CrossRef] [PubMed]
22. Silva, A.; Silva, V.; De Lurdes, M.; Dapkevicius, E.; Azevedo, M.; Cordeiro, R.; Pereira, J.E.; Valentão, P.; Falco, V.; Igrejas, G.; et al. Unveiling Antibiotic Resistance, Clonal Diversity, and Biofilm Formation in *E. Coli* Isolated from Healthy Swine in Portugal. *Pathogens* **2024**, *13*, 305. [CrossRef] [PubMed]
23. Carvalho, I.; Cunha, R.; Martins, C.; Martínez-Álvarez, S.; Chenouf, N.S.; Pimenta, P.; Pereira, A.R.; Ramos, S.; Sadi, M.; Martins, Â.; et al. Antimicrobial Resistance Genes and Diversity of Clones among Faecal ESBL-Producing *Escherichia coli* Isolated from Healthy and Sick Dogs Living in Portugal. *Antibiotics* **2021**, *10*, 1013. [CrossRef]
24. Oniciuc, E.A.; Cerca, N.; Nicolau, A.I. Compositional Analysis of Biofilms Formed by Staphylococcus Aureus Isolated from Food Sources. *Front. Microbiol.* **2016**, *7*, 184310. [CrossRef]
25. Peeters, E.; Nelis, H.J.; Coenye, T. Comparison of Multiple Methods for Quantification of Microbial Biofilms Grown in Microtiter Plates. *J. Microbiol. Methods* **2008**, *72*, 157–165. [CrossRef] [PubMed]
26. Hartinger, S.M.; Medina-Pizzali, M.L.; Salmon-Mulanovich, G.; Larson, A.J.; Pinedo-Bardales, M.; Verastegui, H.; Riberos, M.; Mäusezahl, D. Antimicrobial Resistance in Humans, Animals, Water and Household Environs in Rural Andean Peru: Exploring Dissemination Pathways through the One Health Lens. *Int. J. Environ. Res. Public Health* **2021**, *18*, 4604. [CrossRef] [PubMed]
27. Mondal, A.H.; Siddiqui, M.T.; Sultan, I.; Haq, Q.M.R. Prevalence and Diversity of BlaTEM, BlaSHV and BlaCTX-M Variants among Multidrug Resistant *Klebsiella* spp. from an Urban Riverine Environment in India. *Int. J. Environ. Health Res.* **2018**, *29*, 117–129. [CrossRef] [PubMed]
28. Altayb, H.N.; Elbadawi, H.S.; Alzahrani, F.A.; Baothman, O.; Kazmi, I.; Nadeem, M.S.; Hosawi, S.; Chaieb, K. Co-Occurrence of β-Lactam and Aminoglycoside Resistance Determinants among Clinical and Environmental Isolates of *Klebsiella pneumoniae* and *Escherichia coli*: A Genomic Approach. *Pharmaceuticals* **2022**, *15*, 1011. [CrossRef] [PubMed]
29. Hooban, B.; Fitzhenry, K.; Cahill, N.; Joyce, A.; O' Connor, L.; Bray, J.E.; Brisse, S.; Passet, V.; Abbas Syed, R.; Cormican, M.; et al. A Point Prevalence Survey of Antibiotic Resistance in the Irish Environment, 2018–2019. *Environ. Int.* **2021**, *152*, 106466. [CrossRef] [PubMed]
30. Caltagirone, M.; Nucleo, E.; Spalla, M.; Zara, F.; Novazzi, F.; Marchetti, V.M.; Piazza, A.; Bitar, I.; De Cicco, M.; Paolucci, S.; et al. Occurrence of Extended Spectrum β-Lactamases, KPC-Type, and MCR-1.2-Producing Enterobacteriaceae from Wells, River Water, and Wastewater Treatment Plants in Oltrepò Pavese Area, Northern Italy. *Front. Microbiol.* **2017**, *8*, 272364. [CrossRef]

31. Hassen, B.; Abbassi, M.S.; Benlabidi, S.; Ruiz-Ripa, L.; Mama, O.M.; Ibrahim, C.; Hassen, A.; Hammami, S.; Torres, C. Genetic Characterization of ESBL-Producing *Escherichia coli* and *Klebsiella pneumoniae* Isolated from Wastewater and River Water in Tunisia: Predominance of CTX-M-15 and High Genetic Diversity. *Environ. Sci. Pollut. Res.* **2020**, *27*, 44368–44377. [CrossRef]
32. Nirwati, H.; Sinanjung, K.; Fahrunissa, F.; Wijaya, F.; Napitupulu, S.; Hati, V.P.; Hakim, M.S.; Meliala, A.; Aman, A.T.; Nuryastuti, T. Biofilm Formation and Antibiotic Resistance of *Klebsiella pneumoniae* Isolated from Clinical Samples in a Tertiary Care Hospital, Klaten, Indonesia. *BMC Proc.* **2019**, *13*, 20. [CrossRef]
33. Teixeira, P.; Tacão, M.; Pureza, L.; Gonçalves, J.; Silva, A.; Cruz-Schneider, M.P.; Henriques, I. Occurrence of Carbapenemase-Producing Enterobacteriaceae in a Portuguese River: BlaNDM, BlaKPC and BlaGES among the Detected Genes. *Environ. Pollut.* **2020**, *260*, 113913. [CrossRef] [PubMed]
34. Mahato, S.; Mahato, A.; Pokharel, E.; Tamrakar, A. Detection of Extended-Spectrum Beta-Lactamase-Producing *E. coli* and *Klebsiella* spp. in Effluents of Different Hospitals Sewage in Biratnagar, Nepal. *BMC Res. Notes* **2019**, *12*, 641. [CrossRef] [PubMed]
35. van Duin, D.; Bonomo, R.A. Ceftazidime/Avibactam and Ceftolozane/Tazobactam: Second-Generation β-Lactam/β-Lactamase Inhibitor Combinations. *Clin. Infect. Dis.* **2016**, *63*, 234–241. [CrossRef]
36. Paterson, D.L. Recommendation for Treatment of Severe Infections Caused by Enterobacteriaceae Producing Extended-Spectrum β-Lactamases (ESBLs). *Clin. Microbiol. Infect.* **2000**, *6*, 460–463. [CrossRef] [PubMed]
37. Khan, F.A.; Hellmark, B.; Ehricht, R.; Söderquist, B.; Jass, J. Related Carbapenemase-Producing *Klebsiella* Isolates Detected in Both a Hospital and Associated Aquatic Environment in Sweden. *Eur. J. Clin. Microbiol. Infect. Dis.* **2018**, *37*, 2241–2251. [CrossRef] [PubMed]
38. Shrestha, P.; Prasai Joshi, T.; Nhemhaphuki, S.; Sitoula, K.; Maharjan, J.; Ranjit, R.; Shrestha, P.; Joshi, D.R. Occurrence of Antibiotic-Resistant Bacteria and Their Genes in Bagmati River, Nepal. *Water Air Soil Pollut.* **2023**, *234*, 475. [CrossRef]
39. Falgenhauer, L.; Schwengers, O.; Schmiedel, J.; Baars, C.; Lambrecht, O.; Heß, S.; Berendonk, T.U.; Falgenhauer, J.; Chakraborty, T.; Imirzalioglu, C. Multidrug-Resistant and Clinically Relevant Gram-Negative Bacteria Are Present in German Surface Waters. *Front. Microbiol.* **2019**, *10*, 482269. [CrossRef]
40. Aguilar-Salazar, A.; Martínez-Vázquez, A.V.; Aguilera-Arreola, G.; de Jesus de Luna-Santillana, E.; Cruz-Hernández, M.A.; Escobedo-Bonilla, C.M.; Lara-Ramírez, E.; Sánchez-Sánchez, M.; Guerrero, A.; Rivera, G.; et al. Prevalence of ESKAPE Bacteria in Surface Water and Wastewater Sources: Multidrug Resistance and Molecular Characterization, an Updated Review. *Water* **2023**, *15*, 3200. [CrossRef]
41. Fadare, F.T.; Okoh, A.I. Distribution and Molecular Characterization of ESBL, PAmpC β-Lactamases, and Non-β-Lactam Encoding Genes in Enterobacteriaceae Isolated from Hospital Wastewater in Eastern Cape Province, South Africa. *PLoS ONE* **2021**, *16*, e0254753. [CrossRef]
42. Nascimento, T.; Cantamessa, R.; Melo, L.; Lincopan, N.; Fernandes, M.R.; Cerdeira, L.; Fraga, E.; Dropa, M.; Sato, M.I.Z. International High-Risk Clones of *Klebsiella pneumoniae* KPC-2/CC258 and *Escherichia coli* CTX-M-15/CC10 in Urban Lake Waters. *Sci. Total Environ.* **2017**, *598*, 910–915. [CrossRef] [PubMed]
43. Hoffmann, M.; Fischer, M.A.; Neumann, B.; Kiesewetter, K.; Hoffmann, I.; Werner, G.; Pfeifer, Y.; Lübbert, C. Carbapenemase-Producing Gram-Negative Bacteria in Hospital Wastewater, Wastewater Treatment Plants and Surface Waters in a Metropolitan Area in Germany, 2020. *Sci. Total. Environ.* **2023**, *890*, 164179. [CrossRef] [PubMed]
44. Müller, H.; Sib, E.; Gajdiss, M.; Klanke, U.; Lenz-Plet, F.; Barabasch, V.; Albert, C.; Schallenberg, A.; Timm, C.; Zacharias, N.; et al. Dissemination of Multi-Resistant Gram-Negative Bacteria into German Wastewater and Surface Waters. *FEMS Microbiol. Ecol.* **2018**, *94*, 5. [CrossRef] [PubMed]
45. López-Banda, D.A.; Carrillo-Casas, E.M.; Leyva-Leyva, M.; Orozco-Hoyuela, G.; Manjarrez-Hernández, Á.H.; Arroyo-Escalante, S.; Moncada-Barrón, D.; Villanueva-Recillas, S.; Xicohtencatl-Cortes, J.; Hernández-Castro, R. Identification of Virulence Factors Genes in *Escherichia coli* Isolates from Women with Urinary Tract Infection in Mexico. *Biomed. Res. Int.* **2014**, *2014*, 959206. [CrossRef] [PubMed]
46. Liu, J.; Xu, Z.; Li, H.; Chen, F.; Han, K.; Hu, X.; Fang, Y.; Chen, D. Metagenomic Approaches Reveal Strain Profiling and Genotyping of Klebsiella Pneumoniae from Hospitalized Patients in China. *Microbiol. Spectr.* **2022**, *10*, e0219021. [CrossRef]
47. Özad Düzgün, A.; Yüksel, G. Detection of Virulence Factor Genes, Antibiotic Resistance Genes and Biofilm Formation in Clinical Gram-Negative Bacteria and First Report from Türkiye of *K. oxytoca* Carrying Both $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-51}}$ Genes. *Biologia* **2023**, *78*, 2245–2251. [CrossRef]
48. Hassan, R.; El Naggar, W.; El Sawy, E.; El Mahdy, A. Characterization of Some Virulence Factors Associated with Enterbacteriaceae Isolated from Urinary Tract Infections in Mansoura Hospitals. *Egypt. J. Med. Microbiol.* **2011**, *20*, 9–18.
49. Li, Y.; Ni, M. Regulation of Biofilm Formation in *Klebsiella pneumoniae*. *Front. Microbiol.* **2023**, *14*, 1238482. [CrossRef] [PubMed]
50. Silva, V.; Pereira, J.E.; Maltez, L.; Poeta, P.; Igrejas, G. Influence of Environmental Factors on Biofilm Formation of Staphylococci Isolated from Wastewater and Surface Water. *Pathogens* **2022**, *11*, 1069. [CrossRef]
51. Romero, F.; Acuña, V.; Font, C.; Freixa, A.; Sabater, S. Effects of Multiple Stressors on River Biofilms Depend on the Time Scale. *Sci. Rep.* **2019**, *9*, 15810. [CrossRef]
52. Türkel, İ.; Yıldırım, T.; Yazgan, B.; Bilgin, M.; Başbulut, E. Relationship between Antibiotic Resistance, Efflux Pumps, and Biofilm Formation in Extended-Spectrum β-Lactamase Producing *Klebsiella pneumoniae*. *J. Chemother.* **2018**, *30*, 354–363. [CrossRef] [PubMed]

53. Shadkam, S.; Goli, H.R.; Mirzaei, B.; Gholami, M.; Ahanjan, M. Correlation between Antimicrobial Resistance and Biofilm Formation Capability among *Klebsiella pneumoniae* Strains Isolated from Hospitalized Patients in Iran. *Ann. Clin. Microbiol. Antimicrob.* **2021**, *20*, 13. [CrossRef]
54. Sanchez, C.J.; Mende, K.; Beckius, M.L.; Akers, K.S.; Romano, D.R.; Wenke, J.C.; Murray, C.K. Biofilm Formation by Clinical Isolates and the Implications in Chronic Infections. *BMC Infect. Dis.* **2013**, *13*, 47. [CrossRef]
55. Hasan, M.E.; Shahriar, A.; Shams, F.; Nath, A.K.; Emran, T. Bin Correlation between Biofilm Formation and Antimicrobial Susceptibility Pattern toward Extended Spectrum β-Lactamase (ESBL)- and Non-ESBL-Producing Uropathogenic Bacteria. *J. Basic Clin. Physiol. Pharmacol.* **2021**, *32*, 20190296. [CrossRef]
56. Zong, Z.; Partridge, S.R.; Thomas, L.; Iredell, J.R. Dominance of BlaCTX-M within an Australian Extended-Spectrum β-Lactamase Gene Pool. *Antimicrob. Agents Chemother.* **2008**, *52*, 4198–4202. [CrossRef] [PubMed]
57. Maynard, C.; Bekal, S.; Sanschagrin, F.; Levesque, R.C.; Brousseau, R.; Masson, L.; Larivière, S.; Harel, J. Heterogeneity among Virulence and Antimicrobial Resistance Gene Profiles of Extraintestinal *Escherichia coli* Isolates of Animal and Human Origin. *J. Clin. Microbiol.* **2004**, *42*, 5444–5452. [CrossRef] [PubMed]
58. Carvalho, I.; Chenouf, N.S.; Cunha, R.; Martins, C.; Pimenta, P.; Pereira, A.R.; Martínez-álvarez, S.; Ramos, S.; Silva, V.; Igrejas, G.; et al. Antimicrobial Resistance Genes and Diversity of Clones among ESBL- and Acquired AmpC-Producing *Escherichia coli* Isolated from Fecal Samples of Healthy and Sick Cats in Portugal. *Antibiotics* **2021**, *10*, 262. [CrossRef]
59. Garcês, A.; Correia, S.; Amorim, F.; Pereira, J.E.; Igrejas, G.; Poeta, P. First Report on Extended-Spectrum Beta-Lactamase (ESBL) Producing *Escherichia coli* from European Free-Tailed Bats (*Tadarida teniotis*) in Portugal: A One-Health Approach of a Hidden Contamination Problem. *J. Hazard. Mater.* **2019**, *370*, 219–224. [CrossRef] [PubMed]
60. Caroff, N.; Espaze, E.; Berard, I.; Richet, H.; Reynaud, A. Mutations in the AmpC Promoter of *Escherichia coli* Isolates Resistant to Oxyiminocephalosporins without Extended Spectrum β-Lactamase Production. *FEMS Microbiol. Lett.* **1999**, *173*, 459–465. [CrossRef] [PubMed]
61. Vanhoof, R.; Content, J.; Van Bossuyt, E.; Dewit, L.; Hannecart-pokorni, E. Identification of the AadB Gene Coding for the Aminoglycoside-2″-O-Nucleotidyltraiisferase, ANT(2″), by Means of the Polymerase Chain Reaction. *J. Antimicrob. Chemother.* **1992**, *29*, 365–374. [CrossRef] [PubMed]
62. Sáenz, Y.; Briñas, L.; Domínguez, E.; Ruiz, J.; Zarazaga, M.; Vila, J.; Torres, C. Mechanisms of Resistance in Multiple-Antibiotic-Resistant *Escherichia coli* Strains of Human, Animal, and Food Origins. *Antimicrob. Agents Chemother.* **2004**, *48*, 3996–4001. [CrossRef] [PubMed]
63. Wei, Q.; Jiang, X.; Yang, Z.; Chen, N.; Chen, X.; Li, G.; Lu, Y. DfrA27, a New Integron-Associated Trimethoprim Resistance Gene from *Escherichia coli*. *J. Antimicrob. Chemother.* **2009**, *63*, 405–406. [CrossRef] [PubMed]
64. Mazel, D.; Dychinco, B.; Webb, V.A.; Davies, J. Antibiotic Resistance in the ECOR Collection: Integrons and Identification of a Novel Aad Gene. *Antimicrob. Agents Chemother.* **2000**, *44*, 1568–1574. [CrossRef] [PubMed]
65. Aarestrup, F.M.; Agerso, Y.; Gerner-Smidt, P.; Madsen, M.; Jensen, L.B. Comparison of Antimicrobial Resistance Phenotypes and Resistance Genes in *Enterococcus faecalis* and *Enterococcus faecium* from Humans in the Community, Broilers, and Pigs in Denmark. *Diagn. Microbiol. Infect. Dis.* **2000**, *37*, 127–137. [CrossRef] [PubMed]
66. Neyestanaki, D.K.; Mirsalehian, A.; Rezagholizadeh, F.; Jabalameli, F.; Taherikalani, M.; Emaneini, M. Determination of Extended Spectrum Beta-Lactamases, Metallo-Beta-Lactamases and AmpC-Beta-Lactamases among Carbapenem Resistant *Pseudomonas aeruginosa* Isolated from Burn Patients. *Burns* **2014**, *40*, 1556–1561. [CrossRef] [PubMed]
67. Vila, J.; Ruiz, J.; Goñi, P.; Jimenez De Anta, M.T. Detection of Mutations in ParC in Quinolone-Resistant Clinical Isolates of *Escherichia coli*. *Antimicrob. Agents Chemother.* **1996**, *40*, 491–493. [CrossRef]
68. Rita Rebelo, A.; Bortolaia, V.; Kjeldgaard, J.S.; Pedersen, S.K.; Leekitcharoenphon, P.; Hansen, I.M.; Guerra, B.; Malorny, B.; Borowiak, M.; Andre Hammerl, J.; et al. 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. [CrossRef] [PubMed]
69. Bardiau, M.; Labrozzo, S.; Mainil, J.G. Study of Polymorphisms in Tir, Eae and TccP2 Genes in Enterohaemorrhagic and Enteropathogenic *Escherichia coli* of Serogroup O26. *BMC Microbiol.* **2011**, *11*, 124. [CrossRef]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.