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Article

Comprehensive Analysis of *Acinetobacter baumannii* in Aquatic Environments and Fish Microbiota: Integrating Culture-Dependent, 16S Metagenomics, and Antibiotic Resistance Profiling

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Abstract: Background: This study provides a comprehensive analysis of *Acinetobacter baumannii* in aquatic environments and fish microbiota by integrating culture-dependent methods, 16S metagenomics, and antibiotic resistance profiling. **Methods**: A total of 83 *A. baumannii* isolates were recovered using culture-dependent methods from intra-hospital infections (IHI), wastewater (WW) and surface water (SW) samples from two southern Romanian cities in august 2022. The antibiotic susceptibility was screened using disc diffusion, microdilution, PCR and Whole Genome Sequencing assays. **Results**: The highest microbial load in the analyzed samples was found in Glina, Bucharest for both WW and SW samples across all investigated phenotypes. For Bucharest isolates, the resistance levels corresponded to fluoroquinolones > aminoglycosides > β-lactam antibiotics. In contrast, *A. baumannii* from upstream SW samples in Targoviste showed the highest resistance to aminoglycosides. The *bla*$_{\text{OXA-23}}$ gene was frequently detected in IHI, WW, and SW isolates in Bucharest but was absent in Targoviste. Molecular phylogeny revealed presence of ST10 in Târgoviște isolates and ST2 in Bucharest isolates, while other minor STs were not specifically correlated with a sampling point. Using 16S rRNA sequencing, significant differences in microbial populations between the two locations was identified. The low abundance of *Alphaproteobacteria* and *Actinobacteria* in both locations suggests environmental pressures or contamination events. **Conclusions**: These findings indicate significant fecal contamination and potential public health risks, emphasizing the need for improved water quality monitoring and management.

Keywords: resistant *Acinetobacter baumannii* isolates; wastewater and surface water microbial load; surface water microbiome; pangenome analysis; Southern Romanian cities

1. Introduction

Increasing concerns about antibiotic resistance (AR) have led numerous groups of researchers to inquire about the effects of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) on human and animal health, agriculture, food production and waste management from the perspective of the One Health concept [1]. The One Health approach aims to achieve excellent health for people, animals, and the environment by addressing the spread of emerging infectious diseases at the animal-human-environment interface. Effective solutions require understanding and managing the complex interactions among these interconnected domains. The "One World – One

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Health" approach is based on four key components: ecological, geographic, human activities, and food-agricultural. Food processors, growers, and merchants are responsible for ensuring product quality. This quality encompasses not only the absence of pathogens but also the consideration of risks to humans at the top of the food chain. Preventive measures should begin at the start of the chain, with feed given to animals being free from contaminants such as mycotoxins and antibiotics [2]. AR is one of the major global healthcare crises of the 21st century. The imprudent use of antibiotics in both humans and animals has led to the emergence of antibiotic resistant bacteria (ARB). Wastewater treatment plants (WWTPs) and hospital environments, due to their high microbial load, have become reservoirs for ARGs and hotspots for the dissemination of AR into the environment. Conventional mechanical and biological wastewater treatment processes cannot completely eliminate all pollutants, leading to the release of pollutants into surface water bodies along with treated wastewater. Additionally, the disposal of waste and treated water from urban areas further increases the presence of resistance genes in surface water [3]. Transmission routes into the environment include: the use of raw or digested manure or sewage sludge as fertilizers on agricultural sectors, the use of treated wastewater for irrigation fields and discharging effluent from the WWTPs into the natural ecosystems [4]. During wastewater treatment, surplus sludge is produced, which highlights a high diversity of microorganisms, including pathogenic ones. Nosocomial infections are rapidly transmissible from one patient to another or even through medical employers. *A. baumannii* is an opportunistic nosocomial pathogen responsible for a wide range of infections, of which pneumonia and septicemia are the most common and included in the World Health Organization's (WHO) priority list for research on drug-resistant bacteria and AR [5,6]. Multidrug resistance (MDR) is caused by several resistance markers in *A. baumannii* isolates, such as β-lactamase encoding genes (*bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA-24}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{VEB}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{GES}}$ and *bla*$_{\text{PER}}$). *A. baumannii* can develop resistance due to the chemical similarity between β-lactamase inhibitors and β-lactams [7].

However, the primary mechanism behind β-lactam resistance in *A. baumannii* involves the production of class D β lactamases. β-lactamases are bacterial hydrolases that bind and acylate β-lactam antibiotics. β-lactamases divide into four classes: the active-site serine β-lactamases (classes A, C and D) and the zinc-dependent or (metallo-β-lactamases, or MBLs) [8]. Class D β-lactamases (OXA enzymes) are narrow-spectrum lactamases that provide resistance to cefotaxime and ceftazidime. For *A. baumannii* the most common are class D oxacillinases (OXA type) β-lactamases (OXA-23, OXA-24, OXA-51, OXA-58, OXA-235, OXA-40) [9]. Class A β-lactamases identified in *Acinetobacter* spp. include β-lactamases such as TEM, SHV, CARB, CTX-M, PER, VEB, GES și KPC [10]. Among the MBLs detected in *A. baumannii* are IMP, VIM, SIM-1, SPM-1 and NDM [8]. Class C β-lactamases are cephalosporinases that hydrolyze most penicillins and narrow-spectrum cephalosporins [11]. Genotypic resistance may be the result of chromosomal mutations or may be due to the acquisition of genetic determinants. *A. baumannii* can acquire resistance to various antibiotic classes through chromosomal mutations and the horizontal transfer of ARGs. Mobile genetic elements are represented by transposons (Tn), insertion sequences (SI) integrons and resistance islands [12]. Four transposons carrying *bla*$_{\text{OXA-23}}$ gene have been reported: Tn*2006*, Tn*2007*, Tn*2008*, and Tn*2009*. Tn*2006* and Tn*2008*, associated with IS*Aba1* contribute to the dissemination of *bla*$_{\text{OXA-23}}$. [13]. A variety of resistance islands were identified in *A. baumannii*, including Aba*R1*, Aba*R3*, Aba*R5*, Aba*R6*, Aba*R7*, Aba*R8*, Aba*R9*, and Aba*R10*. Specifically, the Aba*R1* resistance island contains genes such as *tet(A)* a tetracycline efflux pump which confer tetracycline resistance, and respectively *strA*, *strB*, *aphA1*, and *aac69*, responsible for aminoglycosides resistance [14,15].

In recent years, international authorities have made significant efforts to enhance the monitoring of ARBs and ARGs across various environments. A key strategy in these efforts involves mapping the distribution of MDR nosocomial pathogens in different clinical settings and WWTPs [16]. Effluents from WWTPs are released to the surface water level. Therefore, a major risk factor that can affect human health is the contamination with antibiotic-resistant pathogens of plant crops irrigated with water from contaminated rivers [17].

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For 2022, Romania reported very high levels of resistance in *A. baumannii* to fluoroquinolones, aminoglycosides, and carbapenems (ranked fourth and third, respectively, after countries such as Croatia, Greece, Cyprus, and Italy), according to the ECDC [18].

In this context, this study aims to provide a comprehensive analysis of *Acinetobacter baumannii* in aquatic environments and fish microbiota by integrating culture-dependent methods, 16S metagenomics, and antibiotic resistance profiling of recently isolated isolates from different sources in southern Romania.

2. Materials and Methods

2.1. Water Sampling Campaign

On 01.08.2022 and respectively 09.08.2022, 2 liters of wastewater and surface water samples were collected from two wastewater treatment plants (WWTPs) in southern Romania: Glina (n=4 samples), which collect wastewater from Bucharest (the capital city with 1.72 million inhabitants), and Târgoviște (n=4 samples, having 79,610 inhabitants). Samples were taken from the influent, IN; activated sludge, AS from the aeration tank; effluent, EF of both locations; and respectively upstream, UP (200 m) and downstream, DO (200 m) regions of the sampled WWTPs (Dâmbovița and Ialomița rivers, respectively) and transported at 4°C till the microbiology laboratory of the Faculty of Biology, University of Bucharest, Romania.

2.2. Strains Isolation, Quantification, Identification and Antimicrobial Susceptibility Profiles

The diluted samples up to a factor of 10^-5 were filtered through membrane filtration technique, and the filters were inoculated on chromogenic media (CHROMagar Acinetobacter, Paris, France) and on chromogenic media supplemented with carbapenem, cephalosporin and polymyxin antibiotics (CHROMagar CARBA; CHROMagar ESBL and CHROMagar Colistin, Paris, France), incubated at 37 °C for 24 h under aerobic conditions followed by determination of the colony-forming units number (CFU/100 mL) belonging to *A. baumannii* and to the Gram-negative non-fermenting bacilli (NF-GNB), considering filters with a number of white colonies ≤200 per culture medium and using the following relationship:

$$D = \frac{\sum N_1 \dots N_4}{\sum V_1 \dots V_4} = \frac{Ntot}{Vtot} * 100$$

D- density or microbial load; N-total number of the colonies; V-volume x dilution

Next step was represented by the confirmation of carbapenemase (CP) and extended-spectrum β-lactamase (ESBL) producing isolates, and respectively colistin resistant ones by culturing up to 6 colonies for each phenotype on the same culture media and taxonomic identification of wastewater and surface water isolates, carried out using MALDI-TOF MS (Bruker, Germany). The isolates were preserved on broth (Mueller Hinton, Liofilchem, Italy) culture medium supplemented with 20% glycerol at -80°C.

During the same timeframe, a total of 17 *A. baumannii* isolates from intra-hospital infections (IHI) were isolated and identified using automated systems (VitekII Compact).

A total number of 83 *A. baumannii* isolates recovered from aquatic and clinical samples were tested for antibiotic susceptibility using the standard disc diffusion method, following the protocols outlined in the current editions of the Clinical and Laboratory Standards Institute (CLSI) guidelines pertinent to the isolation year [19]. The antibiotic susceptibility profiles of these isolates were tested to the following antibiotics: amikacin (30 μg); ampicillin-sulbactam (20 μg); aztreonam (30 μg); cefepime (30 μg); ceftazidime (30 μg); ciprofloxacin (5 μg); doripenem (10 μg); imipenem (10 μg); meropenem (10 μg); gentamicin (10 μg); and minocycline (30 μg). The antibiotic susceptibility results were interpreted according to the antibiotic classes, respectively for β-lactams, fluoroquinolones, aminoglycosides and tetracyclines.

For colistin susceptibility the microdilution method in Cation-Adjusted Mueller-Hinton Broth medium (CAMHB, OXOID, England) using standard 96-well microtiter plates by performing serial two-fold microdilutions of colistin sulfate (19.000 IU/mg, Sigma-Aldrich, Merck) in 75 μL of CAMHB

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medium (ranged between 128 - 0.25 µg/mL) according to the CLSI, 2022. The media was inoculated in the next step with a 75 µL of 0.5 McFarland suspension from 24-hour cultures grown at 37 °C on Plate Count Agar media. The positive (untreated cultures) and negative controls (sterility control) were included and the minimum inhibitory concentration (MIC) values were determined after incubating for 24 hours at 37 °C as being the last concentration for which no growth was recorded.

2.3. Characterization of Genotypic Resistance Profiles

The presence of carbapenem and cephalosporin encoding genes (*bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{NDM}$, *bla*$_{OXA-23}$, *bla*$_{OXA-24}$, *bla*$_{OXA-58}$, *bla*$_{OXA-235}$, *bla*$_{OXA-51}$, *bla*$_{KPC}$, *bla*$_{GES}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{PER}$, and *bla*$_{VEB}$) was investigated by simplex and multiplex PCR using DNA template extracted through an alkaline extraction method, specific primers and amplification programs and checked by gel electrophoresis [7], [20].

2.4. Whole Genome Sequencing (WGS) and Bioinformatic Analyses of Clinical and Aquatic A. baumannii Isolates

From a total of 83 *A. baumannii* isolates recovered from two WWTPs and from Fundeni Bucharest Hospital in Romania, we performed WGS sequencing for a total of 20 isolates. The selection criteria were based on AR profiles (were selected isolates from all identified phenotypes) and isolation sources (from all sources: IN, EF, AS, UP and DO samples). Total DNA was extracted using DNeasy UltraClean Microbial Kit (Qiagen, Germany), followed by library preparations with Nextera DNA Flex Library Prep Kit (Illumina). The sequencing was performed on Illumina MiSeq and NextSeq platforms (V3, 600 cycles).

Hence, the raw reads were assembled *de novo* using Shovill v1.1.0 pipeline [21]. Furthermore, the resulting sequences were analyzed using ABRicate v1.0.0 [22] tool and the NCBI and VFDB [23] databases to determinate profiles of ARGs and virulence factors (VFs). The Multilocus Sequence Type (MLST) [24] method was utilized to determinate the sequence type (ST) of the isolates, in conformity with the Pasteur scheme. Moreover, Prokka v1.14.6 [25] tool was used to annotate the sequences of the selected isolates. The output generated by Prokka was then utilized as input for Roary v3.13.0 [26]. Newick tree, generated from Roary along with core and accessory genes, was illustrated using Phandango [27] online tool. Roary output was converted with the following script [29] for multidimensional scaling (MDS) and pangenome tree representation by FriPan [28]. Subsequently, the Heaps' law was determined for data set, using Seth Commichaux's Python script [30].

2.5. Metagenomic Analysis of Surface Water Samples and Fish Microbiota, to Highlight the Connection between the Environment and Fish Microbiota

To examine the microbiome from fish gut samples, DNA extraction was performed using the Pure Link Microbiome DNA Purification Kit (Invitrogen, Thermo Scientific, USA), following the manufacturer's instructions. DNA extraction from water samples was carried out using the DNA Power Water Kit (Qiagen, Germany), according to the manufacturer's instructions. The 16S rRNA sequences were then amplified using specific primer pairs for the V3-V4 hypervariable region of the 16S rRNA gene. The PCR products resulting from the amplification of the hypervariable regions of the 16S rRNA gene were purified using AmPure XP magnetic beads (Beckman Coulter, Inc.). Library preparation was conducted using the Ion Plus Fragment Library Kit (Life Technologies, USA), following the manufacturer's instructions. The obtained amplicon libraries were sequenced on an Ion Torrent 316 chip using the Ion Torrent PGM system and the Ion Sequencing 400 Kit (Life Technologies, USA), adhering to the manufacturer's instructions. The sequencing data obtained were processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline, a tool used for microbiome sequencing data analysis, allowing the determination of microbiota composition and diversity. For calculating diversity measures, operational taxonomic units (OTUs) of the 16S rRNA gene were defined at a sequence similarity of at least 97%. The final analysis of the obtained sequences was performed using Ion Reporter software.

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3. Results

3.1. Phenotypic and Genotypic AR Profiles of A. baumannii Isolates

3.1.1. Isolation and Quantification of A. baumannii from Romanian Wastewater and Surface Water Samples

The inoculation of diluted wastewater and surface water samples collected between 01-09.08.2022 from different sampling points of the investigated WWTPs (influent, active sludge, effluent) and surface water samples collected from the upstream and downstream region of the sampled WWTPs on chromogenic culture media (CHROMagar Acinetobacter for determining the total microbial load corresponding to *A. baumannii*) and chromogenic media supplemented with antibiotics (CHROMagar CARBA; CHROMagar ESBL and CHROMagar Colistin) allowed the determination of microbial load in the collected samples (Figure 1 and 2; Supplementary Tables 3 and 4).

Analysis of the comparative levels of the microbial load in the analyzed samples from the receiving river revealed in decreasing order by the phenotype:

- *CARBA phenotype*: DO Glina, Bucharest > UP Targoviste > UP Glina, Bucharest > DO Targoviste;
- *Colistin phenotype*: DO Glina, Bucharest > DO Targoviste > UP Glina, Bucharest > UP Targoviste;
- *ESBL* and *total Acinetobacter phenotype*: DO Glina, Bucharest > DO Targoviste > UP; (Figure 1 and Supplementary Table 3).

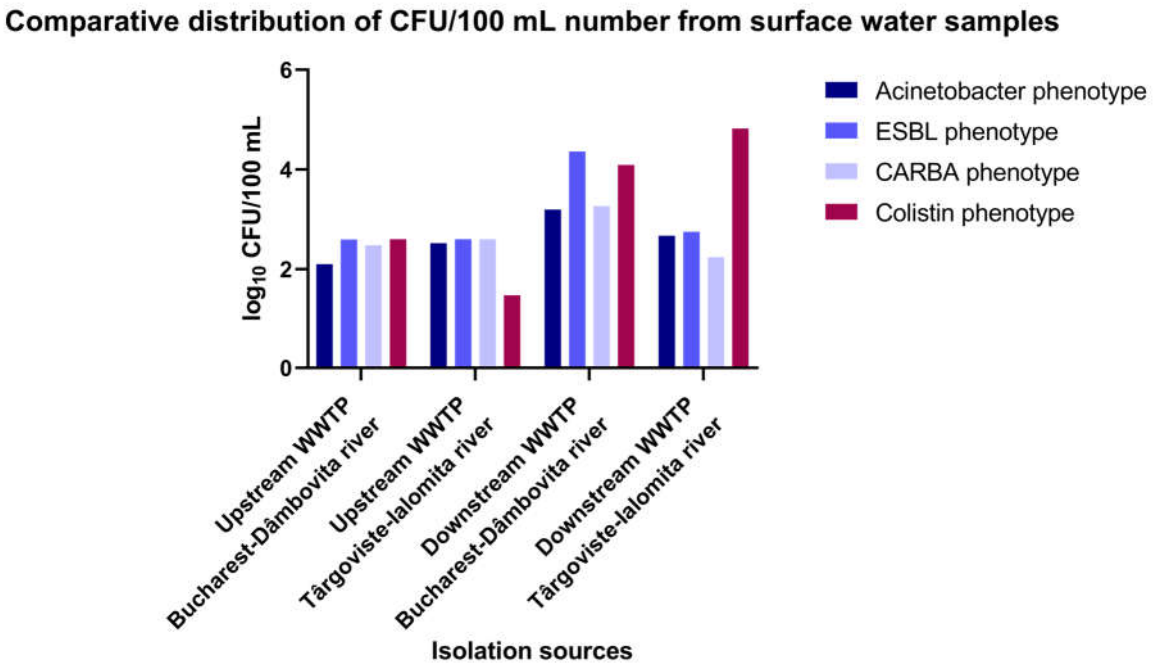

Figure 1. The microbial load of Acinetobacter for the upstream and downstream sampling points of investigated WWTPs in the two locations in southern Romania.

Analysis of the comparative levels of the microbial load in the analyzed samples from the WWTPs revealed in decreasing order by the phenotype:

- *CARBA and ESBL phenotype*: EF Glina, Bucharest > IN Glina, Bucharest > IN Targoviste >AS Glina, Bucharest > EF Targoviste;
- *total Acinetobacter phenotype*: IN Glina, Bucharest > IN Targoviste > AS Glina, Bucharest > EF Glina, Bucharest > EF Targoviste; (Figure 2 and Supplementary Table 4).

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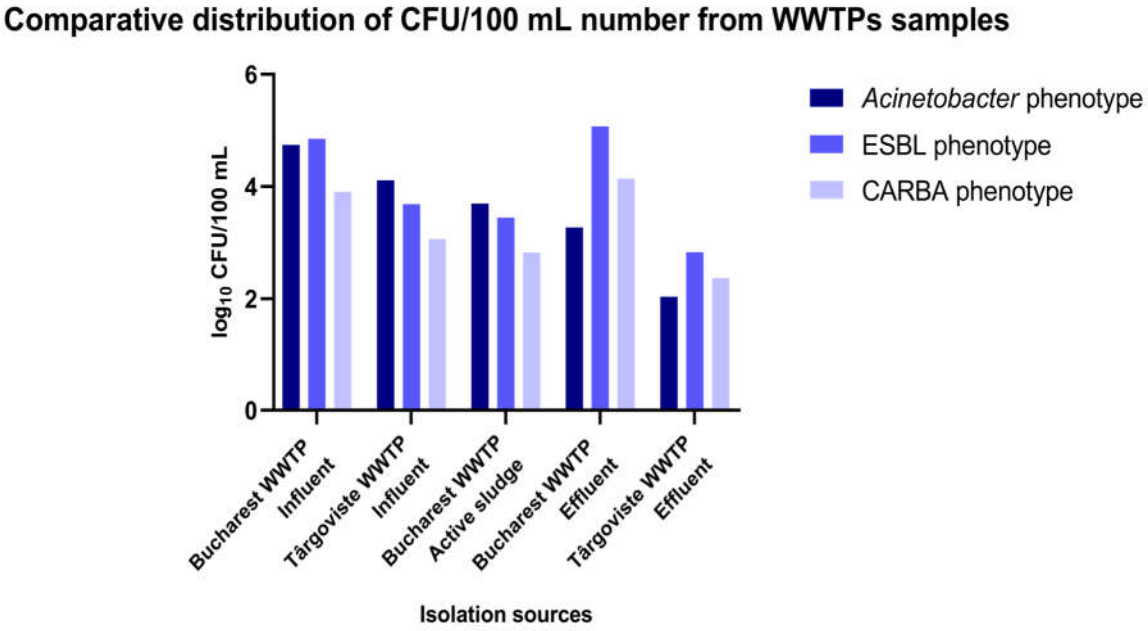

Figure 2. The microbial load with Acinetobacter for the wastewater sample collection inside the investigated WWTPs in the two locations in southern Romania.

3.1.2. Antimicrobial Susceptibilities Profiles of A. baumannii Isolates from Different Isolation Sources and by Geographical Location

In decreasing order, the resistance level of *A. baumannii* isolates recovered from intra-hospital infections (n=17) correspond to fluoroquinolones (100%), β-lactam (98% of the isolates), followed by aminoglycosides and tetracycline antibiotics (94 % of the isolates). The comparative study of the AR profiles according to the isolation sources and location demonstrated the following: for a total of 33 *A. baumannii* isolates recovered from Glina, Bucharest's WWTP and the receiving river the highest resistance level corresponds to aminoglycosides in the case of the AS samples (57%); followed by the isolates isolated from EF (36%), and respectively IN and DO region (43%). For β-lactam antibiotics the resistance levels were in decreasing order as follows: AS, DO (31% of the isolates) > IN (25%) > EF (21%). In the case of fluoroquinolone antibiotics the resistance levels were attributed to the isolates in decreasing order as follows: AS samples (29%) > IN, DO samples (14%) > EF (9% of the isolates). The most susceptible *A. baumannii* correspond to tetracycline antibiotics (Figure 3 and Supplementary Table 5).

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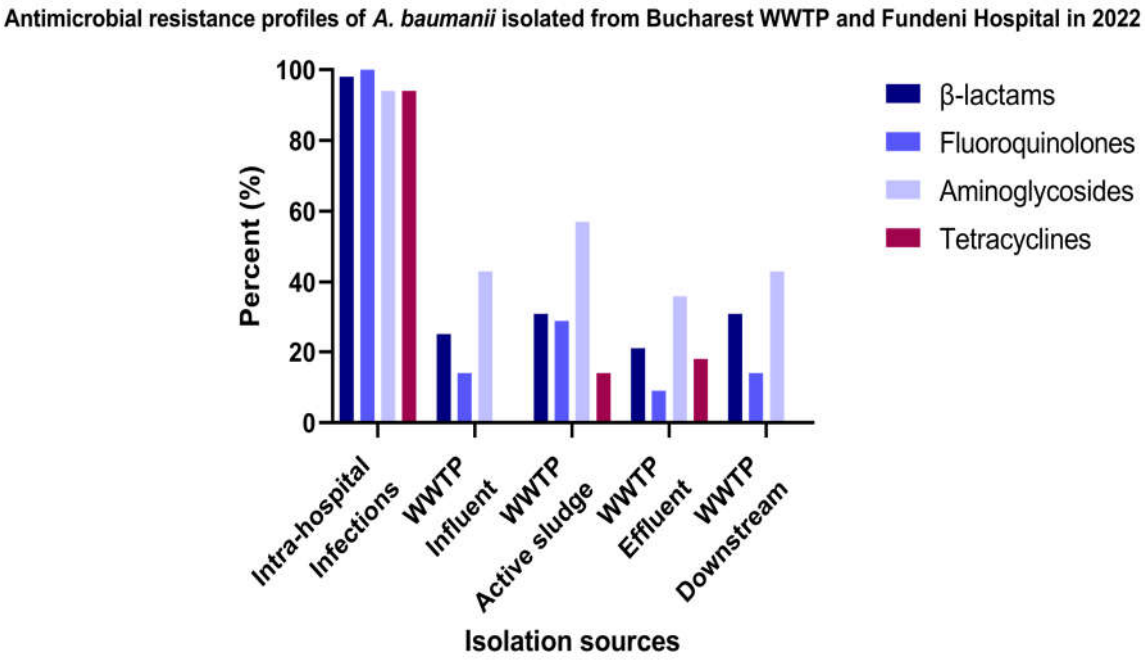

Figure 3. Percentage of A. baumannii isolates isolated from IHIs, WWTP, and surface water samples in Bucharest, categorized according to their resistance profile to different antibiotic classes.

The resistance levels in the case of *A. baumannii* isolated from Targoviste shows that the aminoglycosides resistance was recorded at the top of the resistance level, with 54% of the isolates recovered from surface water sample from UP region, followed by *A. baumannii* isolated from IN, DO (50%) and isolates isolated from EF sample (36%). For β-lactam antibiotics the resistance level was observed to be on the decrease, in the following order: surface water sample from UP region (42% of the isolates) > IN (33%) > EF (31%) and DO (29% of the isolates). The resistance levels identified for fluoroquinolone were as follows: IN samples (33%) > UP samples (17% of the isolates). The most susceptible *A. baumannii* isolates were identified for tetracycline antibiotics, as illustrated in the Figure 4 and Supplementary Table 6.

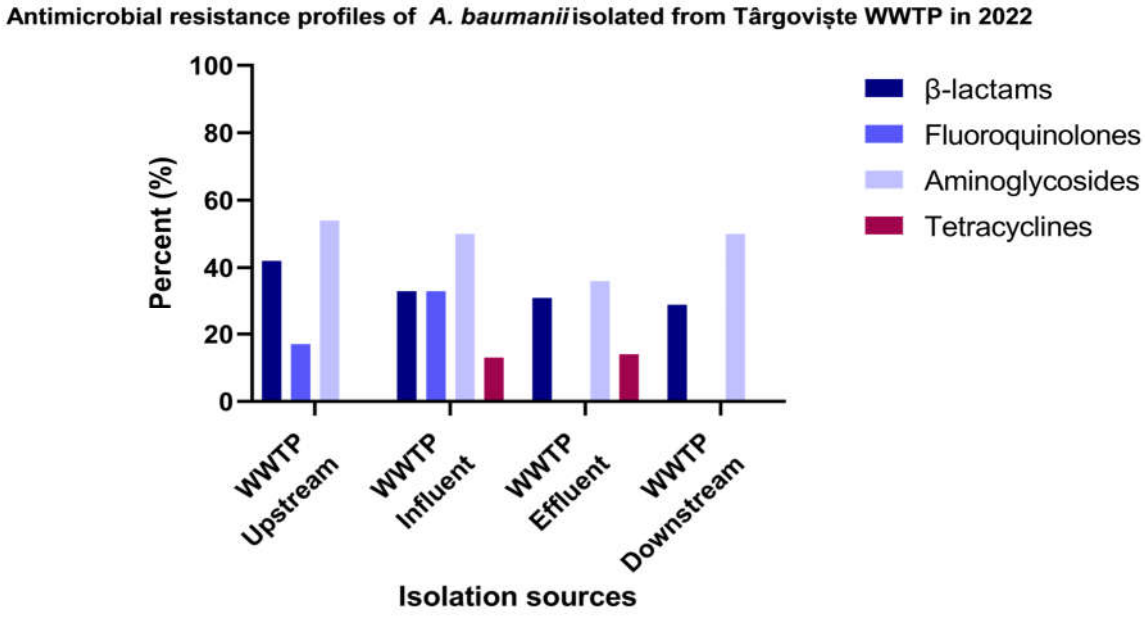

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Figure 4. Percentage of A. baumannii isolates recovered from WWTP and surface water samples in Targoviste, categorized according to their resistance profile to different antibiotic classes.

Using the microdilution method in CAMHB were determined the MIC values for colistin susceptibility as following: in the case of one intra-hospital infection isolate (encoded 24 IHI Buc) within the international clone (IC) 2 (ST2) isolated from Fundeni hospital, was intermediate to colistin with a MIC value of 1 μg/mL. Resistance to colistin may be caused by mutation in genes encoding lipopolysaccharides (LPS), such as *lpxA*, *lpxC* and *lpxD*, as well as genes encoding for phosphoethanolamine transferase (PEtN), such as operon *pmrCAB* as previously demonstrated [31]. In this study, mutations were identified in *lpxC* (N286D), *lpxD* (E117K) and *pmrB* (A138T), the genes were compared to the *A. baumannii* ATCC 1906 genome (GenBank CP045110) [32]. The rest of the clinical and wastewater isolates belonging to epidemic ICs or non-IC were susceptible to colistin (MIC < 0.25 μg/mL) (see Table .1).

Table 1. MIC values for colistin susceptibility in clinical and wastewater A. baumannii isolates from Southern Romania.

ANTIBIOTIC/ ISOLATE	22012-CA5	22012-ENE6	22013-CA5	22013-ENE4	22014-CA2	22014-COLN5	22015-CA3	22015-CA4	22015-ENE6	22016-CA2	22016-CNE3	22016-CNE4	22017-CNE1	22018-CA5	22018-CA6	22019-CNE4	22019-CNE5	24-IHI	3-IHI	49-IHI
COLISTIN (μG/ML)	0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	1	<0.25	<0.25

3.1.3. Genotypic Characterization of β-Lactam Resistance in Clinical, Wastewater and Surface Water A. baumannii Isolates

The comparative molecular study for a total of 33 *A. baumannii* stains recovered from Glina, Bucharest wastewater and surface water samples according to the isolation sources (Figure 4 and Supplementary Table 7) demonstrated the following: the most frequently encountered gene in investigated sources was *bla*$_{\text{OXA-23}}$: AS (28% of the isolates) > EF (19%) and IN, DO (14% of the isolates). As anticipated, the *bla*$_{\text{OXA-51}}$ gene was identified in all isolates isolated from all isolation sources. Nevertheless, isolates isolated from intra-hospital infections were positives for *bla*$_{\text{OXA-23}}$ gene (62%) and *bla*$_{\text{OXA-24}}$ gene (46% of the isolates). Additionally, the presence of the *bla*$_{\text{TEM}}$ gene was confirmed, although at a relatively lower prevalence (ranging from 15 to 14% in IHI and WWTP IF).

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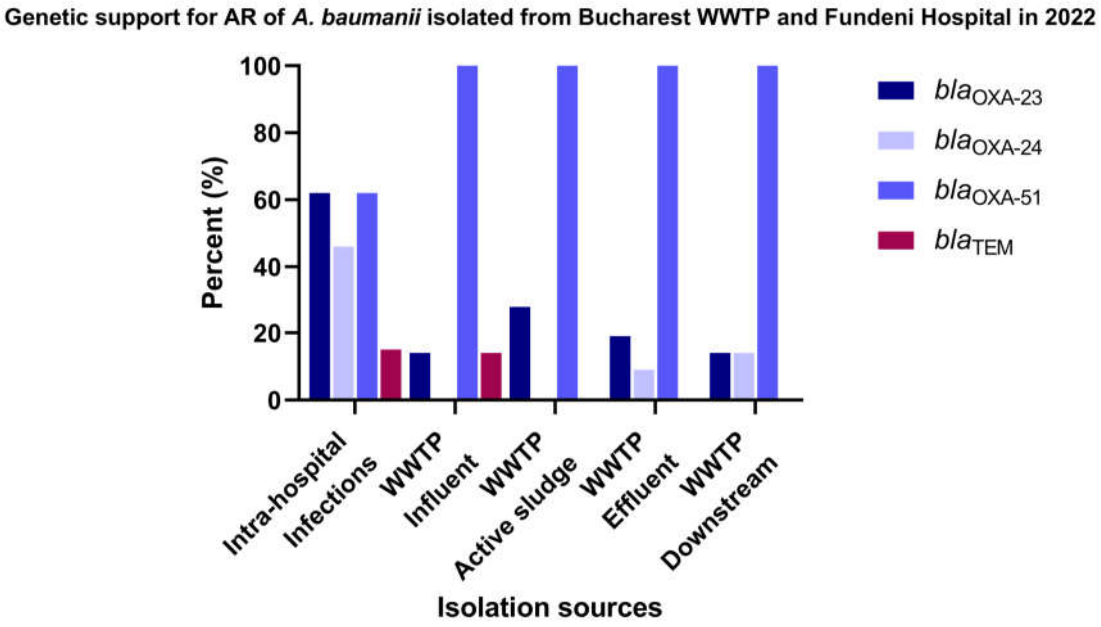

Figure 5. β-lactamase producing A. baumannii isolates from Bucharest, Romania in 2022.

The genotypic characterization of β-lactam resistance performed in 33 isolates of *A. baumannii* isolated from Targoviste wastewater and surface water samples (Figure 6 and Supplementary Table 8) revealed that the *bla*CTX-M gene was identified in 13% of the isolates obtained from surface water samples collected from the DO region of the sampled WWTP. As expected, *bla*OXA-51 gene was identified in 100% of the isolates from all sources.

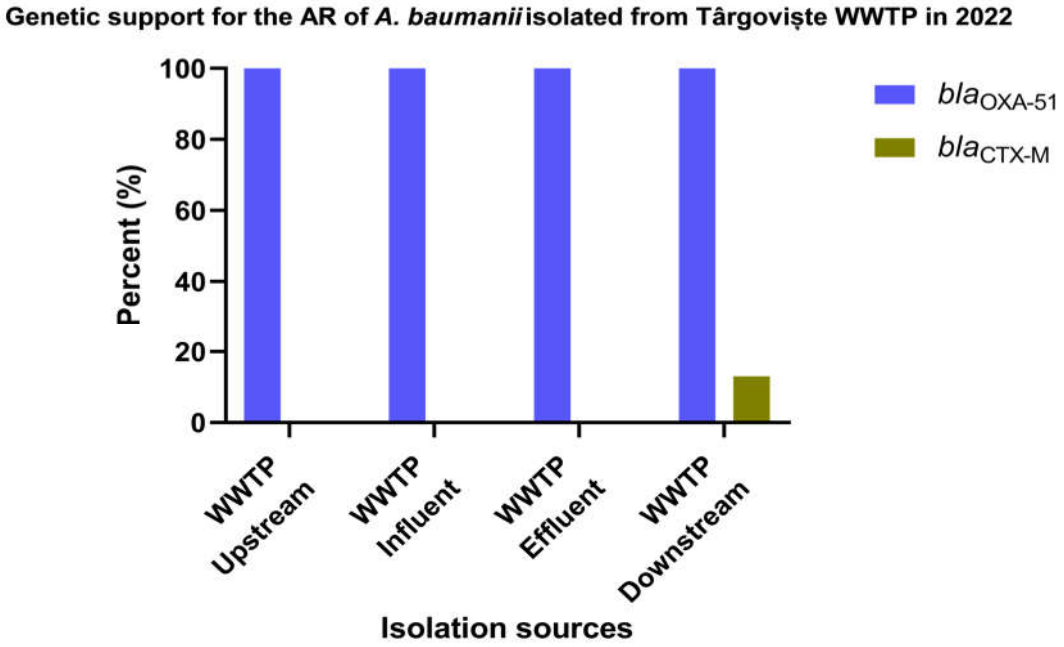

Figure 6. β-lactamase producing A. baumannii isolates isolated from Targoviste, Romania in 2022.

3.1.4. WGS Analysis in Clinical, Wastewater and Surface Water A. baumannii Isolates

WGS analysis of intra-hospital infections, wastewater surface water *A. baumannii* isolates revealed the presence of the following ARGs: *ant(3'')-IIa*, *aph(3'')-Ib*, *aph(3')-VIa*, *aph(6)-Id* and *armA* for aminoglycosides resistance. Macrolide and tetracycline resistance was confirmed by the presence

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of *mph(E)*, *msr(E)* and respectively *tet(B)* genes. In addition, *sul1* and *sul2* genes which encode resistance to sulfonamides, were also identified. Moreover, the genes encoding for chloramphenicol resistance, *catA1* and *cmlB1* genes, were identified in isolates from all sampled sources. Notably, *bla*$_{OXA-23}$ *and bla*$_{OXA-72}$ genes encoding for carbapenem resistance, were present in six of the tested isolates, including isolates from intra-hospital infections and wastewater samples from Bucharest. Furthermore, in one isolate belonging to IC2, isolated from Bucharest WWTP effluent (encoded 22014-CA2), both CP encoding genes mentioned above were identified. Nevertheless, the most prevalent genes, as expected, belonged to the *bla*$_{ADC}$ and *bla*$_{OXA-51}$ families, encoding for classes C and D β-lactamases. Additionally, the *bla*$_{TEM}$ gene, which encodes for class A broad spectrum β-lactamase, has also been identified in a wastewater *A. baumannii* isolate recovered from Bucharest WWTP effluent (Supplementary Table 9).

MLST analyses indicate that the most prevalent clones within the tested isolates from all isolation sources were IC2 (ST2, 25% of the sequenced isolates) followed by the IC8 (ST10, identified in the case of one isolate from wastewater and another one from a surface water samples from Targoviste) and IC7 (ST113 in the case of one wastewater isolate from Targoviste) according Shelenkov et al. classification [33]. Moreover, several other non-IC clones were identified, i.e., ST154 (surface water sample from Glina, Bucharest and wastewater isolate from Targoviste), ST150 (surface water sample from Targoviste), ST32 (wastewater isolate from Glina, Bucharest) (Figure 7).

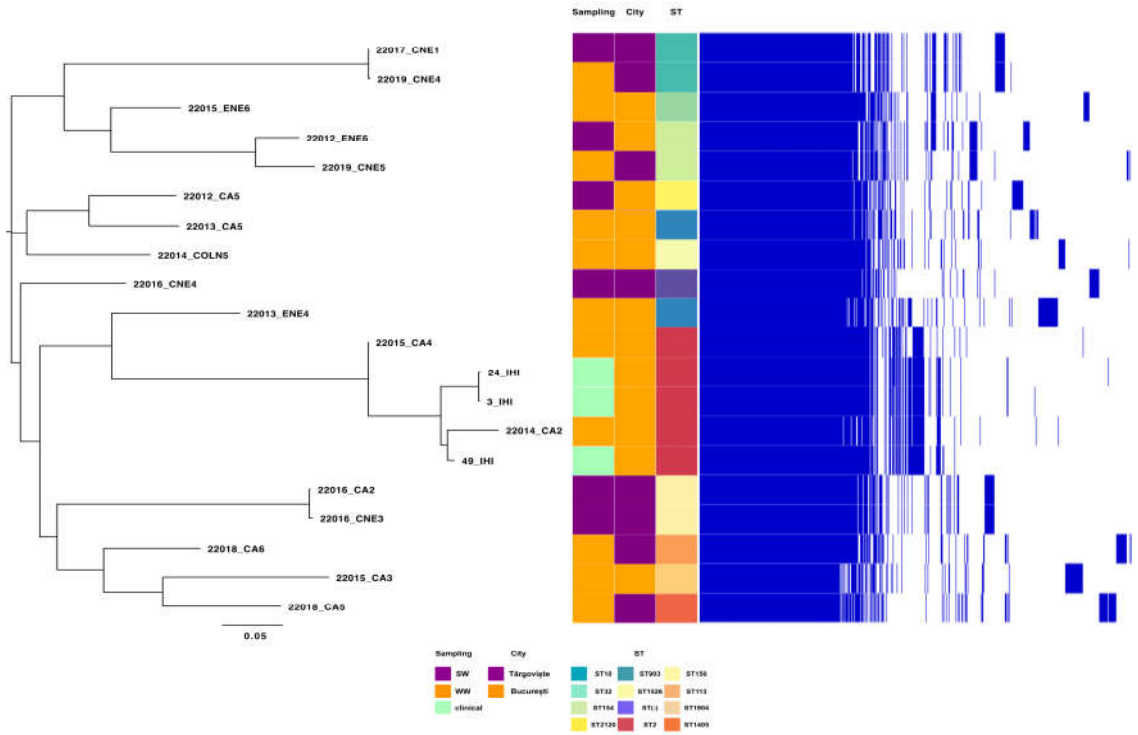

Figure 7. Pangenome analysis of A. baumannii isolates from WW, SW and IHI samples in Southern Romania, based on accesory genes.

Pangenome analysis was undertaken on 20 genomes of *A. baumannii* isolates from WW and SW in two cities in Romania, as well as from IHI. Notably, one cluster stands out from all 20 genomes (red color in Figure 8); it belongs to ST2 and was isolated from WW (n=2) and IHI (n=3) in Bucharest. The other isolates belong to various STs, such as ST150, ST10 and ST154, and correlations between isolations sources could not be established (Figure 7). Moreover, *A. baumannii* pangenome analysis identified a gene pool consisting of 7,644 genes in the sequences of all 20 isolates. Most of these genes (~60%) are classified as hypothetical proteins. The 5-isolate cluster has 224 unique genes, compared to the other 15 isolates in this study. Of these 224 genes, most of them are classified as hypothetical proteins (88%) but there are other genes that could be linked to antibiotic resistance (e.g., *tetA*, *tetR*

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(associated with Tn*10*) and other MGEs (Tn*3* family transposase Tn*2*, Tn*3* family transposase TnAs*3*, IS*66* family transposase IS*Aba17*). Following Heaps' law, the pangenome of *A. baumannii* remains open, characterized by a $\gamma = 0.26$.

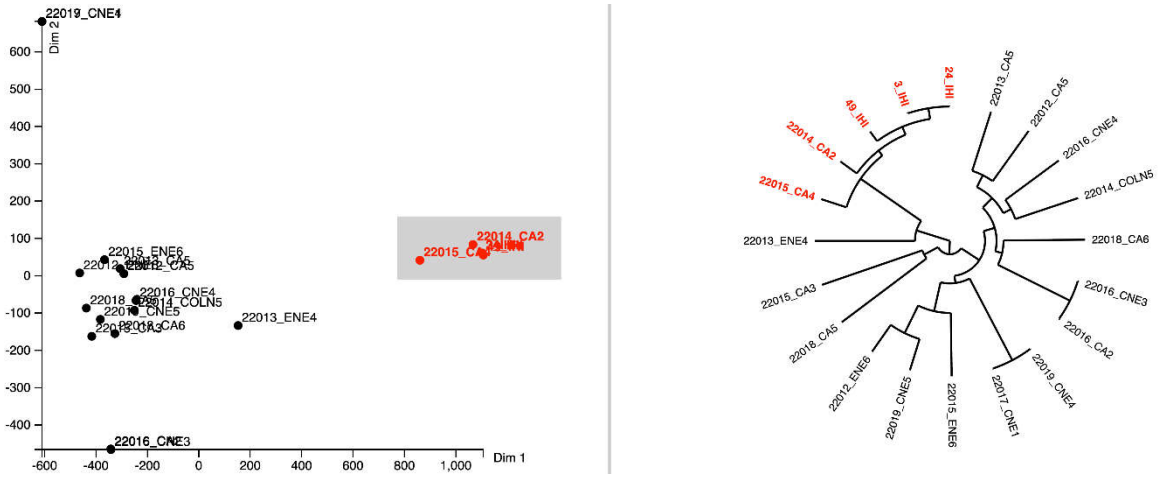

Figure 8. *A. baumannii* isolates' pangenome - FriPan MDS & pangenome tree representation based on accessory genes.

Using the VFDB database it was shown that circulating *A. baumannii* clones in Romanian intra-hospital infections from Bucharest and wastewater from two cities in southern Romania were associated with virulence factors encoding for adherence (*ata, tufA*), biofilm formation (*algW, bap, csuA, csuB, csuC, csuD, csuE*), effector delivery systems (*tssA, B, C, D, E, F, G, K, L, M, tagX*), exoenzymes (*cpaA*), immune modulation (*cps4l, galE, galU, pseB, pseC, pseF, pseG, pseH, pseI, tviB, wbpD*), nutrition/metabolic factors (*bauA, hemO*) [34]. The highest number of virulence factors were associated with clinical and wastewater isolates positives for OXA-23 and OXA23+OXA-72 CPs in the case of wastewater isolate isolated from Bucharest WWTP effluent and belonging to IC2: (40 and respectively 35 encoding genes). An interesting situation was observed in the case of the *cpaA* gene, identified in *A. baumannii* isolates recovered from wastewater and surface water samples from both investigated locations, and associated with ST154, ST903, ST32, and ST10 clones (Supplementary Table S10).

3.2. Metagenomic Analysis of Surface Water Samples and Fish Microbiota, to Highlight the Connection between the Environment and Fish Microbiota

The fish samples collected from Targoviste exhibited a microbiota predominantly composed of *Aeromonadales* species (28%), *Flavobacteriaceae* (10%), *Enterobacteriaceae* (5%), and *Vibrionaceae* (4%) (Figure 9). The phylum *Alpha Proteobacteria* was poorly represented, with an abundance of only 2%. The order *Pseudomonadales* was present in the gut samples with an abundance of 2% of the total number of sequences obtained. The fish samples collected from the Glina, Bucharest site were characterized by lower microbial diversity (based on Shannon diversity index values) (Figure 10). The microbiome at this site was predominantly composed of *Aeromonadaceae* species (56%) and a very high abundance of *Fusobacteriaceae* (15%). The percentage of *Enterobacteriaceae* (8%) was higher compared to that obtained for the samples collected from Targoviste, indicating a degree of fecal contamination, as this family of microorganisms is associated with the human microbiome (Figure 9).

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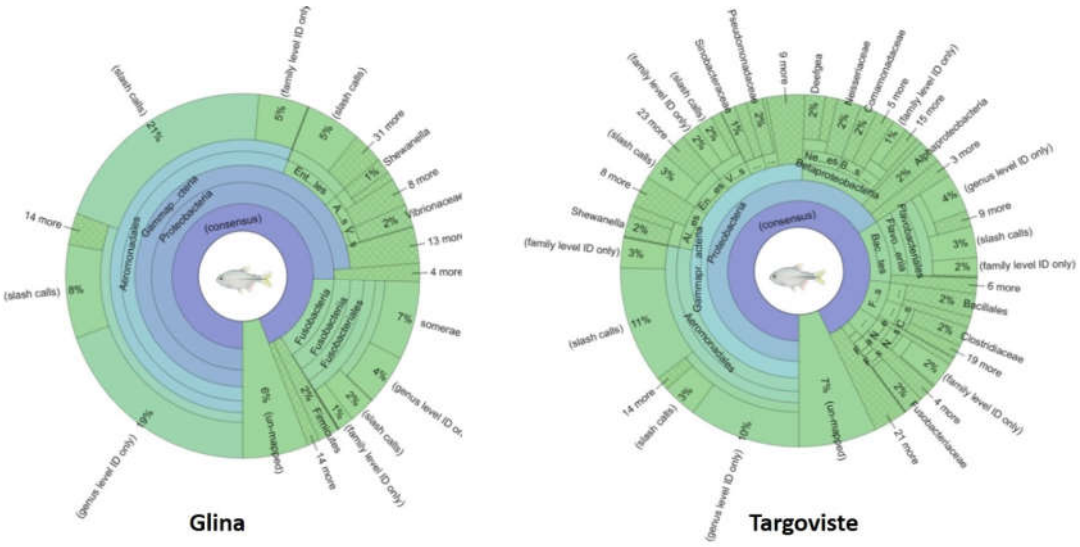

Figure 9. Krona plots illustrating microbial community composition based on 16S rRNA sequencing of fish intestine samples from Glina, Bucharest and Targoviste. (A) Krona plot representing microbial taxa present in fish intestines from Glina, Bucharest. (B) Krona plot representing microbial taxa present in fish intestines from Targoviste. Each segment in the plots represents a taxonomic group at different levels (phylum, class, order, etc.), with the size of the segments corresponding to the relative abundance of that taxon within the sample. Taxonomic labels are color-coded for clarity.

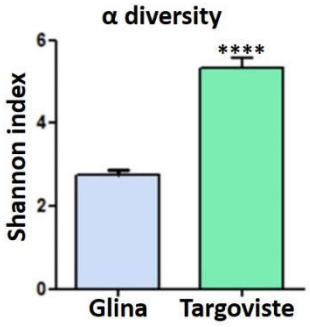

Figure 10. Shannon Diversity Index of microbial communities in fish intestine samples from Glina and Targoviste.

The microbiota of the aquatic environment is typically composed of members of the phyla *Alphaproteobacteria*, *Cytophaga / Flexibacter / Flavobacterium*, and *Actinobacteria*. However, the samples collected from Targoviste and Glina, Bucharest exhibited a low abundance of the phyla Alphaproteobacteria and Actinobacteria.

The surface water samples from the UP region of Targoviste WWTP were characterized by a very high abundance of *Burkholderiales* (38%), *Neisseriales* (19%), and *Pseudomonadales* (9%). Additionally, *Enterobacteriales, Aeromonadales, Alteromonadales*, and *Rhodobacterales* were identified, albeit at lower abundances. The DO samples from Targoviste harbored *Actinomycetales* (6%), *Aeromonadaceae* (5%), and *Flavobacteriaceae* (4%), along with a high presence of *Campylobacteraceae* (6%), a family of microorganisms pathogenic to humans but commensal in some animals (e.g., chickens) (Figure 11 A).

In the UP water samples collected from Glina, Bucharest WWTP, 16S rRNA sequencing revealed high levels of *Burkholderiales* (11%), *Oscillatoriales* (6%), *Flavobacteriales* (4%), *Actinomycetales* (8%), and *Cytophagales* (3%). The DO samples were characterized by the presence of pathogenic microorganisms (*Campylobacteraceae* - 11%) and microorganisms typical of the human microbiota such as

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Enterobacteriaceae, *Bacteroidaceae*, *Prevotellaceae*, and *Lactobacillales*. Additionally, species of the families *Pseudomonadaceae* (4%) and *Moraxellaceae* (6%) were identified (Figure 11 B).

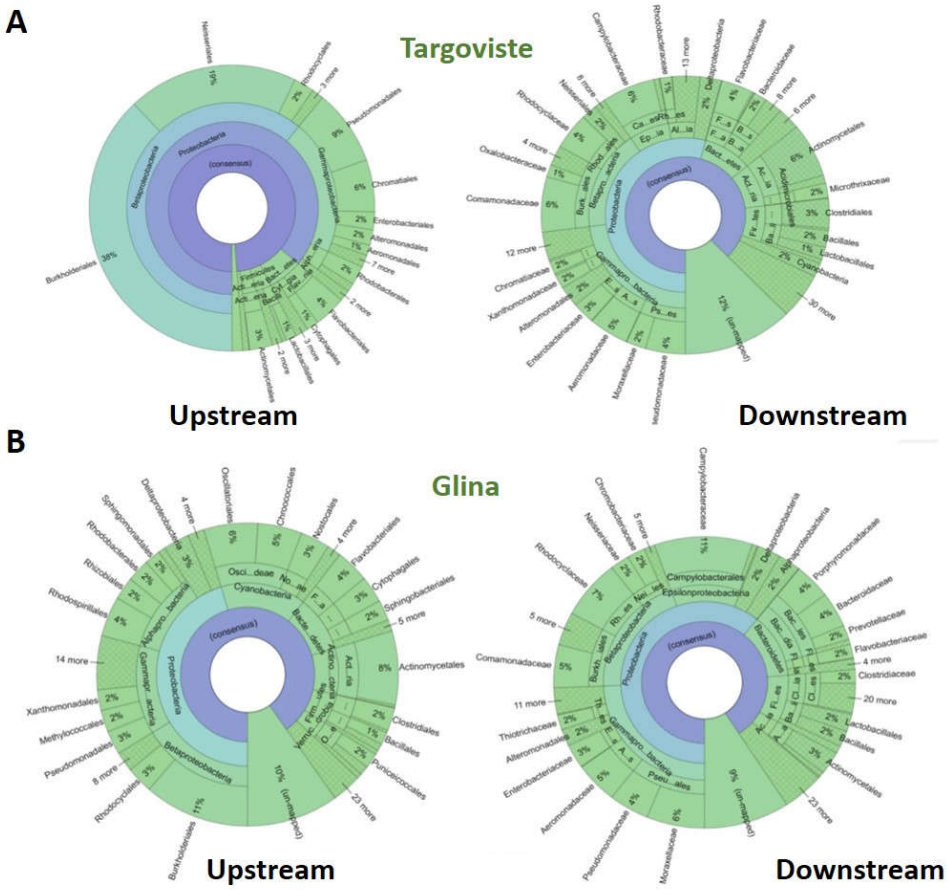

Figure 11. Krona plots illustrating microbial community composition based on 16S rRNA sequencing of upstream and downstream water samples in Targoviste (A) and Glina (B).

Furthermore the analysis of the 16S rRNA metagenomic data from surface water samples from DO regions of Glina, Bucharest and Targoviste WWTPs reveals significant insights into the microbial communities present in these environments. The focus on the *Moraxellaceae* family, particularly the genus *Acinetobacter*, provides a detailed understanding of bacterial diversity and prevalence.

In the water samples collected from DO region of Glina, Bucharest WWTPs *Acinetobacter* was identified as a significant component of the microbial community within the *Moraxellaceae* family. Specifically, *Acinetobacter* constituted 43% of the *Moraxellaceae* reads. This substantial presence highlights the potential environmental impact and resilience of *Acinetobacter* species in this downstream water ecosystem (Figure 12).

Similarly, the water samples from Targoviste revealed an even higher prevalence of *Acinetobacter* within the M*oraxellaceae* family. In this location, *Acinetobacter* accounted for 52% of the *Moraxellaceae* reads. This higher percentage indicates a robust population of *Acinetobacter* species in the Targoviste downstream waters, suggesting possible variations in environmental conditions or anthropogenic influences that favor the proliferation of this genus (Figure 12).

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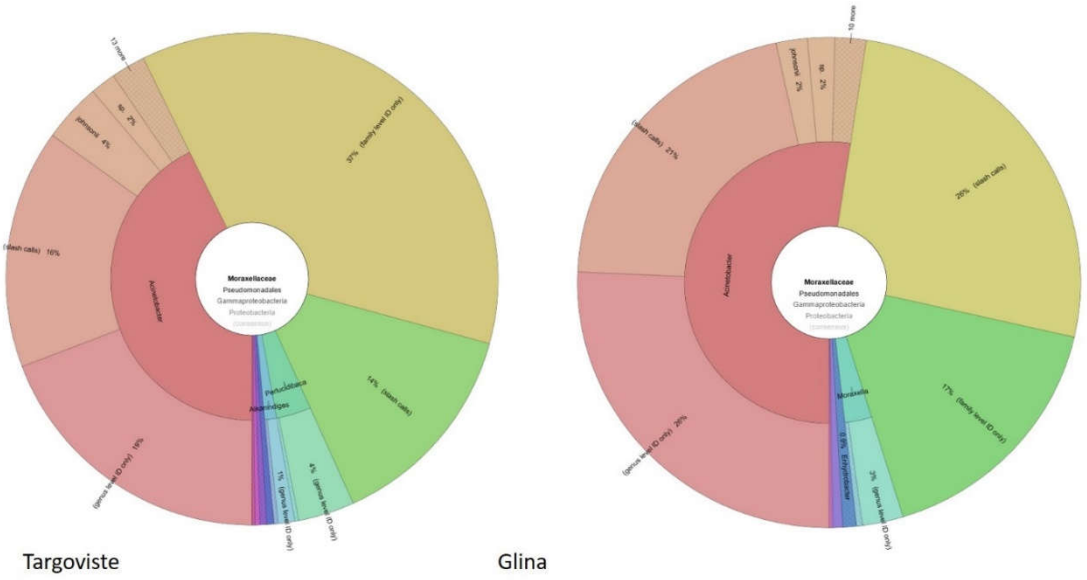

Figure 12. Taxonomic composition of *Moraxellaceae* in surface water samples from DO regions of Glina, Bucharest and Targoviste WWTPs based on 16S rRNA metagenomic analysis.The inner circle represents the taxonomic classification at the family level, while the outer circle provides a more detailed view at the genus level. Glina, Bucharest: *Acinetobacter* (43%), unidentified genera within *Moraxellaceae* (28%), *Moraxella* (7%), *Psychrobacter* (3%), and *Enhydrobacter* (0.9%). Targoviste: *Acinetobacter* (52%), unidentified genera within *Moraxellaceae* (37%), *Moraxella* (7%), and *Enhydrobacter* (2%).

4. Discussion

This paper analyzes *A. baumannii* in aquatic environments and fish microbiota using culture-dependent methods, 16S metagenomics, and antibiotic resistance profiling of recent isolates from Glina, Bucharest and Targoviste WWTP and the receiving rivers (Dambovita and Ialomița) southern Romania. Two WWTPs were selected to represent different pollution sources: urbanized city, wastewater discharges in Bucharest, capital city of Romania and respectively anthropogenic activities and animal waste from a dog shelter în Targoviște. Eight water samples (four from each location) were collected for *A. baumannii* isolation, identified using MALDI-TOF mass spectrometry, further investigated for AR by phenotypic and genotypic assays and 16S metagenomics. Previously, monitoring the quality parameters for five sections of the Dâmbovița river, both upstream and downstream of Bucharest, showed that the river's overall ecological state falls into quality classes III–V (poor to bad quality). The worst conditions corresponded for the DO region of Bucharest, which received partially treated wastewater from the Bucharest WWTP [35]. The Ialomita River's water quality, monitored along its length, ranged from very good to very poor (classes I to V). After 2010, the water quality improved, with only the DO region showing a moderate status [36]. Using culture-dependent assays, we demonstrated that the highest microbial load in the analyzed samples (wastewater from the IN, AS, EF sources, and surface water from the UP and DO regions of the Dambovita and Ialomita rivers) was found in Bucharest for both wastewater (WWTP EF and WWTP IN) and surface water samples (DO region of the WWTP Bucharest) across all investigated phenotypes (CARBA, ESBL, colistin and total *Acinetobacter* population). From both investigated locations a total of 66 *A. baumannii* isolates (33 for each location) were obtained through culture dependent assays from wastewater and surface water samples and compared with 17 isolates recovered in 2022 from IHI to evaluate the circulating clones in different isolation sources. In both WWTPs, the highest resistance levels were found for aminoglycoside antibiotics, followed by β-lactams and fluoroquinolones, with resistance levels varying by location and isolation source. In the case of IHI isolates the resistance levels in decreasing order corresponded to fluoroquinolones > β-lactam > aminoglycosides and tetracycline antibiotics. The investigated isolates for colistin resistance

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have demonstrated that only in the case of an IHI isolate belonging to ST2 isolated from a large hospital in Bucharest, where patients from all over the country are admitted, was intermediate, while the rest of the isolates were sensitive to colistin. Previous data have reported that ST2 is the most prevalent ST associated with colistin resistance in *A. baumannii* across Europe, Asia, Africa, and North and South America [32], [37–48].

In Romania, the most frequently detected bacterial isolates with clinical relevance include *Klebsiella* spp., A. *baumannii*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, all showing MDR phenotype. *A. baumannii*, associated with nosocomial infections such as pneumonia, meningitis, and urinary tract infections, was a focus of a 2018 study aimed at identifying microorganisms responsible for pneumonia in patients at an emergency hospital in Bucharest. Antimicrobial susceptibility testing for *A. baumannii* revealed high resistance rates: 88% to fluoroquinolones (including ciprofloxacin), 86% to β-lactam antibiotics (meropenem), and 86% to aminoglycosides (including amikacin) [49]. The transmission of ARGs among human, animal, and environmental reservoirs is a significant concern, with WWTPs being critical reservoirs for the spread of these genes. For *A. baumannii* isolates isolated from Romanian WWTPs, the highest resistance rates were recorded for fluoroquinolones (87.5% to ciprofloxacin), followed by aminoglycosides (86% to gentamicin and amikacin), and β-lactam antibiotics (84% to aztreonam and meropenem) [17].Viable MDR and carbapenem-resistant *A. baumannii* were detected in urban wastewater, which included hospital wastewater, both before and after secondary wastewater treatment [50]. Other studies have highlighted the presence of putative carbapenem resistant *Acinetobacter* isolates detected in all WWTP samples, except the primary sludge. Also, studies have revealed that *A. baumannii* isolates were resistant to fluoroquinolones, aminoglycosides, β-lactams and polymyxins in different sampling points of urban WWTPs [51].

The WGS analysis of *A. baumannii* isolates from investigated locations revealed both shared and unique characteristics, i.e., *ant(3'')-IIa* gene in all isolation sources from both locations opposite to several CPs: OXA-23 (Bucharest IHI and wastewater); OXA-72 (Bucharest wastewater); OXA-23+OXA-72 (Bucharest wastewater); TEM-1 (Bucharest wastewater); OXA-121 (Targoviste surface water); OXA-120 (Targoviste wastewater). In a study carried out in Croatia on *A. baumannii* isolates recovered from wastewater samples, the carbapenem-resistant isolates were positive for $bla_{\text{OXA-23}}$ gene and belonged similarly to our obtained results to IC2 and the susceptible ones to IC5. Furthermore, these isolates revealed resistance genes encoding for chloramphenicol, aminoglycosides and tetracycline antibiotics [52]. In another study, in eastern Poland using conventional methods and metagenomic assays were demonstrated the presence of *Acinetobacter* spp. and *A. baumannii* isolates carrying MBL (VIM2, NDM and IMP-1)) and class D β-lactamases (OXA-23, OXA-24, OXA-51, OXA-58) in wastewater and river water samples collected in June and September in 2019. High frequency of isolation of *A. baumannii* in IHI, positive for OXA-23 CP and belonging to ST2, was described also in two Bulgarian hospitals, Romania's neighboring country. The CHLDs linked to IC2 were reported also in clinical *A. baumannii* in other neighboring countries of Romania: OXA-23 and OXA-72 in Serbia; and OXA-23 in Albania, OXA-23, OXA-58, and OXA-72 CPs in Croatia, Serbia, Bosnia and Herzegovina.

Molecular typing of *A. baumannii* isolates revealed the presence of four distinct clusters: the ST2 cluster was found in isolates from Bucharest, indicating a localized prevalence in this region, while the cluster containing the ST10 clone was primarily associated with isolates from Targoviște, suggesting a regional specificity for this sequence type. *A. baumannii* ST10 has been found in clinical and community acquired infections globally, including USA [53], Vietnam [54], Iran [55], Australia [56], Belgium [57] and Germany [58]. Moreover, pangenome analysis demonstrated that the genomes of *A. baumannii* are open, as indicated by Heaps' law ($\gamma = 0.26$). An open genome indicates that the gene pool of a isolate has not reached an upper limit, thus allowing the acquisition of new genes through transposable elements [59]. This finding was corroborated by Gherghe-Barbu and collaborators, who used the same tool (e.g., Seth Commichaux's Python script) to analyze pangenome of *A. baumannii* isolated from WW and clinical samples in Targoviste and Ramnicu Valcea, where the value was $\gamma = 0.41$ [60].

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The low abundance of *Alphaproteobacteria* and *Actinobacteria* in both Targoviste and Glina, Bucharest samples contrasts with the typical composition of aquatic microbiota [61]. This deviation could be indicative of specific environmental pressures or contamination events affecting these communities. Factors such as pollution, nutrient loads, or other anthropogenic activities could be influencing the microbial balance.

The dominance of certain bacterial orders in UP *versus* DO samples highlights the impact of local environmental conditions and potential sources of contamination. The presence of pathogenic bacteria like *Campylobacteraceae* in both locations underscores public health concerns, especially regarding the use of these water bodies for recreational or agricultural purposes.

The elevated levels of *Enterobacteriaceae* and other human-associated bacteria in DO samples suggest fecal contamination, likely from sewage discharge or runoff from agricultural lands. The presence of pathogenic bacteria like *Campylobacteraceae* indicates a risk of waterborne diseases, necessitating stringent water quality monitoring and management strategies [62].

The combined analysis of 16S rRNA metagenomic data and chromogenic culture media findings underscores the significant presence of *Acinetobacter* in surface water samples from DO region of Glina, Bucharest and Targoviste WWTPs. This high prevalence is associated with elevated microbial loads and significant resistance phenotypes (CARBA, ESBL, and colistin), especially in the receiving river from DO regions. These insights are important for developing strategies to monitor and mitigate the spread of ARB in the environment, ensuring public health safety and effective wastewater treatment practices.

Limitations of this study may arise from the fact that samples were collected at a single point in time, which does not account for seasonal or temporal variations in microbial load and resistance patterns, that could affect the generalizability of the findings, as well as the fact that the study did not extensively analyze environmental factors such as water temperature, pH, or nutrient levels, which could influence the microbial communities and antibiotic resistance patterns.

5. Conclusions

The findings further underscore WWTPs as reservoirs for MDR *A. baumannii*, highlighting the potential for environmental dissemination and public health risks. Deviations in microbial community composition suggest specific environmental pressures, necessitating stringent water quality monitoring and integrated surveillance strategies to mitigate public health risks associated with fecal contamination and pathogenic bacteria.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: *A. baumannii* isolates from Bucharest wastewater, surface water and intra-hospital infection samples; Table S2: *A. baumannii* isolates from Targoviste wastewater and surface water samples; Table S3: CFU/100 mL in surface water samples; Table 4: CFU/100 mL in wastewater samples; Table 5: Antimicrobial resistance profiles of *A. baumannii* isolated from Bucharest WWTP and Fundeni Hospital in 2022; Table S6: Antimicrobial resistance profiles of *A. baumannii* isolated from Târgoviște WWTP in 2022; Table S7: Genetic support for AR of *A. baumannii* isolated from Bucharest WWTP and Fundeni Hospital in 2022; Table S8: Genetic support for the AR of *A. baumanii* isolated from Târgoviște WWTP în 2022; Table S9: ARGs profiles for *A. baumannii* isolated from WWTPs and Fundeni Hospital in Romania; Table S10: Virulence factors profiles for *A. baumannii* isolated intra-hospital infections and WWTPs in Romania.

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Data Availability Statement: *A. baumannii* isolates and data sets regarding the results are available from the authors.

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

References

1. Drane, K.; Sheehan, M.; Whelan, A.; Ariel, E.; Kinobe, R. The Role of Wastewater Treatment Plants in Dissemination of Antibiotic Resistance: Source, Measurement, Removal and Risk Assessment. *Antibiotics* **2024**, *13* (7). https://doi.org/10.3390/antibiotics13070668.
2. Calistri, P.; Iannetti, S.; L. Danzetta, M.; Narcisi, V.; Cito, F.; Di Sabatino, D.; Bruno, R.; Sauro, F.; Atzeni, M.; Carvelli, A.; Giovannini, A. The Components of "One World - One Health" Approach. *Transboundary and Emerging Diseases*. November 2013, pp 4–13. https://doi.org/10.1111/tbed.12145.
3. Amos, G. C. A.; Ploumakis, S.; Zhang, L.; Hawkey, P. M.; Gaze, W. H.; Wellington, E. M. H. The Widespread Dissemination of Integrons throughout Bacterial Communities in a Riverine System. *ISME Journal* **2018**, *12* (3), 681–691. https://doi.org/10.1038/s41396-017-0030-8.
4. Pérez-Valera, E.; de Melo Rangel, W.; Elhottová, D. Cattle Manure Application Triggers Short-Term Dominance of *Acinetobacter* in Soil Microbial Communities. *Applied Soil Ecology* **2022**, *176*, 104466. https://doi.org/https://doi.org/10.1016/j.apsoil.2022.104466.
5. Poirel, L.; Naas, T.; Nordmann, P. Diversity, Epidemiology, and Genetics of Class D β-Lactamases. *Antimicrob Agents Chemother* **2010**, *54* (1), 24–38. https://doi.org/10.1128/aac.01512-08.
6. Blanco, N.; Harris, A. D.; Rock, C.; Johnson, J. K.; Pineles, L.; Bonomo, R. A.; Srinivasan, A.; Pettigrew, M. M.; Thom, K. A. Risk Factors and Outcomes Associated with Multidrug-Resistant *Acinetobacter baumannii* upon Intensive Care Unit Admission. *Antimicrob Agents Chemother* **2018**, *62* (1), 10.1128/aac.01631-17. https://doi.org/10.1128/aac.01631-17.
7. Gheorghe, I.; Cristea, V. C.; Marutescu, L.; Popa, M.; Murariu, C.; Trusca, B. S.; Borcan, E.; Ghita, C.; Lazar, V.; Chifiriuc, M. C. Resistance and Virulence Features in Carbapenem-Resistant *Acinetobacter* baumannii Community Acquired and Nosocomial Isolates in Romania. https://doi.org/10.37358/RC.19.10.7584.
8. Tooke, C. L.; Hinchliffe, P.; Bragginton, E. C.; Colenso, C. K.; Hirvonen, V. H. A.; Takebayashi, Y.; Spencer, J. β-Lactamases and β-Lactamase Inhibitors in the 21st Century. *J Mol Biol* **2019**, *431* (18), 3472–3500. https://doi.org/https://doi.org/10.1016/j.jmb.2019.04.002.
9. Nigro, S. J.; Hall, R. M. Structure and Context of *Acinetobacter* Transposons Carrying the Oxa23 Carbapenemase Gene. *Journal of Antimicrobial Chemotherapy* **2016**, *71* (5), 1135–1147. https://doi.org/10.1093/jac/dkv440.
10. Philippon, A.; Slama, P.; Dény, P.; Labia, R. A Structure-Based Classification of Class A β-Lactamases, a Broadly Diverse Family of Enzymes. *Clin Microbiol Rev* **2016**, *29* (1), 29–57. https://doi.org/10.1128/cmr.00019-15.
11. Bouza, A. A.; Swanson, H. C.; Smolen, K. A.; VanDine, A. L.; Taracila, M. A.; Romagnoli, C.; Caselli, E.; Prati, F.; Bonomo, R. A.; Powers, R. A.; Wallar, B. J. Structure-Based Analysis of Boronic Acids as Inhibitors of *Acinetobacter*-Derived Cephalosporinase-7, a Unique Class C β-Lactamase. *ACS Infect Dis* **2018**, *4* (3), 325–336. https://doi.org/10.1021/acsinfecdis.7b00152.
12. Vrancianu, C. O.; Pelcaru, C. F.; Alistar, A.; Gheorghe, I.; Marutescu, L.; Popa, M.; Czobor, I.; Gradisteanu, G.; Dobre, E. G.; Chifiriuc, M. C. Escaping from ESKAPE. Clinical Significance and Antibiotic Resistance Mechanisms in *Acinetobacter baumannii*: A Review. *Biointerface Research in Applied Chemistry*. AMG Transcend Association January 1, 2021, pp 8190–8203. https://doi.org/10.33263/BRIAC111.81908203.
13. Chen, Y.; Gao, J.; Zhang, H.; Ying, C. Spread of the *bla*$_{OXA-23}$-Containing Tn*2008* in Carbapenem-Resistant *Acinetobacter baumannii* Isolates Grouped in CC92 from China. *Front Microbiol* **2017**, *8*. https://doi.org/10.3389/fmicb.2017.00163.
14. Naderi, G.; Talebi, M.; Gheybizadeh, R.; Seifi, A.; Ghourchian, S.; Rahbar, M.; Abdollahi, A.; Naseri, A.; Eslami, P.; Douraghi, M. Mobile Genetic Elements Carrying Aminoglycoside Resistance Genes in *Acinetobacter baumannii* Isolates Belonging to Global Clone 2. *Front Microbiol* **2023**, *14*. https://doi.org/10.3389/fmicb.2023.1172861.
15. Fournier, P.-E.; Vallenet, D.; Barbe, V.; Audic, S.; Ogata, H.; Poirel, L.; Richet, H.; Robert, C.; Mangenot, S.; Abergel, C.; Nordmann, P.; Weissenbach, J.; Raoult, D.; Claverie, J.-M. Comparative Genomics of Multidrug Resistance in *Acinetobacter baumannii*. *PLoS Genet* **2006**, *2* (1), e7-.
16. Barbu, I. C.; Gheorghe-Barbu, I.; Grigore, G. A.; Vrancianu, C. O.; Chifiriuc, M. C. Antimicrobial Resistance in Romania: Updates on Gram-Negative ESCAPE Pathogens in the Clinical, Veterinary, and Aquatic Sectors. *Int J Mol Sci* **2023**, *24* (9). https://doi.org/10.3390/ijms24097892.
17. Rosenberg Goldstein, R. E.; Micallef, S. A.; Gibbs, S. G.; George, A.; Claye, E.; Sapkota, A.; Joseph, S. W.; Sapkota, A. R. Detection of Vancomycin-Resistant Enterococci (VRE) at Four U.S. Wastewater Treatment

Plants That Provide Effluent for Reuse. *Science of The Total Environment* **2014**, *466–467*, 404–411. https://doi.org/https://doi.org/10.1016/j.scitotenv.2013.07.039.
18. https://atlas.ecdc.europa.eu/public/index.aspx (Accessed 15 July 2024).
19. CLSI, Performance Standards for Antimicrobial Susceptibility Testing. 32nd Ed. CLSI Supplement M100, Clinical Laboratory Standard Institute, 2022.
20. Gheorghe-Barbu, I.; Corbu, V. M.; Vrancianu, C. O.; Marinas, I. C.; Popa, M.; Dumbravă, A. Ștefania; Niță-Lazăr, M.; Pecete, I.; Muntean, A. A.; Popa, M. I.; Marinescu, L.; Ficai, D.; Ficai, A.; Czobor Barbu, I. Phenotypic and Genotypic Characterization of Recently Isolated Multidrug-Resistant *Acinetobacter baumannii* Clinical and Aquatic Isolates and Demonstration of Silver Nanoparticle Potency. *Microorganisms* **2023**, *11* (10). https://doi.org/10.3390/microorganisms11102439.
21. https://github.com/tseemann/shovill (Accessed 2 July 2024).
22. https://github.com/tseemann/abricate (Accessed 2 July 2024).
23. Chen, L.; Zheng, D.; Liu, B.; Yang, J.; Jin, Q. VFDB 2016: Hierarchical and Refined Dataset for Big Data Analysis—10 Years On. *Nucleic Acids Res* **2016**, *44* (D1), D694–D697. https://doi.org/10.1093/nar/gkv1239.
24. T. Seemann, Mlst (v2.19.0), 2024. Github, https://github.com/tseemann/mlst, (Accessed 1 July 2024).
25. Seemann, T. Prokka: Rapid Prokaryotic Genome Annotation. *Bioinformatics* **2014**, *30* (14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
26. Page, A. J.; Cummins, C. A.; Hunt, M.; Wong, V. K.; Reuter, S.; Holden, M. T. G.; Fookes, M.; Falush, D.; Keane, J. A.; Parkhill, J. Roary: Rapid Large-Scale Prokaryote Pan Genome Analysis. *Bioinformatics* **2015**, *31* (22), 3691–3693. https://doi.org/10.1093/bioinformatics/btv421.
27. Hadfield, J.; Croucher, N. J.; Goater, R. J.; Abudahab, K.; Aanensen, D. M.; Harris, S. R. Phandango: An Interactive Viewer for Bacterial Population Genomics. *Bioinformatics* **2018**, *34* (2), 292–293. https://doi.org/10.1093/bioinformatics/btx610.
28. https://drpowell.github.io/FriPan/ (Accessed 12 July 2024).
29. https://github.com/kwongj/roary2fripan (Accessed 12 July 2024).
30. https://github.com/SethCommichaux/Heap_Law_for_Roary (Accessed 3 July 2024).
31. Novović, K.; Jovčić, B. Colistin Resistance in *Acinetobacter baumannii*: Molecular Mechanisms and Epidemiology. *Antibiotics*. MDPI March 1, 2023. https://doi.org/10.3390/antibiotics12030516.
32. Jovcic, B.; Novovic, K.; Dekic, S.; Hrenovic, J. Colistin Resistance in Environmental Isolates of *Acinetobacter baumannii*. *Microbial Drug Resistance* **2021**, *27* (3), 328–336. https://doi.org/10.1089/mdr.2020.0188.
33. Shelenkov, A.; Akimkin, V.; Mikhaylova, Y. International Clones of High Risk of Acinetobacter Baumannii—Definitions, History, Properties and Perspectives. *Microorganisms* **2023**, *11* (8). https://doi.org/10.3390/microorganisms11082115.
34. http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Acinetobacter. (Accessed 1 July 2024).
35. Zaharia, L.; Ioana-toroimac, G.; Cocoş, O.; Ghiţă, F. A.; Mailat, E. Urbanization Effects on the River Systems in the Bucharest City Region (Romania). *Ecosystem Health and Sustainability* **2016**, *2* (11), e01247. https://doi.org/10.1002/ehs2.1247.
36. Manoiu, V.-M. Water Quality Changes in Ialomiţa River under the Influence of Human Settlements and Activities. In *Aerul şi Apa: Componente ale Mediului*; **2017**; Vol. 2017, pp 317–324. https://doi.org/10.24193/AWC2017_40.
37. Ušjak, D.; Novović, K.; Filipić, B.; Kojić, M.; Filipović, N.; Stevanović, M. M.; Milenković, M. T. In Vitro Colistin Susceptibility of Pandrug-resistant *Acinetobacter baumannii* Is Restored in the Presence of Selenium Nanoparticles. *J Appl Microbiol* **2022**, *133* (3), 1197–1206. https://doi.org/10.1111/jam.15638.
38. Cafiso, V.; Stracquadanio, S.; Lo Verde, F.; Gabriele, G.; Mezzatesta, M. L.; Caio, C.; Pigola, G.; Ferro, A.; Stefani, S. Colistin Resistant *Acinetobacter baumannii*: Genomic and Transcriptomic Traits Acquired Under Colistin Therapy. *Front Microbiol* **2019**, *9*. https://doi.org/10.3389/fmicb.2018.03195.
39. Kabic, J.; Novovic, K.; Kekic, D.; Trudic, A.; Opavski, N.; Dimkic, I.; Jovcic, B.; Gajic, I. Comparative Genomics and Molecular Epidemiology of Colistin-Resistant *Acinetobacter baumannii*. *Comput Struct Biotechnol J* **2023**, *21*, 574–585. https://doi.org/https://doi.org/10.1016/j.csbj.2022.12.045.
40. Mustapha, M. M.; Li, B.; Pacey, M. P.; Mettus, R. T.; McElheny, C. L.; Marshall, C. W.; Ernst, R. K.; Cooper, V. S.; Doi, Y. Phylogenomics of Colistin-Susceptible and Resistant XDR *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* **2018**, *73* (11), 2952–2959. https://doi.org/10.1093/jac/dky290.
41. Trebosc, V.; Gartenmann, S.; Tötzl, M.; Lucchini, V.; Schellhorn, B.; Pieren, M.; Lociuro, S.; Gitzinger, M.; Tigges, M.; Bumann, D.; Kemmer, C. Dissecting Colistin Resistance Mechanisms in Extensively Drug-Resistant *Acinetobacter baumannii* Clinical Isolates. *mBio* **2019**, *10* (4), 10.1128/mbio.01083-19. https://doi.org/10.1128/mbio.01083-19.
42. Fam, N. S.; Gamal, D.; Mohamed, S. H.; Wasfy, R. M.; Soliman, M. S.; El-Kholy, A. A.; Higgins, P. G. Molecular Characterization of Carbapenem/ Colistin-Resistant *Acinetobacter baumannii* Clinical Isolates from Egypt by Whole-Genome Sequencing. *Infect Drug Resist* **2020**, *13*, 4487–4493. https://doi.org/10.2147/IDR.S288865.

43. Palmieri, M.; D'Andrea, M. M.; Pelegrin, A. C.; Perrot, N.; Mirande, C.; Blanc, B.; Legakis, N.; Goossens, H.; Rossolini, G. M.; van Belkum, A. Abundance of Colistin-Resistant, OXA-23- and ArmA-Producing *Acinetobacter baumannii* Belonging to International Clone 2 in Greece. *Front Microbiol* **2020**, *11*. https://doi.org/10.3389/fmicb.2020.00668.
44. Thadtapong, N.; Chaturongakul, S.; Soodvilai, S.; Dubbs, P. Colistin and Carbapenem-Resistant *Acinetobacter baumannii* Aci46 in Thailand: Genome Analysis and Antibiotic Resistance Profiling. *Antibiotics* **2021**, *10* (9). https://doi.org/10.3390/antibiotics10091054.
45. Pournaras, S.; Poulou, A.; Dafopoulou, K.; Chabane, Y. N.; Kristo, I.; Makris, D.; Hardouin, J.; Cosette, P.; Tsakris, A.; Dé, E. Growth Retardation, Reduced Invasiveness, and Impaired Colistin-Mediated Cell Death Associated with Colistin Resistance Development in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **2014**, *58* (2), 828–832. https://doi.org/10.1128/aac.01439-13.
46. Agodi, A.; Voulgari, E.; Barchitta, M.; Quattrocchi, A.; Bellocchi, P.; Poulou, A.; Santangelo, C.; Castiglione, G.; Giaquinta, L.; Romeo, M. A.; Vrioni, G.; Tsakris, A. Spread of a Carbapenem- and Colistin-Resistant *Acinetobacter baumannii* ST2 Clonal Isolate Causing Outbreaks in Two Sicilian Hospitals. *Journal of Hospital Infection* **2014**, *86* (4), 260–266. https://doi.org/https://doi.org/10.1016/j.jhin.2014.02.001.
47. Lowe, M.; Singh-Moodley, A.; Ismail, H.; Thomas, T.; Chibabhai, V.; Nana, T.; Lowman, W.; Ismail, A.; Chan, W. Y.; Perovic, O. Molecular Characterisation of *Acinetobacter baumannii* Isolates from Bloodstream Infections in a Tertiary-Level Hospital in South Africa. *Front Microbiol* **2022**, *13*. https://doi.org/10.3389/fmicb.2022.863129.
48. Nogbou, N.-D.; Ramashia, M.; Nkawane, G. M.; Allam, M.; Obi, C. L.; Musyoki, A. M. Whole-Genome Sequencing of a Colistin-Resistant *Acinetobacter baumannii* Isolate Isolated at a Tertiary Health Facility in Pretoria, South Africa. *Antibiotics* **2022**, *11* (5). https://doi.org/10.3390/antibiotics11050594.
49. Blejan, I. E.; Diaconu, C. E.; Arsene, A. L.; Udeanu, D. I.; Ghica, M.; Drăgănescu, D.; Dragomiroiu, G. T. A. B.; Rădulescu, M.; Maltezou, H. C.; Tsatsakis, A. M.; Papasavva, M.; Drakoulis, N.; Popa, D. E. Antibiotic Resistance in Community-Acquired Pneumonia. A Romanian Perspective. *Farmacia* **2020**, *68* (3), 512–520. https://doi.org/10.31925/farmacia.2020.3.17.
50. Goic-Barisic, I.; Hrenovic, J.; Kovacic, A.; Musić, M. Š. Emergence of Oxacillinases in Environmental Carbapenem-Resistant *Acinetobacter baumannii* Associated with Clinical Isolates. *Microbial Drug Resistance* **2016**, *22* (7), 559–563. https://doi.org/10.1089/mdr.2015.0275.
51. Pulami, D.; Kämpfer, P.; Glaeser, S. P. High Diversity of the Emerging Pathogen Acinetobacter Baumannii and Other *Acinetobacter* Spp. in Raw Manure, Biogas Plants Digestates, and Rural and Urban Wastewater Treatment Plants with System Specific Antimicrobial Resistance Profiles. *Science of The Total Environment* **2023**, *859*, 160182. https://doi.org/https://doi.org/10.1016/j.scitotenv.2022.160182.
52. Higgins, P. G.; Hrenovic, J.; Seifert, H.; Dekic, S. Characterization of *Acinetobacter baumannii* from Water and Sludge Line of Secondary Wastewater Treatment Plant. *Water Res* **2018**, *140*, 261–267. https://doi.org/https://doi.org/10.1016/j.watres.2018.04.057.
53. Jones, C. L.; Clancy, M.; Honnold, C.; Singh, S.; Snesrud, E.; Onmus-Leone, F.; McGann, P.; Ong, A. C.; Kwak, Y.; Waterman, P.; Zurawski, D. V; Clifford, R. J.; Lesho, E. Fatal Outbreak of an Emerging Clone of Extensively Drug-Resistant *Acinetobacter baumannii* With Enhanced Virulence. *Clinical Infectious Diseases* **2015**, *61* (2), 145–154. https://doi.org/10.1093/cid/civ225.
54. Schultz, M. B.; Pham Thanh, D.; Tran Do Hoan, N.; Wick, R. R.; Ingle, D. J.; Hawkey, J.; Edwards, D. J.; Kenyon, J. J.; Phu Huong Lan, N.; Campbell, J. I.; Thwaites, G.; Thi Khanh Nhu, N.; Hall, R. M.; Fournier-Level, A.; Baker, S.; Holt, K. E. Repeated Local Emergence of Carbapenem-Resistant *Acinetobacter baumannii* in a Single Hospital Ward. *Microb Genom* **2016**, *2* (3). https://doi.org/https://doi.org/10.1099/mgen.0.000050.
55. Abhari, S. S.; Badmasti, F.; Modiri, L.; Aslani, M. M.; Asmar, M. Circulation of Imipenem-Resistant *Acinetobacter baumannii* ST10, ST2 and ST3 in a University Teaching Hospital from Tehran, Iran. *J Med Microbiol* **2019**, *68* (6), 860—865. https://doi.org/10.1099/jmm.0.000987.
56. Meumann, E. M.; Anstey, N. M.; Currie, B. J.; Piera, K. A.; Kenyon, J. J.; Hall, R. M.; Davis, J. S.; Sarovich, D. S. Genomic Epidemiology of Severe Community-Onset *Acinetobacter baumannii* Infection. *Microb Genom* **2019**, *5* (3). https://doi.org/https://doi.org/10.1099/mgen.0.000258.
57. Valcek, A.; Nesporova, K.; Whiteway, C.; Pooter, T. De; Coster, W. De; Strazisar, M.; der Henst, C. Van. Genomic Analysis of a Isolate Collection Containing Multidrug-, Extensively Drug-, Pandrug-, and Carbapenem-Resistant Modern Clinical Isolates of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **2022**, *66* (9), e00892-22. https://doi.org/10.1128/aac.00892-22.
58. Savin, M.; Sib, E.; Heinemann, C.; Eichel, V. M.; Nurjadi, D.; Klose, M.; Andre Hammerl, J.; Binsker, U.; Mutters, N. T. Tracing Clinically-Relevant Antimicrobial Resistances in *Acinetobacter baumannii* - *calcoaceticus* Complex across Diverse Environments: A Study Spanning Clinical, Livestock, and Wastewater Treatment Settings. *Environ Int* **2024**, *186*, 108603. https://doi.org/https://doi.org/10.1016/j.envint.2024.108603.

59. Park, S. C.; Lee, K.; Kim, Y. O.; Won, S.; Chun, J. Large-Scale Genomics Reveals the Genetic Characteristics of Seven Species and Importance of Phylogenetic Distance for Estimating Pan-Genome Size. *Front Microbiol* **2019**, *10* (APR). https://doi.org/10.3389/fmicb.2019.00834.
60. Gheorghe-Barbu, I.; Surleac, M.; Barbu, I. C.; Paraschiv, S.; Bănică, L. M.; Rotaru, L. I.; Vrâncianu, C. O.; Niță Lazăr, M.; Oțelea, D.; Chifiriuc, M. C. Decoding the Resistome, Virulome and Mobilome of Clinical versus Aquatic *Acinetobacter baumannii* in Southern Romania. *Heliyon* **2024**, *10* (13). https://doi.org/10.1016/j.heliyon.2024.e33372.
61. Sehnal, L.; Brammer-Robbins, E.; Wormington, A. M.; Blaha, L.; Bisesi, J.; Larkin, I.; Martyniuk, C. J.; Simonin, M.; Adamovsky, O. Microbiome Composition and Function in Aquatic Vertebrates: Small Organisms Making Big Impacts on Aquatic Animal Health. *Front Microbiol* **2021**, *12*. https://doi.org/10.3389/fmicb.2021.567408.
62. Shayo, G. M.; Elimbinzi, E.; Shao, G. N.; Fabian, C. Severity of Waterborne Diseases in Developing Countries and the Effectiveness of Ceramic Filters for Improving Water Quality. *Bull Natl Res Cent* **2023**, *47* (1), 113. https://doi.org/10.1186/s42269-023-01088-9.