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Special Issue Reprint

The Spread of Antibiotic Resistance in Natural Environments

Edited by
Daniel Gyamfi Amoako

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Guest Editor

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About the Editor

Daniel Gyamfi Amoako

Daniel Gyamfi Amoako is a researcher in the Department of Pathobiology at the University of Guelph, Canada, and the Antimicrobial Research Unit at the University of KwaZulu-Natal, South Africa. His research interests encompass microbial genomics, bioinformatics, antimicrobial resistance, molecular epidemiology, pathogen evolution, and One Health. He applies genome sequencing and computational approaches to investigate the resistance, virulence, transmission, and evolutionary dynamics of emerging pathogens across human, animal, food, and environmental systems. He has contributed to major international initiatives, including the Africa Pathogen Genomics Initiative, SEQAFRICA, Network for Genomic Surveillance in South Africa, COG-Train, Mobilome Ontology Working Group, Diversity of Antibiotic Resistance genes and Transfer Elements-Quantitative Monitoring (DARTE-QM), and the ECDC–WHO RSV Genotyping Consortium, supporting genomic surveillance, training, and capacity development worldwide. To date, Amoako has co/authored over 130 publications in leading peer-reviewed journals, with over 11000 citations and an H-index of 45. Additionally, he has successfully supervised several postgraduate students, contributing to the advancement of scientific knowledge in his field.

Preface

"What enters the environment does not simply disappear; it may persist, change, and travel." This principle is especially relevant to antimicrobial resistance, which does not remain confined to the hospitals, households, farms, or industries in which antimicrobial agents are used. Resistant bacteria, antibiotic resistance genes, mobile genetic elements, and antimicrobial residues can move through wastewater, rivers, soils, food-production systems, air, and wildlife-associated environments. These interconnected pathways make the environment an active component of antimicrobial resistance emergence and transmission rather than merely the final recipient of human and agricultural waste.

This Reprint, entitled "The Spread of Antibiotic Resistance in Natural Environments", examines the ecological, genomic, and public health dimensions of environmental antimicrobial resistance. It brings together eight contributions from authors affiliated with institutions across four continents. The collection includes original research, reviews, and a conceptual contribution addressing wastewater-treatment systems, urban rivers, aquatic environments, food-production settings, and human–animal–environment interfaces. Collectively, these works examine how pollution sources, treatment processes, microbial communities, mobile genetic elements, virulence determinants, and co-selective pressures influence the persistence and movement of resistance.

A central motivation for assembling this collection is the need to move environmental antimicrobial resistance research beyond detection alone. Identifying a resistance gene establishes its presence but does not, by itself, define its public health significance. Meaningful interpretation also requires knowledge of its microbial host, genetic context, mobility, viability, environmental persistence, and potential routes of exposure. The contributions demonstrate the complementary value of metagenomics, whole-genome sequencing, culture-based investigation, resistance phenotyping, plasmid analysis, and ecological assessment. They also show why treatment performance cannot be judged solely by reductions in bacterial abundance, as viable resistant organisms and transferable resistance determinants may persist or become differently distributed after treatment.

The aim is to provide an integrated account of the environmental pathways through which antimicrobial resistance is maintained, reshaped, and disseminated. Particular attention is given to source-resolved surveillance, standardized methods, robust assessment of treatment systems, and risk frameworks that connect genomic evidence with credible exposure pathways and practical interventions. By bringing together perspectives from microbiology, genomics, environmental science, public health, food safety, and One Health, the collection encourages collaboration among disciplines that have often examined different parts of the same transmission network.

The intended readership includes microbiologists, genomic scientists, environmental researchers, epidemiologists, clinicians, veterinarians, engineers, food-safety professionals, policymakers, students, and early-career researchers. We hope that the evidence and perspectives assembled here will stimulate further investigation, strengthen environmental surveillance, and direct attention toward points where the spread of resistance can be interrupted. The enduring lesson is clear: protecting the effectiveness of antimicrobials requires us to understand not only where resistance originates, but also the environments through which it evolves and moves.

Daniel Gyamfi Amoako
Guest Editor

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Editorial

The Spread of Antibiotic Resistance in Natural Environments

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Antibiotics can be administered to individual patients or animals, but their evolutionary consequences extend across ecosystems. Antimicrobial residues, resistant bacteria, antibiotic resistance genes (ARGs), and mobile genetic elements released from healthcare facilities, households, farms, and pharmaceutical production enter connected environmental pathways that link human, animal, and ecosystem health [1–4]. Wastewater, surface waters, soils, air, food-production systems, and wildlife-associated environments may act as reservoirs and routes of dissemination. Selective pressures from antimicrobials and co-selective agents can favour the persistence and enrichment of resistance, while diverse microbial communities provide opportunities for horizontal gene transfer [1,2]. The environment is therefore more than a recipient of waste: it is an evolutionary setting in which resistance can persist, change, and circulate across the One Health continuum [2–4]. This Special Issue brings together eight contributions from 50 authors across four continents and examines the ecological, genomic, and public health dimensions of environmental AMR.

Shouqair et al. used source-resolved shotgun metagenomics to compare community sewer nodes, hospital outflows, and wastewater-treatment-plant influent and effluent in Dubai. Hospital wastewater was enriched in clinically important class D beta-lactamases, whereas community wastewater and plant influent shared prominent macrolide- and aminoglycoside-resistance genes. Treatment reduced bacterial diversity, but ARG diversity was not significantly lower in effluent, and resistance determinants remained detectable. Virulence functions also shifted toward traits associated with biofilm formation and persistence. These findings show why plant influent alone cannot represent the distinct risks carried by hospital and community wastewater.

Park et al. complemented this metagenomic evidence with a culture-based analysis of 804 Enterococcus isolates from the influent and effluent of 11 wastewater-treatment plants in South Korea. Overall, resistance and biofilm formation did not differ significantly between stages, but individual phenotypes and genes followed different trajectories. Resistance to ampicillin, ciprofloxacin, and gentamicin, combined with *tetM* and *qnrS*, were increased in effluent, whereas other resistance phenotypes and optrA declined. Treatment performance therefore cannot be inferred from a single aggregate measure or from microbial reduction alone.

Mendoza-Guido et al. examined enterobacteria along a pollution gradient in a tropical river. Isolates from the most contaminated site carried more ARGs and plasmids and exhibited broader resistance. One *Escherichia coli* isolate was resistant to seven antibiotics and harboured a plasmid containing 12 ARGs that was closely related to an *IncH* plasmid found in *Salmonella enterica*. The study shows how phenotyping, environmental gradients, whole-genome sequencing, and plasmid analysis can jointly reveal pathways of transferable resistance.

Nnah et al. assessed the virulomes of multidrug-resistant *E. coli* groups representing human, animal, and environmental sources. The three sectors shared a substantial core virulome containing determinants for adhesion, serum resistance, iron acquisition, and stress adaptation. Environmental groups contained the most extraintestinal pathogenic *E. coli*-associated determinants, whereas diarrhoeagenic markers were most frequent in animal groups. The consistently high predicted pathogenicity indicates that resistance surveillance should include virulence monitoring. Isolate-resolved and long-read analyses are now needed to determine whether resistance, virulence, and mobility determinants occur in the same organisms or genetic elements.

The three reviews place these findings within broader environmental pathways. Sassi et al. examine water, soil, and air as reservoirs and transmission routes, emphasizing pharmaceutical discharge, agricultural runoff, sewage, airborne emissions, biofilms, and horizontal gene transfer. They also examine metals and disinfectants as co-selective agents and microplastics as surfaces that may promote persistence and gene exchange. Meradji et al. focus on water systems, tracing connections created by hospital and municipal effluent, stormwater, agricultural runoff, water reuse, and irrigation return flows. Ifedinezi et al. link environmental AMR to food safety and public health and emphasize stewardship, surveillance, regulation, and One Health coordination. Together, the reviews locate AMR control within pollution prevention, sanitation, water treatment and reuse, agriculture, and food-chain protection.

Yuan et al. extend the discussion through the One Earth–One Health framework. Their communication places AMR within ecosystem self-regulation and reconstruction and proposes a dual-mutation concept and mathematical model that combine evolutionary and niche-construction perspectives. The framework recognizes that resistance predates modern antibiotic use and argues that control measures should account for ecological processes that generate, disseminate, and remove resistant organisms and genes. Its proposed mechanisms remain to be tested empirically, but the contribution broadens the discussion beyond the detection of individual determinants.

Several priorities emerge when the contributions are considered together. Environmental surveillance should be organized around transmission pathways, using source-resolved, longitudinal sampling to determine where resistance enters, persists, or becomes enriched. Harmonized metadata, quality controls, analytical pipelines, and reporting standards are essential for comparing these patterns across sites and over time. Treatment performance must then be judged by risk reduction rather than by bacterial removal or gene detection alone. This requires measurements of absolute abundance and viability alongside community composition, resistance phenotypes, and mobile genetic elements. The analytical methods should serve complementary purposes: metagenomics defines resistome diversity; culture and phenotyping confirm viable resistance; long-read sequencing and plasmid reconstruction link determinants to their hosts and genetic contexts; and transfer experiments test their mobility. Parallel measurements of antibiotics, metals, biocides, and other contaminants can help distinguish selection pressure from simple co-occurrence with pollution. These findings should inform risk assessments that integrate pathogenicity, mobility, environmental persistence, exposure route and dose, and available treatment options. This evidence chain can establish whether interventions reduce viable resistant organisms and transferable ARGs under operational conditions. Building it requires coordination across health, agriculture, environmental management, engineering, food safety, and regulation.

Overall, the environment emerges as a major component of AMR transmission. The central task is to connect source attribution and genomic evidence with treatment performance and credible exposure pathways, enabling surveillance to identify where transmission can be interrupted rather than simply where resistance is present. Embed-

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ding this evidence within One Health policy is essential to protect human, animal, and ecosystem health.

Conflicts of Interest: The author declares no conflicts of interest.

List of Contributions

1. Shouqair, D.; Verma, S.; Alghafri, R.; Nassar, R.; Mohamed, L.; Al Dhaheri, F.; Everett, D.; Shibl, A.A.; Rodríguez, J.; Moradigaravand, D.; Khan, M.; Goering, R.; Senok, A. Source-Resolved Wastewater Metagenomics Reveals Distinct Resistome and Virulome Landscapes Across an Urban Wastewater Continuum. *Antibiotics* **2026**, *15*, 817. https://doi.org/10.3390/antibiotics15090817.
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Article

Source-Resolved Wastewater Metagenomics Reveals Distinct Resistome and Virulome Landscapes Across an Urban Wastewater Continuum

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Abstract

Background/Objectives: Wastewater-based antimicrobial resistance (AMR) surveillance typically relies on treatment plant influent as a single integrated matrix, obscuring source-specific signals. In arid settings where treated effluent is reused, understanding how resistomes and virulomes are structured across wastewater compartments is essential for One Health monitoring. Methods: Shotgun metagenomic sequencing was applied to 57 wastewater samples collected in Dubai, United Arab Emirates, between October 2024 and January 2025. Samples represented nine community sewer nodes, two tertiary-care hospital outflows, and influent and effluent from two wastewater treatment plants (WWTP). Datasets were used for taxonomic, resistome, and virulome profiling. Alpha diversity was compared using Wilcoxon rank-sum tests, beta-diversity differences were assessed using permutational multivariate analysis of variance, and source-associated AMR genes were identified using linear discriminant analysis effect size analysis. Results: A total of 1470 bacterial species, 822 antimicrobial resistance genes (ARGs), and 1554 virulence factor genes

were identified. Bacterial diversity was significantly lower in WWTP effluent than in other compartments. Hospital wastewater was enriched for class D β-lactamases, including multiple bla_{OXA} variants, whereas community wastewater and WWTP influent shared dominant macrolide and aminoglycoside resistance genes including *msr*(*E*), *mph*(*E*), *strB* and *aadA1*. Despite marked reductions in bacterial diversity after treatment, no significant difference in ARG diversity was observed between WWTP influent and WWTP effluent ($p = 0.558$), with resistance genes such as bla_{VEB}, *msr*(*E*), *mph*(*E*) detected in the latter. Virulome profiles shifted from fimbrial gene dominance in untreated sources toward biofilm- and persistence-associated genes in WWTP effluent. ARG alpha diversity varied over time, whereas taxonomic and virulome diversity remained stable. Conclusions: Community and influent wastewater capture population-level AMR carriage, hospital outflows concentrate clinically relevant resistance determinants, and WWTP effluent retains resistance markers despite microbial biomass reduction. Compartment-resolved metagenomic surveillance provides a practical One Health framework for identifying high-value monitoring points.

Keywords: antimicrobial resistance; wastewater; shotgun metagenomics; One Health; WWTP; resistome; virulome

1. Introduction

Antimicrobial resistance (AMR) is one of the defining health threats of the twenty-first century, with an estimated 1.27 million deaths attributed to bacterial AMR globally in 2019 [1]. However, in most settings, surveillance remains largely organized around clinical detection, even though AMR is sustained by connected human and environmental systems [2]. Across these interconnected niches, resistant microorganisms, antimicrobial resistance genes (ARGs), virulence factor genes (VFGs) and mobile genetic elements persist and spread [3,4]. In this context, wastewater-based surveillance has become an important approach for tracking population-level dynamics of infectious diseases [5]. Urban wastewater pools microbial and genetic material from households, healthcare facilities and the wider community into a single readout, while functioning as a One Health interface for environmental AMR surveillance [6].

Within wastewater systems, ARGs are not only detected as isolated markers but form part of a broader resistome that may be maintained and disseminated through the wastewater mobilome [7,8]. Mobile genetic elements including plasmids, integrons, transposons, insertion sequences and phage-associated elements, can facilitate horizontal gene transfer between bacterial hosts [9,10]. The high microbial density and diversity of wastewater, together with residual antimicrobials, disinfectants, metals and other selective pressures, may therefore support the persistence and movement of clinically relevant resistance determinants. This makes wastewater an important environmental reservoir and potential transmission hub for AMR, in addition to its role as a surveillance matrix [7,8].

However, urban wastewater is not a homogeneous surveillance matrix. It includes community wastewater, which reflects population-level microbial carriage and antimicrobial exposure, and hospital wastewater, which concentrates clinically relevant organisms and resistance genes under sustained antimicrobial selection [11]. At wastewater treatment plants (WWTPs), these streams converge with broader city-scale inputs, diluting source-specific signals while generating a biologically complex influent. Treatment at WWTPs reduces microbial biomass and restructures community composition but can retain, enrich or selectively filter ARGs and VFGs in treated effluent [12,13]. This is consequential in arid and rapidly urbanizing regions, where WWTP effluent is increasingly reused for

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irrigation, landscaping and other non-potable applications [14]. In these settings, WWTP effluent is not only the endpoint of treatment; it may represent a route through which residual microbial communities, resistance determinants, and virulence-associated genes are reintroduced into the environment [15]. Despite this relevance, there remains a paucity of data on wastewater-based AMR surveillance in the Arabian Gulf region where rapid urbanization, high population mobility and expanding water reuse make it a distinctive setting for environmental AMR monitoring [16].

Analytical approaches for wastewater-based AMR surveillance include targeted molecular assays and untargeted sequencing approaches [17]. Targeted methods such as quantitative PCR are sensitive, relatively low-cost and effective for monitoring predefined organisms or resistance determinants, but they are constrained by prior target selection and provide limited insight into the broader microbial and functional context of complex wastewater communities [18]. However, shotgun metagenomics sequencing provides a hypothesis-independent, agnostic approach for gene-level profiling, enabling characterization of microbial community structure, ARGs, and VFGs in wastewater samples [19]. This broader resolution is particularly suited to compartment-resolved surveillance, enabling comparison of microbial communities, resistomes and virulomes across wastewater sources.

In this study, we applied shotgun metagenomic sequencing to wastewater sampled from community sewer nodes, tertiary-care hospital outflows, and WWTP influent and effluent in Dubai, United Arab Emirates (UAE), to characterize how microbial communities, resistomes, and virulomes are structured across distinct compartments of an arid urban system. By integrating taxonomic, ARG and VFG profiling, this approach supports a compartment-resolved interpretation of wastewater-based AMR surveillance.

2. Results

A total of 60 wastewater samples comprising 36 community wastewater samples from nine sewer nodes, eight hospital wastewater samples from two tertiary-care hospitals, eight WWTP influent samples and eight WWTP effluent samples were obtained. Three WWTP effluent samples were excluded due to insufficient DNA yield for reliable shotgun metagenomic sequencing, hence 57 metagenomes were included in the analysis. Median sequencing depth was 232.63 million paired-end reads (IQR: 174.62–306.15 million); ranging from: 11.11 to 560.55 million reads.

Microbial community composition: Taxonomic profiling identified 1470 bacterial species across all wastewater samples. Cumulative species richness was highest in community wastewater (1135 species), followed by hospital (851), WWTP influent (646) and WWTP effluent (387). Median species richness per sample was 392.5 (interquartile range (IQR) 326.5–442) in hospital wastewater, 353 (286–397) in community wastewater, 258.5 (205–352.5) in WWTP influent and 142 (84.5–254.5) in WWTP effluent. Across all sources, 246 species were shared among the four groups (Figure 1).

Wastewater communities were dominated by Actinomycetota, Pseudomonadota, Bacillota, and Bacteroidota. In community wastewater, there was a predominance of *Bifidobacterium adolescentis* (11.92%) and *B. longum* (6.06%), with additional contributions from *Bifidobacterium* sp. 002742445 (3.53%) and *Aliarcobacter cryaerophilus_A* (2.38%). Hospital wastewater exhibited a distinct profile driven by *Neisseria suis* (6.84%), alongside *B. adolescentis* (5.26%) and *B. longum* (2.98%). WWTP influent closely resembled the community composition, with *B. adolescentis* (13.67%) and *B. longum* (5.64%) remaining dominant alongside *A. cryaerophilus_A* (3.71%). In contrast, WWTP effluent showed a shift in dominant taxa, characterized by *Ochrobactrum pseudintermedium* (9.48%), with contributions from *B. adolescentis* (8.23%) and *Mycobacterium fortuitum* (5.69%) (Figure 2). Several clinically relevant organisms were detected across wastewater sources and species-level relative

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abundances are provided in shown in Supplementary Figures S1 and S2. Differences in *Enterococcus faecium* and *Enterobacter* spp. were not statistically significant between compartments. In contrast, *Escherichia coli* was significantly enriched in WWTP effluent, whereas *Streptococcus* spp. showed higher relative abundances in community and hospital wastewater (Supplementary Figure S1).

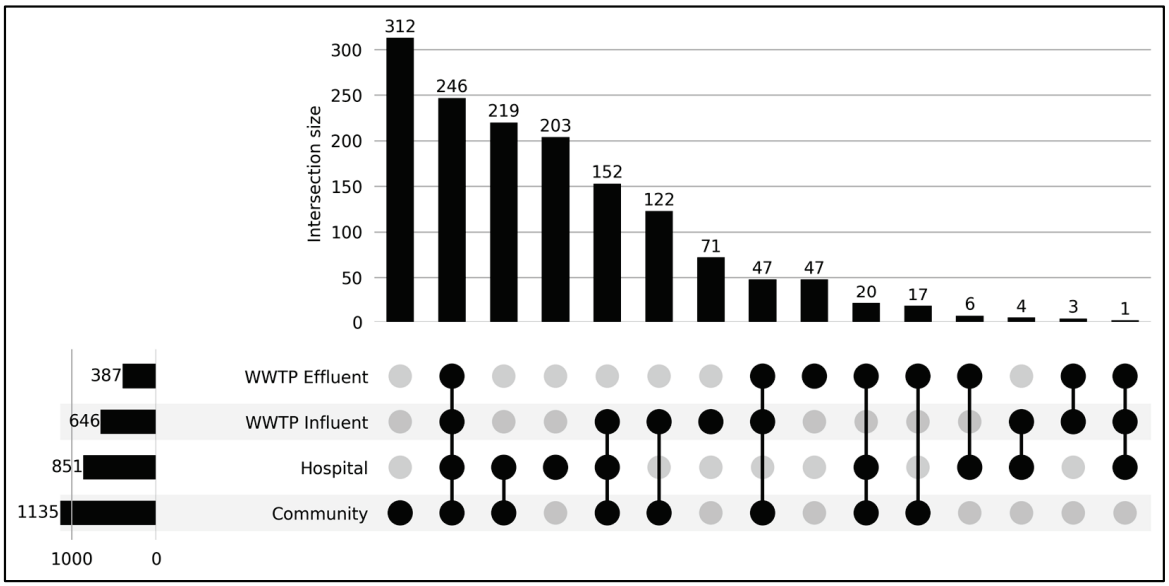

Figure 1. Distribution of bacterial species across wastewater sources. Black circles indicate sources included in each intersection, grey circles indicate sources not included, and connecting lines denote shared intersections.

Alpha diversity of bacterial communities varied across wastewater sources (Shannon index 3.74–4.80; Figure 3), with the highest values in hospital samples, followed by community and WWTP influent. Differences between these three sources were not statistically significant. WWTP effluent showed the lowest diversity, significantly lower than that of all other sources.

Principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarities showed source-specific separation, with PC1 and PC2 explaining 14.74% and 13.51% of the variance (Figure 4). Hospital samples clustered separately, whereas community and WWTP influent samples partially overlapped; wastewater effluent samples were more dispersed with limited overlap. Permutational multivariate analysis of variance (PERMANOVA) confirmed a significant cohort effect ($p = 0.001$).

Antimicrobial resistance genes (ARGs): Resistome profiling across the metagenomes identified 822 ARGs, with the highest number detected in community wastewater ($n = 716$), followed by hospital wastewater ($n = 436$), WWTP influent ($n = 404$) and WWTP effluent ($n = 326$) (Figure 5). ARGs spanning multiple antimicrobial classes were detected across all sources, including macrolide–lincosamide–streptogramin B (MLSB), aminoglycosides, β-lactams, tetracyclines and sulfonamides resistance genes. Figure 5 shows ARGs and antibiotic classes with ARGs across wastewater sources.

Community wastewater was dominated by macrolide and aminoglycoside resistance genes, with *msr*(*E*) and *mph*(*E*) as the most abundant markers, followed by *strB* and *aadA1* with median relative abundances (IQR) of 9.62 (7.46–11.60), 8.80 (6.66–11.04), 3.04 (2.67–3.98) and 2.22 (1.79–2.54), respectively. A similar profile was observed in WWTP influent, where *msr*(*E*) and *mph*(*E*) remained dominant, with median relative abundances (IQR) of 8.97 (6.19–9.72) and 8.56 (5.64–9.10), respectively, alongside increased contributions from *strB* [3.75 (3.12–6.15)] and *aadA1* [2.93 (2.59–3.45)], and persistent detection of

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bla_{LCR} [2.02 (1.45–2.22)]. In contrast, hospital wastewater exhibited a distinct resistome characterized by significant enrichment of class D β-lactamases, which reached 32.63% relative abundance and were significantly higher than in community wastewater, WWTP influent, and WWTP effluent ($p < 0.001$, $p < 0.01$, and $p < 0.01$, respectively). Detected variants included $bla_{OXA-233}$, bla_{OXA-16}, $bla_{OXA-454}$, $bla_{OXA-368}$, and $bla_{OXA-251}$.

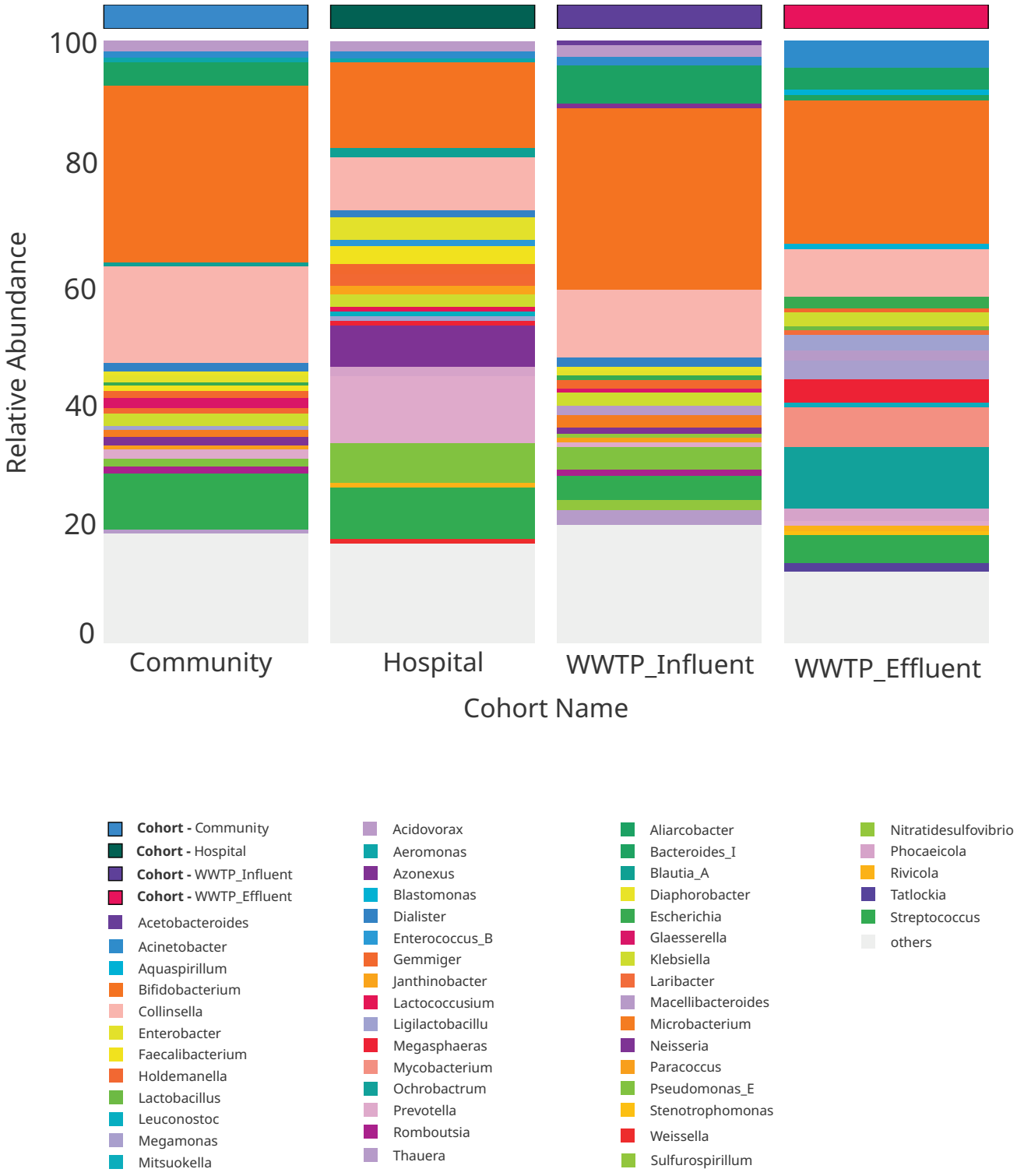

Figure 2. Genus-level taxonomic composition of the top 25 genera across wastewater sources.

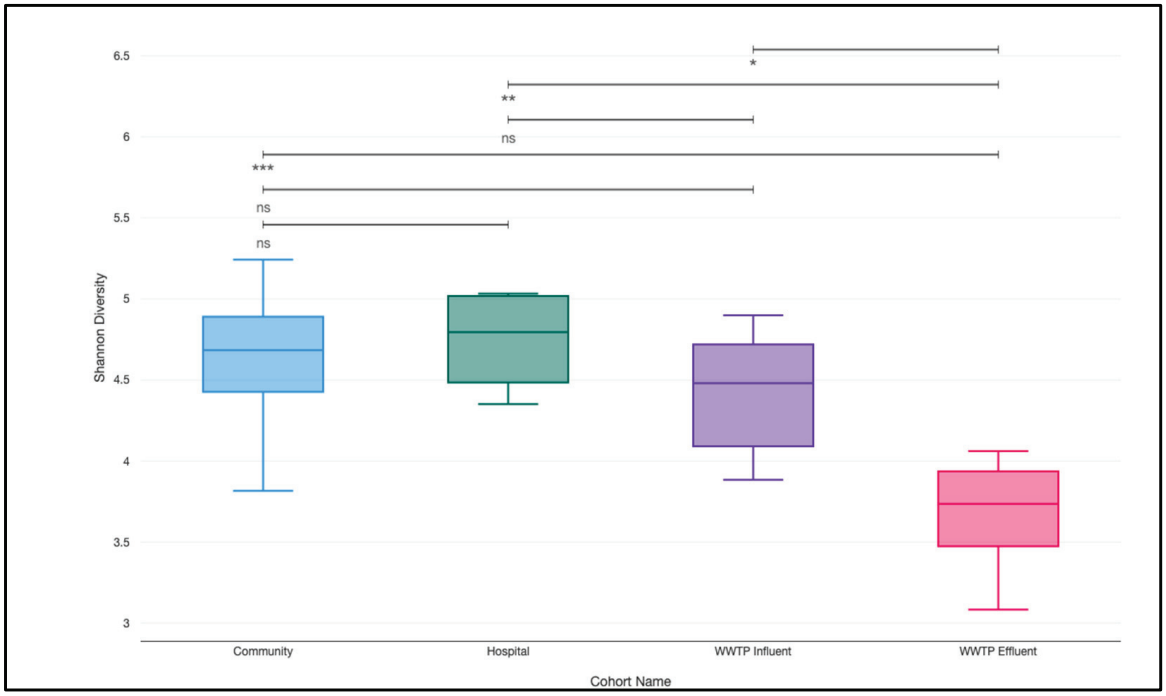

Figure 3. Alpha diversity of bacterial communities across cohorts measured by the Shannon index. Boxplots show the distribution of diversity in community, hospital, WWTP influent and WWTP effluent samples. Significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns indicates a non-significant difference.

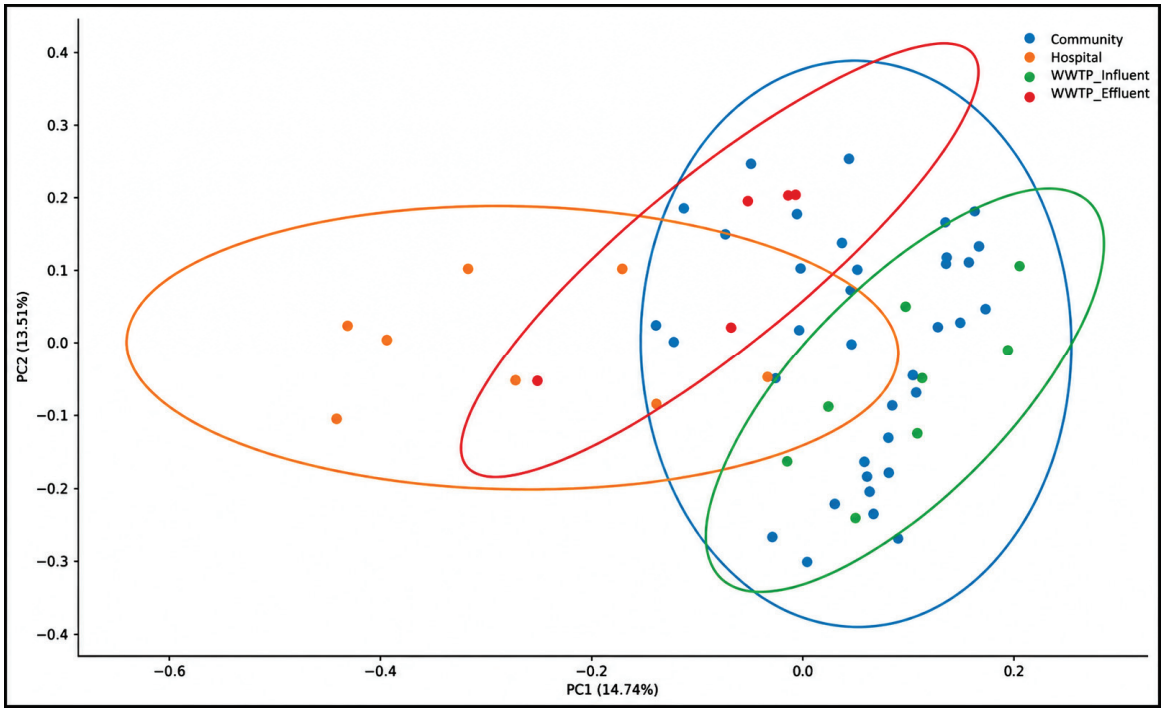

Figure 4. PCoA of Bray–Curtis dissimilarities of bacterial species based on relative abundance. Samples are coloured by cohort (community, hospital, WWTP influent and WWTP effluent). The first two principal coordinates explain 14.74% (PC1) and 13.51% (PC2) of the variance. Ellipses represent 95% confidence intervals around group centroids.

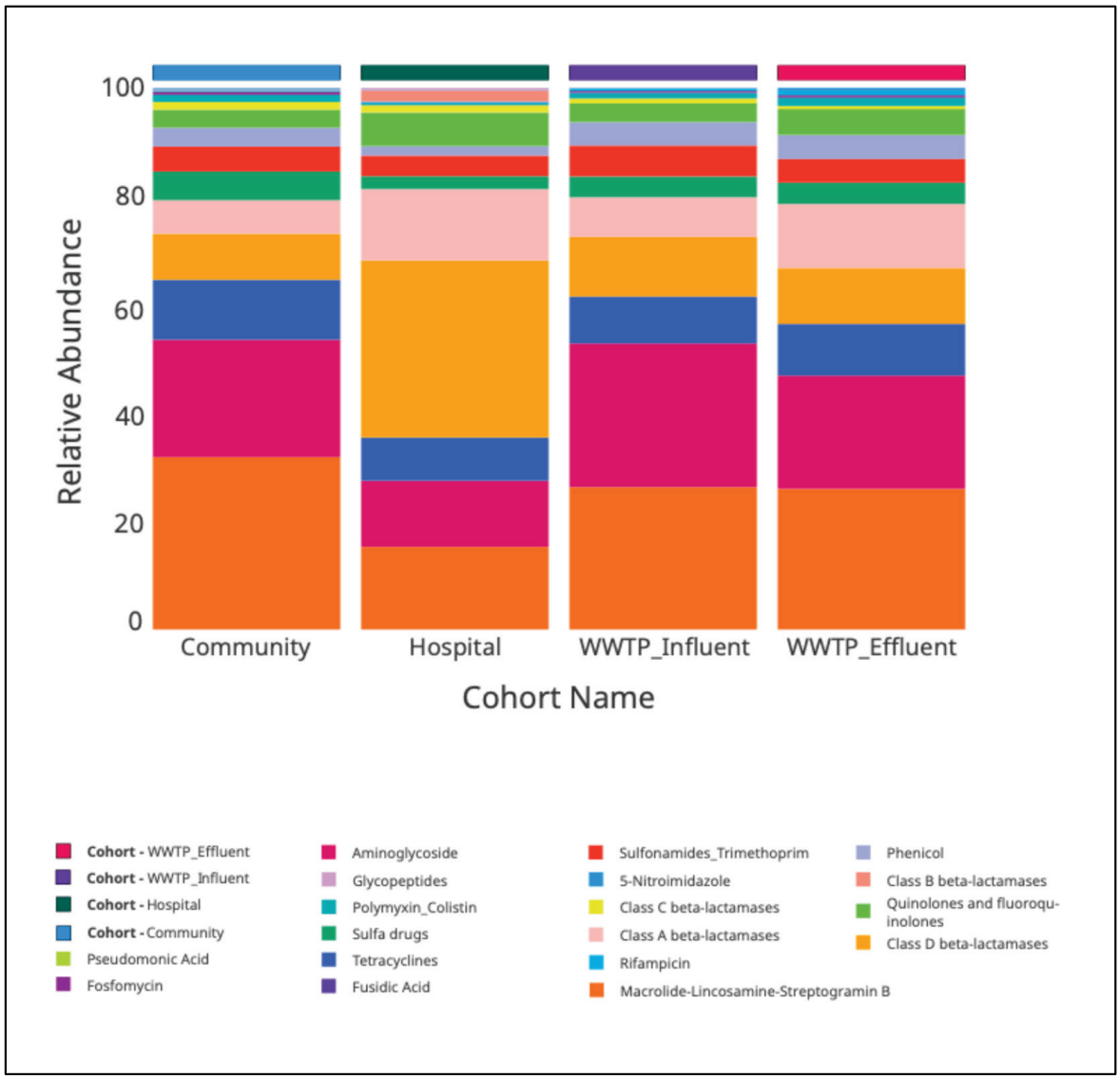

Figure 5. Relative abundance of ARGs and Antibiotic classes with ARGs across wastewater sources.

Hierarchical clustering of ARG relative abundances showed no clear separation between WWTP influent and effluent samples (Supplementary Figure S3), although influent samples displayed slightly higher overall relative abundances across multiple ARGs. Key ARGs shared between influent and effluent included the *msr*(*E*), *mph*(*E*), *strB* and *aadA*. WWTP influent samples showed enrichment of *sul1*, whereas WWTP effluent samples featured β-lactamase genes such as $bla_{\text{VEB-3}}$ and $bla_{\text{VEB-6}}$. Median relative abundances were 0.09 (0.05–3.95), 2.28 (1.76–4.83) and 3.95 (2.25–5.66), respectively. ARG diversity, as measured by the Shannon index, did not differ significantly between WWTP influent and effluent samples ($p = 0.558$).

Bray–Curtis PCoA showed significant source-associated differences in resistome composition ($p = 0.001$; Figure 6). Hospital samples formed a distinct cluster, whereas community and WWTP influent samples overlapped substantially. The WWTP effluent samples were more dispersed and partially overlapped with non-hospital groups.

Linear discriminant analysis effect size (LefSe) analysis further resolved the ARGs driving source-associated resistome differences (LDA > 3.75; Supplementary Figure S4). Hospital wastewater was distinguished by class D β-lactamases, including bla_{OXA} variants, together with *qnrD1*, whereas community wastewater and WWTP influent were charac-

terized by macrolide and aminoglycoside resistance determinants. Effluent-associated resistance determinants included persistent macrolide and aminoglycoside genes, alongside the chloramphenicol resistance gene *cmlA1*.

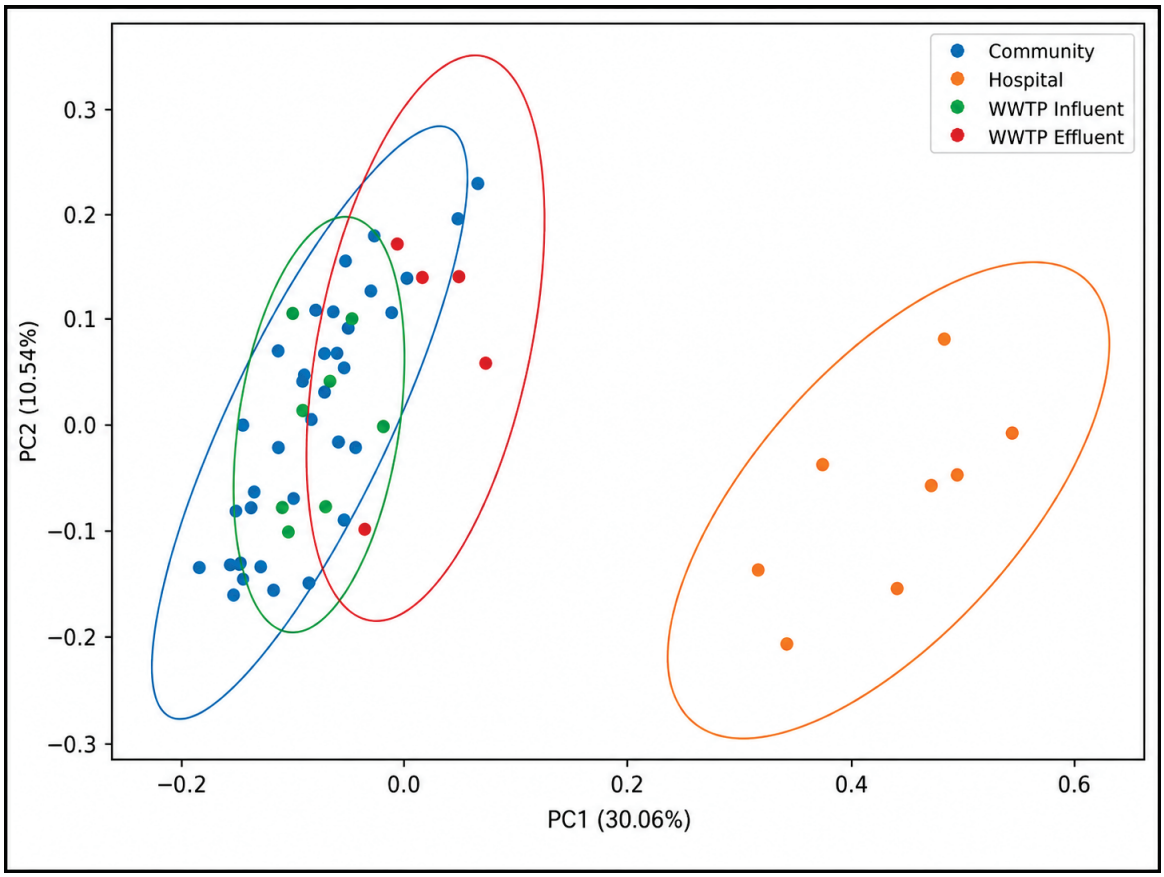

Figure 6. Bray–Curtis PCoA of ARG profiles across wastewater sources. Points represent individual samples coloured by source, with ellipses indicating 95% confidence intervals.

Virulence factor genes (VFGs): VFGs profiling identified 1554 genes and revealed source-specific virulome patterns. Community, hospital and WWTP influent samples were dominated by the fimbrial operon genes *mrkC*, *mrkB*, *mrkF*, *mrkA*, *mrkD* and *mrkJ*. These had comparable abundance in community wastewater and WWTP influent, but were significantly enriched in hospital wastewater ($p < 0.05$). Additional virulence genes, including *fimD* and the iron acquisition genes *entF* and *fepD*, were also detected in these samples, but at lower abundance. In contrast, WWTP effluent showed reduced overall VFGs abundance and a distinct profile with enrichment of the lipid A acyltransferase gene *acpXL* (median relative abundance [IQR]; 11.73 [0.08–23.37]). WWTP effluent samples also exhibited increased abundance of curli-associated genes (*csgE*, *csgB* and *csgD*), while *mrk* genes remained detectable (Figure 7).

PCoA showed significant source-associated differences in virulome composition (PERMANOVA, $p = 0.001$; Figure 8). Hospital wastewater formed a distinct and more dispersed cluster, whereas community wastewater and WWTP influent overlapped substantially and did not differ significantly ($p = 0.201$). WWTP effluent samples clustered more tightly, with limited overlap with the other sources (Figure 8).

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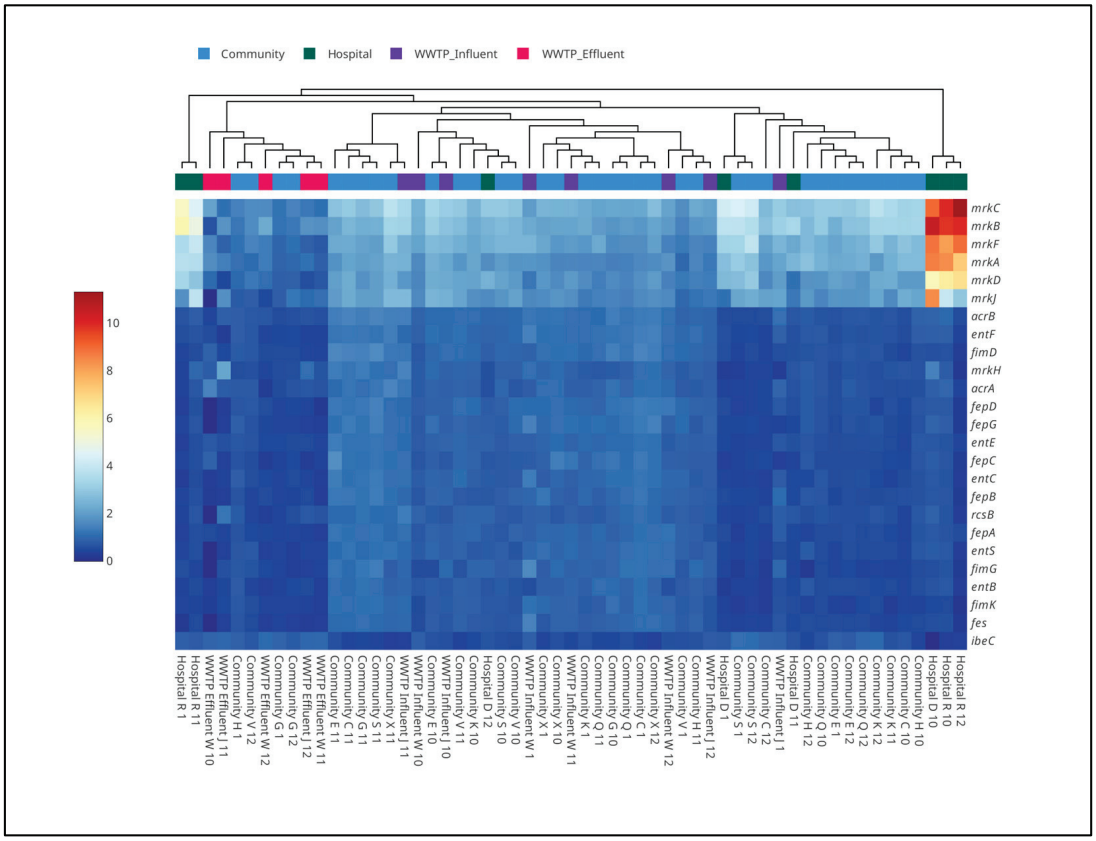

Figure 7. Relative abundances of VFGs across wastewater samples, with hierarchical clustering of samples.

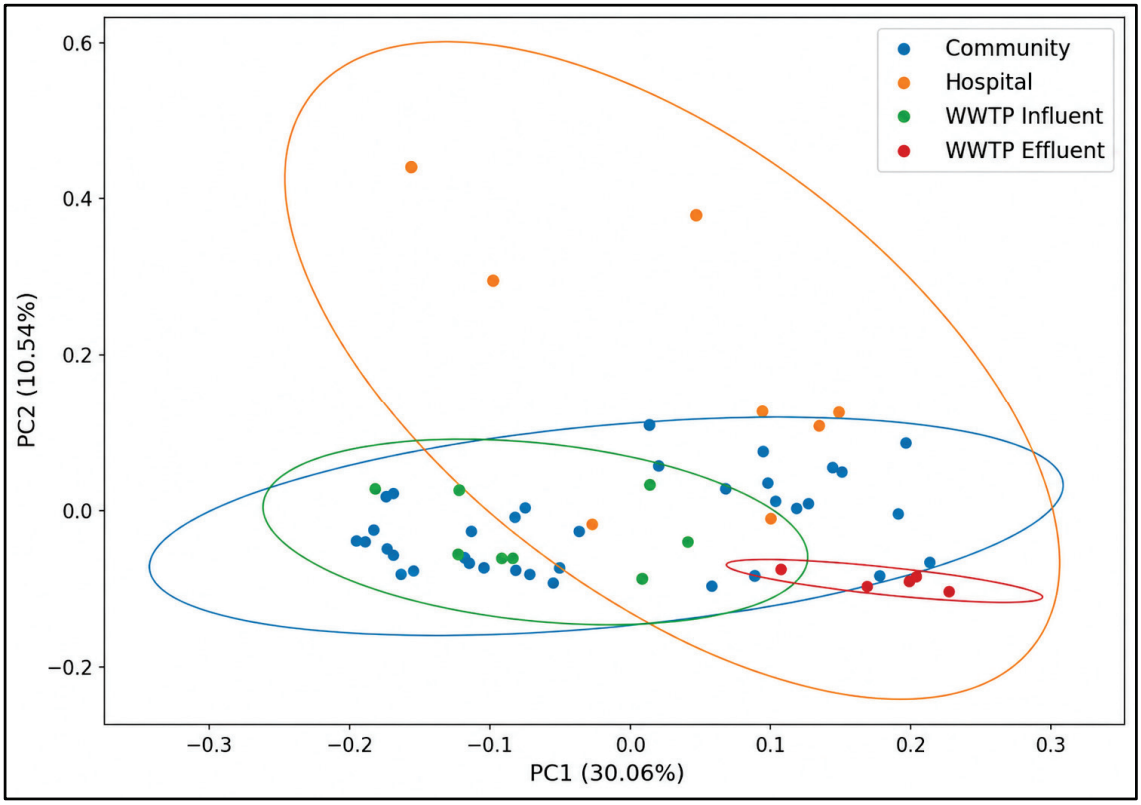

Figure 8. Bray–Curtis PCoA of VFG profiles across wastewater sources. Points represent the individual samples coloured by source, and ellipses indicate 95% confidence intervals.

Across all 57 samples, ARGs and VFGs Bray–Curtis dissimilarities matrices were significantly positively correlated by Mantel tests using both Pearson and Spearman coefficients (Pearson $r = 0.58$, $p = 0.001$; Spearman $\rho = 0.59$, $p = 0.001$).

Alpha diversity (Shannon index) was assessed across taxonomic, resistome, and virulome profiles over the sampling periods. Taxonomic and VFG diversity remained stable with no significant temporal variation observed. In contrast, ARG alpha diversity exhibited temporal variation with Wilcoxon Rank Sum pairwise comparisons revealing significant differences across most sampling months ($p < 0.05$; Supplementary Figure S5).

3. Discussion

The present study demonstrates that the urban wastewater continuum comprises distinct microbial, resistome, and virulome niches, each with its own ecological signature [20]. Community wastewater and WWTP influent reflected population-level carriage, hospital wastewater concentrated healthcare-associated resistance determinants, and WWTP effluent represented the residual biological footprint released after treatment [19,20]. Sampling these compartments separately revealed heterogeneity that is obscured when surveillance relies on WWTP influent samples alone [20]. This resolution is particularly relevant in settings with extensive treated wastewater reuse, where engineered water systems intersect with environmental and human health pathways, underscoring the importance of compartment-specific surveillance within a One Health framework [6,19].

Across the 57 metagenomes analyzed, bacterial richness was high throughout the wastewater network but partitioned distinctly by compartment. Community wastewater showed the highest cumulative richness, consistent with the breadth and heterogeneity of the residential catchments sampled, whereas hospital wastewater showed the highest median richness per sample, reflecting concentrated within-site complexity [21]. WWTP effluent had the lowest richness and Shannon diversity, indicating marked treatment-associated restructuring [19]. These patterns indicate that wastewater compartments are not interchangeable matrices, as each captures a biologically distinct surveillance signal. Community wastewater and WWTP influent were dominated by gut-associated taxa, notably *B. adolescentis* and *B. longum*, with an overlapping community structure [22]. Whilst this finding supports the use of WWTP influent as a broad urban composite, it underscores the added value of upstream community-node sampling for finer source resolution. In contrast, WWTP effluent composition was consistent with a selected residual community shaped by post-treatment biomass reduction [19].

Clinically relevant taxa, including ESKAPE-associated organisms, were detected across the wastewater continuum, demonstrating the capacity of metagenomic surveillance to identify clinically important microorganisms within complex mixed communities [19,23]. However, many of these taxa occurred at low relative abundance and showed limited compartment-specific enrichment. Their patterns were not uniformly source-specific, suggesting contributions from both community and healthcare inputs, as well as treatment-related shifts. This supports compartment-resolved surveillance, as reliance on a single wastewater matrix may obscure organism-specific enrichment and persistence across the urban wastewater system.

The resistome analysis provided the most direct AMR surveillance signal. Community wastewater and WWTP influent shared dominant macrolide and aminoglycoside resistance genes, including *msr*(*E*), *mph*(*E*), *strB*, and *aadA1*, indicating continuity between upstream catchment community inputs and WWTP influent profiles [24]. Hospital wastewater yielded a clinically enriched resistome signal, including significant enrichment of class D β-lactamase genes, and detection of *qnrD1*. As shotgun metagenomic sequencing detects genetic material rather than viable organisms or active gene expression, this finding is

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indicative of enrichment of clinically relevant resistance determinants rather than direct evidence of viable resistant pathogens or transmission risk [25]. This supports hospital wastewater as a high-yield sentinel matrix for clinically relevant resistance determinants before downstream dilution [26]. Importantly, this enrichment does not imply that hospitals are the sole source of AMR within urban wastewater systems. Rather, it indicates that hospital outflows act as concentrated reservoirs of healthcare-associated resistomes, whereas community wastewater and WWTP influent remain critical for capturing broader population-level signals. Although WWTP effluent had the lowest bacterial richness and diversity, ARG diversity did not differ significantly between influent and effluent samples. This suggests that microbial reduction did not translate into proportional reductions in the resistome profiles [27]. Residual ARGs may persist through surviving taxa, particle or biofilm-associated DNA, or compositional shifts caused by treatment or selective pressure [15,28]. The retention of selected macrolide and aminoglycoside resistance determinants and β-lactamase signals, including bla_{VEB} variants, identifies treated effluent as a critical endpoint for AMR monitoring. These findings do not imply inadequate treatment, as conventional WWTPs are not designed to eliminate ARGs or VFGs, rather, they define the residual AMR signal at the point where treated wastewater may reconnect with environmental pathways through discharge or reuse [29].

Virulome profiling provided important functional context to the resistome. Untreated wastewater compartments were dominated by fimbrial and adhesion-associated genes, particularly members of the *mrk* operon, with the strongest signals observed in hospital wastewater [30]. In contrast, WWTP effluent was enriched for *acpXL* and curli-associated genes, suggesting that the residual post-treatment community is shaped less by host-associated virulence traits and more by ecological characteristics that favour membrane adaptation, surface attachment, biofilm formation, and persistence in engineered and environmental habitats [28]. Although the detection of virulence factor genes does not imply gene expression, pathogenicity, or disease risk [31], these profiles provide insight into the functional attributes that may support microbial persistence and host–environment interactions within wastewater ecosystems. Temporal analysis added a dynamic monitoring layer. ARG alpha diversity varied significantly across the four-month sampling period, whereas taxonomic and virulome diversity remained comparatively stable. This suggests that resistome profiles may respond more rapidly than broad microbial community structure to short-term changes in antimicrobial use, healthcare activity, infection pressure, population mobility, or catchment inputs [32]. Although significant month-to-month differences in ARG diversity were observed, the four-month sampling window limits conclusions regarding broader temporal trends or seasonality. Therefore, these findings should be interpreted as short-term temporal variation within the study period, and longer longitudinal surveillance will be required to determine whether such patterns persist over time [24,33,34].

Collectively, these findings demonstrate the value of a layered metagenomic approach for AMR surveillance across the urban wastewater continuum. Taxonomic profiling resolved compartment-specific microbial communities, resistome analysis identified distinct resistance gene distributions, and virulome profiling provided complementary functional context. Temporal sampling further showed that ARG composition varied more across sampling periods than either taxonomic or virulome profiles. Together, these data reveal compartment-specific AMR signatures that would be obscured by WWTP influent-only surveillance.

Although shotgun metagenomics is a costly and technically demanding approach, it provides hypothesis-independent resolution that is particularly valuable during the discovery phase of wastewater-based AMR surveillance and in regions where baseline

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data are limited. Targeted approaches, such as high-throughput qPCR, can then be used as alternate options for routine or higher-frequency surveillance once key markers have been defined [25].

These findings should be interpreted within the limitations of the study. Community and hospital wastewater were collected as grab samples, whereas WWTP influent and effluent were collected as 24 h composites; this difference in sampling methodology may partly account for observed inter-compartment variation and should be considered alongside biological interpretations. To reduce the influence of short-term temporal variability, samples were collected repeatedly over a four-month study period rather than relying on a single sampling event.

More broadly, shotgun metagenomics detects genetic material rather than viability, gene expression, or transmission potential [35,36]. Consequently, the persistence of ARGs and virulence-associated genes in treated effluent reflects residual genetic signals at the point of environmental discharge rather than demonstrated exposure risk. Future studies integrating absolute quantification, viability-informed approaches, antibiotic residue measurements, mobile genetic element analysis, and linked clinical and treatment-process metadata would strengthen source attribution and risk assessment.

By resolving microbial, resistome, and virulome signatures across community wastewater, hospital wastewater, WWTP influent, and WWTP effluent, this study demonstrates the value of compartment-specific surveillance for understanding AMR dynamics across the wastewater continuum. Such an approach is particularly relevant in water-scarce settings where treated wastewater reuse creates close links between engineered water systems, environmental reservoirs, and human populations, reinforcing the role of wastewater surveillance within a One Health AMR monitoring framework [37].

4. Materials and Methods

Sampling framework: Wastewater was sampled monthly between October 2024 and January 2025 at 13 sites in Dubai, UAE, including nine community pumping stations, two tertiary-care hospital outflows, and two wastewater treatment plants (WWTPs) [25]. To reduce temporal variability, all sites were sampled within the same predefined monthly window, with a maximum interval of 48 h between collections. Community and hospital wastewater samples were obtained as grab samples, whereas WWTP influent and effluent were collected as 24 h flow-composite samples using refrigerated autosamplers (Hach, Loveland, CO, USA). For each sampling event, 1 L of wastewater was collected in sterile wide-mouth bottles (Azlon, Stone, Staffordshire, UK) and transported to the laboratory at 4 °C for downstream processing.

DNA extraction: Total community DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions [38–40]. Purified DNA was washed, eluted, and quantified using a Qubit Flex fluorometer and the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) [39].

Shotgun metagenomic sequencing: DNA libraries were prepared using the Watchmaker DNA Library Prep Kit with Fragmentation (7K0019-1K; Watchmaker Genomics, Boulder, CO, USA) according to CosmosID validated workflow (CosmosID, Germantown, MD, USA) [38,39]. Briefly, genomic DNA (1 ng) underwent enzymatic fragmentation, end repair, and A-tailing using a master mix of Watchmaker Frag/AT Buffer and Frag/AT Enzyme Mix, with incubation at 37 °C for 5 min followed by 65 °C for 30 min. IDT xGen Stubby Adapters were ligated to the processed DNA using the kit-supplied ligation reagents at 20 °C for 15 min. Adapter-ligated libraries were purified using CleanNGS magnetic beads (CleanNA, Waddinxveen, The Netherlands) and eluted in 10 mM Tris-HCl

(pH 8.0). The IDT xGen UDI Primers and the kit-supplied amplification mix were then added, and the libraries were amplified for 16 PCR cycles. The amplified libraries were purified using CleanNGS magnetic beads and eluted in nuclease-free water. Final library concentrations were quantified using the Qubit fluorometer dsDNA High Sensitivity Assay (Thermo Fisher Scientific, Waltham, MA, USA). Prior to sequencing, libraries were converted for platform compatibility using the Adept workflow (Element Biosciences, San Diego, CA, USA) [38,39]. Paired-end sequencing (2 × 150 bp) was performed on an Element AVITI platform using Cloudbreak chemistry (Element Biosciences, San Diego, CA, USA) [38–41].

Bioinformatic analysis: Shotgun metagenomic data were analyzed using the CosmosID bioinformatics platform (v2.0; CosmosID, Germantown, MD, USA) for taxonomic, resistome, and virulome profiling. Taxonomic classification was performed using the Taxa-Kepler Domain workflow (v1.1.0; database v3.0.0), while resistome and virulome profiling were performed using the AMR and Virulence Marker workflow (v1.1.0; database v1.1.0). After quality filtering, reads were matched against curated reference databases using a k-mer-based algorithm for microbial classification and functional gene detection. Taxa were assigned to the species level when supported by sequence data and reference coverage, with strain-level resolution reported where applicable. Relative abundance values were calculated as reads per million and normalized to total prokaryotic reads. ARGs and VFGs were identified using the ResFinder and VFDB databases through the CosmosID Kepler-AMR/VF workflow.

Alpha diversity was assessed using the Shannon index and compared across cohorts using the Wilcoxon rank-sum test. Beta diversity was assessed using Bray–Curtis dissimilarity and visualized by principal coordinate analysis (PCoA), with statistical significance determined by PERMANOVA. To assess whether ARGs and VFGs profiles co-varied across the wastewater continuum, Mantel tests were performed between the Bray–Curtis dissimilarity matrices of ARGs and VFGs relative abundance profiles across all 57 samples. Both Pearson and Spearman correlation coefficients were calculated with 999 permutations to assess significance using Python SciPy v1.17.1. Linear discriminant analysis effect size (LEfSe) was performed on ARG profiles to assess differential abundance across wastewater sources. Statistical significance was set at $p < 0.05$, and an LDA score threshold of 3.75 was applied.

5. Conclusions

This study demonstrates that compartment-resolved wastewater metagenomics can reveal distinct microbial, resistome, and virulome signatures across the wastewater continuum. Community wastewater and WWTP influent captured broad population-level AMR signals, hospital wastewater highlighted clinically enriched resistance determinants, and treated effluent retained residual resistance and virulence-associated markers despite reduced bacterial diversity. These findings show that wastewater compartments are not interchangeable surveillance matrices and that influent-only monitoring may obscure source-specific AMR patterns.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics15090817/s1.

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A.A.S., J.R., D.M., D.E., R.G. and M.K. All authors have read and agreed to the published version of the manuscript.

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

Abbreviations

The following abbreviations are used in this manuscript:

AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
bp	Base pair
CRI	Collaborative Research Initiative
DNA	Deoxyribonucleic acid
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*,*Pseudomonas aeruginosa*, and *Enterobacter* spp.
IDT	Integrated DNA Technologies
IQR	Interquartile range
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
MGE	Mobile genetic element
MLSB	Macrolide–lincosamide–streptogramin B
NCBI	National Center for Biotechnology Information
NIH	National Institutes of Health
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SRA	Sequence Read Archive
UAE	United Arab Emirates
UDI	Unique dual index
VFDB	Virulence Factor Database
VFG	Virulence factor gene
WWTP	Wastewater treatment plant

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Article

Virulome Landscape of Multidrug-Resistant *Escherichia coli* Across Human, Animal, and Environmental Reservoirs

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Abstract

Background/Objectives: *Escherichia coli* (*E. coli*) spans commensal, intestinal pathogenic, and extraintestinal pathogenic lineages distributed across human, animal, and environmental reservoirs, yet the extent to which virulence architectures are shared across these compartments remains incompletely understood. Using a One Health framework, we profiled putative virulence determinants in pooled multidrug-resistant (MDR) *E. coli* source groups representing human, animal, and environmental sectors. **Methods:** Virulence genes were predicted with VirulenceFinder, and presence–absence profiles were integrated to define functional composition, sector overlap, source-group distribution breadth, and pathotype-associated signatures. Predicted pathogenic potential was assessed with PathogenFinder and compared with pathogenic family richness. **Results:** Overall, 114 putative virulence genes were detected, with adhesion/colonization functions dominating the virulome (33/114), followed by toxin-associated genes (12/114). A conserved core of 50 virulence genes was shared across all three sectors, including determinants linked to serum resistance (*iss*, *ompT*, *traT*), adhesion (*csgA*, *fimH*), stress adaptation (*terC*), and iron acquisition (*sitA*, *iutA*, *fyuA*). ExPEC-associated determinants were most numerous in environmental source groups (n = 52), whereas diarrheagenic *E. coli* markers were most frequent in animal-associated groups (n = 42). LEE-associated effectors were infrequent and largely absent from human source groups. Despite ecological differences in virulence composition, pathogenicity scores remained consistently high across sectors (0.83–0.92) and showed no significant association with pathogenic family richness (Spearman's $\rho = 0.197$, $p = 0.392$). **Conclusions:** Within the limits of pooled source-group analysis, these findings suggest that MDR *E. coli* across One Health compartments shares a broadly distributed, ExPEC-associated virulence repertoire overlaid with sector-specific pathotype signals, underscoring the value of integrated genomic surveillance while highlighting the need for isolate-resolved analysis.

Keywords: *Escherichia coli*; virulence genes; One Health; ExPEC; DEC; pathogenicity score; environmental reservoirs

1. Introduction

Escherichia coli (*E. coli*) is an exceptionally diverse bacterial species that functions as both a commensal and a pathogen. Commensal strains colonize the gastrointestinal tract, supporting digestion and microbial balance. In contrast, pathogenic lineages cause diverse diseases, including diarrhea, urinary tract infections, septicemia, and neonatal meningitis [1]. Virulence factors mediate the ability of *E. coli* to cause disease. They code for adhesins, toxins, invasins, iron acquisition systems, and other factors that help the bacteria colonize, evade the immune system, and damage tissue [2]. These genes, frequently situated on mobile genetic elements such as plasmids, bacteriophages, and pathogenicity islands, form the basis for the classification of *E. coli* into distinct pathotypes, including enteropathogenic (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC), and extraintestinal pathogenic *E. coli* (ExPEC) [3].

The locus of enterocyte effacement (LEE) is a widely researched pathogenicity island that encodes the type III secretion system (T3SS) and associated effectors that cause the attaching and effacing (A/E) lesions seen in EPEC and EHEC. These lesions allow for close adhesion to intestinal epithelial cells and disruption of the host cytoskeleton, which causes diarrhea and serious risks such as hemolytic uremic syndrome [4,5]. Key LEE-associated genes include *eae* (encoding intimin) and *tir* (encoding the translocated intimin receptor), which are involved in host cell attachment. The *espA/B/F/J* genes are structural components of the T3SS and effector translocation machinery, whereas non-LEE-encoded effectors (*nleA/B/C*) and *etpD* regulate host signaling and immunological responses. Additionally, *tccP* stimulates actin polymerization and pedestal formation, which improves colonization efficiency [4,5].

The One Health paradigm provides a valuable framework for understanding how human, animal, and environmental health are interconnected, particularly for organisms such as *E. coli*, which circulate across all three compartments [6,7]. Within this continuum, livestock and companion animals can act as reservoirs of pathogenic lineages, while environmental matrices, including surface waters, wastewater, and soils, provide important settings for persistence, amplification, and dissemination [8,9]. Human infection may therefore arise through multiple, overlapping routes, including direct transmission, foodborne exposure, zoonotic contact, and environmentally mediated pathways. Against this background, defining how virulence determinants are distributed across sectors is critical for understanding shared pathogenic potential and for strengthening surveillance strategies within a One Health context.

Importantly, cross-compartment comparisons of virulence gene distribution can distinguish broadly shared from compartment-associated signatures across ecologically linked reservoirs. Such patterns are informative for assessing reservoir connectivity and the broader architecture of pathogen risk, even though they do not in themselves resolve direct transmission pathways or transmission directionality. In this study, the analytical focus was therefore placed on the broader virulence gene repertoire detected across pooled source groups, with pathotype-related interpretation applied subsequently through annotations linked to DEC-, ExPEC-, and other virulence-associated markers. Moreover, despite major advances in genomic approaches, virulence-focused studies of *E. coli* remain disproportionately centered on human clinical isolates, with comparatively limited characterization of animal and environmental reservoirs. This imbalance restricts One Health-level insight into how virulence traits are distributed across ecologically connected compartments [10–12]. To address this gap, we characterized the virulence gene repertoire detected across pooled MDR *E. coli* source groups from human, animal, and environmental compartments. Additionally, rather than analyzing predefined pathotypes as separate cohorts, we profiled the broader virulence gene repertoire across pooled multidrug-resistant *E. coli* source groups

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and subsequently interpreted selected determinants using pathotype-associated annotations, including DEC- and ExPEC-linked markers. Within this framework, LEE-associated genes are considered pathotype-linked virulence signals embedded within the broader virulome, rather than evidence of isolate-level pathotype assignment or pathotype-specific cohort design.

2. Results

2.1. Distribution of Virulence Factors

Virulome analysis identified 114 putative virulence genes, annotated by functional category and pathotype association, across the 21 source groups representing human (n = 2), animal (n = 10), and environmental (n = 9) compartments. Functional classification showed that adhesion- and colonization-associated genes were the most abundant group (n = 33), followed by toxin-associated genes (n = 12) and microbial competition genes (n = 12) (Figure 1). Overall, the detected repertoire was dominated by determinants linked to colonization, persistence, and host interaction rather than by invasion-associated features alone. The full list of detected genes and their functional assignments is provided in Supplementary Tables S1 and S2.

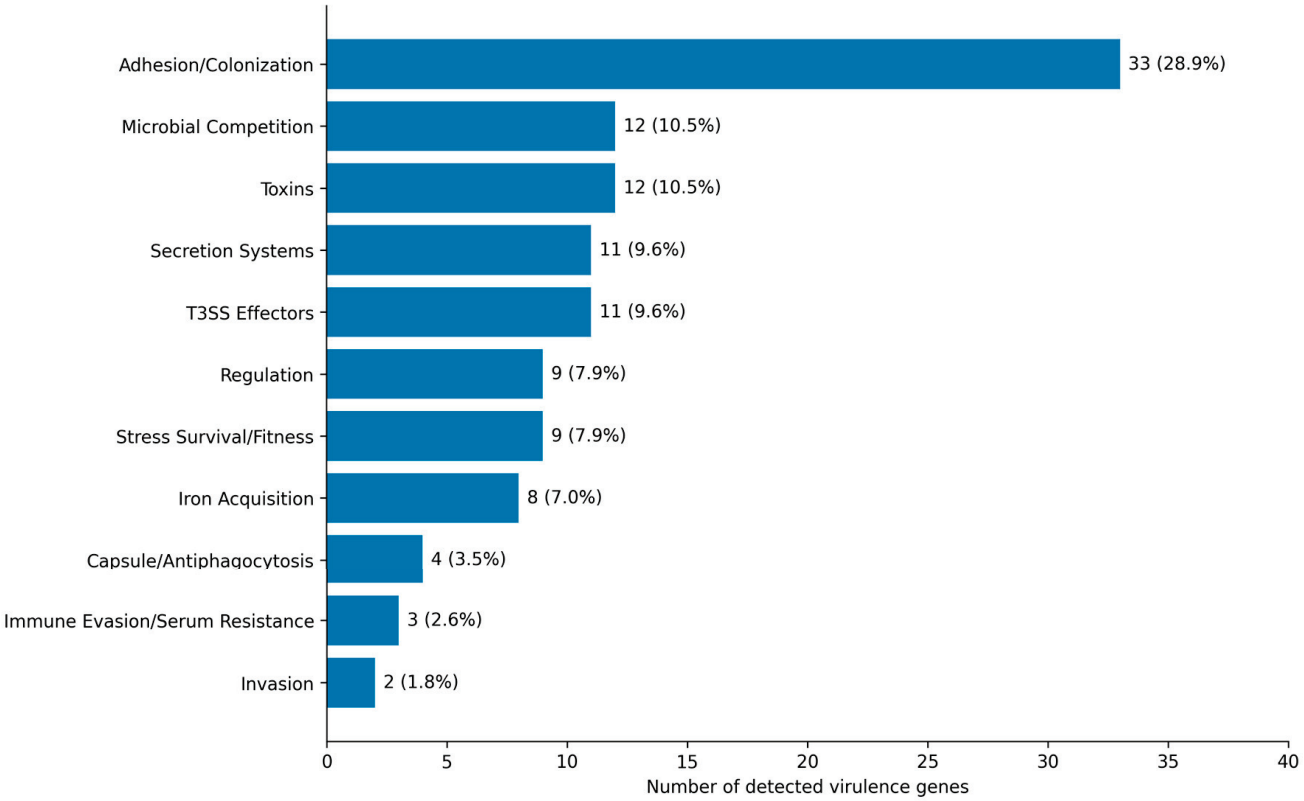

Figure 1. Distribution of virulence genes by function. Horizontal bars show the number and percentage of the 114 detected virulence genes assigned to each functional class. Bars are ordered from most to least abundant functional class.

2.2. Distribution Across One Health Compartments and Overlap Structure

Virulence gene richness varied across One Health compartments, with 62 distinct virulence genes detected in human-associated source groups, compared with 98 in animal-associated and 92 in environmental source groups. Despite these differences, the sector-level virulome was anchored by a substantial three-way shared core of 50 genes present across all compartments (Figure 2; Table S3). This shared set included determinants related to adhesion/colonization, iron acquisition, serum resistance, and stress adaptation/fitness.

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Relative to total richness, the shared core accounted for 80.6% of the human-associated virulome (50/62), compared with 51.0% in animal-associated sources (50/98) and 54.3% in environmental sources (50/92), indicating that human-associated profiles were largely nested within a broader virulence repertoire observed in non-human compartments.

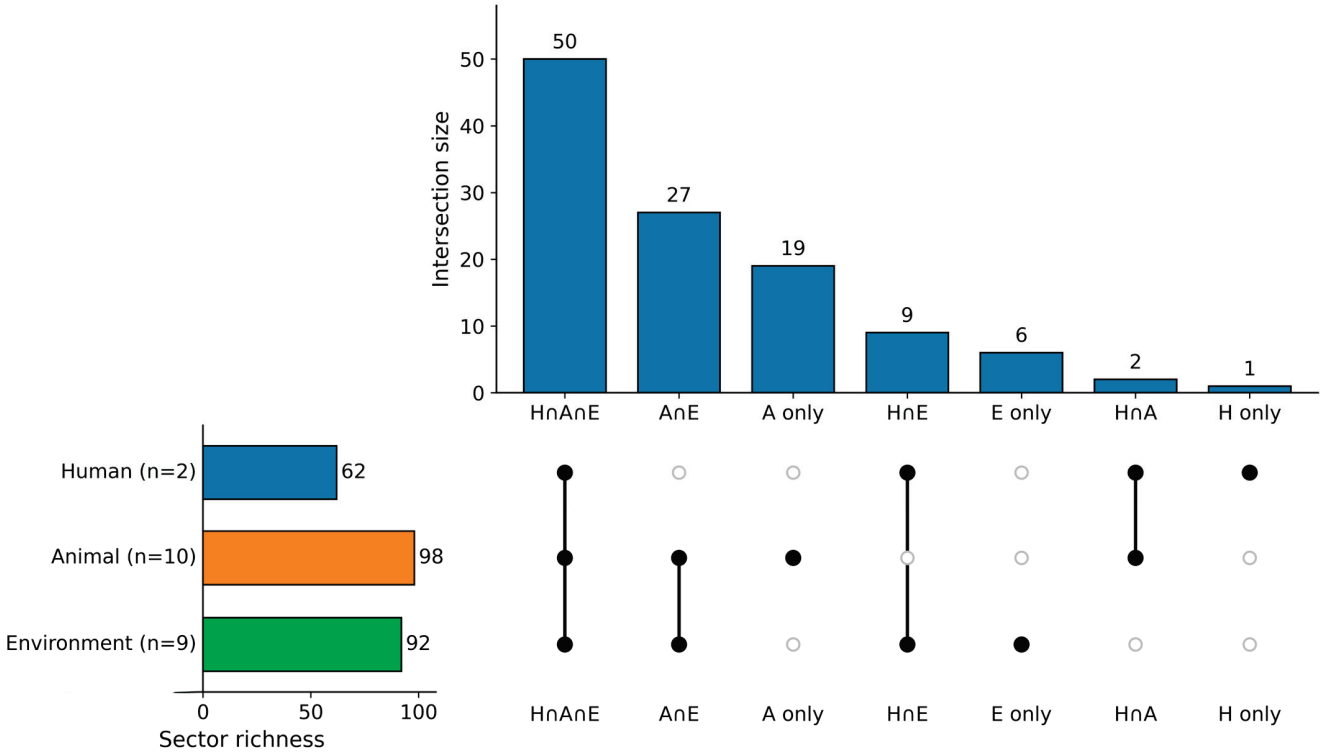

Figure 2. Integrated overlap structure of virulence genes across One Health sectors. The UpSet plot shows sector-level presence/absence intersections for 114 detected virulence genes, including the three-way shared core, pairwise overlaps, and compartment-specific components. The shared core comprised 50 genes detected across human, animal, and environmental sectors, including determinants associated with adhesion/colonization, iron acquisition, serum resistance, and stress adaptation/fitness. H: human; A: animal; E: environment.

Beyond this common core, additional virulence diversity was concentrated in animal and environmental source groups, which contributed larger exclusive and pairwise-overlap gene sets, whereas human-associated sources contributed very few unique detections (Figure 2). These patterns indicate substantial cross-compartment similarity in virulence profiles at the source-group level, together with a possible asymmetric expansion of accessory virulence content in non-human reservoirs. Given the smaller number of human-associated source groups and the broader heterogeneity of animal and environmental sources, these differences should be interpreted cautiously.

2.3. Broadly Distributed Virulence Genes Across Source Groups

Comparison across One Health compartments revealed a subset of virulence genes with broad distribution across human, animal, and environmental source groups (Figure 3). Among the 15 most prevalent genes, four (*ompT*, *iss*, *csgA*, and *terC*) were detected in all 21 source groups, while most of the remaining top-ranked genes were present in at least 17 groups spanning all three compartments. These rankings were based on the number of positive source groups in which each gene was detected (0–21) and therefore reflect source-group-level distribution breadth rather than isolate-level prevalence. The most prevalent genes were broadly shared across compartments, whereas lower-frequency genes showed greater variability in their distribution across source groups.

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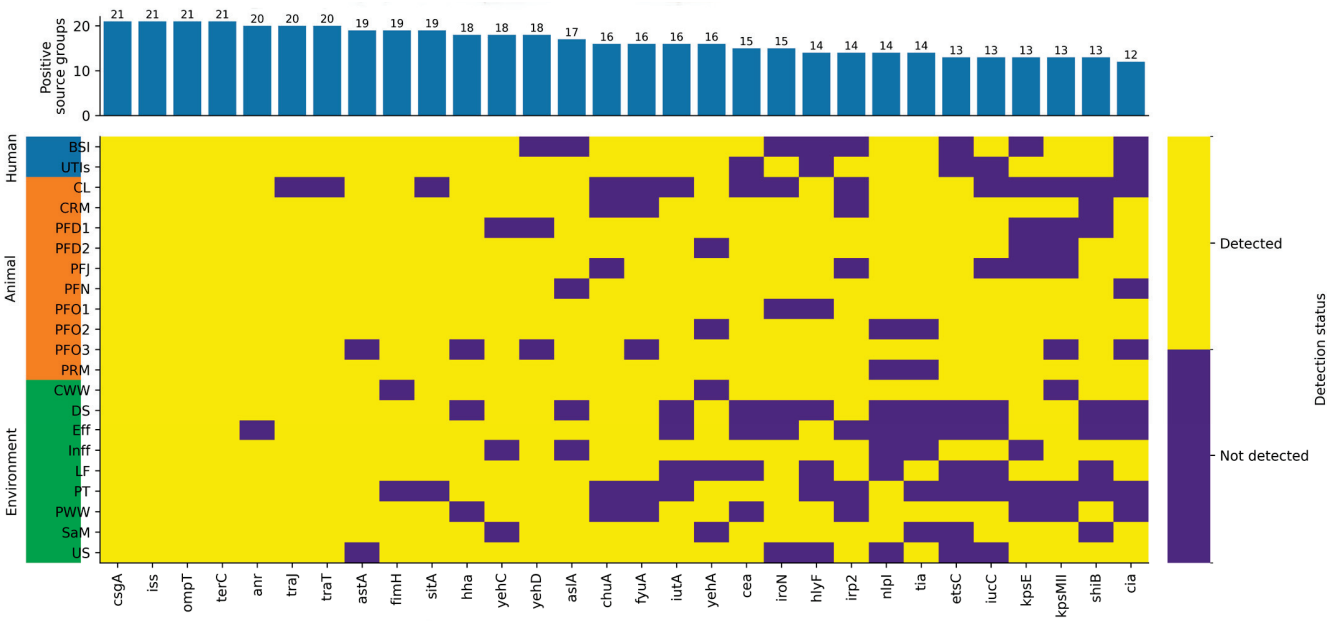

Figure 3. Joint distribution of the 30 most common virulence genes across individual source groups. Columns indicate virulence genes ranked by the number of positive source groups (0–21), not by the number of individual isolates carrying each gene, while rows represent source groups from the human, animal, and environmental sectors. The heatmap displays source-group-level presence/absence patterns and should be interpreted as pooled-source virulence profiles rather than isolate-resolved genotypes. Yellow denotes the presence of a gene, while purple indicates its absence in each source group. Bloodstream infection BSI; urinary tract infection UTI; pig feces December 1 PFD1; pig feces December 2 PFD2; pig feces January PFJ; pig feces November PFN; pig feces October 1 PFO1; pig feces October 2 PFO2; pig feces October 3 PFO3; pig retail meat PRM; chicken litter CL; chicken retail meat CRM; downstream DS; effluent Eff; influent Inf; litter fertilizer LF; pig truck PT; chicken wastewater CWW; pig wastewater PWW; soil after manure application SaM; upstream US.

2.4. DEC- and ExPEC-Associated Patterns, LEE-Associated Markers, and Core ExPEC Suites Across Compartments

Cross-compartment comparison of pathotype-associated virulence markers revealed clear sector-level differences between DEC- and ExPEC-linked determinants (Figure 4). DEC-associated markers were most numerous in animal-associated source groups (42), with lower counts in environmental (29) and human-associated groups (17). By contrast, ExPEC-associated determinants were detected across all three compartments, with the highest counts in environmental source groups (52), followed by animal (46) and human-associated groups (37).

In contrast, LEE-associated markers were comparatively rare and unevenly distributed. *eae* was detected in five source groups across animal and environmental compartments but was absent from human-associated sources, whereas *tir* was restricted to four animal-associated groups. Similarly, *espF* was identified only in animal and environmental sources, spanning six groups overall, while *tccP* occurred at low frequency across all three sectors. The canonical ExPEC determinants, however, were consistently detected across human, animal, and environmental reservoirs. These included adhesins (*fimH*, *papA*, *papC*), iron-acquisition systems (*fyuA*, *chuA*, *iutA*, *sitA*, *iroN*), serum-resistance and outer membrane-associated factors (*iss*, *ompT*), plasmid-linked transfer determinants (*traT*, *traJ*), and the fitness-associated gene *terC*. Notably, *iss*, *ompT*, and *terC* were each detected in all source groups, showing the broad distribution of this ExPEC-associated repertoire.

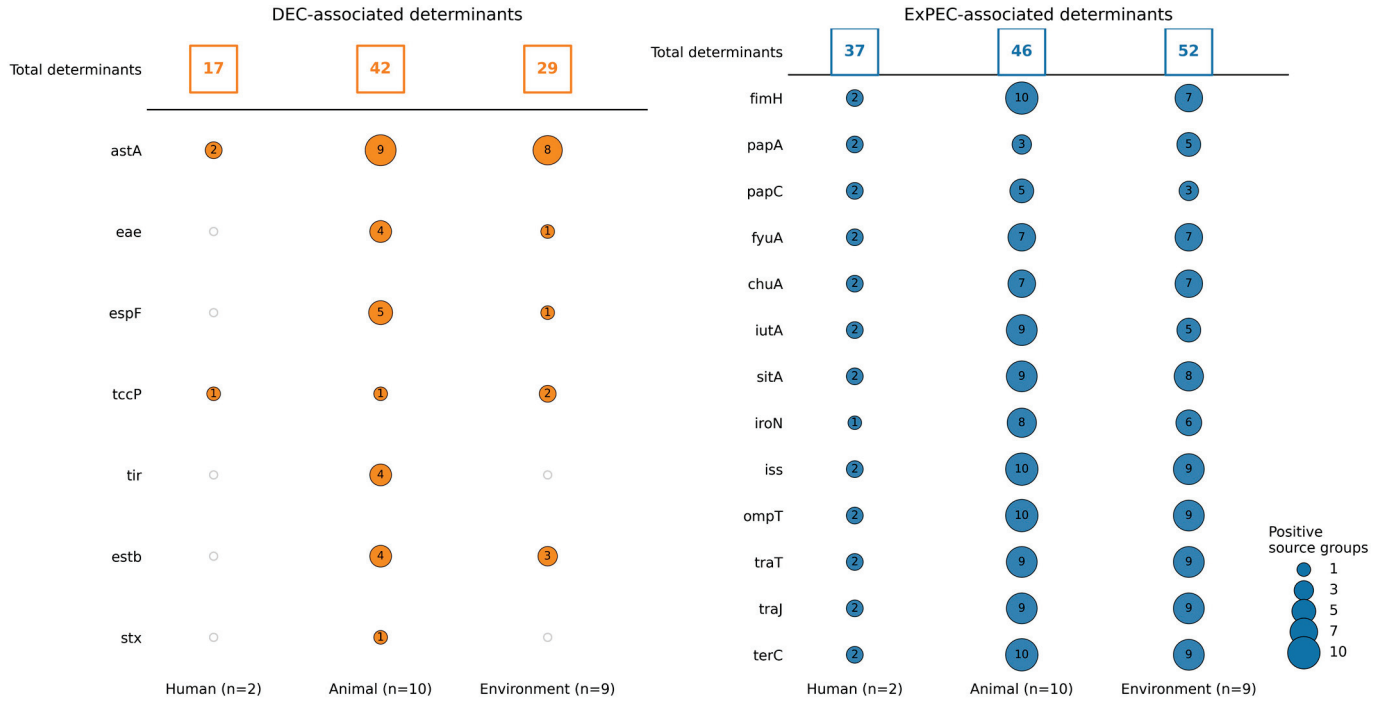

Figure 4. Joint analysis of pathotype-associated virulence determinants and source structure. Two-panel dot plot summarizing DEC- and ExPEC-associated virulence genes across human, animal, and environmental sectors. The top row in each panel shows the total number of associated determinants per sector, and the lower rows show representative hallmark genes, with dot size indicating the number of positive source groups. The figure summarizes sector-level counts and representative markers across compartments. These sector totals reflect source-group-level detection aggregated to the compartment level, based on source-group presence/absence profiles, and should not be interpreted as prevalence estimates.

2.5. Predicted Pathogenic Potential Across One Health Sectors

Predicted pathogenicity scores, estimated from assembled source-groups (Methods, Section 4.5), were consistently high across human, animal, and environmental source groups, with only modest variation among sectors (Table S4). Pathogenic family richness, defined here as the number of virulence family classifications represented in the composite pathogenicity analysis, varied across source groups (Figure 5), whereas sector-level pathogenicity means remained comparable, with no significant differences detected among compartments (Kruskal–Wallis, $p = 0.651$) (Tables S4–S6). At the source-group level, animal and environmental groups showed greater dispersion than human-associated groups, which clustered more tightly. This contrast may reflect both biological heterogeneity across source types and uneven source-group representation, particularly the smaller number of human-associated groups.

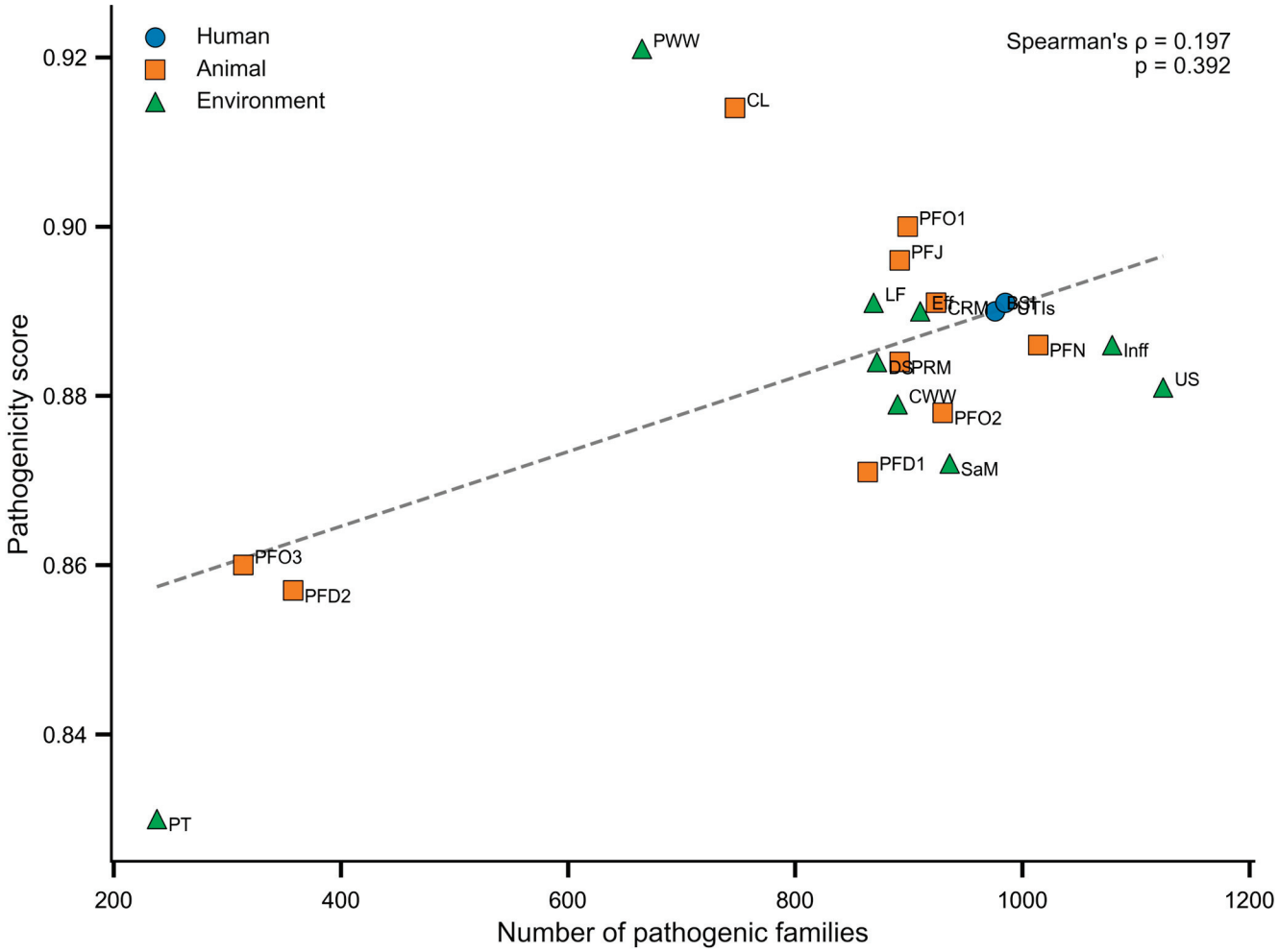

Figure 5. Relationship between pathogenicity score and pathogenic family richness across human, animal, and environmental source groups. Each point represents a source group. Spearman's correlation showed a weak, non-significant positive association (ρ = 0.197, p = 0.392). Bloodstream infection BSI; urinary tract infection UTI; pig feces December 1 PFD1; pig feces December 2 PFD2; pig feces January PFJ; pig feces November PFN; pig feces October 1 PFO1; pig feces October 2 PFO2; pig feces October 3 PFO3; pig retail meat PRM; chicken litter CL; chicken retail meat CRM; downstream DS; effluent Eff; influent Inf; litter fertilizer LF; pig truck PT; chicken wastewater CWW; pig wastewater PWW; soil after manure application SaM; upstream US.

3. Discussion

Using a One Health framework, we mapped virulence genes in drug-resistant *E. coli* across human, animal, and environmental reservoirs by integrating overlap structure, source-group distribution patterns, and pathotype-associated signatures. The pathotype-associated labels were applied after broad virulence screening to aid interpretation of detected determinants, rather than to define predefined analytical cohorts. The resulting virulome showed extensive cross-compartment overlap, with a substantial shared core and only limited compartment-exclusive content, indicating that many virulence-associated traits are distributed across ecologically linked reservoirs rather than confined to a single sector [6,7].

The shared core genes, together with the broad distribution of the most prevalent determinants across source groups, suggest that a large component of the virulome is composed of functions associated with colonization, persistence, host interaction, and ecological fitness. Recurrent detection of adhesion-associated genes such as *csgA* and *fimH*, serum-resistance determinants including *iss*, *ompT*, and *traT*, the fitness-associated gene

terC, and iron-acquisition systems such as *iutA*, *iroN*, *fyuA*, *chuA*, and *sitA* indicates that these traits are not restricted to human-associated isolates. Similar broad distributions of virulence-linked functions have been reported across human, animal, and environmental *E. coli* backgrounds [7,11,13–15], and our data extend these observations by showing source-group-level continuity of these traits within a single regional One Health setting.

At the same time, the data do not support a completely uniform virulence landscape. ExPEC-associated determinants were more numerous in environmental and animal sectors, whereas DEC-associated markers were most concentrated in animal-associated sources. This asymmetry suggests that broadly shared virulence architecture is overlaid by narrower compartment-associated patterns. The environmental enrichment of ExPEC-linked traits and the concentration of DEC-associated markers in animal sources are consistent with reports that wastewater, food animals, and related interfaces can harbor clinically relevant virulence repertoires beyond classical human clinical contexts [3,11,16–20]. However, these contrasts should be interpreted cautiously, as they may reflect not only biological differences across reservoirs but also unequal sampling depth, source heterogeneity, and the pooled source-group design.

LEE-associated markers formed a comparatively limited component of the detected virulome and were largely skewed toward non-human source groups. In particular, *tir* was restricted to animal-associated groups, whereas *eae* and other *esp/nle* markers were not detected in human-associated groups. Given that the sampled human source groups represented bloodstream and urinary tract infection contexts, this pattern is compatible with a predominance of extraintestinal rather than attaching-and-effacing disease biology, although the small number of human-associated source groups limits stronger inference. In contrast to studies in which LEE-associated markers define STEC/EHEC/EPEC-related lineages [3,21], this dataset was characterized more strongly by a broadly distributed ExPEC-associated background than by prominent LEE-associated signatures.

The pathogenicity-score analysis adds a complementary perspective to the gene-distribution results. Although pathogenicity scores remained consistently high across sectors, their weak and non-significant association with pathogenic family richness indicates that inferred pathogenic potential was shaped less by the number of virulence-family classifications than by their composition. Several animal and environmental source groups showed high predicted pathogenicity despite only moderate pathogenic-family richness, suggesting that composite risk may be concentrated in particular virulence configurations rather than scaling directly with family counts. This interpretation is consistent with previous observations that plasmid- and prophage-associated virulence content can amplify pathogenic potential in non-human reservoirs [11].

Several limitations should be considered when interpreting these findings. First, the analyses were conducted at the pooled source-group presence/absence level rather than at the level of isolate prevalence or individual-genome carriage, and sector-level presence was defined by detection in at least one constituent source group. Accordingly, the results describe source-group-level virulence profiles and should not be interpreted as isolate-level prevalence estimates, strain-resolved genotypes, or direct evidence of transmission. Second, the smaller number of human-associated source groups, limited to bloodstream infection and urinary tract infection pools, together with uneven isolate representation across sources, reduced statistical power and comparative resolution for human-sector and source-specific comparisons. This may have constrained detection of human-associated virulence diversity and limited the strength of source-level One Health inference. Third, the greater virulence gene richness observed in animal and environmental compartments may partly reflect deeper sampling, larger numbers of source groups, and greater ecological heterogeneity, rather than intrinsic differences in virulence potential alone. Finally, although

all isolates included in this study were multidrug-resistant, the analytical framework was designed to examine virulence gene distribution and predicted pathogenicity rather than resistome composition or resistance–virulence relationships. As a result, the study did not assess co-occurrence, within-genome co-carriage, or potential co-selection between antimicrobial resistance genes, virulence determinants, and mobile genetic elements such as plasmids, integrons, or transposons. Because pooled source-group assemblies were analyzed rather than isolate-resolved genomes, clonal relatedness, resistance–virulence linkage, mobile genetic element context, and transmission directionality could not be resolved. Future studies incorporating balanced isolate-level whole-genome sequencing, integrated resistome–virulome analysis, plasmid reconstruction, and phylogenomics will be required to determine whether multidrug resistance, virulence, and mobile genetic elements are co-selected or co-transmitted across One Health compartments.

However, even within these constraints, the results support a One Health surveillance framework that integrates gene-content profiles with composite pathogenicity measures and genomic context. This combined perspective was more informative than gene counts alone because it highlighted high-scoring non-human source groups that would have been less apparent from richness alone [15,22].

4. Materials and Methods

4.1. Ethical Consideration

This study is part of a larger program on Antibiotic Resistance and One Health. The Biomedical Research Ethics Committee (reference: BCA444/16) and the Animal Research Ethics Committee (reference: AREC/007/018) at the University of KwaZulu-Natal and the KwaZulu-Natal Provincial Health Ethics Committee (Reference: HRKM108/17KZ_2017RP3 _437) provided ethical approval. The Department of Agriculture, Forestry, and Fisheries (DAFF) gave permission to sample animals (Ref 12/11/1/5), while Umgeni Water granted authorization to sample water. The KwaZulu-Natal Department of Health, the uMgungundlovu District Manager, and health facility managers all provided gatekeeper permission.

4.2. Study Isolates

This study included 555 multi-drug-resistant (MDR) *E. coli* isolates from earlier studies conducted in the uMgungundlovu District, KwaZulu-Natal Province, South Africa [23–28]. The isolates included 12 from humans (isolates implicated in bloodstream and urinary tract infections), 385 from food animals (pigs and poultry samples from farm to table), and 159 from the environment (soil and water bodies). Isolates were preserved at −80 °C at the Antimicrobial Research Unit, University of KwaZulu-Natal. To resuscitate, frozen stocks were thawed and plated onto Eosin Methylene Blue (EMB) agar, which was then incubated at 37 °C for 18–24 h. Distinct *E. coli* colonies were then sub-cultured on nutrient agar to produce pure cultures. Individual colonies were collected with sterile pipette tips and transferred to sterile Eppendorf tubes. To enable comparative analysis across multiple One Health compartments while remaining within sequencing resource constraints, isolates were pooled by source group prior to genomic analysis. Each pooled sample, therefore, represents a composite source group rather than individual isolates. A total of 21 pooled source groups were generated across human, animal, and environmental compartments (Table S7). Pool sizes ranged from 3 to 46 colonies per source group, depending on sample availability. Human-associated pools comprised urinary tract infection (UTI; n = 5) and bloodstream infection (BSI; n = 7) isolates. Animal-associated pools included chicken retail meat (CRM; n = 10), chicken litter (CL; n = 6), pig retail meat (PRM; n = 42), pig feces October 1 (PFO1; n = 40), pig feces October 2 (PFO2; n = 40), pig feces October 3 (PFO3;

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n = 29), pig feces November (PFN; n = 45), pig feces December 1 (PFD1; n = 44), pig feces December 2 (PFD2; n = 46), and pig feces January (PFJ, n = 42). Environmental pools comprised chicken wastewater (CWW; n = 3), pig wastewater (PWW; n = 38), pig truck (PT; n = 16), upstream (US; n = 36), downstream (DS; n = 10), influent (Inf; n = 25), effluent (Eff; n = 34), soil after manure application (SaM; n = 28), and litter fertilizer (LF; n = 9). Colonies within each source group were combined to form a single composite pool for downstream DNA extraction and sequencing.

This source-group pooling framework was selected as an exploratory design to capture broad virulence gene distribution across One Health compartments rather than to estimate isolate-level prevalence or resolve strain-level relationships. It was intended for distribution-focused virulome mapping rather than balanced isolate-level whole-genome sequencing for prevalence estimation, phylogenomic reconstruction, or isolate-resolved comparative genomics. Pigs are widely known as large reservoirs of virulent *E. coli* strains that can spread to humans via gastrointestinal routes, direct contact, or environmental contamination [29–31]. To capture the diversity and evaluate its contribution to pathogenicity, we carried out extensive sampling of pig feces.

4.3. Genomic DNA Extraction and Quality Assessment

The GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, MO, USA) was used to extract genomic DNA from each pooled *E. coli* source group, following the manufacturer's instructions. DNA concentration and purity were assessed using the NanoDrop™ 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Colonies were pooled prior to extraction, and where feasible, approximately balanced colony input was used to reduce over-representation of individual colonies within a pool. However, because isolate biomass and DNA yield can vary, perfect isolate-level normalization was not achievable, and downstream analyses were therefore interpreted qualitatively at the source-group presence/absence level rather than as isolate-level quantitative frequencies.

4.4. Library Preparation and Sequencing of Pooled E. coli Source Groups

Genomic libraries were prepared from DNA extracted from pooled *E. coli* source groups using the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA), which enables efficient DNA fragmentation and indexing for high-throughput sequencing [32]. Paired-end sequencing was then performed on the Illumina MiSeq platform [33] at the National Institute for Communicable Diseases (NICD) Sequencing Core Facility in Johannesburg, South Africa.

4.5. Genomic Data Analysis

Raw sequencing reads generated from pooled *E. coli* source groups were quality-checked using FastQC and trimmed with Trimmomatic by the sequencing facility at the National Institute for Communicable Diseases. Filtered high-quality reads were assembled with SPAdes through the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) assembly pipeline. The resulting assembled contigs from each pooled source-group were used for downstream virulence screening and pathogenicity prediction. Virulence determinants were identified from pooled-source assemblies using VirulenceFinder v2.0 from the Center for Genomic Epidemiology, applying a minimum identity threshold of 90% and a minimum coverage threshold of 60%. Pathogenicity scores were estimated with PathogenFinder [34] using assembled contigs generated for each pooled source-group assembly.

For sector-level analysis, a virulence gene was considered present if it was detected in at least one constituent source group within that sector. Binary presence/absence matrices were used to identify compartment-exclusive genes, pairwise overlaps, and the three-way

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shared core. Distribution breadth was quantified as the number of positive source groups per gene across all 21 source groups. Unless otherwise specified, gene detections reported in the Results and figure legends refer to source-group-level presence/absence derived from pooled assemblies rather than isolate-level frequency.

Virulence genes annotated as associated with diarrheagenic *Escherichia coli* (DEC) or extraintestinal pathogenic *E. coli* (ExPEC) were further evaluated by integrating pathotype annotations with sector-level presence/absence profiles. Locus of enterocyte effacement (LEE)-associated markers, including *eae*, *tir*, *espA*, *espB*, *espF*, *espJ*, *nleA*, *nleB*, *nleC*, *etpD*, and *tccP*, were specifically screened in the LEE module summary [4]. Overlap structure was visualized using an UpSet plot, broadly distributed genes were summarized in a heatmap, and DEC-/ExPEC-associated determinants were compared across sectors using a dot plot. Data analysis was performed in Python (version 3.11) [35–38].

4.6. Statistical Analysis

Associations between pathogenicity score and pathogenic family richness, defined here as the number of virulence family classifications represented for each source group in the composite pathogenicity analysis, were assessed across the 21 source groups using Spearman's rank correlation coefficient (ρ) [39]. Sector-level differences in pathogenicity scores among human, animal, and environmental compartments were tested using the Kruskal–Wallis test [40], with Bonferroni-adjusted post hoc pairwise comparisons applied where relevant. All analyses were two-tailed, and statistical significance was set at $\alpha = 0.05$. The analyses were conducted in Python (version 3.11) using the scientific libraries Pandas, NumPy, SciPy, Seaborn, and Matplotlib. Scatterplots were generated to visualize the relationship between pathogenic family richness and pathogenicity score, with the corresponding test statistics displayed. Virulence gene richness was defined as the number of distinct virulence genes detected within the relevant analytical unit. Accordingly, at the sector level, it represents the number of unique virulence genes identified within a sector based on detection in at least one constituent source group, whereas at the source-group level it denotes the number of distinct virulence genes detected within an individual source group.

5. Conclusions

This exploratory study indicates that the virulome of multidrug-resistant *Escherichia coli* is broadly distributed across human, animal, and environmental reservoirs within the sampled One Health framework. At the pooled source-group level, the detected repertoire included a substantial shared core of determinants linked to colonization, persistence, and pathogenic potential. ExPEC-associated determinants were widely represented across compartments, whereas DEC-associated signals were more localized, particularly in animal-associated sources. These patterns suggest that broadly shared virulence architecture coexists with narrower compartment-associated signatures across the human–animal–environment interface. At the same time, these findings should be interpreted as source-group-level distribution patterns rather than as evidence of isolate-level prevalence, direct transmission, or causal reservoir relationships. The consistently high pathogenicity scores observed across sectors further support the view that predicted pathogenic potential is not confined to human-associated sources.

This underscores the value of integrated One Health surveillance for capturing how virulence-associated risk is distributed across linked reservoirs. However, because the present analysis was based on pooled source-group assemblies, it could not resolve strain-level diversity, clonal relatedness, within-genome co-carriage, or resistance–virulence linkage. Future work should therefore prioritize balanced, isolate-resolved genomic approaches

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that integrate phylogenomics, resistome–virulome profiling, plasmid reconstruction, and mobile genetic element characterization to determine how multidrug resistance and virulence are maintained, associated, and potentially disseminated across interconnected One Health systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics15050512/s1, Table S1: Functional categories of virulence determinants: definitions, counts, and genes; Table S2: Gene-level mapping to functional categories and associated pathotypes, with aggregated detections by sector; Table S3: 50 common virulence genes across sources; Table S4: Pathogenicity scores, number of pathogenic families, and virulence gene richness across sources and sectors. Table S5: Spearman correlation and Kruskal–Wallis test results for pathogenicity score and pathogenic family richness across One Health sectors. Table S6: Descriptive statistics of pathogenicity scores across One Health sectors. Table S7: Number of composite E. coli colonies per pooled source group.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the relevant institutional Ethics Committees (BCA444/16, 17 March 2017; AREC/007/018, 9 February 2018; HRKM108/17KZ_2017RP3_437, 29 March 2017; DAFF 12/11/1/5, 9 September 2018).

Informed Consent Statement: Patient consent was not required, as this study analyzed bio-banked *Escherichia coli* isolates and associated laboratory data collected as part of routine clinical care, and all data were handled in an anonymous manner, in accordance with approvals from the Biomedical Research Ethics Committee, Animal Research Ethics Committee, Department of Agriculture, Forestry and Fisheries, and the KwaZulu-Natal Provincial Health Ethics Committee.

Data Availability Statement: All sequencing reads generated from the pooled *E. coli* source groups have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject ID [PRJNA1431768].

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Conflicts of Interest: All other authors declare that they have no conflict of interest.

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One Earth–One Health to Combat Antimicrobial Resistance Having a Dual Mutation Pattern, Based on the Robust Self-Regulation and Perpetual Reconstruction of Ecosystems

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Abstract

Background/Objectives: Antimicrobial resistance (AMR) has emerged as a grave threat to human health, and a One Earth–One Health (OE-OH) concept was proposed for addressing this challenge in 2024. Here, this concept was systematically defined, clarified, and refined, for better understanding, interpreting related results, and taking some measures to combat the crisis. **Methods**: Using logical reasoning and deductive methods, a dual mutation pattern was put forward for microbial resistance, adhering to the principle of parsimony and integrating Lamarckian, Darwinian, and Niche construction theories, and the evolutionary origins of current AMR were schematically presented. Subsequently, its theoretical foundation, together with a fundamental mathematical model, was defined and clarified based on the robust self-regulation and perpetual reconstruction of ecosystems, and then the generation, dissemination, and elimination of AMR and antibiotic resistance genes (ARGs) were sorted out and elucidated from abiotic and biotic factors. Finally, learning from the approach of problem management, some crucial measures are suggested for the research and development, application, and management of antibiotics, emphasizing the key role of simulating and utilizing the self-regulation of ecosystems. **Results**: A dual mutation pattern of microbial resistance and the evolutionary origins of current AMR was put forward. The theoretical foundation of the OE-OH concept, together with a fundamental mathematical model, was presented. Some unique perspectives, such as the emergence of AMR and ARGs 3.5 billion years ago and their ubiquity across the globe prior to antibiotic use, were clarified. Moreover, some crucial measures are proposed for addressing AMR. **Conclusions**: It is essential to implement the OH Joint Plan of Action from the OE-OH perspective, strongly emphasizing the key role of simulating and utilizing the self-regulation of ecosystems on addressing AMR.

Keywords: OE-OH; one health; antibiotic resistance; microbe; plant; Lamarck; niche; Darwin; environment; combination

1. Introduction

Antimicrobial resistance (AMR) has emerged as a serious threat to global public health and economic development, and the COVID-19 pandemic has further exacerbated this crisis [1–3]. It is shown that AMR has rapidly spread from diverse settings where antibiotics are used into surrounding environments [4,5]. Moreover, antibiotic resistance genes

(ARGs) can be extensively detected in a wide range of water and soil environments [4–7], with their presence even being on the north slope of Mount Everest [8]. In light of these alarming trends, the World Health Organization (WHO) has projected that, without any intervention, antibiotic resistance would lead to 10 million deaths annually by 2050 [9,10]. In response to this crisis, the Food and Agriculture Organization of the United Nations (FAO), the United Nations Environment Programme (UNEP), the WHO, and the World Organisation for Animal Health (WOAH, founded as OIE) have developed the One Health Joint Plan of Action (2022–2026) (OH JPA) and appeal working together for the health of humans, animals, plants, and the environment [7,11]. The OH JPA outlines the commitment of the four organizations to collectively advocate and support the implementation of One Health (OH).

As is widely acknowledged, the use of antibiotics, particularly their overuse and abuse, has caused the crisis of AMR. However, it is worth exploring whether these reports can accurately reflect the actual situation of AMR. On the one hand, many studies, shaped by the interests of research, focus on the spread and evolution of AMR among microorganisms and in the environment, understudying the self-regulatory capacity of the Earth's ecosystem in weakening and eliminating AMR and ARGs [12–15]. This self-regulatory capacity encompasses the complex interactions among humans, plants, animals, microorganisms, and the environment, as well as the functional redundancy and removal of ARGs [4,16–18]. On the other hand, it is also worth contemplating whether the mere detection of ARGs implies that AMR has been transmitted to the sampling site. Both aspects have prompted a more profound re-evaluation for the strategies and measures aimed at addressing AMR. Based on the discoveries of various laws on drug combinations preventing AMR and the effects of numerous plant metabolites on reversing AMR [12,19–22], a One Earth–One Health (OE-OH) concept was put forward for preventing AMR, without details, during the 6th International Caparica Conference in Antibiotic Resistance 2024 (IC^2AR 2024) and in a following publication [12,23].

OE-OH is a systems engineering approach and concept that takes niche construction as its core theoretical foundation. It progresses from exploring, understanding, and interpreting the self-regulation and continuous reconstruction mechanisms of ecosystems to simulating and applying these mechanisms, ultimately aiming to promote human health and sustainable development, particularly in addressing the crisis posed by AMR [12,23]. It adheres to holism and systems thinking, regarding the Earth as a giant ecosystem composed of countless sub-ecosystems that operate through analogous mechanisms and involve diverse abiotic and biotic factors such as soil, climate, air, sunlight, water, plate tectonics, geological disasters, microbes, plants, animals, and/or humans. It believes and emphasizes the strong regulatory capacity of the Earth's ecosystem and advocates the adoption of eco-mimetic methods and strategies in addressing health threats.

The OH concept posits that AMR encompasses multiple facets, including humans, animals, plants, and the environment, emphasizing the pivotal role of humans in combating AMR. In contrast, OE-OH places greater emphasis on the robust self-regulatory capacity of the Earth's ecosystem and its sub-ecosystems in relation to AMR. Specifically, it accords equal importance to the generation, spread, weakening, and elimination of AMR within the ecosystem, and regards the production and use of antibiotics as an intervention on the ecosystem's AMR dynamics. Moreover, in approaches and strategies for addressing AMR, OE-OH attaches great importance to simulating and applying the regulatory mechanisms of ecosystems. Therefore, OE-OH can serve as an important reference in approach and concept for ensuring the scientific, rational, and sustainable implementation of the OH JPA. Herein, the theoretical foundation of this concept, together with many innovative prospectives, reasoning, deductions, and inductions concerning AMR and ARGs, are presented, from the OE-OH concept, as follows. Based on these, some distinctive strategies for combatting AMR are subsequently proposed and elaborated.

2. The OE-OH Concept

2.1. A Dual Mutation Pattern of Microbial Resistance

In accordance with the principle of parsimony [24,25], also known as Occam's Razor, the mutation theory of bacterial resistance can be reconsidered. We believe that microbial resistance predominantly engages in proactive evolution by adaptive mechanisms, which are designed to avert the unnecessary expenditure of energy and resources that are typically associated with random mutations. This assertion aligns with Lamarck's theory, which has also garnered support from recent research endeavors [26]. However, there may also be minor imprecise mutants during the course of proactive resistance mutations. Simultaneously, some ARG-carrying mutants can also emerge through the occasional and non-adaptive random mutations and be passively screened by natural selection in accordance with Darwinian evolution. From these, ARG-carrying mutants originate from two distinct types of mutations, as shown in Figure 1A. The dual mutation pattern of microbial resistance, characterized primarily by proactive evolution and occasionally by passive selection, exhibits a striking congruence with the niche construction theory, which pertains to ecosystems [27,28]. From this pattern, the evolutionary origins of current AMR were deduced and is shown in Figure 1B. Moreover, although proactive mutation is the primary pattern of microbial resistance to antibiotics, a large number of antibiotic-resistant microbes can still be found prior to exposure to antibiotics. This is due to the substantial accumulation of ARG-carrying pathogens in the Earth's ecosystem over a long period of time.

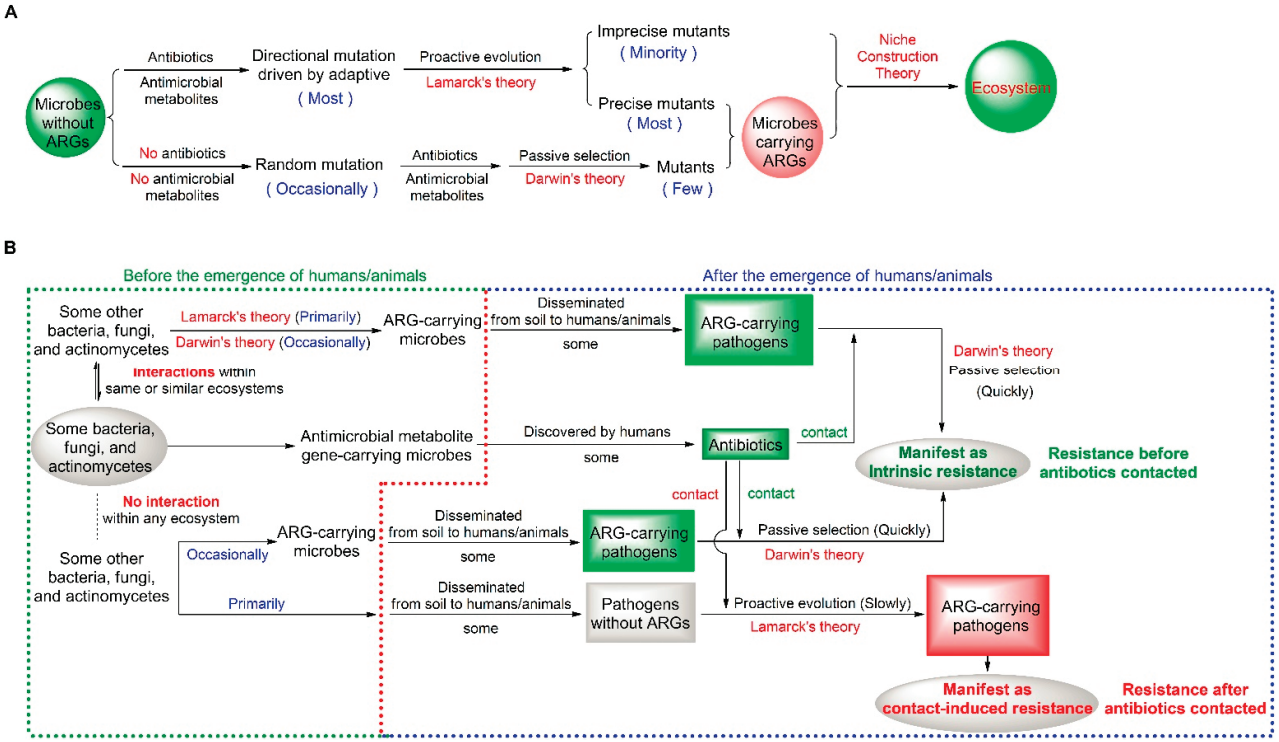

Figure 1. A dual mutation pattern of microbial resistance (**A**) and the evolutionary origins of current antimicrobial resistance (AMR) (**B**). ARGs, antibiotic resistance genes.

2.2. Theoretical Logic of the OE-OH Concept Based on Ecosystems

Inspired by the dual mutation pattern illustrated in Figure 1, it can be inferred that antimicrobial metabolites produced by microbes (some of which are termed antibiotics now), microbial resistance, and ARGs likely emerged concurrently with the formation of microbial ecosystems. This is because they arise from the competition among microorganisms within ecosystems and are renewed as ecosystems undergo reconstruction [29–32]. Namely,

ARG-carrying bacteria likely first emerged when certain microbial ecosystems were formed 3.5 billion years ago [33] and have been continuously spreading and renewing ever since (Figure 2). This was also supported by a recent publication [34]. Simultaneously, the Earth, including soil, various environments, and all the organisms that inhabit it, can be regarded as a giant ecosystem composed of countless sub-ecosystems that operate through similar mechanisms. Therefore, the robust self-regulatory capacity and perpetual reconstruction of ecosystems, particularly those of the Earth's [13–15], form the theoretical logic of the OE-OH concept. From this perspective, various aspects related to microbial resistance can be reexamined.

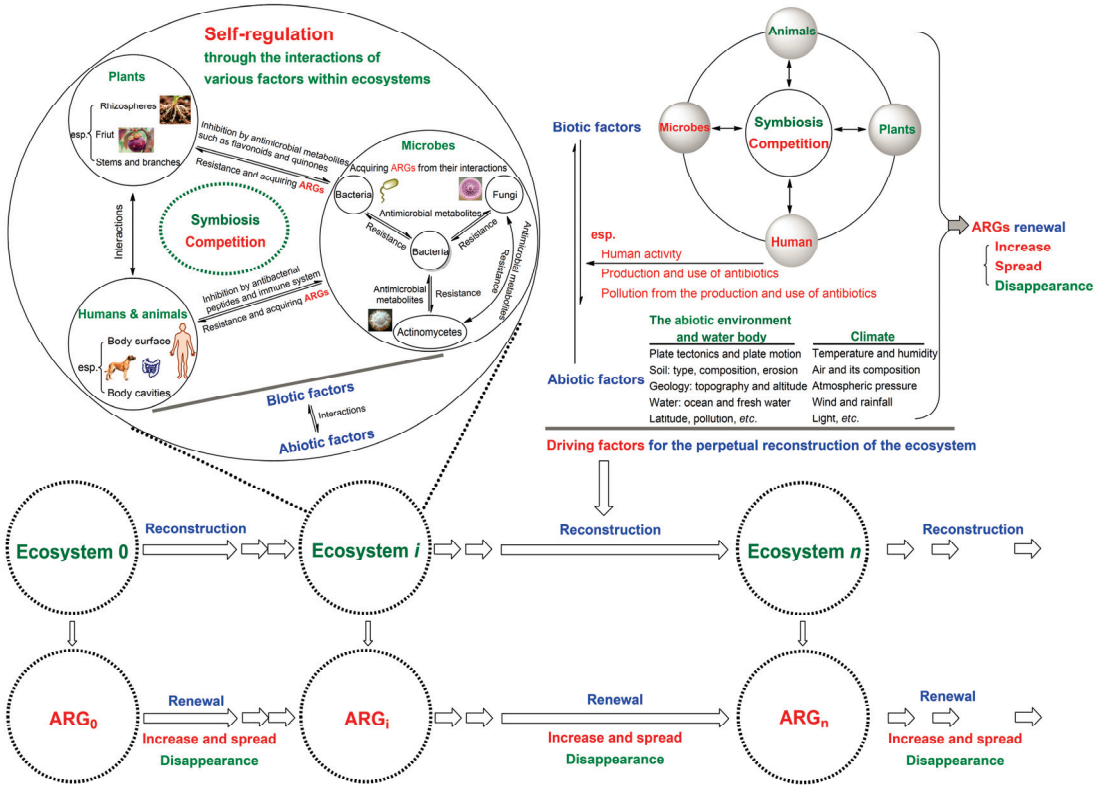

Figure 2. Schematic for the theoretical foundation of the One Earth–One Health concept. This includes the theoretical logic and driving factors of ARG renewal within an ecosystem based on the robust self-regulatory capacity and perpetual reconstruction of ecosystems; the ecosystem can be the entire Earth's ecosystem or its various sub-ecosystems; ecosystems 0, i, and n indicate the ecosystem at different time points during its evolution, and their antibiotic resistance genes (ARGs) correspondingly renew with the perpetual reconstruction of the ecosystem.

2.3. A Fundamental Mathematical Model for the ARGs Renewing with the Ecosystem

The OE-OH concept envisions the Earth as a giant ecosystem, intricately woven from a myriad of sub-ecosystems that operate through similar mechanisms. The amount of ARGs within an ecosystem can be articulated through a fundamental mathematical model as follows.

$$ARG_i = ARG_0 + (ARG_1{}^{In} + ARG_2{}^{In} + \ldots\ldots + ARG_i{}^{In} + \ldots\ldots + ARG_n{}^{In}) + (ARG_1{}^{De} + ARG_2{}^{De} + \ldots\ldots + ARG_i{}^{De} + \ldots\ldots + ARG_n{}^{De})$$

$$ARG_n = ARG_0 + \sum_{i=1}^{n} ARG_i^{In} + \sum_{i=1}^{n} ARG_i^{De}$$

where ARG_i and ARG_n are the amount of ARGs at two time points (Figures 2 and 3) of the ecosystem, which can be the Earth's ecosystem or its various sub-ones, and time point i can be equal to n; ARG_0 can be the amount of ARGs at any time point, while time points i and n are more than or equal to time point 0, and especially, the amount of ARG_0 is zero

before the microbial ecosystem emerged on Earth; ARG^{In} is the increased amount of ARGs within the ecosystem, for example, $ARG_1{}^{In}$ is that from time point 0 to 1; ARG^{De} (defined as a negative value) is the decreased amount of ARGs within the ecosystem, for example, $ARG_1{}^{De}$ is that from time point 0 to 1; $\sum_{i=1}^{n} ARG_i^{In}$ is the sum of the increased amount of ARG_s from time point 1 to n, and $\sum_{i=1}^{n} ARG_i^{De}$ is the sum of the decreased amount of ARG_s from time point 1 to n.

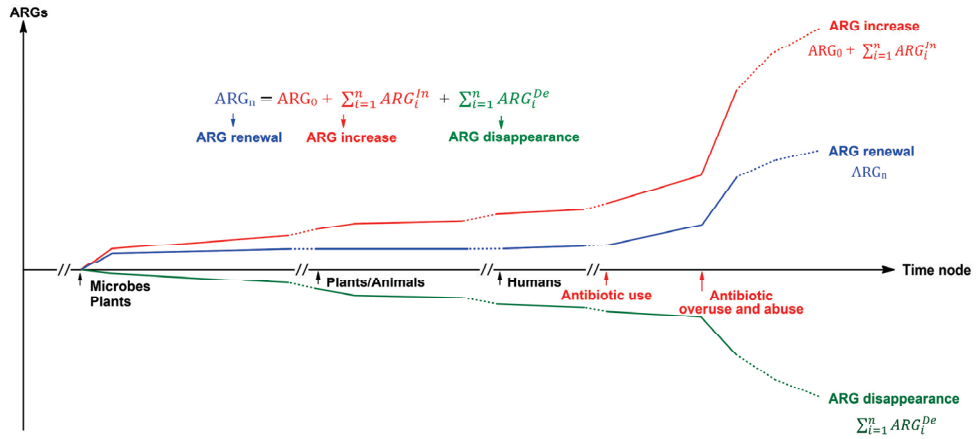

Figure 3. A schematic for the amount of antibiotic resistance genes (ARGs, y) within an ecosystem changing with typical evolutionary time nodes (Time node, x), according to a fundamental mathematical model. The ecosystem can be the entire Earth's ecosystem or its various sub-ecosystems, and the typical time nodes include the emergence of microbes, plants, animals, and humans and the use of antibiotics by humans.

3. ARG Analyses from the OE-OH Concept Based on Ecosystems

3.1. ARGs Emerging Prior to the Emergence of Humans and Existing Everywhere in the World

Antimicrobial metabolites are a category of natural products that generate from the competition for ecological niches among microorganisms within ecosystems or in response to survival stress [29–31]. As noted earlier, microbes emerged 3.5 billion years ago, and by that time, diverse ecosystems teeming with microbial communities had already taken shape [33,34]. After a long period of evolution, there are sufficient reasons to infer that the structural skeletons of most clinical antibiotics can be biosynthesized by environmental microorganisms. This can be confirmed by the fact that the structural skeletons of clinical antibiotics were mostly discovered from soil microorganisms [29]. In other words, microorganisms that carry genetic information for biosynthesizing the structural skeletons of most clinical antibiotics, including microbial strains belonging to the same or different genera and species, are widely distributed in various sub-ecosystems of Earth. Even synthetic quinolone antimicrobial agents bear a resemblance in structural framework to naturally occurring compounds such as plant-derived flavonoids and benzofuranones [12,35]. This is also a reason that natural products with antimicrobial activity can be continuously discovered from environmental microorganisms. Likely, the genetic information of microorganisms producing these natural products has existed on the Earth before the emergence of humans [36–38] but was only discovered and excavated after entering into the 20th century.

It is reported that today's pathogenic microorganisms originate from the soil [39,40]. Therefore, clinical pathogens should be widely distributed across various ecosystems on Earth and likely prior to the emergence of humans [41]. Since antimicrobial metabolites were generated with the formation of microbial ecosystems, it is reasonable to deduce that, due to microbial competition within ecosystems, the ARGs of pathogenic microorganisms evolved in response to the survival stress from antimicrobial metabolites (some of which

are now used as antibiotics) produced by other environmental microbes may have already emerged at that time.

As the Earth's ecosystem and its diverse sub-ecosystems evolve and undergo reconstruction driven by a variety of biotic and abiotic factors [42], new ARGs are perpetually emerging. Moreover, ARGs are continuously spreading and renewing across every corner of the Earth. Therefore, it can be inferred that microorganisms responsible for producing the structural skeleton of certain antibiotics, along with pathogens carrying corresponding ARGs, are located in specific ecosystems. When antibiotics are isolated from soil or marine microbes, it is likely that these pathogens have already encountered these antibiotics during the evolutionary process of the Earth's ecosystem. Through proactive adaptive mutations and the acquisition of heritable ARGs, these pathogens have also developed resistance to these antibiotics. This may explain why ARG-carrying pathogens can sometimes be detected shortly after the introduction of new antibiotics to the market [43], or before they appear to have come into contact with the corresponding antibiotics, sometimes even before the antibiotics are officially approved [44]. Namely, these pathogens have, in fact, been exposed to the corresponding antibiotics long ago.

Given this, it is foreseeable that ARGs can be found not only in extreme environments such as Mount Everest and the Mariana Trench but also in the Antarctic and Arctic regions of the Earth. This notion is also supported by recent research [45–49] and means that ARGs are likely to be detected in varying degrees from every ecological niche containing microbes if the detection methods are sufficiently sensitive. Moreover, it is reasonable to assume that a certain amount of ARGs existed in various ecosystems and across the Earth prior to the production and use of antibiotics by humans (Figure 3).

3.2. ARGs by the Self-Regulation of Ecosystems Before the Use of Antibiotics

Since the emergence of microbes on Earth, diverse ecosystems composed of microbial communities have gradually taken shape and continuously evolved along with the incorporation of new organisms and the elimination of existing ones. Throughout the Earth's evolutionary history, information on antibiotic resistance genes (ARGs) have been in a state of perpetual renewal, driven by the ongoing reconstruction of ecosystems. This renewal process encompasses the emergence and dissemination of new ARGs, as well as the weakening and elimination of existing ones, which is also supported by recent research [4,6,50–54].

As shown in Box 1, a wide range of biotic and abiotic factors, including the abiotic environment, water bodies, climate, microbes, plants, animals, and humans, can impact the renewal of ARGs within the Earth's ecosystem and its various sub-ecosystems. Owing to the sufficient self-regulation, self-balancing, and buffering capacities of the Earth's ecosystem [12–15], the evolution of AMR and ARGs has historically maintained a balanced and controllable state prior to the industrial production and use of antibiotics by humans. However, the situation has become increasingly worrying due to the extensive use of antibiotics, particularly their overuse and abuse in clinical settings, livestock, poultry farming, and aquaculture. This was also reflected in the rapid increase in AMR and the widespread dissemination of ARGs after the industrial production and use of antibiotics.

Box 1. The OE-OH concept based on the self-regulation of the Earth's ecosystem for AMR and ARGs.

$$ARG_n = ARG_0 + \sum_{i=1}^{n} ARG_i^{In} + \sum_{i=1}^{n} ARG_i^{De}$$

Factors for the generation and spread of ARGs (ARG^{In})	Factors for the weakening and elimination of ARGs (ARG^{De})
Abiotic factors **The abiotic environment and water bodies** Plate tectonics and plate motion, geological disasters Soil components, dust, and air [6,39,55–59] Hydrographic system and water flows [5,60,61] Ocean and its currents [62–64] The melting of ice and snow in the Arctic, the Antarctic, and high mountains [8] **Climate** Rainfall, wind, global warming, and drought [42,65] Biotic factors influenced by climate change and climate extremes [66–69]	**Abiotic factors** **The abiotic environment and water bodies** Plate tectonics and plate motion, geological disasters Soil components, dust, and air [6,55,70] Dilution and/or elimination of antibiotics and ARGs [71–74] Dilution, decrease, and disappearance of selection pressure [51] The killing and clearance of ARGs and antibiotic-resistant pathogens [72,75–78] **Climate** Rainfall, wind, global warming, and drought [42,65,76] Biotic factors influenced by climate change and climate extremes [51,67,68]
	
Biotic factors **Microbes** Bacteriophage and virus [79–81] The interaction of various microbes [29–31,67,82,83] Amino acid auxotrophic microbes [84] The activation to ARG expressions by antimicrobial metabolites [18] Horizontal transfer of ARGs according to genetic compatibility and ecological connectivity [54] **Plants** The decrease in plant diversity [85] ARG enrichment in the rhizosphere of plants [86] The activation of ARG expressions by plant antimicrobial metabolites [18] The interactions between plants and microbes **Animals** Activities and migration Foraging and excretion [87–93] The activation of ARG expressions by antimicrobial peptides [18] **Human and human activity** Daily activities, migration, travels, diet, and excretion [3,39,87,88,90,92,94–96] The activation of ARG expressions by antimicrobial peptides [52] Population gathering and domestic sewage [4,97,98] The use and abuse of antimicrobial agents in medical activities, livestock, poultry farming, aquaculture, etc. [4,51,62] The use of air and environmental disinfectants [99] The pollution of air and water from the production and use of antibiotics and environmental disinfectants [3,4,71,99–102] The impacts of human activities on all biotic and abiotic factors that generate and spread AMR and ARGs in the Earth′s ecosystem [63,66,85,103–105]	**Biotic factors** **Microbes** Fitness costs [50] Negative selection and disappearance of selection pressure [16–18,51] Genetic incompatibility inhibiting the horizontal transfer of ARGs [54,106] The degradation of interactions among various microbes with antibiotics [82,107] The killing and inhibition of other microbes and pathogens within ecosystems [4] **Plants** The increase in plant diversity [85] The degradation of antimicrobial agents by plants [108–110] Sensitization of antimicrobial resistance by plant antimicrobial metabolites [12,111] The killing and clearance of plant antimicrobial metabolites to drug-resistant pathogens [4,112] **Animals** The killing and clearance of animal bodies to drug-resistant pathogens including the immune system, antibacterial peptides, beneficial microorganisms, etc. [113] Foraging and sensitization of the intestinal microbial ecology to drug-resistant pathogens [4] **Human and human activity** The killing and clearance of human bodies to antibiotic-resistant pathogens [52,53,114,115] including the immune system, antibacterial peptides, beneficial microorganisms, etc. Diet and sensitization of gut microbial ecology to antibiotic-resistant pathogens [12,116] Degradation, reduction, and removal of antibiotics in waste residues and water from medical activities, livestock, poultry farming, aquaculture, etc. [72,107,108] Disinfection and inactivation of antibiotic-resistant pathogens All other possible human activities on biotic and abiotic factors that clear antibiotics and decrease ARGs within the Earth′s ecosystems [72,104,113,117]

Note: OE-OH, One Earth-One Health; AMR, antimicrobial resistance; ARGs, antibiotic resistance genes; ARG^{In}, the increased amount of ARGs within the ecosystem; and ARG^{De}, the decreased amount of ARGs within the ecosystem.

3.3. Impact of Antibiotic Use on the Self-Regulation of Ecosystems to ARGs

In the 20th century, many secondary metabolites with antimicrobial activities at low concentrations were discovered from environmental microorganisms, particularly actinomycetes, fungi, and bacteria, across diverse habitats such as land, oceans, and the body surfaces and feces of animals and humans [29,31]. Some of these metabolites and their derivatives have been developed as clinical antibiotics. In fact, the genetic information of microorganisms producing these antibiotics, together with the corresponding ARGs carried by pathogens resistant to these antibiotics, was already generated long ago and can be considered as the products of competition among various microorganisms during the Earth's evolutionary history. Although most of the clinical antibiotics currently in use are structural derivatives of these natural antibiotics, they share similar structural skeletons with corresponding naturally sourced antibiotics. Consequently, the information of their corresponding ARGs carried by pathogens has existed in nature long ago. Moreover, pathogens carrying these resistance genes have spread globally, with some disseminating through soil and others colonizing specific parts of the human body [39].

From the above, the widespread use of antibiotics across various domains, including medical practices, livestock breeding, poultry farming, and aquaculture, can be considered as human intervention on the evolution of ARGs within the global ecosystem [82]. In addition, general human activities, as well as the interactions between the human body and microbes, have been driving the spread of ARGs (Box 1) prior to the use of antibiotics; several key activities associated with the industrial production and use of antibiotics by humans have led to a sharp increase in AMR and the rapid spread of ARGs. These activities are as follows: (1) the use, overuse and abuse of antibiotics in medical practices, livestock, poultry farming, and aquaculture, etc. [4,51,62]; (2) the pollution of the Earth's environment caused by wastewater discharged from the settings of antibiotic production and use [3,4,7,62,71,100–102]; (3) the excessive density and size of urban populations, and the large amounts of domestic sewage generated under conditions of antibiotic overuse and misuse [4,97,98]; and (4) the impacts of other human activities on all biotic and abiotic factors that contribute to the generation and spread ARGs within the Earth's ecosystem [63,66,85,103–105].

Theoretically, the wider the application scope of antibiotics and the greater their usage, the larger the intervention intensity on ecosystems. When the intervention intensity keeps within a specific and controllable range, the Earth's ecosystem and its myriad of sub-ecosystems have sufficient self-regulation capabilities to restore or reestablish a new balance (Box 1). But if the intervention intensity exceeds the self-regulation capacity of ecosystems, the balance of these ecosystems will inevitably be disrupted, endangering larger ecosystems and even the entire Earth's ecosystem [13,118]. Therefore, our endeavors to discover new antibiotics seem to have the potential to solve the problem of antibiotic shortage caused by AMR, however they are merely a passive defense strategy even if the development of new antibiotics can be accelerated with the assistance of artificial intelligence (AI) [119]. Although AI has the potential to predict microbial resistance to existing antibiotics and facilitate a proactive defense in the future, the struggle between humans and microbes is at most evenly matched. More significantly, the damage to various ecosystems caused by the overuse and abuse of antibiotics, together with the impact and deterioration of new ecosystem reconstruction on human living conditions, should arouse sufficient attention in this struggle.

Therefore, if the overuse and abuse of antibiotics are not controlled, the approval and application of new antibiotics will only accelerate the spread of AMR and ARGs. This will lead to the continuous destruction of larger and more ecosystems centered on the application environment. Moreover, various ecosystems containing more pathogens carrying

ARGs will be restructured, ultimately posing a threat to the survival and development of humans. Thus, it is imperative to implement scientific and rational measures to control the spread of AMR and ARGs, to maintain the balance of the Earth's ecosystem and its various sub-ecosystems.

4. Measures Combating AMR from the OE-OH Concept Based on Ecosystems

As previously stated, the use of antibiotics can be regarded as an intervention on ecosystems from the OE-OH concept. Thereby, a counteracting intervention aimed at preserving the balance of ecosystems should be taken for effectively combating AMR (Figure 4) [120]. Simultaneously, the self-regulation and -balance capabilities of ecosystems can be fully understood and utilized for risk evaluation on some measures taken for the research and development, application, and management of antibiotics. Learning from the approach of problem management [121,122], some crucial measures from the OE-OH concept, with the support of the literature, are suggested as follows:

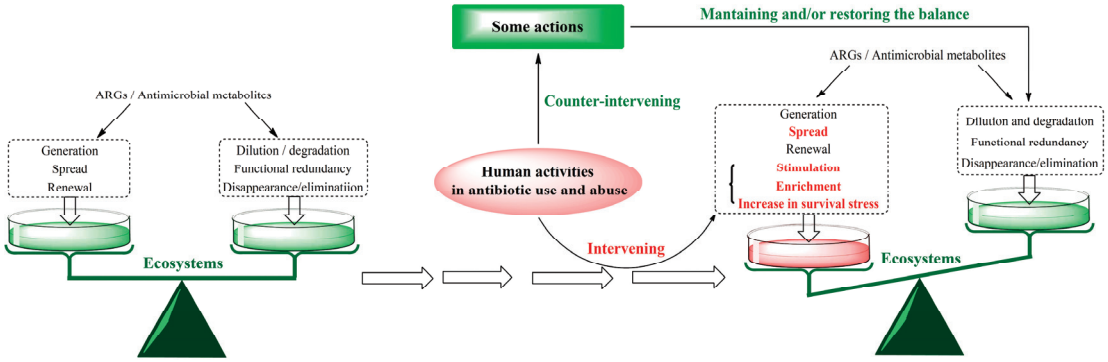

Figure 4. Analysis schematic of some imperative actions taken to offset the imbalance risk of ecosystems caused by the antibiotic use exceeding the self-regulation and balancing capacity of ecosystems, for combating the AMR from the OE-OH concept. ARGs, antibiotic resistance genes.

4.1. Minimizing Antibiotic Use, While Fully Utilizing the Regulatory Role of Plants

From the OE-OH perspective, the bodies of humans or animals themselves can be regarded as ecosystems with sufficient self-regulation capabilities. For instance, their immune systems [52], antimicrobial peptides [114], colonized probiotics, and the competition among microorganisms within the body's ecosystem can eliminate infected pathogens, including those that are antibiotic-resistant. Meanwhile, many plants, such as traditional Chinese medicines and ethnic medicines, also have the potential to regulate the balance between the bodies of humans or animals and microbes [12,112,123]. This can be achieved through their regulatory effects on the body's inflammatory and immune responses, their direct antibacterial, antifungal, and antiviral actions, as well as their ability to enhance the inhibitory activities of metabolites produced by probiotics against antimicrobial-resistant microbes. This has been evidenced in China during the COVID-19 pandemic [124]. Therefore, it is entirely feasible to reduce the use of antibiotics through utilizing the self-regulation and self-balance abilities of both humans and animals themselves, as well as the regulatory effects of plants on these abilities. Furthermore, even in cases where the bodies of humans and animals are infected, the balance between them and microbes can still be regulated or restored by plants. This can help to mitigate the progression of microbial infections, to the greatest extent possible, and avoid the unnecessary use of antibiotics.

4.2. Minimizing Antibiotic Emissions, While Fully Utilizing the Self-Regulation of Ecosystems

From the OE-OH perspective, microbial resistance predominantly follows the proactive pattern of adaptive evolution, occasionally the passive one of random mutation by natural selection. As the Earth continues to evolve and develop, ARG-carrying pathogens that are widely distributed can be activated, screened, and enriched under the stress of antibiotics. Consequently, the emission of antibiotics into the surrounding environment not only stimulates the overexpression of ARGs in pathogens and enriches the information of ARGs but also enables susceptible bacteria to proactively evolve into resistant pathogens. Therefore, it is crucial to minimize the emission of antibiotics and ARGs.

Alternatively, from the OE-OH perspective, the transmission processes of antibiotics and ARGs also involve their dilution, redundancy, or disappearance by various ecosystems. Therefore, when effective control is challenging and emissions are unavoidable, the weakening and elimination capacities of ecosystems to antibiotics and ARGs can be fully utilized [123,125–127]. At this moment, scientific and rational measures should be adopted, based on risk management [128], to implement graded emissions for keeping the emissions of antibiotics and ARGs within the controllable and balanced range of ecosystems, minimizing the damage caused by the excessive accumulation of antibiotics and ARGs to the original ecosystem.

4.3. Avoiding Excessive Density and Size of Urban Population

From the OE-OH perspective, the entire Earth's ecosystem possesses a robust self-regulation capacity to manage the generation, dissemination, enrichment, dilution, weakening, and elimination of ARGs, thereby maintaining its equilibrium. However, the overuse and abuse of antibiotics by humans will lead to the continuous enrichment and spread of ARGs and stimulate their proliferation in the living environment [129], resulting in an increasing diversity and abundance of ARGs around their habitat. If the density and size of urban populations become excessive at this moment, they will significantly surpass the capacity of various biotic and abiotic factors (Box 1) to weaken and eliminate ARGs, as well as the self-regulation ability of ecosystems, leading to an excessive accumulation of ARGs in ecosystems centered around the gathering areas of urban populations. Therefore, it is crucial to avoid the excessive density and size of urban populations [126]. Also, it is better to be kept within reasonable limits for the scale of cities, and the urban layouts should be appropriately decentralized. This can also be indirectly proved by the transmission patterns of the COVID-19 pandemic. Therefore, it may be encouraged to conduct research on the impact of urban size and distribution spacing, as well as the density, distribution, and size of urban populations, on the enrichment and dissemination of ARGs.

4.4. Accelerating the Antibiotic Reserve Based on the Understanding for Microbial Defense Mechanisms

From the OE-OH perspective, it is unrealistic for humans to discover antibiotics to which microorganisms will never develop resistance. This also holds true for multi-target antibiotics whether they are natural or fully synthetic products. Nevertheless, to combat AMR, it remains necessary to develop new antibiotics that are difficult to trigger a resistance to, as much as possible, and a sufficient reserve of these new antibiotics should be ensured. To achieve this, we can gain a comprehensive understanding of the proactive defense mechanisms of microbes in ecosystems [18] and thoroughly explore the unknown ARGs within ecosystems to avoid, as much as possible, using new antibiotics to treat infections caused by pathogens carrying the corresponding ARGs. To attain this, we can fully utilize AI technology to comprehensively predict potential ARGs of pathogens and possible AMR [130,131]. Moreover, it is also encouraged to develop new antibiotics with

high specificity and minimal disruption to gut microbes and organismal ecosystems [132]. In addition, from a policy standpoint, the protection period of patents for new antibiotics can be extended, for reducing the unnecessary use of new antibiotics.

4.5. Encouraging Antibiotics Used in Combination with Plant-Derived Antimicrobial Ingredients

From the OE-OH perspective, the increased application of new antibiotics will accelerate the spread and enrichment of AMR and ARGs if the overuse and abuse of antibiotics are not controlled. Therefore, it is imperative to use antibiotics rationally. Among various strategies for the rational use of antibiotics, combination therapy has the advantages of cost-effectiveness in enhancing the efficacy of antibiotics, reversing microbial resistance, and extending the life cycle of antibiotics, buying more time for the development of new antibiotics. This can also be proved by the clinical practice of combination therapy, such as the combination of sulfamethoxazole and trimethoprim, β-lactamase inhibitors and β-lactam antibiotics, and multiple anti-tuberculosis drugs. Therefore, combination therapy is highly commendable [12,82]. However, it is noteworthy that an inappropriate antibiotic combination would instead increase the risk of AMR, due to the effect of preventing resistance being associated with the fractional inhibitory concentration index, as well as the proportion and concentration of two antibiotics in the combination [19,20,133].

Antibiotics are derived from the competition among microorganisms. Both bacteria/fungi producing the structural skeleton of antibiotics and pathogenic bacteria are classified as microbes. As a result, their individual defense mechanisms are familiar to each other, making it easier for pathogens to develop resistance to antibiotics in the combination. However, the antimicrobial ingredients of plants originate from the interaction between microorganisms and plants within ecosystems, and the defense mechanisms between plants and microbes are less familiar to each other. Simultaneously, plant-derived antimicrobial components generally exhibit weaker antibacterial activity and smaller stresses on microbial survival compared with antibiotics. Therefore, it is more challenging for microbes to develop a resistance to them [20]. Moreover, the combination of plant-derived antibacterial components and antibiotics often has a wide range of synergistic effects [12], and the impact on gut microbes is also milder. Therefore, plant-derived antibacterial components are ideal candidates for combination therapy with antibiotics.

4.6. Simulating the Elimination of Antibiotics and ARGs Within Ecosystems

From the OE-OH perspective, ecosystems have robust capacity to regulate AMR, encompassing the dilution, weakening, and elimination of ARGs. Therefore, it is highly encouraged to simulate the elimination of antibiotics and ARGs within ecosystems based on a thorough understanding of their self-regulation mechanisms [120,134]. For instance, employing microbial ecology to combat ARG dissemination [4], utilizing photocatalysis-enhanced constructed wetlands to remove ARGs [72], simulating sunlight-induced inactivation of tetracycline-resistant bacteria [75], and leveraging bacteria–microalgae–fungi symbionts or plants to remove antibiotics [107,108]. Additionally, these ecological simulation methods and technologies can also, to the greatest extent possible, avoid the potential adverse effects on ecosystems that may be caused by the measures taken.

5. Methods

5.1. The Definition, Refinement and Clarification of the OE-OH Concept

The OE-OH concept was put forward from the previous results and the sufficient self-regulation ability of the Earth's ecosystem in the evolution of AMR [12,23]. Here this concept has been further defined, improved, and clarified using logical reasoning and deductive methods based on the principle of parsimony [24,25], involving the understanding

integration of Lamarck's theory, Darwinian evolution, and the niche construction theory. It includes the dual mutation patterns of microbial resistance, the theoretical underpinnings of the OE-OH concept based on ecosystems, and a basic mathematical model for the ARGs renewing with the ecosystem. The literature supporting this reasoning and deduction, together with all other literature, was unsystematically searched from PubMed database and Google academic search engine, using various relevant keywords. Furthermore, some highly persuasive references in the obtained literature were also tracked.

5.2. Analyses of ARG Generation, Spread and Elimination from the OE-OH Concept

From the OE-OH concept, the generation, spread, and elimination of ARGs along with different time nodes of the Earth's evolution were analyzed and sorted out from various aspects of abiotic and biotic factors [33,34,42], using reasoning, deductive, and inductive methods based on the self-regulation of ecosystems [13–15], together with the supporting literature unsystematically searched from PubMed database and Google academic search engine using some relevant search terms. These especially include the emergence and distribution of ARGs emerging before humans, ARG regulation by ecosystems such as the generation, spread, weakening, and elimination of ARGs before the industrial production and use of antibiotics, and the sharp increase in ARGs after the industrial production and use of antibiotics, which together impact the self-regulation and self-balance of ecosystems for ARGs.

5.3. Measures Combating AMR from the OE-OH Concept Based on Ecosystems

Regarding the use of antibiotics as an intervention on ecosystems, some important measures for the research and development, application, and management of antibiotics are suggested, from the OE-OH prospective, for maintaining the balance of ecosystems regulating ARGs, learning from the approach of problem management [121,122].

6. Future Directions

Drawing on the OE-OH concept, the following research directions are proposed for future exploration: (1) referring to Lamarck's theory and niche construction, emphasizing the interactions among various factors in host ecosystems and strengthening research on the proactive defense mechanisms of microbe, for developing new antibiotics with strong selectivity that cause minimal disruption to human ecosystems; (2) conducting in-depth investigations into ARGs in problem-oriented samples from both designed terrestrial and marine environments, which can not only can help to elucidate the dissemination of ARGs but can also provide additional evidence for understanding the Earth's evolution, plate tectonics, and human migration; (3) emphasizing the crucial role of the self-regulation of ecosystems on addressing AMR, strengthening research on the regulation of plants on the ecosystem of human bodies to prevent microbial infection, and encouraging the research and development of antibiotics in combination with plant-derived antimicrobial ingredients; (4) increasing efforts to study the dilution and degradation of antibiotics in ecosystems, as well as the weakening and elimination of ARGs, and developing methods and technologies that simulate the elimination of antibiotics and ARGs within ecosystems; and (5) based on the self-regulatory capacity of ecosystems in the elimination of antibiotics and ARGs, conducting research on the schemes of population distribution and urban settlements for combatting AMR.

7. Conclusions

The OE-OH concept has been clarified and refined, which includes (1) a dual mutation pattern of primarily proactive evolution aligning with Lamarck's theory and occasionally

passive selection in accordance with Darwinian evolution theory for microbial resistance mutation, adhering to the principle of parsimony; (2) the theoretical logic of this concept, which is based on the robust self-regulatory capacity and perpetual reconstruction of ecosystems, and a fundamental mathematical model for the renewal of ARGs within the ecosystem; and (3) the notion that the farther the evolutionary distance between species, the weaker the antagonistic effect of the secondary metabolites they produce on each other, and the more difficult it is for them to develop resistance to each other. Derived from this concept, it deduced that AMR and ARGs emerged 3.5 billion years ago and existed in every corner of the Earth prior to the use of antibiotics by humans. Regarded as an intervention on ecosystems, the use of antibiotics, particularly their overuse and abuse, has posed a concern that transcends the ecosystem's self-regulatory capacity. Based on these, several crucial measures derived from the OE-OH concept are proposed for combatting AMR. These measures place a strong emphasis on simulating and leveraging the self-regulatory mechanisms of ecosystems, advocating for the minimization of antibiotic use and emissions, preventing the excessive density and size of urban populations, and encouraging antibiotics used in combination with plant-derived antimicrobial ingredients. Finally, it is essential to implement the OH Joint Plan of Action from the OE-OH perspective, emphasizing the key role of utilizing the self-regulation of ecosystems in addressing AMR.

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Review

The Role of the Environment (Water, Air, Soil) in the Emergence and Dissemination of Antimicrobial Resistance: A One Health Perspective

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Abstract

Antimicrobial resistance (AMR) has emerged as a planetary health emergency, driven not only by the clinical misuse of antibiotics but also by diverse environmental dissemination pathways. This review critically examines the role of environmental compartments—water, soil, and air—as dynamic reservoirs and transmission routes for antibiotic-resistant bacteria (ARB) and resistance genes (ARGs). Recent metagenomic, epidemiological, and mechanistic evidence demonstrates that anthropogenic pressures—including pharmaceutical effluents, agricultural runoff, untreated sewage, and airborne emissions—amplify resistance evolution and interspecies gene transfer via horizontal gene transfer mechanisms, biofilms, and mobile genetic elements. Importantly, it is not only highly polluted rivers such as the Ganges that contribute to the spread of AMR; even low concentrations of antibiotics and their metabolites, formed during or after treatment, can significantly promote the selection and dissemination of resistance. Environmental hotspots such as European agricultural soils and airborne particulate zones near wastewater treatment plants further illustrate the complexity and global scope of pollution-driven AMR. The synergistic roles of co-selective agents, including heavy metals, disinfectants, and microplastics, are highlighted for their impact in exacerbating resistance gene propagation across ecological and geographical boundaries. The efficacy and limitations of current mitigation strategies, including advanced wastewater treatments, thermophilic composting, biosensor-based surveillance, and emerging regulatory frameworks, are evaluated. By integrating a One Health perspective, this review underscores the imperative of including environmental considerations in global AMR containment policies and proposes a multidisciplinary roadmap to mitigate resistance spread across interconnected human, animal, and environmental domains.

Keywords: antimicrobial resistance; antibiotic-resistant bacteria; environmental pollution; One Health; horizontal gene transfer; metagenomics; waterborne pathogens

1. Introduction

Antimicrobial resistance (AMR) has become one of the most pressing global health challenges of the 21st century. It undermines the effectiveness of antibiotics, threatens medical advancements, and contributes to increased mortality, morbidity, and healthcare costs worldwide. The World Health Organization (WHO) has designated AMR as a top public health priority, advocating for a coordinated "One Health" approach that integrates human, animal, and environmental health efforts [1].

Although AMR research and surveillance have historically centered on clinical settings, the environmental dimension of resistance is gaining increasing attention. Multiple environmental compartments act as reservoirs and conduits for the spread of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). For instance, aquatic environments polluted by untreated or inadequately treated sewage and pharmaceutical effluents [2], agricultural soils exposed to manure or irrigation runoff containing antimicrobial residues [3], and aerosols originating from livestock operations or wastewater treatment facilities [4] all contribute to the dissemination of resistance determinants. These environmental sources not only harbor resistance elements but also enable their transfer across microbial species and ecosystems through horizontal gene transfer [5]. Figure 1 presents an overview of key environmental pathways involved in the emergence and dissemination of resistance.

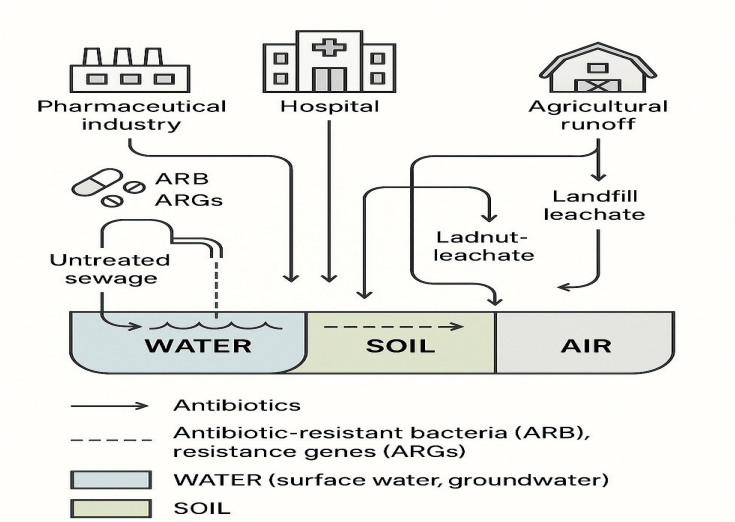

Figure 1. Main entry routes of antibiotics into the environment and their contribution to antimicrobial resistance. Adapted from (Berendonk et al., 2015) [2].

Addressing the environmental drivers of AMR is essential, particularly in regions lacking stringent waste disposal regulations and environmental safeguards [6,7]. Unlike the more controlled conditions of clinical settings, environmental matrices are highly heterogeneous and often less monitored, allowing resistance to persist and evolve unnoticed.

This review provides a critical synthesis of the role of environmental compartments—namely, water, soil, and air—as reservoirs and transmission routes for AMR. It highlights recent research findings related to the distribution and dynamics of resistance in these environments, drawing from diverse methodological approaches, including environmental surveillance, molecular analyses, and case studies from different global contexts. Special attention is paid to pollution-driven AMR hotspots and the challenges they pose for containment. In doing so, this review emphasizes the importance of integrating environmental perspectives into AMR monitoring, prevention, and mitigation strategies on a global scale.

2. Environmental Pathways of Antimicrobial Resistance Transmission

The dissemination of AMR occurs through intricate environmental pathways, enabling ARB and ARGs to persist and spread across diverse ecosystems [8]. These transmission routes encompass aquatic systems, terrestrial reservoirs, and atmospheric dispersion, each playing a pivotal role in the global AMR crisis [2]. The interactions among these pathways exacerbate the risks, fostering the exchange of resistance determinants across environmental, agricultural, and clinical settings.

2.1. Aquatic Ecosystems: Critical Reservoirs and Transmission Pathways for Antimicrobial Resistance

Aquatic environments serve as reservoirs and conduits for AMR, facilitating the persistence and horizontal transfer of ARBs and ARGs [9]. The confluence of urban wastewater, agricultural runoff, and industrial effluents creates a selective pressure that enhances the propagation of resistance determinants in aquatic ecosystems [10]. Even advanced wastewater treatment facilities struggle to eliminate mobile genetic elements encoding resistance, further amplifying the environmental burden of AMR [11].

2.1.1. Key Sources of AMR Contamination in Aquatic Systems

Municipal wastewater treatment plants are significant contributors to the environmental spread of AMR, as they receive high loads of antibiotic residues and resistant bacteria from human excretion [12]. These effluent samples often contain clinically relevant resistance genes such as $bla_{CTX\text{-}M\text{-}15}$, associated with extended-spectrum β-lactamase (ESBL) production, and *mcr-1*, which confers resistance to colistin. Studies have shown that these resistance genes are present in over 80% of effluent samples worldwide, reflecting the pervasive nature of AMR in urban environments [13]. Despite implementing advanced treatment processes, residual antibiotics and mobile genetic elements (MGEs) persist in the treated effluents. These compounds continue circulating through surface waters, facilitating the spread and persistence of resistant pathogens [14]. The presence of these pollutants in the environment underscores the challenge of eliminating AMR from wastewater systems. It highlights the potential for these waters to act as reservoirs and conduits for resistance, ultimately impacting both human and environmental health. The principal entry pathways of antibiotics and resistance genes into aquatic environments are illustrated in Figure 2.

The agricultural sector also plays a crucial role in the dissemination of AMR, particularly through the use of antibiotics in livestock farming. These veterinary antibiotics, such as tetracyclines and macrolides, are commonly administered in food animal production to promote growth and prevent disease [15]. However, the widespread use of these substances has led to the accumulation of resistance determinants in soils, which can leach into nearby water bodies, further exacerbating the issue [16]. Rivers and lakes receiving agricultural runoff have been found to contain significant concentrations of these antibiotics, which in turn drive the selection of resistant pathogens. Notably, strains of *Escherichia coli* ST131, a widely recognized multidrug-resistant pathogen, as well as methicillin-resistant *Staphylococcus aureus* (MRSA), have been isolated from such environments [17,18]. These findings illustrate how agricultural runoff acts as a vector for AMR, contributing to the emergence and spread of resistant organisms that can impact human health and the broader ecosystem.

Pharmaceutical effluents represent another critical source of AMR in the environment, particularly in regions where pharmaceutical manufacturing is concentrated. Wastewater discharged from pharmaceutical manufacturing plants has been identified as a significant hotspot for the selection of AMR [19]. Research conducted in industrial hubs such as Hyderabad, India, has revealed alarmingly high ciprofloxacin concentrations exceeding 1 mg/L, 30,000 times higher than the minimum selective concentration required for resis-

tance development [20]. Similar findings have been reported worldwide. For example, in China, pharmaceutical effluents have reached ciprofloxacin concentrations up to 31 mg/L, and surface waters near manufacturing sites have shown levels as high as 2.5 mg/L [21]. In the United States, elevated concentrations of antibiotics such as ciprofloxacin, azithromycin, and sulfamethoxazole have been detected in surface waters and municipal effluents, especially in the Midwest and Northeast regions [22]. In Europe, a comprehensive survey of the Danube River revealed the presence of multiple antibiotic-resistant bacteria and high levels of pharmaceutical residues along its 2300 km course [22]. A recent global survey found that ciprofloxacin concentrations in rivers exceeded safe levels in 64 countries, with notable hotspots not only in India but also in China, Nigeria, Pakistan, and several European countries [23]. These examples underscore that pharmaceutical pollution and its contribution to environmental antibiotic resistance areglobal phenomena, not limited to any single country or region. These elevated concentrations promote the emergence of hypermutagenic strains, such as *Pseudomonas aeruginosa*, which can rapidly acquire mutations and adapt to the selective pressure exerted by the antibiotics [24]. Such high concentrations of antibiotics in effluents underscore the need for stricter regulations and more effective waste management strategies in pharmaceutical industries to mitigate their contribution to environmental AMR. These industrial discharges are a significant source of AMR contamination, emphasizing the need for comprehensive approaches to address pharmaceutical waste and limit its environmental impact.

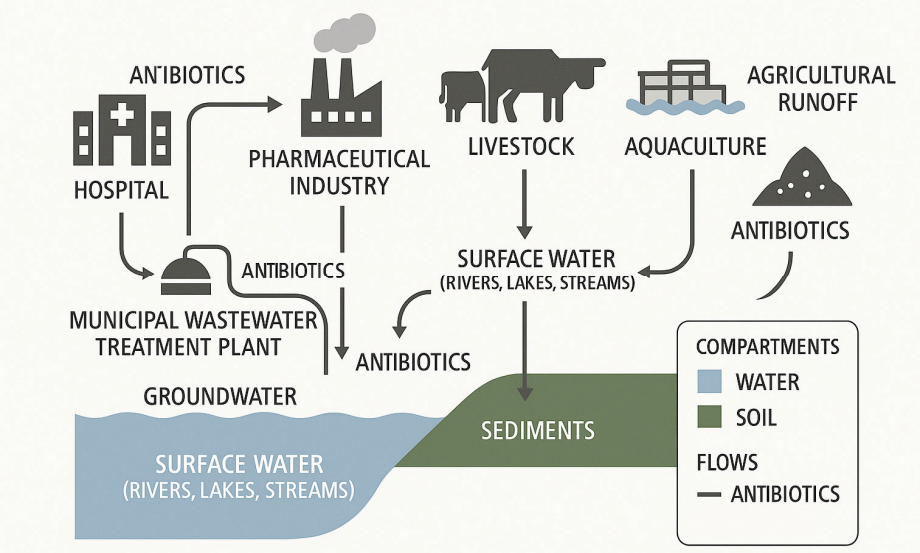

Figure 2. Schematic diagram illustrating the main entry pathways of antibiotics into aquatic environments. Adapted from Maghsodian et al., 2022 [25].

2.1.2. Mechanisms of Resistance Propagation in Aquatic Systems

Horizontal gene transfer (HGT) plays a pivotal role in the spread of AMR in aquatic environments, particularly in biofilms. These biofilms offer a conducive microenvironment for conjugating resistance genes between environmental and pathogenic bacteria, enhancing the horizontal transmission of resistance traits [26,27]. Studies have highlighted the high-frequency transfer of carbapenem resistance genes, such as bla_{NDM-1}, in this context, within wastewater biofilms. Notably, such genes have been transferred on Incx3 plasmids, with conjugation rates reaching as high as 10^2 transconjugants per donor cell, underscoring gene transfer efficiency in these aquatic settings [28,29]. This phenomenon is of significant concern, as it facilitates the rapid dissemination of resistance genes across bacterial popu-

lations, exacerbating the global threat of AMR. The main mechanisms of horizontal gene transfer in aquatic environments are illustrated in Figure 3.

In addition to HGT, sub-inhibitory concentrations of antibiotics present in aquatic environments can exert selection pressure on bacterial populations, even at levels as low as nanograms to micrograms per liter. Such concentrations, often resulting from residual antibiotics from human and agricultural sources, are sufficient to trigger bacterial stress responses, notably the SOS response [30]. This stress response, in turn, leads to an increase in integron activity, which enhances the acquisition of resistance genes [31]. Among the genes acquired under these conditions are *sul1*, which confers resistance to sulfonamides, and *qnrS*, which is associated with quinolone resistance [32]. The presence of sub-inhibitory antibiotics thus acts as a continuous, albeit often unnoticed, driver of resistance gene accumulation, further complicating efforts to combat bacterial resistance.

Furthermore, the persistence of extracellular DNA in the environment significantly contributes to the spread of AMR. MGEs, such as the *tet*(W) gene, can remain stable in aquatic sediments for extended periods, often over 40 days, due to their protection by organic matter and clay particles [33]. This persistence allows the transformation of environmental pathogens, such as *Vibrio cholerae*, with resistance genes that may have been previously acquired by other bacteria in the surrounding environment [33]. The ability of resistance genes to survive outside of bacterial cells and remain viable in the environment further complicates the mitigation of AMR, as it creates a reservoir of genetic material that can potentially be taken up by various pathogens, thereby enhancing the spread of resistance across different bacterial species.

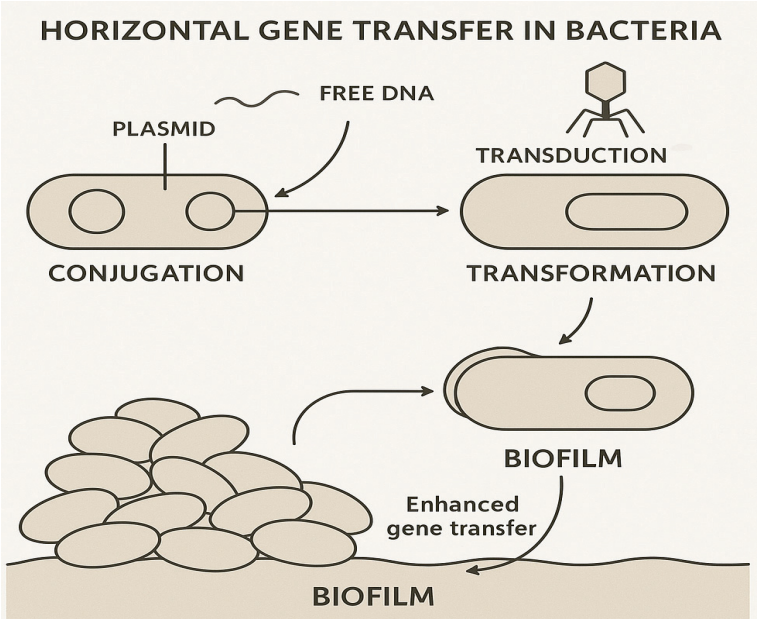

Figure 3. Schematic diagram illustrating the main mechanisms of horizontal gene transfer (HGT) in bacteria: conjugation, transformation, and transduction. Adapted from Kunhikannan et al., 2021 [34].

2.1.3. Challenges in Wastewater Treatment and Mitigation Strategies

Despite significant technological advancements in wastewater treatment processes, WWTPs remain insufficient in eliminating antibiotic residues and AMR determinants from treated effluents. Conventional methods such as ultraviolet (UV) disinfection, which aim to reduce bacterial loads in wastewater, have shown limited success in degrading extracellular DNA that carries antibiotic resistance genes, such as *ermB*, which is associated with macrolide resistance [35]. This failure to eliminate MGEs means that resistance

genes persist in the treated water, allowing resistant bacteria to continue circulating in the environment and potentially leading to the spread of AMR across ecosystems [36].

Further complicating the issue, chlorine disinfection, a widely used method for pathogen control in wastewater, has been observed to select chlorine-resistant strains of *Enterococcus faecium* that harbor *vanA*, a gene responsible for vancomycin resistance [37]. These chlorine-resistant strains can recolonize distribution networks within a mere 72 h after treatment, undermining the effectiveness of disinfection processes and contributing to the persistence and spread of resistance in the environment [38]. This phenomenon underscores the limitations of conventional disinfection techniques in addressing the complexity of AMR in wastewater.

Additionally, recycling biosolids from wastewater treatment, often employed as a means of agricultural fertilization, poses further challenges in combating AMR. The anaerobic digestion of biosolids can enrich spore-forming bacteria, such as *Clostridioides difficile*, which carry resistance determinants like *cfr*, a gene conferring resistance to linezolid [39,40]. When these biosolids are applied to agricultural fields, they can reintroduce AMR into terrestrial ecosystems, thus perpetuating the cycle of resistance. This process highlights a significant environmental pathway through which resistant bacteria and resistance genes are disseminated into the broader ecosystem, exacerbating the global challenge of AMR [14]. Therefore, while advancements in wastewater treatment technologies have made progress in reducing bacterial contamination, the persistence of antibiotic residues, resistance genes, and resistant bacteria in treated effluents and biosolids continues to be a significant concern in the fight against AMR. The main water and wastewater treatment technologies, their advantages, limitations, and potential contribution to AMR are summarized in Table 1.

Table 1. Summary of water and wastewater treatment technologies, their advantages, limitations, and potential contribution to AMR.

Treatment Technology	Main Advantages	Main Limitations	Potential Contribution to AMR	References
Conventional Activated Sludge	Cost-effective, widely used	Incomplete removal of antibiotics and ARGs	Residual antibiotics and ARGs in effluent	[41,42]
Membrane Filtration (MBR)	High removal efficiency for bacteria/particles	High cost, membrane fouling	Reduces ARB/ARGs, but not all; possible biofilm formation	[41,42]
Ozonation/Advanced Oxidation	Effective for many compounds	High energy cost, byproducts	Degrades some antibiotics/ARGs, but not all	[42,43]
UV Disinfection	Effective for bacteria, no chemical residues	Ineffective for extracellular DNA; limited by water turbidity	May not remove free ARGs; possible regrowth	[44]
Chlorination	Broad-spectrum disinfection, low cost	Can select for chlorine-resistant bacteria; toxic byproducts	Selection of resistant strains; incomplete ARG removal	[45]
Composting/Biosolids Recycling	Waste valorization, nutrient recycling	ARGs can persist in biosolids; risk if not fully sanitized	Spreads ARGs if not properly treated	[46]
Thermophilic Composting	High temperature degrades many ARGs	Not all ARGs eliminated; energy-intensive	Reduces but does not eliminate resistance genes	[46]

Table 1. *Cont.*

Treatment Technology	Main Advantages	Main Limitations	Potential Contribution to AMR	References
Biochar Amendment	Reduces extracellular DNA availability	Effectiveness varies with soil type and conditions	Limits horizontal transfer of ARGs	[46]
Gamma-ray Irradiation	Inactivates ARB and ARGs efficiently	High cost, limited large-scale application	Reduces ARGs and ARB in treated effluents	[47]

2.2. Terrestrial Reservoirs: Soil as a Dynamic Reservoir for Antimicrobial Resistance Evolution

Soil ecosystems are not mere passive repositories of ARBs and ARGs but active bioreactors where resistance determinants evolve, recombine, and disseminate. The interplay of agricultural practices, microbial ecology, and anthropogenic pressures transforms soil into a nexus for AMR amplification, with implications spanning environmental and clinical settings [48].

2.2.1. Drivers of ARG Accumulation in Soil

Livestock manure has become a significant vector for introducing antibiotics into agricultural soils, with studies showing that it accounts for approximately 30–90% of the antibiotics administered to animals, including commonly used compounds such as tetracyclines and sulfonamides [49]. When manure is applied to soils, it creates sub-inhibitory concentrations of these antibiotics, typically ranging from 0.1 to 100 μg/kg, which fosters the selective pressure needed to proliferate antibiotic-resistant microorganisms [50,51]. This resistance is not a transient phenomenon, as a single manure application has been reported to deposit between 10^6 and 10^8 copies of ARGs per gram of soil, including genes conferring resistance to tetracycline (*tet*(M)) and macrolides (*erm*(B)) [52]. These ARGs are deposited and integrate stably into the soil's microbial communities, where they can persist for over 20 years, further embedding resistance into the agricultural ecosystem. This long-term persistence raises substantial concerns about the continued presence of these resistance markers in the environment, potentially leading to long-lasting ecological consequences.

In addition to the persistence of resistant genes, research has demonstrated that manure from swine, in particular, significantly enhances the population of *Clostridioides difficile* spores in agricultural soils [53]. This bacterium, which is known to harbor resistance genes such as cfr (linezolid resistance) and *vanA* (vancomycin resistance), can increase by up to 100-fold following manure amendments [54]. The presence of these resistant strains in the soil increases the risk of environmental contamination and poses serious zoonotic risks. These resistant strains can enter the food chain through soil contamination or contact humans and animals directly, amplifying the spread of resistance across various ecological niches.

The use of treated wastewater for irrigation compounds these issues by introducing not only antibiotic residues but also heavy metals like copper and zinc, as well as chemical disinfectants. These substances exert co-selection pressures, where organisms resistant to one agent may also develop resistance to others, particularly when they share similar resistance mechanisms [55].

Heavy metals such as copper and zinc are well-documented co-selective agents for antimicrobial resistance. Their presence in soil and water can select for bacteria carrying both metal resistance genes and antibiotic resistance genes, often located on the same mobile genetic elements (co-resistance), or via shared efflux pumps (cross-resistance) [56–59]. For instance, the czcA gene confers resistance to zinc and cadmium, and is frequently

found in proximity to sul1, a sulfonamide resistance gene, on integrons or plasmids [58]. Experimental studies have shown that exposure to copper at environmentally relevant concentrations (10–100 mg/L) rapidly increases the abundance of both metal and antibiotic resistance genes in bacterial communities, even in the absence of antibiotics [56,58,59]. Similarly, pre-exposure to zinc has been shown to accelerate the development of ciprofloxacin resistance in *E. coli* [57].

Chemical disinfectants, such as quaternary ammonium compounds (QACs), triclosan, and chlorhexidine, are also linked to the selection and persistence of antibiotic resistance [60,61]. Overuse or improper use of these disinfectants can induce genetic adaptations in bacteria—such as mutations in efflux pump genes (e.g., qacA/B, marA, mexA-mexB-oprM)—that confer decreased susceptibility to both biocides and multiple antibiotics [60,61]. This phenomenon, known as cross-resistance, has been documented in clinically relevant strains of *E. coli*, *P. aeruginosa*, and *S. aureus* [61].

High concentrations of resistance genes such as czcA, which confers resistance to zinc and cadmium, and sul1, which provides resistance to sulfonamides, have been found abundant in soils irrigated with treated wastewater. This co-selection process is especially concerning because it can spread multidrug-resistant organisms within agricultural systems, further complicating efforts to manage and mitigate antimicrobial resistance in the environment and human health [58,60,61].

2.2.2. Soil as a Hotspot for HGT

Recent large-scale genomic analyses have revealed the central role of plasmids in facilitating HGT across diverse bacterial taxa and ecological niches. A global study analyzing over 10,000 plasmid genomes uncovered a complex network of plasmid taxonomic units (PTUs), highlighting the evolutionary pathways genes, including those conferring AMR—can disseminate broadly [62]. These PTUs were classified into six host-range grades, from narrow host plasmids (Grade I) to broad-host-range plasmids (Grade VI), the latter capable of moving between bacteria of different phyla. Over 60% of the plasmids examined were found to transcend species boundaries, underlining their role as vectors of gene exchange across taxonomic and ecological barriers. Broad-host-range plasmids such as IncP, IncW, and PromA groups were identified as key mediators of gene flow between environmental and clinical bacterial populations. This capacity enables the rapid spread of AMR genes from ecological reservoirs, such as soil or water, to human pathogens, emphasizing the need to understand plasmid dynamics in the context of One Health [62]. This demonstrates the potential for clinically relevant resistance traits to emerge in environmental reservoirs, with soil as a key medium for exchanging resistance determinants between environmental and pathogenic bacteria. Additionally, prevalent on root surfaces, microbial biofilms further enhance the capacity for plasmid exchange [63]. This interaction highlights the importance of biofilm-mediated gene transfer in facilitating the spread of resistance genes, even in environments that are not directly exposed to antibiotics. The main mechanisms and ecological networks of horizontal gene transfer in soil environments are illustrated in Figure 4.

In addition to conjugation, other mechanisms of HGT, such as transformation and transduction, also play significant roles in the movement of AMR genes within soil ecosystems. Transformation occurs when extracellular DNA carrying resistance genes, such as *sul2* and *intI1* (which encode sulfonamide resistance and class 1 integrons, respectively), binds to soil particles like clay and remains in a viable form for extended periods, often exceeding a year, even under arid conditions [64]. This persistence of extracellular DNA ensures that soil remains a reservoir of transferable genetic material, capable of being incorporated into other bacterial cells under the right conditions. Transduction, another

key gene transfer mechanism, is mediated by bacteriophages that carry genetic material between bacterial species. In soil environments, bacteriophages facilitate the transduction of the *mcr-1* gene, which confers resistance to colistin, between *Enterobacteriaceae* and environmental *Aeromonas* species. This process is particularly pronounced under drought stress, where the frequency of transduction events is elevated, thereby enhancing the spread of resistance genes within microbial communities [65]. These findings emphasize the complex and dynamic nature of HGT in soil ecosystems, where various mechanisms interact to promote the persistence and dissemination of antimicrobial resistance.

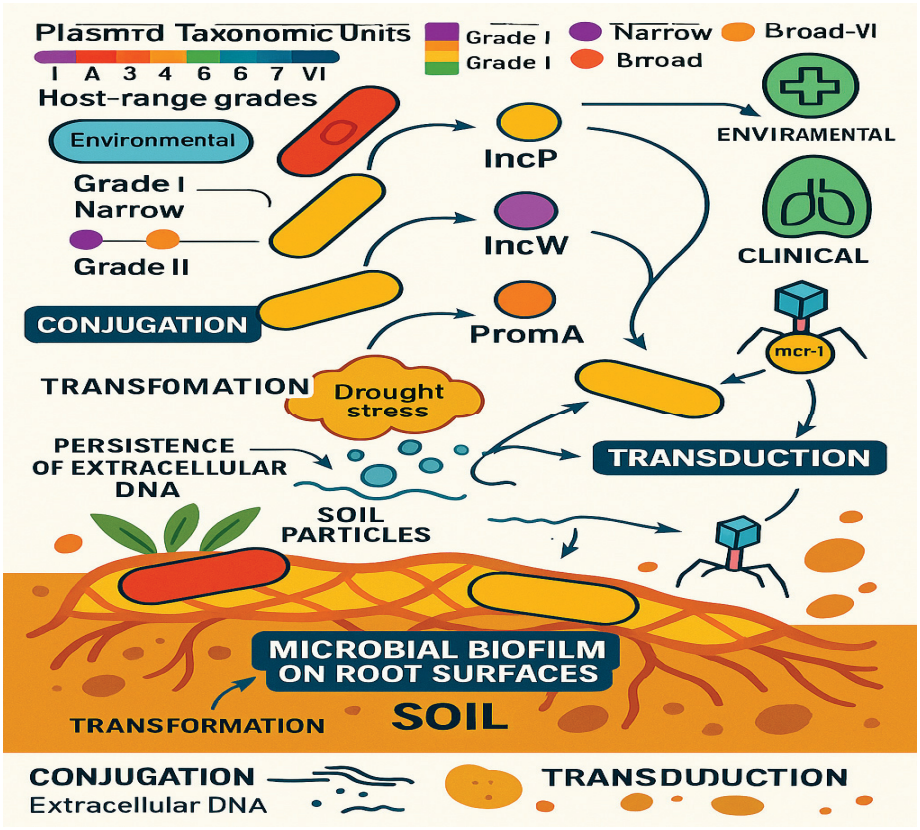

Figure 4. Schematic diagram illustrating HGT in soil environments, including plasmid host-range diversity, biofilm-mediated plasmid exchange, transformation (with extracellular DNA binding to soil particles), and transduction via bacteriophages. Adapted from Ciach et al., 2024 [66]; Liu et al., 2024 [67]; Aminov, 2011 [68]; Li et al., 2023 [69].

2.2.3. Metagenomic Insights into Soil Resistomes

Recent metagenomic studies have revealed significant concerns regarding the complexity and potential risks associated with soil resistomes [70,71]. These studies highlight the considerable diversity of ARGs in agricultural soils, which has become a growing environmental issue. Agricultural soils harbor a range of 200 to 500 distinct ARG subtypes, some clinically significant. Among these, variants such as *vanA* and bla_{KPC} stand out because they confer resistance to critical antibiotics like glycopeptides and carbapenems, which are used to treat various severe infections [72]. The widespread presence of these genes in soil poses a direct threat to human health, as it may contribute to the environmental dissemination of resistance traits that could eventually affect clinical treatment outcomes.

In addition to the sheer diversity of ARGs, cross-kingdom gene transfer has emerged as another alarming trend. Soil fungi, particularly species from the *Aspergillus genus*, have been identified as unexpected reservoirs for bacterial ARGs. A noteworthy example is the *ampC* gene, which confers resistance to β-lactam antibiotics. The detection of ampC within fungal mitochondrial genomes provides strong evidence of horizontal gene transfer between

bacterial and fungal communities in the soil [66,73]. This finding challenges traditional notions about gene transfer and is supported by multiple genomic and metagenomic studies that have identified bacterial antibiotic resistance genes within fungal genomes [66,74–76]. It underscores the need for further investigation into the intricate microbial interactions that may facilitate the spread of resistance genes across different domains of life. The ability of fungi to harbor bacterial resistance genes adds a new layer of complexity to the dynamics of ARG spread in the environment, highlighting the importance of considering all microbial communities when assessing the spread of antimicrobial resistance [66,75–77].

Furthermore, the geographic distribution of ARGs in soil varies significantly depending on farming practices, providing additional insight into the relationship between agricultural activity and the persistence of resistance genes. In Europe, soils located near intensive poultry farms have been found to contain up to ten times higher levels of erm(B), a gene associated with resistance to macrolides, compared to soils from organic farming systems [78–80]. This stark contrast can be directly linked to the extensive use of veterinary antibiotics in conventional farming systems, which are widely used to prevent and treat infections in livestock. Studies have shown that such antibiotic use correlates with increased abundance and persistence of ARGs in soil [51,81,82]. The higher concentration of ARGs in soils from intensive farming systems suggests that antibiotic use in agriculture is critical in fostering the selection and persistence of resistant strains in the environment [83]. Such findings highlight the significant influence of agricultural practices on the spread of antimicrobial resistance and emphasize the need for sustainable farming practices that reduce the reliance on antibiotics.

2.2.4. Atmospheric Transport Dynamics

ARBs exhibit significant resilience and adaptability, enabling their persistence in various environmental conditions. These bacteria can survive in the air for extended periods, ranging from 8 to 72 h, thanks to protective organic coatings, such as lipids derived from manure. These coatings protect the bacteria from harmful ultraviolet (UV) radiation and desiccation, which would otherwise impair their viability [84]. This ability to withstand harsh environmental conditions contributes to the widespread dispersal of ARBs across different ecosystems.

The spread of ARBs, including MRSA, is facilitated by wind-driven processes, further enhancing their transmission on local and continental scales. On a regional scale, studies have shown that MRSA strains, such as MRSA-CC398, originating from pig farms in the Netherlands, can be detected in 30% of residential areas within a 3 km radius of the farms [85]. This finding underscores the significant role of airborne dissemination in propagating resistant strains from animal reservoirs to human populations. On a larger, continental scale, wind-borne particulate matter can transport resistance genes across vast distances. For example, dust storms originating from North African feedlots have been shown to carry the $bla_{CTX\text{-}M}$ genes responsible for ESBL production to Southern Europe. These airborne particles contribute to the environmental burden of antimicrobial resistance, with deposition rates of up to 10^7 ARG copies per square meter per day [86].

In addition to the direct transmission of resistant bacteria, particulate matter, suchas $PM_{2.5}$ and PM_{10}, serves as a vector for genetic material that facilitates the spread of resistance [87]. These fine particles, less than 10 μm in diameter fine particles can carry conjugative plasmids, such as IncHI2, which harbor resistance genes like *mcr-1*, responsible for colistin resistance. The adsorption of these plasmids onto silica surfaces further enhances their stability and mobility in the atmosphere [88]. Moreover, studies have detected extracellular DNA encoding *vanA*, a gene responsible for vancomycin resistance, in urban air samples, highlighting the widespread distribution of genetic material that promotes resistance [89].

Interestingly, particulate matter can also facilitate the long-range dispersal of ARBs through a process known as ice nucleation. Fine particles laden with ARBs can act as nuclei, promoting cloud formation and enabling bacteria to be transported across intercontinental distances via precipitation. For instance, *Klebsiella pneumoniae*, a pathogen associated with antimicrobial resistance, has been recovered from rainfall in pristine Arctic regions, illustrating how airborne bacteria can be carried over vast distances and deposited in remote locations [90]. This highlights the global scale of the problem and the complex mechanisms by which ARBs can spread, potentially impacting regions far removed from their sources. The main mechanisms and pathways of atmospheric transport and dispersal of antimicrobial-resistant bacteria and genes are illustrated in Figure 5.

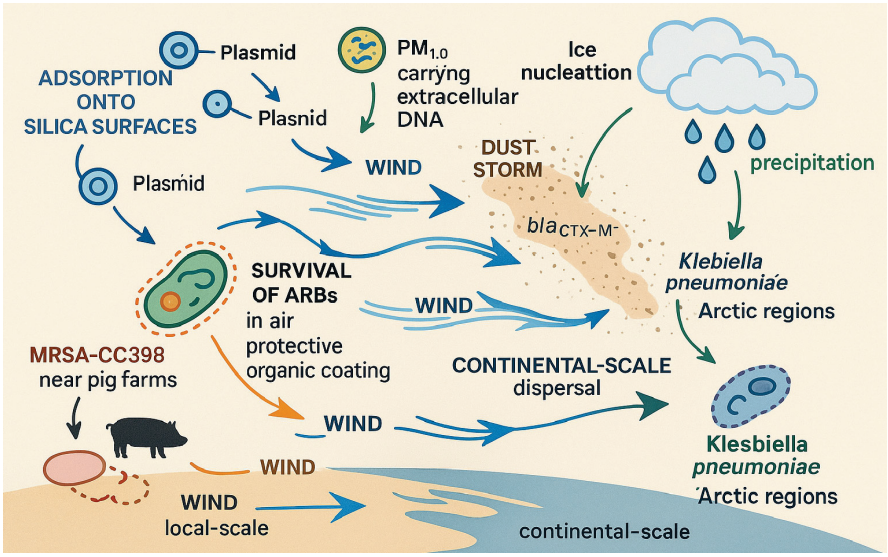

Figure 5. Schematic diagram illustrating the main mechanisms and pathways of atmospheric transport and dispersal of antimicrobial-resistant bacteria (ARBs) and resistance genes, including survival in aerosols, wind-driven dispersal, association with particulate matter, and long-range transport via ice nucleation and precipitation. Adapted from Zhou et al., 2023 [87].

2.2.5. Horizontal Gene Transfer in Air

Emerging evidence increasingly supports the idea that airborne conditions play a significant role in facilitating genetic exchange among bacterial populations, particularly in AMR. Several studies have shown that mechanisms of HGT, such as conjugation and transduction, can occur in airborne particles, contributing to the spread of resistance genes across vast distances [91,92].

One of the primary mechanisms facilitating HGT in the air is conjugation, a process where genetic material is transferred between bacterial cells through direct contact. Studies have shown that *Acinetobacter* species, known for their role in transmitting resistance genes, can transfer plasmids encoding carbapenem resistance, such as the bla_{NDM-1} gene, within bioaerosols [93,94]. For instance, Scoffone et al. demonstrated that *Acinetobacter baumannii*, a significant pathogen in hospital-associated infections, can transfer bla_{NDM-1} plasmids within bioaerosols. This highlights the potential for spreading antibiotic resistance through the atmosphere, especially as wind-borne bioaerosols can carry resistant bacteria over long distances [95].

In addition to conjugation, phage-mediated transduction is another crucial mechanism of genetic exchange in airborne and environmental conditions. Bacteriophages, viruses that infect bacteria, are abundant in various environments and have been detected in the DNA fraction of particulate matter. These phages can carry and transfer ARGs, including those conferring resistance to critical antibiotics. The presence of ARGs, such as *blaTEM*,

blaCTX-M, and qnr, in the bacteriophage DNA fraction obtained from diverse environmental samples—including wastewater and river water—has been demonstrated. This finding highlights the role of phages as vectors for horizontal gene transfer in natural environments, potentially including aerosols, thereby contributing to the dissemination of resistance genes. The capacity of phages to mediate gene transfer enhances bacterial genetic diversity and represents a significant route for the spread of antimicrobial resistance across different ecosystems [96]. The main mechanisms of horizontal gene transfer in airborne environments, including conjugation and phage-mediated transduction, are illustrated in Figure 6.

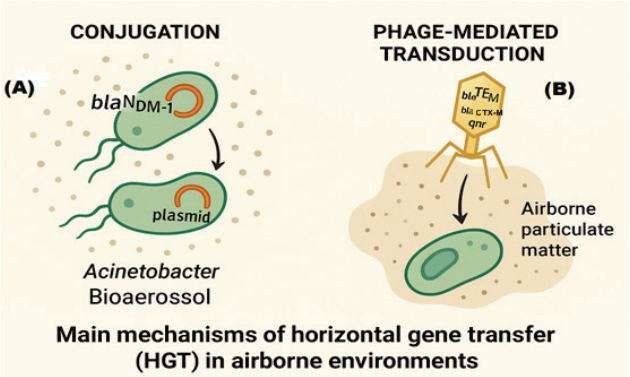

Figure 6. Schematic diagram illustrating the main mechanisms of horizontal gene transfer (HGT) in airborne environments: (**A**) Conjugation between bacteria within bioaerosols, exemplified by *Acinetobacter* species transferring *blaNDM-1* plasmids; (**B**) phage-mediated transduction, where bacteriophages carry and transfer antibiotic resistance genes (ARGs) such as *blaTEM*, *blaCTX-M*, and qnr within airborne particulate matter. Adapted from Colomer-Lluch et al., 2011 [96]; Ye et al., 2021 [97].

3. Impact of Environmental Pollution on AMR

Environmental pollution is a global catalyst for the evolution and dissemination of antimicrobial resistance (AMR), creating selective pressures that favor resistant bacteria and facilitating the horizontal transfer of resistance genes across all ecosystems [98]. The main environmental drivers include pharmaceutical and industrial waste, untreated sewage, agricultural runoff, and air pollution, each contributing uniquely to the selection, persistence, and spread of AMR on every continent [98–102].

3.1. Key Pollutants, Sources, Concentrations, and Global Locations

3.1.1. Pharmaceutical Waste

Pharmaceutical residues are detected worldwide, from rivers in Europe and North America to major rivers in Asia and Africa. For example, a global survey found antibiotics in rivers in over 100 countries, with concentrations of ciprofloxacin reaching up to 31 mg/L in pharmaceutical effluents in India and 1.2 mg/L in the Danube River, Europe [99,100]. In China, concentrations of sulfonamides in surface waters near manufacturing hubs can reach 2.5 mg/L [101]. These sub-inhibitory concentrations (10–100 ng/L or higher) select for resistance genes such as *blaCTX-M*, *qnrS*, and *mcr-1* in environmental bacteria [99,101].

3.1.2. Wastewater (Treated and Untreated)

Both untreated and inadequately treated municipal wastewater are major sources of ARB and ARGs globally, especially in low- and middle-income countries [98,100]. Urban sewage in 60 countries has revealed a clear geographic distinction in AMR gene abundance, with the highest levels in Asia, Africa, and South America [100]. Wastewater—whether treated or not—releases high loads of carbapenem-resistant Enterobacteriaceae (CRE),

vanA-positive *Enterococcus*, and integrons (intI1) into aquatic environments, with ARG concentrations up to 10^8 copies/mL [19]. Discharges can impact both coastal and inland waters.

3.1.3. Agricultural Runoff

Intensive agriculture is a major driver of AMR, with conventionally farmed soils harboring more ARGs than organic systems [98,101]. For example, soils near intensive poultry farms in Europe contain up to ten times higher levels of erm(B) than organic farms [101]. In the US and China, tetracycline and sulfonamide resistance genes persist in soils for decades after manure application [98,101]. Heavy metals like copper and zinc, commonly found in fertilizers and animal feed, co-select for ARGs such as czcA and sul1 [98,101].

3.1.4. Industrial Waste and Microplastics

Industrial discharges, including from textile manufacturing and microplastics, are global issues. In estuaries of the UK and China, industrial legacy pollution correlates with high levels of sul1, ermB, and marA genes [19,98]. Microplastics sampled from rivers in Europe, Asia, and Africa carry ARGs and promote biofilm formation by resistant bacteria [19,98].

3.1.5. Air Pollution

Recent studies show that air pollution (PM2.5 and PM10) can carry ARGs and resistant bacteria across continents [19].

For example, dust storms from North Africa have transported *blaCTX-M* genes to Southern Europe, while ARGs have been detected in urban air samples in China, Europe, and the US [19]. The main environmental pollutants contributing to antimicrobial resistance, their major sources, typical concentrations, and global locations are summarized in Table 2.

Table 2. Key environmental pollutants contributing to AMR (Global Data).

Pollutant Type	Major Sources	Key Resistance Elements	Typical Concentrations	Locations Detected	References
Pharmaceuticals	Drug manufacturing, hospitals	*blaCTX-M*, *qnrS*, *mcr-1*	10–31 mg/L (India), 1.2 mg/L (Europe), 2.5 mg/L (China)	Rivers and lakes in India, Europe, China, Africa, US	[24,32,103]
Wastewater (Treated and Untreated)	Urban wastewater, hospitals	*vanA*, *blaKPC*, *intI1*, ESBLs	Up to 10^8 ARG copies/mL	Coastal and inland waters, global	[10,11]
Agricultural Runoff	Manure, fertilizers, heavy metals	*ermB*, *tetM*, *sul1*, *czcA*	Up to 10× higher than organic soils	Europe, US, China, Africa	[55,88,104]
Industrial Waste and Microplastics	Textiles, plastics, dyes	*sul1*, *ermB*, *marA*, *blaTEM*	Notably high in estuaries, rivers	UK, China, Europe, Africa	[52,89,105]
Air Pollution	PM2.5, PM10, dust storms	*blaCTX-M*, *mcr-1*, *sul1*	Up to 10^7 ARG copies/m^2/day	China, Europe, US, Africa	[19,98]

3.2. Synergistic Effects of Combined Pollutants

While the individual roles of antibiotics, metals, and microplastics in promoting AMR have been discussed above, recent research emphasizes that their combined presence in the environment can have synergistic effects that go beyond the sum of their individual impacts. These interactions can lead to enhanced selection, persistence, and horizontal

transfer of resistance genes, representing an emerging concern for environmental and public health.

For example, co-contamination with metals and antibiotics can significantly amplify resistance gene expression. A recent study demonstrated that a mixture of zinc (10 mg/L) and oxytetracycline (0.1 mg/L) resulted in a 100-fold increase in tetA gene expression in *Bacillus* species from soil, compared to exposure to either contaminant alone [106]. This finding suggests that metal-antibiotic mixtures may accelerate the evolution and dissemination of resistance traits in contaminated soils.

Microplastics act as both vectors and catalysts for gene transfer. Unlike previous sections that focused on their role as passive carriers, new evidence shows that microplastics such as 1 μm polystyrene particles can actively enhance the horizontal transfer of resistance genes. Specifically, the presence of these particles increased the transfer rate of the *blaTEM* gene by 50% between *Vibrio* species in marine environments [107]. This highlights the unique role of microplastics in creating microenvironments that facilitate gene exchange among bacteria.

Overall, these findings underscore the importance of considering pollutant mixtures and their interactions, rather than assessing each contaminant in isolation, when evaluating environmental drivers of AMR. Future mitigation strategies should account for these synergistic effects to more effectively curb the spread of resistance [108].

3.3. Quantitative Evidence of Pollution—AMR Links

The presence and proliferation of AMR genes in environmental reservoirs—such as water, soil, and air—pose a growing threat to public health. Among these, waterborne resistance is particularly concerning. Studies have shown that even low concentrations of antibiotics, such as ciprofloxacin, can exert a selective pressure on microbial communities in aquatic ecosystems.

For instance, Kraupner et al. demonstrated that ciprofloxacin concentrations as low as 1 μg/L significantly altered the composition of complex aquatic bacterial biofilms. This exposure led to the enrichment of mobile quinolone resistance genes, especially *qnrS*, emphasizing that even minimal levels of pharmaceutical contaminants can contribute to the selection and persistence of resistance determinants in natural environments [109]. Similarly, Li et al. (2016) found that in soil amended with manure, lower ciprofloxacin concentrations (0.04–0.4 mg/kg) slowed the dissipation of plasmid-mediated quinolone resistance (PMQR) genes, including *qnrS*, and promoted the survival of resistant bacterial taxa [110].

Similarly, in agricultural soils, manure has been shown to contribute to a significant rise in the levels of sul1, a sulfonamide resistance gene. Within just one year of manure application, sul1 levels in the soil can increase from 10^3 to 10^6 copies per gram, highlighting agricultural practices' rapid and sustained influence on the soil resistome [48]. Furthermore, airborne transmission is a significant vector for the spread of AMR, particularly in agricultural settings.Studies have demonstrated that particulate matter (PM2.5) near livestock farms contains substantial concentrations of ARB.For instance, research has identified the presence of airborne MRSA CC398 in pig farms, indicating a potential risk for airborne transmission of resistant pathogens [111].

4. Antimicrobial Resistance in the Environment: A Global Perspective

4.1. Global Distribution of Environmental AMR Hotspots

The variability in AMR across different regions is influenced by several key drivers that reflect each region's unique socio-economic and infrastructural contexts. In low- and middle-income countries (LMICs), sanitation gaps represent a major contributor to

the spread of ARB. Around 80% of wastewater in these countries is estimated to remain untreated, releasing substantial quantities of ARB into rivers used for drinking and irrigation. This untreated wastewater, with concentrations ranging from 10^6 to 10^8 ARB per milliliter, provides an environment for the proliferation and transmission of resistant strains, significantly impacting public health, especially in communities dependent on these water sources for daily consumption and agricultural purposes [112]. In addition to sanitation issues, agricultural pressures in LMICs further exacerbate the problem of AMR. Projections indicate that veterinary antibiotic use, especially in poultry and aquaculture, will rise by 67% by 2030. This increase is expected to enhance the selective pressure on microbial populations, fostering the development and spread of resistant strains in animal and environmental reservoirs [113].

Conversely, high-income countries (HICs) face different yet significant challenges related to the persistence and spread of AMR. Industrialized farming practices in HICs contribute substantially to the environmental burden of antimicrobial resistance. Despite regulatory measures, it is estimated that 70% of medically necessary antibiotics in the United States are used in livestock farming, which continues to enrich agricultural soils with resistance genes, particularly erm(B) and tet(M). These genes confer resistance to macrolides and tetracyclines, respectively, and are prevalent in both farm environments and the surrounding ecosystems, where they can be disseminated through manure, runoff, and other agricultural activities [114]. In high-income countries (HICs), hospital effluents are recognized as critical sources of AMR dissemination into the environment. These effluents often contain elevated concentrations of carbapenemase genes, including bla_{KPC} and $bla_{OXA\text{-}48}$, which can persist through conventional wastewater treatment processes. A study conducted by Nasri et al. [115]. In Tunisian hospitals, reported concentrations of carbapenemase genes such as $bla_{OXA\text{-}48}$ reaching up to 10^4 copies per milliliter in untreated hospital wastewater. These genes were detected using quantitative PCR techniques, emphasizing their prevalence and potential for environmental dissemination [115]. The global distribution of environmental AMR hotspots, including regions with high prevalence in soil, air, and water, as well as areas impacted by untreated wastewater, intensive agriculture, and pharmaceutical or hospital effluents, is illustrated in Figure 7.

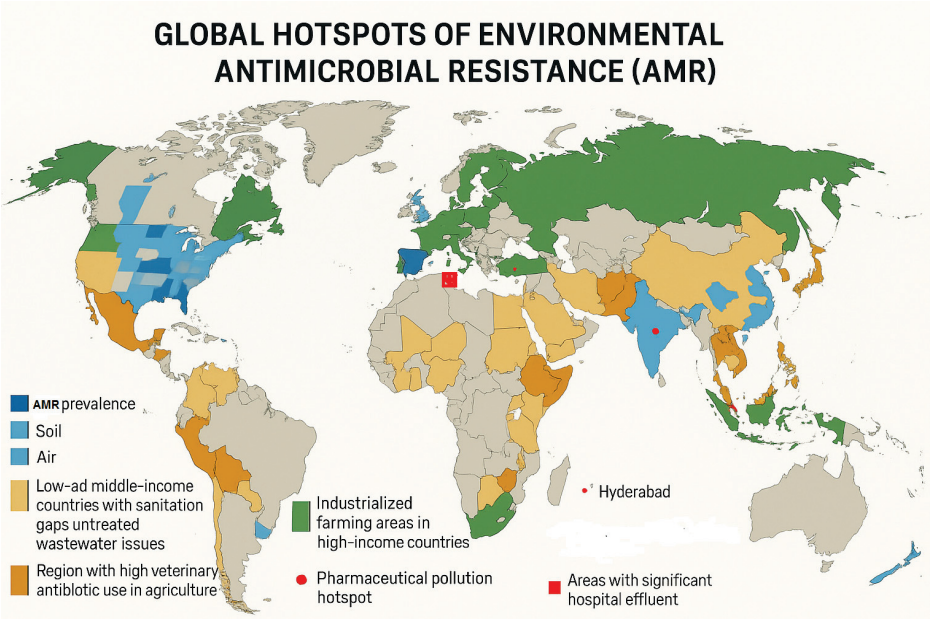

Figure 7. Global hotspots and environmental drivers of antimicrobial resistance (AMR).

World map highlighting regions with high AMR prevalence in soil, air, and water; low- and middle-income countries with sanitation gaps and untreated wastewater; regions with intensive veterinary antibiotic use; industrialized farming areas; pharmaceutical pollution hotspots (e.g., Hyderabad); and areas with significant hospital effluence.

4.2. Regional Case Studies

The presence of AMR in environmental reservoirs is a growing concern across various regions, with substantial implications for public health. In Sub-Saharan Africa, the sanitation crisis plays a critical role in the spread of AMR. In Nigeria, studies have demonstrated the presence of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*, particularly those carrying the $bla_{CTX\text{-}M\text{-}15}$ gene, in drinking water sources.

For example, a study conducted on selected rivers in Ibadan revealed the presence of $bla_{CTX\text{-}M}$ genes in *E. coli* isolates from surface waters, indicating significant environmental contamination and public health risk in communities relying on these water bodies [116].

Additionally, research conducted in Oyo State confirmed the presence of ESBL-producing Gram-negative bacteria, including *E. coli*, in various surface and underground water sources, with a notable prevalence of the $bla_{CTX\text{-}M}$ gene [117].

This widespread contamination highlights the critical intersection between inadequate sanitation infrastructure and AMR proliferation, exacerbating health disparities in low-resource settings. In Ghana, genomic surveillance has revealed the presence of *Klebsiella pneumoniae* strains carrying the $bla_{NDM\text{-}1}$ gene—an enzyme responsible for carbapenem resistance—across hospital, environmental, and animal samples, underscoring the pervasive nature of resistance transmission across One Health domains. These findings raise serious concerns about the ecological dissemination of highly resistant pathogens and their implications for vulnerable populations, including neonates at risk of sepsis-related mortality [118].

In India and Southeast Asia, pharmaceutical pollution and agricultural practices significantly contribute to the environmental load of resistant pathogens. In Hyderabad, India, the pharmaceutical industry discharges effluents containing high concentrations of ciprofloxacin, around 1.5 mg/L, which has been shown to select for *qnrS*—a gene associated with quinolone resistance—in 90% of aquatic *V. cholerae* populations [24]. This exemplifies how pharmaceutical contamination in marine environments can directly impact the microbial resistance profiles of local ecosystems. In Vietnam, the widespread use of oxytetracycline in aquaculture further contributes to AMR. Shrimp farms apply 500 mg/kg of this antibiotic, significantly increasing the prevalence of antibiotic resistance genes. A study by Le and Munekage (2004) [119] observed that antibiotic residues, including tetracyclines, were present in water and sediment samples from shrimp ponds in mangrove areas, raising concerns about these practices' environmental impact. Although the specific prevalence of the *tetA* gene was not quantified in the study, such residues can foster the development and persistence of resistance in these environments. This, in turn, exacerbates the spread of tetracycline-resistant bacteria, contributing to a growing environmental health risk [119].

In Europe and North America, where regulatory frameworks exist to control AMR, paradoxical trends emerge in the face of agricultural runoff and wastewater management limitations. In the Netherlands, pig farms near agrarian soils have been found to harbor *mcr-1*, a gene conferring resistance to colistin, at concentrations as high as 10^6 copies per gram of soil. Furthermore, identical plasmids carrying *mcr-1* were detected in human clinical isolates, suggesting that agricultural runoff contributes to transmitting resistant pathogens from the environment to human populations [120]. Similarly, WWTPs have been identified as critical sources of environmental antibiotic resistance in the United

States. Although designed to reduce microbial contaminants, these facilities often cannot eliminate clinically relevant resistance genes such as $bla_{CTX\text{-}M}$, which are associated with resistance to third-generation cephalosporins. Recent studies indicate that ARGs persist through treatment processes, with effluents discharging substantial quantities of resistance genes into surrounding aquatic environments, including major bodies of water like the Great Lakes. This highlights the limitations of current wastewater management strategies and underscores the complexity of mitigating antimicrobial resistance through regulatory frameworks alone [121]. The main regional drivers, transmission pathways, dominant resistance genes, and associated human health impacts of environmental AMR are summarized in Table 3.

Table 3. Regional drivers and pathways of environmental AMR.

Region	Key Drivers	Primary Pathways	Dominant ARB/ARGs	Human Health Impact	References
Sub-Saharan Africa	Untreated sewage	Water contamination	*blaCTX-M-15*, *mcr-1*	Rise in pediatric MDR diarrhea	[116]
India and SE Asia	Pharmaceutical effluents, intensive aquaculture	River systems, aquaculture	*blaNDM-1*, *qnrS*	High rates of untreatable UTIs, aquaculture-associated resistance	[24,119]
Europe/N. America	Intensive farming, WWTP effluents	Agricultural runoff, WWTP effluents	*erm(B)*, *tet(M)*, *blaCTX-M*	MRSA in farmworkers; environmental dissemination of ESBLs	[120,121]
Latin America	Urban slums	Urban waterways	*blaOXA-48*, *blaKPC*, *blaVIM-2*, *qnrS*	Hospital CRE outbreaks, community transmission	[122]

4.3. Transboundary AMR Spread

Global trade and airborne transmission are two critical vectors contributing to the spread of AMR on a global scale. The international movement of goods, particularly food products, plays a significant role in disseminating resistant pathogens. In Vietnam, colistin resistance genes, such as *mcr-1* and *mcr-3*, have beenidentified in ESBL-producing *E. coli* isolated from food sold in Ho Chi Minh City, indicating a reservoir of resistance genes within the food chain [123]. These resistant strains pose a risk of transboundary transmission when such food products are exported.

A notable example of this risk is observed in Europe, where *mcr-3*-positive Salmonellastrainswere identified in human infections in Denmark between 2009 and 2017. Genomic analysis revealed that some of these cases were linked to recent travel to Southeast Asia, including Vietnam, suggesting a route of transmission facilitated by international travel and trade. These isolates also carried multiple resistance genes, further amplifying the public health threat [124].

The spread of such resistant pathogens through international trade highlights the importance of monitoring and regulating the movement of goods to mitigate the global transmission of AMR.

In addition to trade, airborne transmission has emerged as another significant pathway for the spread of resistant bacteria. Dust storms, particularly those originating from poultry farms in China, have been identified as a means by which AMR bacteria, such as *Streptococcus* spp. carrying *erm*(F) genes, including the Pacific, are dispersed across vast

distances. These storms carry particulate matter laden with resistant pathogens, facilitating their introduction into new environments where they can proliferate and spread further. One study found that airborne ARGs were detected up to 10 km downwind from poultry farms, suggesting that wind patterns can facilitate the spread of resistant bacteria into surrounding environments [125].

5. Strategies for Mitigating Environmental AMR: A Multidisciplinary Roadmap

The fight against environmental AMR demands coordinated, science-driven strategies that address contamination sources, transmission routes, and global inequities. Below, we synthesize evidence-based interventions, technological innovations, and policy frameworks critical for curbing environmental AMR. The main pillars of these strategies are illustrated in Figure 8.



Figure 8. Global strategies for addressing environmental AMR.

Schematic representation of key strategies to mitigate environmental antimicrobial resistance: prevention of infections through improved hygiene and sanitation; implementing policy and regulatory frameworks, including the One Health approach; enhancing public awareness and education; promoting research and development of new drugs and alternative therapies; and strengthening surveillance and monitoring systems for antimicrobial resistance.

5.1. Regulatory and Policy Interventions

Restricting agricultural antibiotic use has emerged as a critical strategy in addressing AMR in environmental reservoirs, particularly agrarian soils. A notable example of this approach is the European Union's Veterinary Medicinal Products Regulation (EU 2019/6), which has been instrumental in reducing veterinary antibiotic sales by 43% between 2019 and 2023. This regulation significantly reduced this by banning the prophylactic use of antibiotics in livestock and mandating that veterinary prescriptions be required for antibiotic treatments [126,127]. These regulatory measures are aimed at controlling antibiotic use and minimizing the development and spread of resistance genes in agricultural environments.

Similar policies adopted globally, such as the U.S. FDA Guidance #213, have demonstrated the efficacy of such interventions, with studies indicating a 35% reduction in antibiotic resistance gene (ARG) abundance in agricultural soils following these restrictions [128].

Pharmaceutical emission standards are another critical component of AMR mitigation efforts, particularly concerning contaminating water sources with pharmaceutical residues. India's Zero Liquid Discharge Policy represents a significant regulatory initiative aimed at addressing the environmental impacts of pharmaceutical manufacturing. The policy mandates that drug manufacturers treat effluents to a maximum of <1 μg/L of antibiotic residues, a measure that has proven effective in reducing the presence of the bla_{NDM-1} gene in local waterways by 60% near Hyderabad [11]. This reduction in antimicrobial contamination is crucial for preventing the environmental dissemination of resistance genes, which can enter the food chain and exacerbate public health threats. In Europe, the EU has implemented a comprehensive surveillance strategy through its Watch List, which monitors the presence of antibiotics such as ciprofloxacin in surface waters. By enforcing strict thresholds of $\leq$0.1 μg/L, this initiative plays a pivotal role in limiting the environmental spread of pharmaceutical pollutants. These combined efforts to regulate pharmaceutical emissions are essential for reducing the environmental burden of AMR, ensuring that the aquatic microbiomes remain less conducive to the selection and dissemination of resistant strains [129].

5.2. Technological Innovations in Wastewater and Manure Treatment

Advanced wastewater treatment technologies play a crucial role in reducing the dissemination of ARGs in the environment. Among these techniques, the combination of ozonation and activated carbon adsorption has proven particularly effective. Studies conducted in Germany have shown that this process can eliminate up to 99.9% of ARGs, including the β-lactamase gene $bla_{CTX-M-15}$, with a treatment cost of approximately €0.05 per cubic meter of water. This method has been widely implemented in various wastewater treatment plants, demonstrating its high efficiency and cost-effectiveness [130].

Membrane bioreactors (MBRs) represent another advanced treatment strategy. For instance, the NEWater plant in Singapore uses a 0.1 μm ultrafiltration membrane to achieve a significant reduction in the *mcr-1* gene, which is associated with colistin resistance. MBRs combine biological treatment with physical separation, thereby enhancing water quality and significantly reducing the risk of ARG transmission into the environment [131].

Regarding the treatment of animal manure, thermophilic composting has been shown to be effective. Heating manure to 55 °C for seven days can degrade the *tet(M)* gene—linked to tetracycline resistance—by up to 99%. This high-temperature process accelerates the breakdown of resistance determinants, thereby reducing the likelihood of gene transfer to soil and water ecosystems [132].

Soil amendment with biochar is also emerging as a promising strategy to limit the horizontal transfer of ARGs. Adding 5% (*w*/*w*) of biochar to soil can significantly reduce the availability of extracellular DNA, a key vector in gene transfer between bacteria. This approach not only helps mitigate the spread of ARGs but also enhances soil fertility and promotes carbon sequestration, making it a sustainable option for managing manure and organic waste [133].

Additionally, *gamma-ray irradiation* has been identified as an effective method for mitigating ARB and ARGs in aquatic environments. A study by Zhang et al. (2024) [47] demonstrated that gamma-ray irradiation significantly reduced ARGs, including *tetO*, *tetA*, and bla_{TEM-1}, in secondary effluents from municipal wastewater treatment plants. The irradiation process led to up to 90.5% removal of these genes and resulted in no resurgence of the ARGs after exposure to gamma rays. This technique not only inactivated ARB but

also reduced the extracellular DNA responsible for horizontal gene transfer, thus mitigating the environmental risks associated with antibiotic resistance [47].

5.3. One Health Integration

Global coordination efforts to combat AMR have seen significant strides, particularly through initiatives like the WHO-FAO-OIE Tripartite Collaboration. This collaboration launched the Global AMR Surveillance System (GLASS), which has been instrumental in integrating environmental data from over 90 countries, providing a comprehensive global overview of AMR trends and challenges [134]. Such initiatives are crucial in ensuring a coordinated response to AMR, facilitating data sharing, and fostering international cooperation. At a regional level, the European Union's Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) has also contributed by funding transdisciplinary projects aimed at reducing antibiotic use in agriculture. For example, the DISARM project is one such initiative that focuses on mitigating antibiotic dependence in farming through the adoption of alternative strategies [135].

On the national level, countries like the Netherlands and Thailand have set exemplary models in their efforts to combat AMR. The Netherlands, through its "Farm-to-Fork" model, has achieved a remarkable reduction in veterinary antibiotic usage by 71% between 2009 and 2023. This policy shift has had a significant impact on the prevalence of MRSA in pigs, decreasing it by 90% [136]. This success underscores the effectiveness of integrated approaches to AMR, particularly when coupled with stringent regulations and a commitment to reducing antibiotic use in livestock. Similarly, Thailand's reforms in the aquaculture sector have led to the replacement of 50% of antibiotics used in shrimp farming with probiotics. This transition has not only improved farm productivity but has also resulted in a 40% reduction in the prevalence of the *qnrS* gene in coastal sediments, a significant marker for fluoroquinolone resistance [137]. These national success stories highlight the potential of well-coordinated, locally tailored interventions in the fight against AMR. Through the implementation of innovative practices and regulations, countries can effectively reduce the environmental burden of resistance, contributing to a global reduction in AMR.

5.4. Global Surveillance and Monitoring Networks

Environmental DNA (eDNA) tracking has emerged as a powerful tool in the surveillance of ARGs in various environmental reservoirs, including water, soil, and air. One notable initiative in this field is the MARA project (Monitoring of Antibiotic Resistance in the Environment), which analyzed metagenomic data from over 1400 environmental samples collected across 18 provinces in China between 2013 and 2020. This large-scale study identified and quantified 290 ARGs and 30 mobile genetic elements, including clinically relevant genes such as bla_{NDM} and *mcr* [138]. By aggregating data from diverse ecological compartments such as surface water, sediment, dust, and soil, the MARA project provides a robust framework for understanding the environmental distribution of resistance genes and identifying potential hotspots of AMR. This effort contributes significantly to global AMR surveillance and supports evidence-based strategies for mitigating the spread of resistant pathogens [139].

In addition to global efforts, regional projects such as ResistoMap have taken eDNA tracking a step further by offering a real-time dashboard that monitors ARG hotspots in European rivers. This system enables continuous tracking of resistance gene dynamics in water bodies, where resistance genes can persist and disseminate, impacting both aquatic ecosystems and human populations. The ResistoMap project provides an important resource for policymakers and environmental health professionals, helping to pinpoint regions with elevated ARG levels, thus facilitating targeted interventions in the management

of waterborne resistance. The ability to track ARGs in real time also enables a more rapid response to emerging threats, further enhancing public health preparedness in the face of rising AMR [140].

Furthermore, citizen science initiatives, such as the mBioTracker App, have empowered local communities to contribute to the tracking of ARGs, thus expanding the reach of surveillance efforts. In Kenya, for example, the app allows farmers to upload data on the presence of ARGs in manure, facilitating localized interventions to reduce the spread of resistance within agricultural settings. This collaborative approach not only enhances the spatial and temporal coverage of AMR monitoring but also fosters community involvement in addressing a global health challenge. By leveraging the power of citizen science, the mBioTracker App represents a novel way to integrate grassroots participation into the broader scientific endeavor to combat antimicrobial resistance, especially in regions where resources for traditional monitoring may be limited [141].

5.5. Emerging Solutions and Future Directions

Managing and mitigating AMR through innovative strategies is crucial for addressing the growing environmental threats posed by resistant pathogens. One promising approach involves phage-based biocontrol, where engineered bacteriophages have been used to target specific pathogens such as *P. aeruginosa*. In controlled trials, these phages significantly reduced the bacterial population in wastewater systems. A study by Kauppinen et al. (2021) [142] showed that the use of bacteriophages *vB_PaeM_V523* and *vB_PaeM_V524* led to a reduction of more than 2.4 log10 in *P. aeruginosa* populations, highlighting the potential of phages to control resistant strains in environmental reservoirs. This reduction not only underscores the potential of phages as a tool for environmental AMR control but also emphasizes the importance of targeting resistant strains directly in their ecological niches, such as wastewater, where they are prevalent [142].

Another critical intervention in reducing AMR in agricultural settings is vaccine-driven stewardship. For example, the use of poultry vaccines targeting *E. coli* has led to a notable reduction in the reliance on antibiotics in broiler production systems. In a field experiment, the vaccination of broiler chickens with a live attenuated *E. coli* vaccine resulted in improved general health parameters and a significant reduction in the incidence of *E. coli* infections, leading to less antibiotic treatment. This reduction in antibiotic consumption directly impacted the environmental spread of resistance, particularly in agricultural runoff, where the prevalence of resistant *E. coli* strains, including those with the *tet(A)* gene, was lower than in unvaccinated flocks. Such vaccines represent an effective strategy to protect animal health and reduce the ecological burden of AMR, highlighting the role of preventive measures in managing AMR across ecosystems [143].

In addition to biological interventions, technological advancements such as artificial intelligence (AI) are increasingly vital in predicting and managing AMR. Machine learning models, such as DeepARG, have shown exceptional promise in forecasting the emergence of ARGs in environmental soils. With an impressive accuracy rate of 92%, these AI-powered systems enable researchers and policymakers to anticipate potential hotspots of ARG accumulation, allowing for more targeted interventions and more effective stewardship programs. The integration of AI into AMR management strategies offers a new frontier in predictive epidemiology, where data-driven approaches can guide proactive environmental health measures, ultimately reducing the spread of resistance genes across various ecosystems [144].

6. Conclusions

Environmental AMR represents an escalating global crisis, driven mainly by anthropogenic pollution. Water, soil, and air have become interconnected reservoirs for ARGs and ARBs, contributing to the widespread dissemination of resistance across ecosystems. The primary forces behind this phenomenon include pharmaceutical waste, agricultural runoff, and inadequate sanitation systems, all of which create selective pressures that accelerate the evolution of resistance in microbial populations. These pressures are particularly potent in environments contaminated by the overuse and misuse of antibiotics and the lack of proper waste management and water treatment infrastructure. In high-income regions, environmental AMR is exacerbated by legacy pollution and intensive agricultural practices, which continue to contribute to the accumulation of antibiotic residues in the environment. In contrast, low-resource settings are often disproportionately affected by unregulated antibiotic use in human healthcare and animal husbandry, combined with poor infrastructure that hampers adequate sanitation and waste treatment efforts.

The environmental transmission of AMR necessitates immediate and coordinated global efforts to mitigate its spread and impact. Addressing this challenge requires robust policy enforcement at the international level, particularly by implementing regulations that limit antibiotic use in agriculture. For instance, the European Union has successfully reduced veterinary antibiotic use by 71%, setting an important precedent for global efforts to regulate antimicrobial consumption in farming. Alongside these regulatory measures, pharmaceutical emission standards must be established to minimize the release of antibiotic residues into the environment, particularly through industrial processes and waste disposal practices. Integrating innovative technologies also plays a critical role in combating environmental AMR. Advanced wastewater treatment techniques, such as ozonation and the use of biochar, can significantly reduce the levels of ARGs and ARBs in water systems. Real-time surveillance systems for tracking ARGs are also vital tools for monitoring resistance trends and enabling rapid response to emerging threats. Furthermore, addressing environmental AMR demands a "One Health" approach that bridges human, animal, and environmental health. Initiatives like the World Health Organization's Global Antimicrobial Resistance Surveillance System (GLASS) network exemplify this integrated approach, which fosters collaboration across sectors and enhances the global capacity to combat AMR in all its environmental reservoirs. These collective efforts are essential to mitigating the planetary threat AMR poses and ensuring antibiotics' continued effectiveness in treating infectious diseases worldwide.

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Review

The Role of Water as a Reservoir for Antibiotic-Resistant Bacteria

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Abstract

Water systems serve as multifaceted environmental pools for antibiotic-resistant bacteria (ARB) and resistance genes (ARGs), influencing human, animal, and ecosystem health. This review synthesizes current understanding of how antibiotics, ARB, and ARGs enter surface, ground, and drinking waters via wastewater discharge, agricultural runoff, hospital effluents, and urban stormwater. We highlight key mechanisms of biofilm formation, horizontal gene transfer, and co-selection by chemical stressors that facilitate persistence and spread. Case studies illustrate widespread detection of clinically meaningful ARB (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) and mobile ARGs (e.g., *sul1/2*, *tet*, *bla* variants) in treated effluents, recycled water, and irrigation return flows. The interplay between treatment inefficiencies and environmental processes underscores the need for advanced treatment technologies, integrated monitoring, and policy interventions. Addressing these challenges is critical to curbing the environmental dissemination of resistance and protecting human and ecosystem health.

Keywords: antibiotic resistance genes; antibiotic-resistant bacteria; spread mechanisms; environmental pathways of transmission; waterborne pathogens

1. Introduction

Water, one of the most critical environmental reservoirs, acts as both a direct habitat and transmission route for antibiotic-resistant bacteria [1,2]. Water bodies such as rivers, lakes, oceans, and groundwater are susceptible to contamination with antibiotic residues, bacteria, and resistance genes through multiple sources [3,4]. These sources include agricultural runoff from livestock farming, untreated human waste, pharmaceutical industries, and wastewater treatment plants that often fail to remove all pharmaceutical contaminants.

Antibiotic residues in water create selective pressure, fostering the survival and proliferation of resistant strains of bacteria [5,6]. For instance, bacteria like *Escherichia coli* [7,8], *Salmonella* spp. [9,10], and *Pseudomonas aeruginosa* [9], which are typically found in wastewater or contaminated water sources, can harbor bacteria resistant to multiple antibiotics, such as β-lactams and tetracyclines [10]. These resistant bacteria can be transmitted to humans

and animals through contaminated drinking water [11], recreational water activities, or by consuming contaminated food [12], such as fish or vegetables irrigated with polluted water [13,14].

Research employing traditional culture-based methods has shown that resistant strains can persist in water for extended periods, challenging eradication efforts. For example, a study by Zhang et al. reported the presence of over 150 distinct resistance genes in river water samples taken from urban and rural locations. Culture-independent methods, like metagenomics, have revealed a broader diversity of resistant bacteria and their genes within aquatic environments, offering a clearer picture of how water ecosystems harbor and propagate antimicrobial resistance (AMR) [15].

Waterborne diseases remain a key concern in developing regions where water treatment facilities may be inadequate, underscoring the need for better management practices. Addressing antibiotic pollution in water bodies, such as improved wastewater treatment and restrictions on pharmaceutical discharge, is vital in curbing the spread of antibiotic-resistant bacteria (ARB) [16].

Water plays a significant role as a reservoir for ARB, contributing to the spread and persistence of these pathogens in the environment (Figure 1). The presence of ARB in water bodies such as rivers, lakes, wastewater, and drinking water poses a serious public health threat [17] due to the potential for human exposure [18].

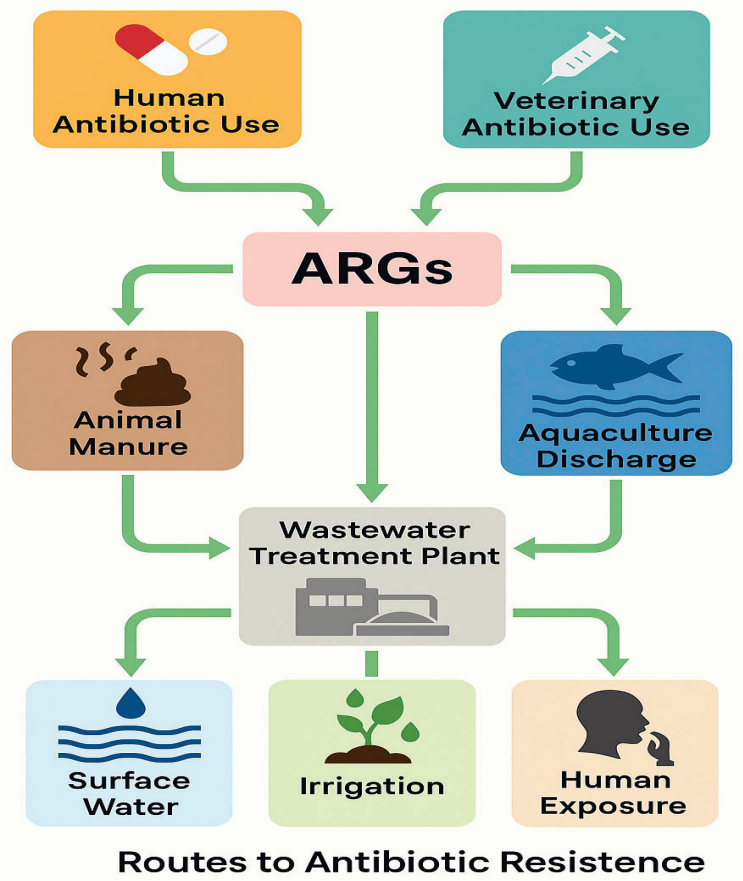

Figure 1. Environmental, clinical, and agricultural sources contribute to the circulation of ARGs.

Historical and Anthropogenic Drivers of Antibiotic Resistance and Environmental Dissemination

Antibiotic resistance genes (ARGs) have deep evolutionary roots. Functional metagenomic analyses have revealed that resistance mechanisms to major antibiotic classes including β-lactams, tetracyclines, and glycopeptides exist in microbial communities long before the advent of human antibiotic use. ARGs have been identified in 30,000-year-old

permafrost sediments, demonstrating that these genes originally served ecological functions unrelated to medical or veterinary contexts. Horizontal gene transfer (HGT) later facilitated their spread across diverse bacterial populations [17,19,20].

In the modern era, the widespread and unregulated use of antibiotics in human and animal sectors has accelerated the dissemination of ARGs. A major contributor is the agricultural sector. In livestock systems, notably poultry, swine, and cattle, antibiotics such as tetracyclines, macrolides, sulfonamides, and β-lactams are routinely used for both treatment and growth promotion. This continuous selective pressure favors resistant bacteria such as *Escherichia coli*, *Salmonella* spp., and *Enterococcus* spp. These are shed in manure, which is commonly applied to agricultural fields, leading to contamination of soil, crops, and nearby water bodies. Genes like *blaCTX-M*, *mcr-1*, and *tet(M)* have been recovered from both livestock waste and human clinical isolates, demonstrating direct zoonotic and environmental transfer [21].

In aquaculture, antibiotics such as oxytetracycline, florfenicol, and sulfadimethoxine are used to prevent bacterial infections in fish and shrimp. These drugs persist in aquatic environments, selecting for ARGs like *tet*, *sul*, and *qnr*, which can accumulate in sediments and be transferred to native or opportunistic bacteria. Pathogens such as *Vibrio* and *Aeromonas*, commonly found in aquaculture settings, have been shown to harbor these ARGs, creating potential routes for resistance transmission to humans via seafood or contact with contaminated water [22,23].

Plant agriculture also contributes to ARG dissemination. In the United States, China, and other countries, antibiotics such as streptomycin and oxytetracycline are used to manage bacterial diseases in crops like apples, pears, and citrus. These treatments have led to the emergence of resistant plant pathogens such as *Erwinia amylovora*. Resistance genes including *strA* and *strB* have also been identified in non-pathogenic, plant-associated bacteria like *Pseudomonas* spp. and *Xanthomonas* spp., which can potentially transfer ARGs to human pathogens via plasmids and integrons [24,25].

The World Health Organization has highlighted the severity of the antimicrobial resistance crisis through its global priority pathogen list, which includes critical threats such as carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*. These organisms are increasingly found outside clinical settings, including in agricultural and aquatic environments, where antibiotic residues and ARGs create ideal conditions for their selection and spread [26].

Collectively, these examples from livestock, aquaculture, and crop production underscore how anthropogenic use of antibiotics in non-clinical settings contributes to the environmental resistome. ARGs from these systems can ultimately reach humans through interconnected pathways including food consumption, direct contact, and contaminated water, reinforcing the importance of a "One Health" approach to antimicrobial resistance containment.

2. Sources of Antibiotic-Resistant Bacteria in Water

One health issue influencing life across humans, animals, and the environment is antibiotic resistance, which is found in one of the most basic resources for life water [27]. Antibiotic-resistant pathogens and their genes have been found in streams, lakes [28], rivers [29], and oceans [30]. They can often be traced back to hospital discharge [31], farms, or sewage systems (Table 1) [32]. Even properly functioning wastewater treatment systems may not fully remove resistant pathogens and their genes [33].

ARB in water environments originates from various sources, including wastewater treatment plants [34], agricultural runoff [11], urban stormwater [35], and landfill leachates [36]. Additionally, it can include human and animal excreta too [37]. This oc-

currence is primarily because the bacteria in these wastes are often exposed to antibiotics, whether for treatment, prophylaxis, or as growth promoters in livestock farming [33].

Table 1. Comparative summary of predominant ARGs, associated pathogens, regions, and detection methods by water source.

Water Source	Main ARGs	Associated Pathogens	Regions	Detection Methods	References
Wastewater Treatment Plants	*sul1*,*tet*, *blaCTX-M*	*E.coli*, *K. pneumoniae*, *P. aeruginosa*	Europe, Asia, Africa	qPCR, metagenomics	[34,38]
Hospital Effluents	*blaNDM*, *blaOXA-48*, *mcr-1*	ESKAPE pathogens, *Enterococcus* spp.	Middle East, Africa	Culture, PCR, WGS	[39,40]
Agricultural Runoff	*tetM*, *sul1*, *ermB*	*E. coli*, *Enterococcus* spp., *Salmonella* spp.	N. America, Asia	qPCR, metagenomics	[41,42]
Urban Stormwater	*sul1*, *tetA*, *blaTEM*	*E. coli*, *S. aureus*	USA, China	qPCR, MST	[43,44]
Drinking Water	*sul1*, *tetA*, *blaTEM*	*P. aeruginosa*, *Legionella* spp.	Global	Culture, qPCR	[45,46]
Recycled Water	*blaCTX-M*, *sul1*, *qnrS*	*E. coli*, *P. aeruginosa*	Europe, Australia	qPCR, culture	[47,48]

2.1. Wastewater Treatment Plants (WWTPs)

WWTPs are complex microbial environments with a mix of pathogenic and environmental bacteria. They constitute reservoirs for ARBs and antibiotic resistance genes (ARGs). While designed to treat sewage, conventional treatment processes often fail to eliminate ARBs and ARGs, releasing them into receiving water bodies. Studies show that WWTPs are both points of antibiotic accumulation and hotspots for ARBs and ARGs [38].

Since antibiotics are not completely broken down in the body, they are excreted via urine and feces, eventually reaching wastewater treatment plants (WWTPs) [49]. If these plants do not effectively remove the contaminants, antibiotics and resistant bacteria may be released into freshwater systems like rivers and lakes [50]. Biological treatment systems like activated sludge may inadvertently promote horizontal gene transfer under antibiotic-selective pressures [51]. Moreover, standard disinfection processes (e.g., chlorination, UV) are not fully effective at degrading extracellular DNA, allowing ARGs to persist in effluents. Advanced technologies such as ozonation, membrane bioreactors, and UV/H_2O_2 oxidation achieve higher ARG removal efficiencies, but their adoption is limited by cost, energy needs, and maintenance complexity, particularly in LMIC settings [51].

For example, Okoh et al. studied WWTPs in the Eastern Cape and highlighted their role as significant pollution sources for pathogens and antimicrobial resistance genes [36]. These results indicated that WWTPs in the Eastern Cape were identified as important sources of antimicrobial-resistant *E. coli*. They found that 32.7% of *E. coli* isolates exhibited multidrug resistance to 15 antibiotics, with resistance genes like *mcr-1* and *ermA* detected in effluent samples [52].

This finding is supported by several studies from developing and developed regions [35,36], which discussed the spread of antimicrobial resistance due to inadequate removal during treatment processes. Bacteria identified in this study include *P. aeruginosa*,

Klebsiella pneumoniae, and various *Enterococcus* spp. The study conducted by Yang et al. took place in a large-scale municipal wastewater treatment plant located in Chuzhou, China. The researchers used Illumina MiSeq sequencing to analyze the structure of microbial communities in the activated sludge of this facility. The results revealed that the dominant phyla were Proteobacteria, Bacteroidetes, and Actinobacteria, with a notable presence of Firmicutes and Chloroflexi [53].

Additionally, research shows that the coexistence of pharmaceuticals and personal care products (PPCPs), biocides, metals, and agrochemicals in these environments further contributes to the development of antibiotic resistance among bacterial populations [54]. In many WWTPs, the combined presence of these chemicals creates significant evolutionary pressure, thereby promoting the selection and propagation of resistant bacteria. In South-eastern China, a study on animal farms found 22 ARGs conferring resistance to major antibiotics, including tetracyclines, sulfonamides, quinolones, aminoglycosides, and macrolides. The spread of these ARGs was dominated by *sul* genes, followed by *tet* and *erm* genes. The presence of metals like copper, arsenic, and zinc in agricultural settings may trigger co-selection for these ARGs, enhancing their persistence in the environment [55]. A study conducted in Tunisia, on sewage sludge-amended soils, revealed varying indices of antibiotic resistance, with the highest values observed for amoxicillin (AMX), doxycycline (DOX), and azithromycin (AZI). The prevalence of these resistant bacteria was influenced by factors such as antibiotic consumption patterns, wastewater treatment processes, and the storage conditions of sewage sludge [56]. A systematic review analyzed the impact of point sources such as wastewater treatment facilities and agricultural operations on the prevalence of ARB in natural environments. The study highlighted the need for improved study designs and analytical methods to quantify the effect of these point sources on ARB dissemination [57]. While WWTPs represent centralized, high-load entry points, agricultural runoff introduces diffused but sustained ARG pressure into surface and groundwaters. This transition from point to non-point sources highlights the synergistic role of farming practices in amplifying environmental resistance.

2.2. Agricultural Runoff

The extensive use of antibiotics in agriculture, particularly in livestock farming, contributes to the presence of ARB in water systems. When used as fertilizer or improperly managed, manure from treated animals can introduce ARB and ARGs into surface and groundwater through runoff [58]. Runoff during rainfall can transport these resistant elements into nearly all water bodies, increasing the environmental load of ARB.

Recent studies have underscored that agricultural runoff is a significant pathway for disseminating antimicrobial-resistant bacteria and resistance genes into the environment. For instance, Zhu et al. demonstrated that animal manure, especially from swine farms, is a reservoir of diverse and abundant antibiotic resistance genes, which can be mobilized into water systems through runoff [41]. The research was conducted in China, targeting three major swine-producing regions: Beijing, Jiaxing, and Pu Tian. These farms collectively housed over 10,000 pigs annually, representing intensive farming practices typical in China. The study identified 149 ARGs across manure samples, with resistance mechanisms spanning multiple antibiotic classes, including tetracyclines, sulfonamides, β-lactams, and macrolides [41]. Heuer et al. focused on EU countries (Germany, the Netherlands), where manure application increased *tet* and *sul* genes in agricultural soils. Dairy cow manure was a major reservoir of *erm* genes [42]. Mobile genetic elements (plasmids, transposons) facilitated ARG exchange between manure-borne *E. coli* and soil bacteria like *Pseudomonas* spp. [59]. Co-selection via heavy metals (copper, zinc) in manure amplified ARG persistence.

Complementing these findings, Guo et al. provided evidence of seasonal and spatial variability in the abundance of resistance genes within agricultural watersheds, highlighting that runoff events can sustain elevated levels of these genes in surface waters [60]. Similarly, Cheng et al. found that rural watersheds receiving agricultural runoff contain multiple resistance genes, directly linking these findings to farming practices, particularly the widespread use of antibiotics in livestock production [61]. This finding emphasizes the critical need for improved management practices to mitigate the environmental and public health risks associated with the spread of antibiotic resistance from agricultural sources.

2.3. Urban Stormwater

Urban runoff is a notable pathway for the dissemination of ARB and ARGs into aquatic environments. Factors such as combined sewer overflows, improper medication disposal, and surface runoff contribute to the presence of these contaminants in urban waterways [62]. Several studies have characterized its role in spreading ARB into downstream water bodies, highlighting the multifaceted nature of resistance dissemination.

For example, O'Malley et al. reviewed how urban stormwater acts as a hotspot for ARGs due to the convergence of pollutants, mobile genetic elements, and selective pressures such as heavy metals. Their work synthesized data from 12 separate storm–sewer outfalls in the Tampa Bay metropolitan area of Florida, USA. In that Tampa Bay study, the sulfonamide resistance gene *sul1* and the tetracycline resistance gene *tetA* were highly prevalent, being detected in over 95% of all grab-sampled stormwater outfalls, which emphasizes that traditional gray stormwater infrastructure may inadvertently facilitate the transport of these resistance elements into recreational and drinking water sources [62]. While the review aggregates global data, specific studies in Milwaukee highlight localized impacts, such as ARG transport into Lake Michigan [63]. The confluence of MGEs (e.g., plasmids, integrons) and contaminants in stormwater creates a reservoir for horizontal gene transfer, posing risks to public health through recreational or drinking water exposure [62]. Additionally, O'Malley et al. distinguished between intracellular and extracellular ARGs in stormwater, revealing distinct seasonal and spatial patterns that further complicate the overall resistome profile of urban runoff [64].

Similarly, Lee et al. profiled four municipal separate storm–sewer (MS4) outfalls during spring–summer 2017 in Columbus, OH, USA, and showed that wet-weather (storm) flows discharged much higher densities of *E. coli* than baseflow; microbial-source tracking (MST) found ruminant fecal markers in 91% of samples. Metagenomic analysis revealed a resistome dominated by sulfonamide resistance genes (*sul1*, *sul2*), which were strongly associated with urban and agricultural runoff, as well as tetracycline resistance genes (*tetA*, *tetC*, *tet(O)*, *tet(W)*), which correlated with fecal indicators like *E. coli*. Beta-lactam resistance genes such as bla_{TEM} (indicative of resistance to penicillin-type antibiotics) and macrolide resistance genes like *ermF* (frequently found in extracellular DNA) were also widespread. These ARGs were often co-located with bacterial pathogens, including *Staphylococcus aureus* and *Salmonella enterica Typhimurium*, highlighting risks of co-transfer between resistance and pathogenicity traits [43].

In another study, stormwater runoff in humid Chinese cities contained 1.8× higher micropollutant concentrations (antibiotics, endocrine disruptors) during wet weather compared to dry conditions. ARGs such as *sul1* and *tetC* were prevalent, with stormwater discharge contributing significantly to surface water contamination, particularly in areas with combined sewer overflows [65].

Di Cesare et al. evaluated the impact of a moderate rain event (4 mm/h) on ARG levels in a large subalpine river in the Italian Alps. They used qPCR to track five ARGs—*tetA* (tetracycline), *ermB* (macrolide), $\mathrm{bla}_{\mathrm{CTX\text{-}M}}$ (β-lactam), *sulII* (sulfonamide), and *qnrS*

(quinolone)—and found that total ARG abundance increased 24-fold during the rain compared to the annual average. This spike coincided with higher concentrations of total phosphorus, ammonium-N, and microbial aggregates, implicating overland runoff and catchment sources in ARG mobilization [66].

Ahmed et al. were the first to link sewage-associated marker genes with ARGs in an Australian urban river, sampling untreated sewage influent and 12 sites along the Brisbane River and its tributaries under baseflow and stormflow. They quantified fecal indicators (*E. coli*, enterococci), sewage markers (e.g., HF183), and ARGs such as *sul1*, *intI1*, *tetW*, and *ermF*. Stormflow samples showed markedly higher abundances of both sewage markers and ARGs compared to dry weather, with strong correlations ($r > 0.8$) between HF183 and multiple ARGs, highlighting rain-driven mobilization of human-derived resistance elements [67]. In contrast to the broader environmental contributions, hospital effluents introduce highly concentrated loads of multidrug-resistant pathogens and clinically relevant ARGs into wastewater networks.

2.4. Hospital Effluents

Due to medical treatments and laboratory activities, wastewater from healthcare facilities often contains high concentrations of antibiotics and ARB. If not adequately treated before discharge, these effluents can introduce resistant bacteria into municipal sewage systems and natural water bodies [68].

Hospital wastewater is a recognized hotspot for antibiotic-resistant bacteria due to the heavy use of antibiotics in clinical settings. Multiple studies have shown that hospital effluents contain high levels of antibiotic residues and resistant pathogens. For example, a study conducted in Egypt identified MDR bacteria in hospital WWTP effluents, with isolates including *Staphylococcus haemolyticus*, *Enterococcus faecalis*, and *E. coli* being recovered via 16S rRNA gene sequencing. These isolates resisted standard antibiotics such as tetracycline, ampicillin, and ceftazidime at concentrations up to 25 ppm, although they were susceptible at higher concentrations [39].

Another study from Ethiopia focused on the bacteriological quality of hospital wastewater and found that the most frequently isolated pathogens were *S. aureus*, *E. coli*, *Citrobacter* spp., and *Acinetobacter* spp. [69]. Notably, *K. pneumoniae* and *P. aeruginosa* have also been reported in hospital effluents, critical ESKAPE pathogens responsible for hospital-acquired infections. For instance, research from Benin documented a predominance of non-enterobacteria and *Acinetobacter* spp. in hospital effluents, highlighting their potential to disseminate resistance genes into the environment [70]. In addition, recent metagenomic approaches have revealed that hospital effluents are reservoirs for clinically significant resistance determinants, including extended-spectrum beta-lactamases (ESBLs) and carbapenemases [71]. As treated wastewater is increasingly reused or discharged into potable water sources, understanding how ARB and ARGs persist or are inadequately removed during advanced water treatment becomes crucial.

2.5. Drinking Water Treatment and Distribution Systems (DWDS)

Even after treatment, drinking water can harbor ARB and ARGs. Factors such as biofilm formation within distribution systems and using disinfectants like chlorine can influence the prevalence and persistence of these contaminants in potable water [72]. Another review discussed how conventional drinking water treatment processes might not effectively eliminate ARB, potentially extending antibiotic resistance and posing risks to human health. The authors emphasized the importance of understanding the frequency and quantity of bacteria with antibiotic-resistant mutations in drinking water systems [73].

DWDS are critical for ensuring safe water; however, several investigations have detected low levels of ARB and ARGs in both finished drinking water and tap water, even after conventional treatment processes such as coagulation, sedimentation, filtration, and disinfection.

For instance, Xi et al. examined drinking water treatment plants in Michigan and Ohio using culture-based and molecular methods. Their work revealed that although the overall bacterial load decreased significantly during treatment, the relative abundance of ARGs increased in tap water compared to source water. The study identified a range of ARGs associated with resistance to beta-lactams, aminoglycosides, tetracyclines, and fluoroquinolones. Moreover, regrowth of heterotrophic bacteria in the distribution system was observed, with species such as *Pseudomonas* spp. and *Acinetobacter* spp. emerging as a notable ARB [46].

Another study by Sanganyado and Gwenzi reviewed ARB's occurrence, removal, and human health risks in drinking water systems. Their review provides a global synthesis of ARB and antibiotic resistance genes ARGs in drinking water systems, highlighting case studies from China, sub-Saharan Africa, Europe, and North America and emphasizing both conventional and advanced treatment processes. The most commonly detected ARGs across these systems include sulfonamide (*sul1*, *sul2*), tetracycline (*tetA*, *tetM*), β-lactam (*ampC*, bla_{TEM}), macrolide (*ermB*), and the class 1 integron integrase gene (*intI1*), with opportunistic pathogens such as *P. aeruginosa*, *Legionella pneumophila*, non-tuberculous mycobacteria, and ESBL-producing *E. coli* frequently persisting throughout treatment. This work underscored that conventional disinfection (e.g., chlorination) may not effectively remove ARGs and, in some cases, may even select for resistant populations [45]. Similarly, a study by Chen et al. conducted targeted qPCR and 16S rRNA sequencing on bulk water and biofilm samples from a full-scale DWTP and distribution system in Shanghai, China, using Yangtze River source water. They quantified six key markers, *sul1*, *sul2*, *tetA*, *tetM*, *ampC*, and *intI1*, and found that although absolute ARG abundances dropped post-treatment, relative abundances in biofilms remained high. Opportunistic pathogens detected by qPCR included *Mycobacteria* spp., *Legionella* spp., *L. pneumophila*, *P. aeruginosa*, and *Hartmannella vermiformis*, all of which correlated strongly with ARG levels, underscoring biofilms as reservoirs for ARG dissemination [74].

These studies have highlighted that while treatment processes effectively reduce overall microbial counts, the selective pressures exerted during treatment and within distribution pipelines may foster the proliferation of ARB. Notably, ARB pathogens, such as *E. coli*, *P. aeruginosa*, and *Acinetobacter baumannii*, that can cause infections in vulnerable populations have been identified in tap water. In some instances, the levels of ARGs related to multidrug resistance and specific antibiotic classes (e.g., beta-lactams) were higher in distributed water than in finished water directly from treatment plants.

2.6. Recycled Water

Reusing treated wastewater for purposes like irrigation can be a source of ARB exposure. Studies have detected various resistant bacteria in recycled water, highlighting the need for comprehensive risk assessments and effective treatment strategies to mitigate potential health risks [47].

Recycled water, which is increasingly used for irrigation and industrial applications, has been shown to harbor ARB and ARGs [75,76]. Several studies have demonstrated that even after treatment, recycled wastewater may retain or even select for resistant bacteria due to suboptimal removal of antibiotics and the persistence of biofilms [77].

For example, a study by Chen et al. detected a diverse array of ARGs in recycled wastewater used for agricultural irrigation. In this work, researchers identified clinically

relevant bacteria, including multidrug-resistant *E. coli*, *E. faecalis*, and *P. aeruginosa*, in samples collected from recycled water systems [78]. Similarly, Drigo et al. reported the effectiveness of recycled water treatment plants in eliminating human opportunistic pathogens and antimicrobial-resistant microorganisms. The study identified several enteric opportunistic pathogens, including *A. baumannii*, *E. coli*, *E. faecalis*, *K. pneumoniae*, and *P. aeruginosa*. The findings indicated that while the treatment plants removed over 95% of these pathogens and resistance genes, some subpopulations survived disinfection and had the potential to regrow within the recycled water systems. This suggests that despite high-quality treatment, certain antibiotic-resistant bacteria may persist in recycled water, posing potential risks if the water is reused [48].

2.7. Irrigation and Non-Potable Uses

Recycled, non-potable water used for irrigation can be a significant source of ARB that may be transferred to crops and, ultimately, to humans. Several studies have investigated this issue.

A study by Odonkor and Addo examined water sources used for irrigation in rural settings in Ghana and reported that surface water sources, particularly dams, exhibited high *E. coli* contamination (up to 3.22×10^5 CFU/100 mL) and multidrug resistance (49.48% of isolates). Resistance to penicillin (32.99%) and tetracycline (21.45%) was common, with *sul1* and *tet* genes frequently detected. Their findings highlighted that even water not intended for direct human consumption can serve as a reservoir for ARB, raising concerns about foodborne exposure [79]. Similarly, Larson et al. conducted research in Peru and detected multidrug-resistant *E. coli* and *Klebsiella* spp. on vegetables irrigated with reclaimed water, suggesting that recycled water used in agriculture may facilitate the transfer of resistance genes onto produce [80]. In addition, research in Nigeria has identified antibiotic-resistant *Enterococcus* spp. and *Salmonella* spp. in water sources used for vegetable irrigation, highlighting risks to food safety and public health. Key studies focus on regions such as Lagos State and Northwest Nigeria, where agricultural runoff, untreated wastewater, and livestock activities contribute to contamination of irrigation water with ARB [81].

In Idaho, USA (Snake River Watershed), irrigation return flows (IRFs) contained *E. coli* and *enterococci* with resistance to tetracycline (13%) and macrolides (*ermF*). MDR patterns were observed in 12% of isolates, linked to agricultural runoff and livestock activities [82].

The presence of foodborne pathogens in irrigation water is common. These organisms could remain viable and survive for a very long time and develop antibiotic resistance, posing a great threat to public health. Pathotypes of *E. coli* and *Enterococcus* spp. are microorganisms that survive under these conditions [83]. *Salmonella* spp. can also be found in the agricultural environment and is one of the most common causes of severe foodborne diseases.

Among the diverse sources contributing to ARG dissemination in aquatic environments, hospital effluents are consistently reported to have the highest concentrations of clinically significant resistance genes, such as *blaNDM*, *blaOXA-48*, and *mcr-1* [40]. Though volumetrically smaller than agricultural runoff, these effluents are enriched in multidrug-resistant pathogens and last-resort resistance genes. In contrast, agricultural runoff, particularly from intensive livestock operations, contributes a broader volume of ARGs such as *tet*, *sul*, and *erm* due to the scale of antibiotic application in animal production [42]. Urban stormwater runoff has also emerged as a significant source, containing mixtures of human and animal waste, pharmaceuticals, and metals, all of which drive co-selection [51]. Treated recycled water may harbor lower ARG levels but still poses a risk of ARG persistence, especially where advanced treatments are lacking.

2.8. Regional Case Study

2.8.1. Antibiotic Resistance in North African Water Bodies

In a recent survey, qPCR and metagenomic surveys across North Africa have revealed that sulfonamide (*sul1*), tetracycline (*tetM*), and extended-spectrum β-lactamase ($bla_{\text{CTX-M}}$) genes are not only widespread but also often highly abundant in both the water column and underlying sediments of coastal lagoons, estuaries, and river systems. Tunisia (Bizerte Lagoon): In sediment cores and surface waters of this heavily urbanized Mediterranean lagoon, *sul1* copy numbers reached (sediment) and (water), with *tetM* and *blaCTX-M* detected at similarly elevated levels [84]. In Morocco (Oued Sebou Estuary): Surveys here found *sul1* and *tetM* in 100% of sediment–water-paired samples, with *blaCTX-M-15* present in over 60% of isolates, highlighting the impact of upstream agricultural runoff and untreated urban discharges [85]. *Vibrio alginolyticus* isolated from sea bream in Egyptian aquaculture farms near Port Said (Nile Delta) harbored *sul1* (28.8% of isolates), *tetA*, and *blaTEM* genes. This study underscores marine ecosystems' role as AMR reservoirs, with multidrug-resistant bacteria likely spreading to coastal waters through aquaculture effluents [86]. Recent investigations of Algerian water systems have revealed the presence and environmental persistence of clinically important β-lactamase and colistin resistance determinants. A broader survey in Batna targeting hospital tap water, sewage, and environmental effluents reported a high level of resistance in both tap water and wastewater. The most commonly found carbapenem resistance mechanism was the *OXA-48* enzyme, but other carbapenemases were also detected. In addition, the *mcr-1* gene was detected in 18 *E. coli* of different sequence types [87]. In addition, along the Soummam River near Béjaïa, screening of 12 river water samples recovered 20 carbapenem-resistant *Enterobacteriaceae* isolates, 17 of which carried *blaOXA-48*-like and 3 harbored *blaOXA-244* genes [88].

Together, these studies underscore that both freshwater and coastal systems in Algeria act as reservoirs for ESBLs, MBLs, carbapenemases, and plasmid-mediated colistin resistance, highlighting the urgent need for reinforced wastewater treatment and continuous ARG surveillance to mitigate environmental and public health risks.

2.8.2. Antibiotic Resistance in European Water Bodies

A comprehensive survey across ten European countries analyzed effluents from 16 wastewater treatment plants (WWTPs) and their receiving rivers. Quantitative PCR (qPCR) detected antibiotic resistance genes (ARGs) such as *sul1*, *intI1*, and *blaOXA-58* in all samples. The study found that the abundance of these genes was inversely correlated with the number of biological treatment steps implemented at the WWTPs, suggesting that advanced wastewater treatment processes play a key role in mitigating ARG dissemination into the environment [89].

2.8.3. Southern Germany: River Biofilms and Sediments

A targeted study of a river influenced by a small WWTP in Southern Germany examined surface water, sediment, and river biofilms. qPCR analyses revealed the presence of *blaTEM*, *ermB*, *tetM*, and *sul1*. ARG concentrations were significantly higher downstream of the WWTP, indicating that treated effluent discharge is a major contributor to ARG propagation in fluvial ecosystems [90].

2.8.4. Italy: Maiella National Park

Even in protected areas like Maiella National Park in Italy, ARGs were prevalent. Multidrug-resistant bacteria were isolated from water and fecal samples of both livestock and wildlife. Detected resistance genes included *mcr-4*, *blaOXA-48*, *blaTEM*, and *tetB*. This underscores the pervasiveness of ARGs even in relatively pristine environments,

highlighting the role of human and animal activities in spreading resistance determinants into natural ecosystems [91].

2.8.5. Antibiotic Resistance in Asian Water Bodies

In Asia, aquatic environments are increasingly recognized as significant reservoirs of antibiotic resistance genes (ARGs), driven by human activity and aquaculture. In South Korea, a study conducted in South Jeolla Province and Jeju Island assessed effluents from coastal aquaculture farms. Quantitative PCR (qPCR) analysis detected 22 distinct ARGs, with *tetB* and *tetD* being the most prevalent. These resistance genes were strongly associated with bacterial genera such as *Vibrio* spp. and *Marinomonas* spp., underscoring the role of aquaculture effluents in facilitating the spread of ARGs in marine ecosystems [92]. Similarly, investigations in Xiamen City, China, revealed widespread ARG contamination in lagoon waters impacted by urban stormwater runoff. Using high-throughput qPCR and metagenomic techniques, researchers identified 285 ARGs, including *sul1*, *tetO*, and *qnrS*, indicating that rapid urbanization and insufficient wastewater management are key contributors to ARG dissemination in Chinese aquatic systems [44]. These findings highlight how both aquaculture and urban development are critical drivers of environmental antibiotic resistance in Asia.

Interpreting the environmental burden of ARGs requires careful evaluation of detection methods. The following section critically examines the methodological strengths and limitations that influence how resistance is quantified across water systems.

3. Methodological Considerations

Understanding the strengths and limitations of various ARG detection methods is essential for interpreting findings across environmental studies. The primary approaches include culture-based isolation, quantitative PCR (qPCR), and metagenomics, each with distinct biases and constraints.

Culture-based methods allow isolation of live bacterial strains for phenotypic resistance profiling and downstream molecular analyses. However, they capture only a small fraction of environmental microbial diversity, typically <1% of total bacteria, and fail to detect viable but non-culturable (VBNC) organisms or extracellular ARGs (eARGs) [93]. Their use can thus lead to significant underestimation of the resistome in aquatic environments.

Quantitative PCR (qPCR) offers high sensitivity and specificity for detecting known ARGs in complex environmental samples [94]. It is widely used for quantifying genes such as *sul1*, *intI1*, *blaTEM*, and *tetA* in wastewater and surface water [95]. However, qPCR is inherently limited to predefined targets and is vulnerable to primer-template mismatches, amplification biases, and the inability to distinguish intracellular ARGs from free extracellular DNA [96]. This can lead to the overestimation of the abundance of functional resistance if eARGs persist post-treatment [97].

Shotgun metagenomics enables untargeted, high-resolution profiling of entire microbial communities and their associated resistomes [98]. It is especially valuable for identifying novel or rare ARGs as well as co-occurring mobile genetic elements (e.g., integrons, plasmids). However, accurate quantification depends on sequencing depth, bioinformatic pipeline choices, and the completeness of reference databases like CARD, ResFinder, or ARGs-OAP [99]. Misannotation or false positives can arise when ARG homologs are misclassified.

In summary, no single method provides a complete picture of environmental AMR. Combining multiple approaches or interpreting results within their methodological context is essential. This triangulation is reflected in our comparative synthesis (Table 2) and global detection map (Figure 2), which integrate findings across platforms and regions.

Table 2. Comparative ARG patterns.

Water Source	Region	Top ARG Families	Detection Methods
WWTP effluents	Europe, Asia	*sul1*, *blaTEM*, *tetA*	qPCR, metagenomics
Agricultural runoff	North America, EU	*tetM*, *sul2*, *ermB*	culture, qPCR
Hospital effluents	North Africa, Asia	*blaNDM*, *mcr-1*, *intI1*	metagenomics
Recycled water	South America	*sul1*, *qnrS*, *blaCTX-M*	qPCR

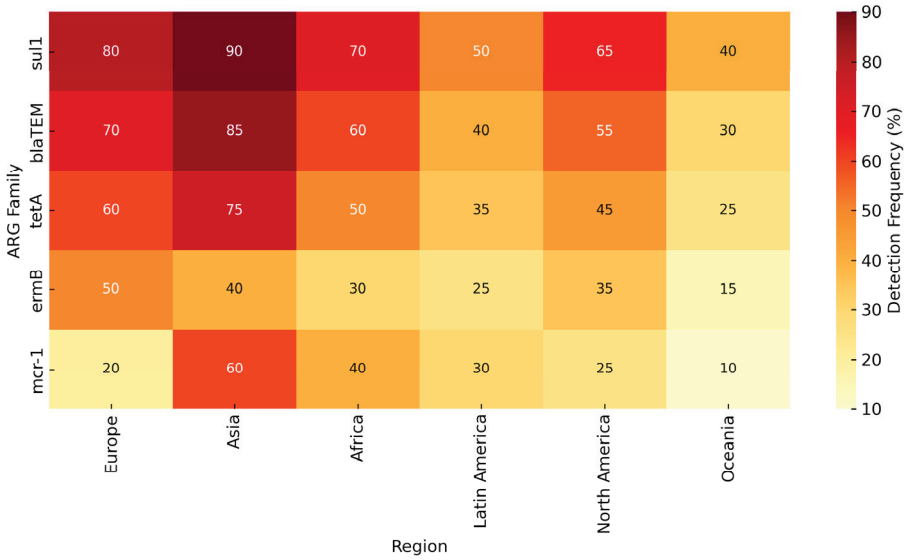

Figure 2. Heat-map-style diagram showing ARG prevalence across continents and detection methods (culture, qPCR, metagenomics).

Given the variability in methods and environmental contexts, synthesizing ARG prevalence across regions and water sources provides a clearer understanding of global resistance patterns.

The presence of ARB in water systems is not solely dependent on source inputs but also on microbial and physicochemical mechanisms that promote persistence and spread. We now shift focus to key ecological and molecular drivers such as biofilm formation, and microplastic-mediated co-selection.

4. Mechanisms of Persistence and Spread in Water

4.1. Biofilm Formation in Natural and Engineered Systems

Bacteria readily form biofilms on surfaces such as sediments, pipes, and microplastics in aquatic environments. These biofilms provide a protective environment where bacteria can survive adverse conditions, including disinfection, and maintain high cell densities. Biofilms act as reservoirs, periodically releasing ARB into water supplies through detachment, posing direct public health risks [100].

Biofilms in aquatic environments are indeed highly conducive to horizontal gene transfer (HGT) due to the dense microbial communities they support and the extracellular polymeric substances (EPS) matrix that enhances survival and communication [101]. This has been demonstrated in various studies focusing on both natural and engineered aquatic systems, including those involving microplastics, which serve as novel substrates for biofilm growth and ARG exchange [102,103]. This phenomenon is evident in drinking

water distribution systems and natural water bodies [74]. HGT is further amplified in biofilms due to high cell density and proximity, allowing ARGs to transfer between species, including uptake of free DNA from dead cells by living bacteria, perpetuating resistance even after disinfection [74].

A study demonstrates that biofilms on plastic debris transport ARB/ARGs across oceans. A study found *mcr-1* in biofilms on polyethylene particles in the Mediterranean Sea. Biofilms on sediment particles act as ARG reservoirs. *E. faecalis* with *vanA* (vancomycin resistance) transfers genes to aquatic *Vibrio* spp., creating hybrid pathogens [104]. Biofilms in distribution systems release ARB (e.g., *L. pneumophila* with *ermB*) into tap water, especially in aging infrastructures. In addition, biofilms in trickling filters or membrane bioreactors inadvertently enrich ARGs (bla_{NDM}, *sul1*) due to prolonged exposure to sub-inhibitory antibiotics [105].

Biofilms on root surfaces facilitate ARG exchange between soil bacteria and plant endophytes, enabling food chain contamination. In addition, biofilms accumulate metals (Zn, Cu) and disinfectants, co-selecting for resistance. For example, *czcA* (zinc resistance) and *mecA* (methicillin resistance) are linked on the same plasmid in *Staphylococcus* biofilms [103].

4.2. Microplastics as Novel Reservoirs and Vectors of ARGs

Microplastics have rapidly become recognized as a distinct ecological niche for antibiotic-resistant bacteria and genes. Their hydrophobic surfaces concentrate organic pollutants, heavy metals, and residual antibiotics, creating microzones of intense selective pressure. In the Danube River, Nguyen et al. found that microplastic-associated biofilms harbored ARG densities up to five times higher than surrounding suspended particles, with integron-bearing *Pseudomonas* spp. and *Aeromonas* spp. dominating the plastisphere community. Moreover, ocean currents can transport these colonized plastics thousands of kilometers, effectively shuttling resistance traits across biogeographic boundaries and connecting distant ecosystems in a single resistome network [104].

In addition, emerging research indicates bacteria colonizing these plastic particles benefit from a stable niche that can protect them from environmental stressors and promote gene exchange [105]. Studies have shown that biofilms on microplastics tend to be thicker and harbor higher densities of ARB compared to those on natural surfaces, potentially increasing the risk of ARG dissemination [106,107].

Research highlights the unique microbial communities found on microplastics, including pathogens such as *Vibrio* spp., *Pseudomonas* spp., and even human and fish pathogens, which tend to be more abundant in plastic biofilms than in natural environments. These biofilms also facilitate horizontal gene transfer (HGT), increasing the spread of ARGs within aquatic ecosystems [107,108].

Additionally, the movement and residence time of microplastics in water bodies shaped by hydrological and physical processes affect how microbial communities form and change on plastic surfaces, influencing ARG dissemination potential further [109].

4.3. Co-Selection by Emerging Chemical Stressors

Beyond antibiotics and metals, various emerging pollutants, including quaternary ammonium biocides, nanoparticle disinfectants, and pharmaceutical metabolites, can co-select for multidrug resistance. Bahded et al. demonstrated in laboratory experiments that exposure to sub-lethal concentrations of silver nanoparticles induces efflux pump overexpression in *A. baumannii*, which simultaneously confers cross-resistance to fluoroquinolones [110]. Field studies by Russo et al. linked high concentrations of benzalkonium chloride (a common surfactant) in Italian hospital stormwater to elevated class 1 integron

prevalence. These findings underscore that controlling AMR in water requires a broad lens on all co-selective agents, and not just conventional antibiotics [111].

Other pollutants, such as heavy metals (e.g., mercury, arsenic) and disinfectant by-products, also contribute to selecting resistant bacteria. These substances can co-select for ARGs because resistance mechanisms (such as efflux pumps) can confer protection against multiple types of stressors [112]. Heavy metals in water, often from industrial discharge or pipe corrosion, further enrich the resistome by favoring bacteria harboring metal resistance genes and ARGs [113].

Co-selection, where non-antibiotic environmental stressors select for antibiotic resistance, is increasingly recognized as a major contributor to ARG persistence in water environments. Heavy metals such as copper, zinc, and mercury, as well as disinfectants like quaternary ammonium compounds (QACs), are known to exert selective pressures via shared resistance mechanisms. These include multidrug efflux pumps (e.g., *MexAB-OprM*), stress-response regulators (*SoxRS*, *MarA*), and co-located resistance genes on integrative conjugative elements or plasmids (e.g., *czcA*, *qacEΔ1*, *mecA*) [114,115]. Diverse ARGs associated with metals have been discovered, such as coupling of resistance genes for methicillin with Zn [116], tetracycline with Cu [117], and multiple antibiotics with Hg [118]. For instance, *Acinetobacter baumannii* exposed to silver nanoparticles developed fluoroquinolone resistance through efflux activation, while QAC exposure was linked to class 1 integron enrichment in hospital wastewater [119]. These mechanisms underscore the need to regulate both antibiotics and chemical co-selectors in wastewater discharge.

5. Public Health and Food Safety Impacts

ARB in water create a multifaceted threat to human health and food safety by acting as both infection sources and environmental reservoirs of resistance. Infected drinking or recreational water can introduce multidrug-resistant pathogens that evade standard treatments. For example, surveys of community water sources have found that 50% of *E. coli* isolates were MDR, and many had high multi-antibiotic resistance indices [79]. Larson et al. directly linked ARB in household drinking water to human carriage of resistant *E. coli*, highlighting water as a transmission route in rural communities [80]. Irrigation amplifies the problem: Weidhaas et al. detected multiple resistance gene families on vegetables irrigated with reclaimed wastewater, demonstrating that ARB on crops introduce resistance into the food chain [120]. Such findings implicate reclaimed or contaminated irrigation water in foodborne exposures to ARB. At the same time, natural and engineered water systems spread resistance more broadly: La Rosa et al. emphasized that wastewater treatment plants accumulate ARB/ARGs and then disseminate them via treated effluent, irrigation, and water reuse into agricultural lands and aquatic ecosystems [38]. Similarly, Fahrenfeld et al. observed that treated wastewater distribution often increases ARG diversity (via microbial regrowth), meaning end-use water (including for irrigation) can carry more resistance than the source [77]. Significantly, even disinfection steps can backfire: Sanganyado et al. noted that chlorination may promote horizontal transfer of resistance genes from environmental ARB to human pathogens in distribution systems [45]. These studies link ARB in water to elevated human infection risk and foodborne transmission, while also showing that water bodies and reuse practices serve as ecological reservoirs that spread resistance across human and environmental interfaces [33,120].

6. Environmental Transmission Pathways

Environmental pathways play a key role in the transmission and persistence of AMR across ecosystems, linking clinical settings with natural environments (Figure 3). Several studies have examined these pathways and identified various ARB that move between

water, soil, wildlife, and the human gut. For example, Xi et al. demonstrated that drinking water treatment and distribution systems could serve as reservoirs for ARGs and ARB, with species such as *E. coli*, *P. aeruginosa*, and *A. baumannii* being detected even after treatment [46]. Similarly, studies in wastewater treatment plants have shown that hospital effluents and agricultural runoff contribute significantly to environmental AMR; research by Sanganyado et al. reviewed how ARB, such as *Enterococcus* spp. and *K. pneumoniae*, are disseminated via treated wastewater into natural water bodies [45].

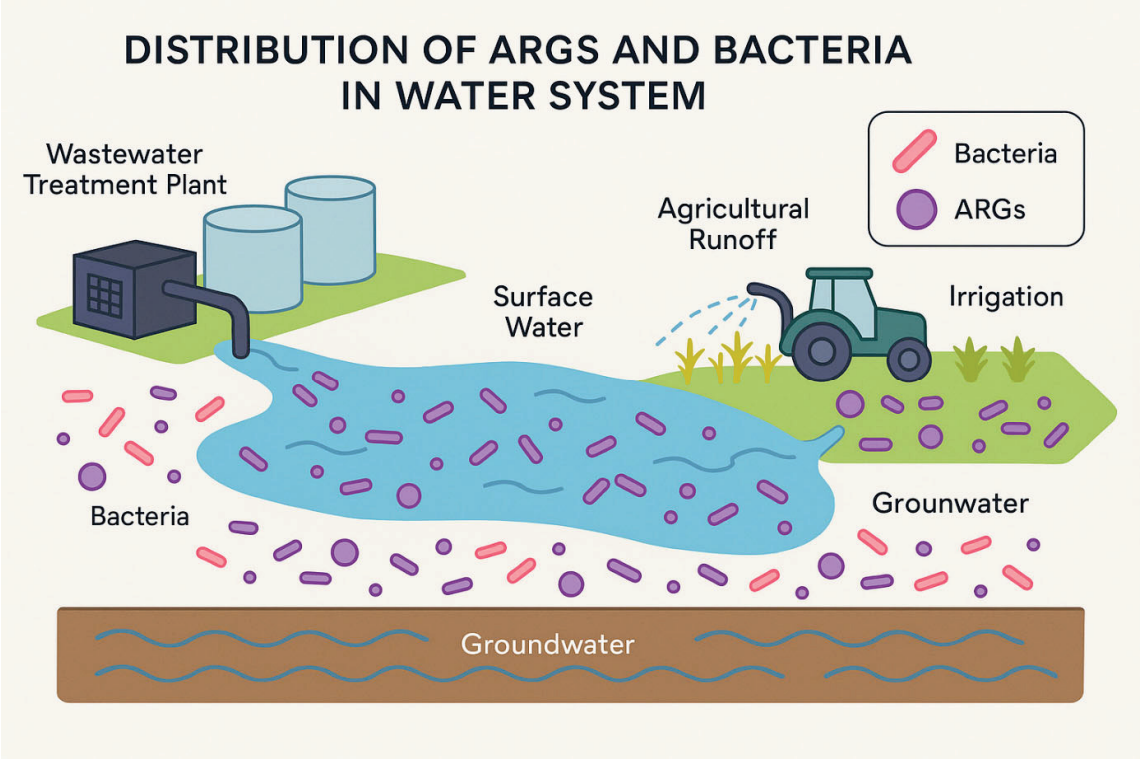

Figure 3. Schematic of potential AMR bacteria and ARGs transmission pathways in the water system.

Moreover, research investigating recycled water and irrigation has found that water reused for agricultural purposes can harbor multidrug-resistant *E. coli*, *Enterobacter* spp., and even *Salmonella* spp., suggesting that these water sources are key vectors for transferring resistance genes to crops and, ultimately, to humans [121]. In soil, antibiotic residues from manure and wastewater can select for resistant organisms, with studies revealing high levels of resistance in environmental isolates of *Pseudomonas* spp. and *Acinetobacter* spp. from agricultural lands [122]. Additional pathways include the persistence of ARGs in biofilms along water distribution systems and in sediments of rivers and lakes [123].

ARGs have been detected in biofilms from various freshwater ecosystems, including rivers and wetlands, indicating their role as reservoirs of resistance in aquatic environments [124].

AMR is no longer confined to clinical settings; it is now recognized as a global problem, with environmental pathways playing a crucial role in the dissemination and persistence of antibiotic-resistant bacteria and antibiotic resistance genes. A study conducted by Kusi et al. provided a comprehensive review of how surface waters become hotspots for AMR and examined the public health implications of these environmental reservoirs. The authors described several interconnected pathways through which antimicrobial resistance is developed and transmitted in aquatic environments [1]. Although numerous studies have documented the presence of ARGs in surface waters, sediments, and treated effluents, establishing a direct, causal relationship between environmental exposure and resistant

infections in humans remains scientifically complex. This is primarily due to methodological challenges such as the absence of longitudinal cohort data, the inability to track specific mobile genetic elements from environmental to clinical isolates, and confounding exposure routes such as foodborne or zoonotic pathways [125,126]. For instance, metagenomic analyses in Ghana and Peru have shown overlaps in resistant *E. coli* genotypes in human stool and household water, but lacked molecular resolution to confirm direct transmission [127]. A promising frontier involves integrating high-resolution, whole-genome sequencing with wastewater-based epidemiology and clinical surveillance, though such efforts remain logistically and economically constrained in many regions [128].

6.1. Wastewater Discharge and Effluent Release

Hospital wastewater is a significant reservoir of multidrug-resistant pathogens (e.g., *K. pneumoniae*, *P. aeruginosa*) and clinically relevant ARGs, such as carbapenemases (bla_{KPC}, bla_{NDM}) and ESBL ($bla_{\text{CTX-M}}$). These effluents often contain high concentrations of antibiotics, disinfectants, and biocides, which exert selective pressure, enriching resistant strains [38,129]. Urban sewage systems collect antibiotics from human excretion, while industrial effluents (e.g., pharmaceutical manufacturing, livestock farms) introduce antibiotics, heavy metals, and ARGs. For example, tetracycline and sulfonamide residues are frequently detected in municipal wastewater, promoting ARG transfer via HGT [130]. When this partially treated water is discharged into rivers, lakes, or coastal waters, it acts as a direct input of resistance determinants into the natural environment [131]. These effluents may come from clinical settings where high antibiotic use selects for resistant strains, thereby enriching the environmental resistome [132].

6.2. Agricultural Runoff and Manure Application

The use of antibiotics in livestock (e.g., tetracyclines, macrolides) results in up to 90% of the administered drugs ending up in manure. When applied untreated to fields, manure introduces residual antibiotics, ARB, and ARGs into soils, which are subsequently dispersed via rainfall runoff or wind erosion [133,134]. ARGs like *ermB* (macrolide resistance) and *qnrS* (quinolone resistance) adhere to crops such as lettuce and spinach, posing foodborne transmission risks [133,135]. Birds and rodents grazing on manure-amended fields spread ARB (e.g., *E. faecalis* with *vanA*) to distant ecosystems [136]. Heavy metals in manure (e.g., zinc from feed additives) co-select for metal tolerance genes (*czcA*) and ARGs (bla_{TEM}), amplifying resistance.

6.3. Sediments as Long-Term Reservoirs

Sediments in rivers, lakes, and coastal areas accumulate bacteria and organic matter over time. They act as long-term reservoirs for ARB and ARGs because the conditions in sediments (e.g., low oxygen, high nutrient content) support bacterial survival and even growth. These reservoirs can slowly release resistant bacteria into the water column, especially during disturbances like storms or human activity [53,137]. For example, *E. coli* with $bla_{\text{CTX-M-15}}$ persists in river sediment biofilms despite seasonal fluctuations in antibiotic concentrations [103].

6.4. Soil–Water Interactions and Irrigation Return Flows

When treated or untreated wastewater is applied to agricultural fields or used for irrigation, ARGs and ARB infiltrate soils and leach into groundwater. In arid regions, irrigation return flows (IRFs) have been shown to carry multidrug-resistant *E. coli* and *Enterococcus* spp. into adjacent streams, creating a cyclical exchange between soil and surface water that amplifies environmental resistance burdens [82].

6.5. Wildlife-Mediated Dissemination

Wild birds, fish, and small mammals frequent multiple water bodies and can pick up ARB from one site (e.g., a polluted wetland) and deposit them in another (e.g., a pristine lake). For instance, migratory waterfowl have been found to carry class 1 integron-bearing bacteria in their feces, effectively linking disparate aquatic ecosystems in a single resistance network [136].

6.6. Aerosolization and Recreational Exposure

High-pressure water sprays in irrigation or desalination plants and wave action along coasts can generate aerosols laden with ARB and ARGs. Inhalation or contact with these bioaerosols represents an underappreciated transmission route, especially for occupational and recreational water users, and warrants targeted study of airborne resistance in coastal and agricultural settings [102].

These environmental pathways illustrate that aquatic ecosystems serve as interconnected reservoirs for AMR. Wastewater discharge, agricultural runoff, biofilm formation, sediment accumulation, microplastics, chemical stressors, and robust horizontal gene transfer contribute to ARB and ARGs' persistence and spread in natural waters [138]. The interplay of these factors increases the risk of resistant infections via direct exposure (e.g., through drinking, recreation, or irrigation) and facilitates the integration of environmental resistance into human pathogens, posing a serious public health challenge [1].

Recent studies have shed light on how aquatic environments act as dynamic reservoirs and transmission pathways for AMR. Research indicates that antibiotic residues, heavy metals, and other pollutants discharged from municipal, hospital, and industrial sources persist in rivers, lakes, and even drinking water systems, creating selective pressures that favor ARB and facilitate horizontal gene transfer of ARGs [139,140].

For example, Xi et al. showed that although drinking water treatment processes reduce overall microbial loads, regrowth in the distribution system, often within biofilms, can lead to higher relative abundances of ARGs [46]. Similarly, studies by Chen et al. and Sanganyado & Gwenzi have documented that conventional disinfection methods (like chlorination) may not completely eradicate ARB; instead, they can sometimes select for resistant populations that persist in treated water [45,74].

Xu et al. employed high-throughput sequencing to profile ARGs in drinking water treatment plants and distribution networks. Their work showed that conventional processes, while reducing overall bacterial counts, may inadvertently select for resistant organisms [141]. Similarly, Lu et al. examined sand settling reservoirs. They found that ARGs associated with β-lactam and tetracycline resistance were consistently present, suggesting that natural sediments act as long-term reservoirs [142]. Moreover, Karkman et al. highlighted that fecal pollution from human and animal sources significantly contributes to the ARG abundance in surface waters, linking agricultural runoff and wastewater discharge to environmental AMR [143].

Additional work has assessed the role of emerging pollutants. For example, studies on microplastics [144] indicate that these particles provide a robust substrate for biofilm formation, thereby enhancing the horizontal transfer of ARGs among bacteria such as *E. coli* and *P. aeruginosa* [144]. Furthermore, investigations into heavy metal pollution [114] have shown that metals like mercury and arsenic co-select for antibiotic resistance, as resistance mechanisms are often genetically linked on mobile elements [114].

7. Mitigation Strategies and Future Directions

7.1. Wastewater-Based Epidemiology (WBE) as a Surveillance Tool

Over the past five years, wastewater-based epidemiology has emerged as a powerful approach for tracking viral outbreaks and monitoring antibiotic resistance trends in real time. By sequencing metagenomes from raw influent at key WWTPs, researchers can quantify hundreds of ARG families and correlate their abundance with antibiotic consumption data from pharmacy records [145]. For example, researchers used WBE in Barcelona to detect early rises in carbapenem resistance genes two weeks before hospital admissions increased, providing a critical window for public health intervention. Integrating WBE into national AMR action plans could offer continuous, community-level insights into emerging resistance hotspots and treatment failures [146].

7.2. Advanced Treatment Technologies

Advanced treatment technologies are increasingly critical for mitigating antibiotic resistance in wastewater effluents. Advanced oxidation processes (AOPs), including ozonation, UV/H_2O_2 oxidation, and Fenton reactions, generate non-selective hydroxyl radicals capable of degrading over 90% of common antibiotics such as tetracyclines and sulfonamides, while also reducing mobile genetic elements like class 1 integrons [147]. Pilot-scale systems coupling ozone with UV irradiation have shown enhanced micropollutant breakdown and a marked decrease in the regrowth potential of ARB [148]. Similarly, membrane bioreactors (MBRs), which combine biological treatment with microfiltration or ultrafiltration, can achieve 4–6 log reductions in ARB and substantial declines in antibiotic resistance gene (ARG) abundance when operated under optimized conditions [34]. However, biofilm formation on membrane surfaces can inadvertently enrich ARGs such as bla_{NDM} and *sul1*, necessitating rigorous cleaning protocols [78]. To overcome this, sequential MBR–AOP trains are deployed to ensure oxidative destruction of any ARGs surviving primary filtration [149]. In addition, adsorption onto granular activated carbon (GAC) effectively removes trace antibiotics and extracellular DNA fragments, reducing concentrations to nanogram-per-liter levels through pore diffusion and surface interactions. Recent research on engineered sorbents like functionalized biochars and metal–organic frameworks further demonstrates that tailoring surface chemistry enhances pollutant uptake and enables sorbent regeneration, offering a sustainable, low-energy option for polishing effluent before environmental discharge [149].

Looking forward, emerging technologies and transdisciplinary strategies hold promise in reshaping how we monitor, manage, and mitigate aquatic antibiotic resistance.

8. Future Directions

The escalating threat of antibiotic resistance in aquatic environments demands a paradigm shift toward innovative, interdisciplinary solutions. A critical priority lies in advancing material science to revolutionize water treatment technologies. Functionalized nanoparticles, graphene oxide membranes, and catalytic composites show promise in selectively degrading antibiotic residues and extracellular resistance genes, particularly for recalcitrant pollutants that evade conventional methods. Concurrently, integrating artificial intelligence with wastewater-based epidemiology could transform surveillance capabilities. Machine learning algorithms trained on metagenomic datasets from wastewater may predict emerging resistance trends, such as the spread of carbapenemase (bla_{NDM}) or colistin (*mcr-1*) genes, enabling preemptive public health interventions. Equally urgent is elucidating the role of microplastics as vectors for resistance dissemination. Research must quantify how plastisphere biofilms facilitate horizontal gene transfer between envi-

ronmental and pathogenic bacteria, particularly in marine ecosystems where microplastic pollution intersects with aquaculture and coastal urbanization.

While technological and policy advancements are essential, they must be contextualized within global socio-economic inequities. Many low- and middle-income countries (LMICs) lack infrastructure for centralized wastewater treatment and antimicrobial monitoring. In such settings, low-cost interventions are critical. Promising solutions include solar-driven advanced oxidation processes, constructed wetlands, gravity-fed sand or biochar filters, and natural coagulants, all of which have shown efficacy in reducing ARGs and antibiotic residues [149]. Additionally, international cooperation must support regulatory enforcement, sustainable pharmaceutical waste disposal, and capacity building for microbial surveillance. One Health frameworks should prioritize equitable access to these technologies and ensure that LMICs are not left behind in global AMR mitigation.

We acknowledge the limited representation of studies from North America, Latin America, and Oceania in this review. Future research should prioritize targeted surveillance in these underrepresented regions to build a more globally comprehensive understanding of aquatic antibiotic resistance. These regions often have distinct environmental pressures and antimicrobial usage profiles, yet remain poorly characterized. Coordinated international efforts and expanded surveillance initiatives aligned with WHO GLASS and UNEP are needed to close these geographic data gaps.

Additionally, international cooperation must support regulatory enforcement, sustainable pharmaceutical waste disposal, and capacity building for microbial surveillance. One Health frameworks should prioritize equitable access to these technologies and ensure that LMICs are not left behind in global AMR mitigation.

Longitudinal ecological assessments are needed to evaluate the cascading impacts of antibiotic resistance accumulation in aquatic systems. These studies should address how sediment-bound resistance genes alter microbial community dynamics, disrupt nutrient cycling, and propagate resistance to non-target species, including keystone microorganisms and aquatic fauna. Parallel efforts must address behavioral and regulatory gaps. Public education campaigns to curb antibiotic misuse in healthcare and agriculture should be reinforced by policies enforcing stricter pharmaceutical discharge limits and "polluter pays" principles for industries contributing to aquatic AMR. However, technological and policy advances alone will fail without addressing global inequities. Low-resource regions urgently require decentralized, cost-effective solutions, such as solar-driven advanced oxidation processes or biochar filtration systems, supported by international funding and technology transfer programs. Finally, a One Health framework is indispensable for harmonizing antibiotic usage data across human, veterinary, and environmental sectors.

9. Conclusions

As dynamic reservoirs of antibiotic resistance, water systems pose a formidable challenge to global health. This review underscores that wastewater discharges, agricultural runoff, and inadequate treatment processes perpetuate the environmental persistence of ARBs and ARGs. Key mechanisms—such as biofilm-mediated gene transfer, microplastic-facilitated dissemination, and co-selection by chemical stressors—amplify resistance spread across ecosystems. Clinically relevant pathogens like *E. coli*, *P. aeruginosa*, and *K. pneumoniae* persist in drinking water, irrigation sources, and recreational waters, directly threatening human health through exposure and foodborne transmission.

Current mitigation strategies, including advanced oxidation processes and membrane bioreactors, show promise but require optimization to address regrowth risks and infrastructure limitations. Furthermore, emerging pollutants such as microplastics and nanoparticles' role in driving resistance remains poorly understood. A paradigm shift

toward One Health approaches is imperative, integrating surveillance, policy, and technology to disrupt environmental transmission pathways. Without coordinated global action, the silent proliferation of resistance in water will undermine decades of medical progress. Safeguarding aquatic ecosystems is not merely an environmental imperative but a cornerstone of planetary health.

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The Impact of Urban Pollution on Plasmid-Mediated Resistance Acquisition in Enterobacteria from a Tropical River

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Abstract: Background: The exposure of environmental bacteria to contaminants in aquatic ecosystems accelerates the dissemination of antibiotic-resistance genes (ARGs) through horizontal gene transfer (HGT). **Methods:** In this study, we sampled three locations along a contamination gradient of a polluted river, focusing on isolating Enterobacteria from the surface waters to investigate the relationship between urban pollution and antibiotic resistance. The genomes of 15 isolates (5 per site) were sequenced to identify plasmid-borne ARGs and their association with resistance phenotypes. **Results:** Isolates from the site with the highest contamination (Site 3) showeda larger number of ARGs, plasmids, and resistance phenotypes. Notably, one of the isolates analyzed, *E. coli* A231-12, exhibited phenotypic resistance to seven antibiotics, presumably conferred by a single plasmid carrying 12 ARGs. Comparative analysis of this plasmid revealed its close evolutionary relationship with another IncH plasmid hosted by *Salmonella enterica*, underscoring its high ARG burden in the aquatic environment. Other plasmids identified in our isolates carried *sul* and *dfrA* genes, conferring resistance to trimethoprim/sulfamethoxazole, a commonly prescribed antibiotic combination in clinical settings. **Conclusions:** These results highlight the critical need to expand research on the link between pollution and plasmid-mediated antimicrobial resistance in aquatic ecosystems, which can act as reservoirs of ARGs.

Keywords: Enterobacteria; Costa Rica; integrons; plasmid evolution; antibiotic resistance; horizontal gene transfer; *Escherichia coli*; *Klebsiella*; IncH plasmids; beta-lactamase

1. Introduction

The contamination of water resources with organic compounds of anthropogenic origin has exceeded the capacity to monitor and evaluate their harmful effects on environmental health [1]. The presence of chemical contaminants, including emerging pollutants (EPs) such as microplastics, pharmaceuticals, pesticides, and heavy metals, can elevate the abundance of antibiotic-resistance genes (ARGs) in aquatic environments, even without antibiotics. Experimental studies have shown that non-antibiotic compounds can impact horizontal gene transfer (HGT) dynamics [2,3], increasing plasmid transfer rates across diverse bacterial taxa. These contaminants exert selective pressures that not only promote ARG proliferation but also generate mutations in antibiotic target genes. For instance, they can induce oxidative stress in bacteria, triggering DNA damage and activating repair pathways like the SOS response, facilitating the acquisition of new mutations. Concurrently,

oxidative stress has been observed to alter cell membrane permeability and to up-regulate genes associated with conjugation, thus enhancing both plasmid conjugation rates and transformation frequencies [2–4].

Wastewater (with or without treatment) contains complex mixtures of chemical residues used by the human population (e.g., pesticides, antibiotics, and other pharmaceutically active compounds) that are accumulated and released into the environment. Urban growth increases the sources of these pollutants in river basins significantly, and urban populations will only continue to increase [5]. Further, in economies with scarce wastewater management, resistance-selecting chemicals can be detected at higher concentrations in surface waters [6,7]. This might cause selective pressure over the exposed microbial communities, leading to the dissemination of ARGs in the ecosystem.

Bacterial species commonly found in aquatic environments, such as *Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas* spp., are frequently associated with plasmids that carry antibiotic-resistance genes (ARGs). In aquatic settings, biofilms are crucial in enhancing plasmid retention and transfer. Bacteria in biofilms, due to their close physical proximity, experience higher rates of conjugation, allowing plasmids to be transmitted more efficiently compared with planktonic (free-floating) bacterial populations [8,9]. Due to that, biofilms can serve as reservoirs for plasmids with ARGs, protecting them from being lost in the absence of antibiotics, but they also help to preserve genes required for plasmid transfer. This contrasts with planktonic populations, where plasmids are often lost due to selective sweeps or genetic deletions that eliminate transfer regions [8]. The spatial structure and local competition within biofilms enable bacteria to maintain these plasmids longer, contributing to the persistence of ARGs in aquatic systems, even without antibiotics. This dynamic highlights the biofilm's critical role in sustaining plasmid-mediated antibiotic resistance in bacterial communities.

Although antibiotic resistance has emerged across various bacterial groups, bacteria from the family Enterobacteriaceae are of particular concern due to their critical role in the intestinal microbiota and significance in human and animal health. Certain species within this group exhibit high levels of antimicrobial resistance, raising substantial concerns regarding their potential impact on human health [10]. In this study, we aimed to characterize the diversity of plasmid-mediated ARGs in Enterobacteriaceae isolated from water column samples along a gradient in pollution and anthropogenic activities (urbanization). Samples were collected from three sites along the main course of the Virilla River, situated in the Western Central Valley of Costa Rica (Supplementary Figure S1). The Virilla River is part of the larger Grande de Tárcoles River Watershed, which flows into the Pacific Ocean. Urbanization and shifts in land use within this watershed have significantly increased pollution levels, mainlydue to untreated municipal sewage and industrial discharges [11,12]. These findings emphasize the potential role of urban pollution in driving the spread of antibiotic resistance in clinically important bacteria, highlighting the key role that plasmids play in this process.

2. Results

2.1. Genome Characterization and Taxonomic Identification

In Table 1, we present the characteristics of the assembled genomes and their taxonomic classification as determined using the Type Strain Genome Server (TYGS) [13]. Eight of the 15 genomes analyzed were identified as *Escherichia coli* based on TYGS, while 4 additional isolates were classified as *Klebsiella pneumoniae*, 1 as *Klebsiella variicola*, and another as *Klebsiella quasipneumoniae* (Supplementary Figure S2). TYGS could not identify the isolate A223-7 at the species level but associated its genome with the *Citrobacter* genus; however, the GTDB tool indicated a close relationship to *Citrobacter gillenii*, with a 98.96% average nucleotide identity (ANI).

To further clarify the isolates' taxonomic assignment and genomic relationships, we conducted a phylogenomic analysis using 908 single-copy core genes. We show in Figure 1 the phylogenomic relationships between the isolates and related genomes obtained from

the NCBI database, including reference genomes for each species and representative strains for different *Escherichia coli* phylogroups and *Shigella* subclades. The NCBI assembly names of all the external genomes used in this phylogenomic analysis are listed in Supplementary Table S1.

Table 1. Characteristics of the assembled genomes. Size, N50, L50, and GC content were determined with Quast; the closest taxonomy was obtained using the Type Strain Genome Server and the Completeness and Contamination with Checkm2.

Isolate (Site)	Sequencing Method	Contigs	Size (Mb)	N50	L50	%GC	Completeness	Contamination	Taxonomy According to TYGS (dDDH in %)
A222-7 (1)	Illumina	73	4.90	130,684	11	50.61	100	0.05	*Escherichia coli* (73.8)
A229-5 (1)	Illumina	88	4.86	111,924	14	50.76	100	0.04	*Escherichia coli* (73.8)
A229-12 (1)	Illumina	99	4.75	103,479	15	50.78	100	0.09	*Escherichia coli* (74.7)
A236-7 (1)	Illumina	64	4.80	167,951	10	50.7	100	0.15	*Escherichia coli* (74)
A236-12 (1)	Illumina	73	4.7	147,946	8	50.59	100	0.19	*Escherichia coli* (75.1)
A223-7 (2)	Illumina	45	4.97	413,561	6	52.48	100	0.05	*Citrobacter* sp. (42.5) *
A230-5 (2)	Illumina	40	5.41	296,086	6	57.74	100	0.09	*Klebsiella quasipneumoniae* (71.4)
A230-7 (2)	Illumina	50	5.48	349,092	6	57.22	100	0.23	*Klebsiella pneumoniae* (93.4)
A230-8 (2)	Illumina	159	4.96	77,565	23	50.62	100	0.12	*Escherichia coli* (74.1)
A237-6 (2)	Illumina	67	5.22	188,161	10	57.56	100	0.85	*Klebsiella pneumoniae* (93.4)
A224-7 (3)	Illumina	84	5.60	141,929	14	57.21	100	0.13	*Klebsiella variicola* (92.2)
A231-5 (3)	Illumina	51	5.47	215,170	9	57.27	100	0.26	*Klebsiella pneumoniae* (93.5)
A238-6 (3)	Illumina	75	5.54	191,124	10	57.19	100	0.19	*Klebsiella pneumoniae* (93)
A224-8 (3)	Illumina and PacBio	5	4.70	4,712,205	1	50.73	100	0.04	*Escherichia coli* (75.2)
A231-12 (3)	Illumina and PacBio	4	5.08	4,757,699	1	50.61	100	0.02	*Escherichia coli* (73.8)

* dDDH was calculated against the *Citrobacter tructae* genome (closest match).

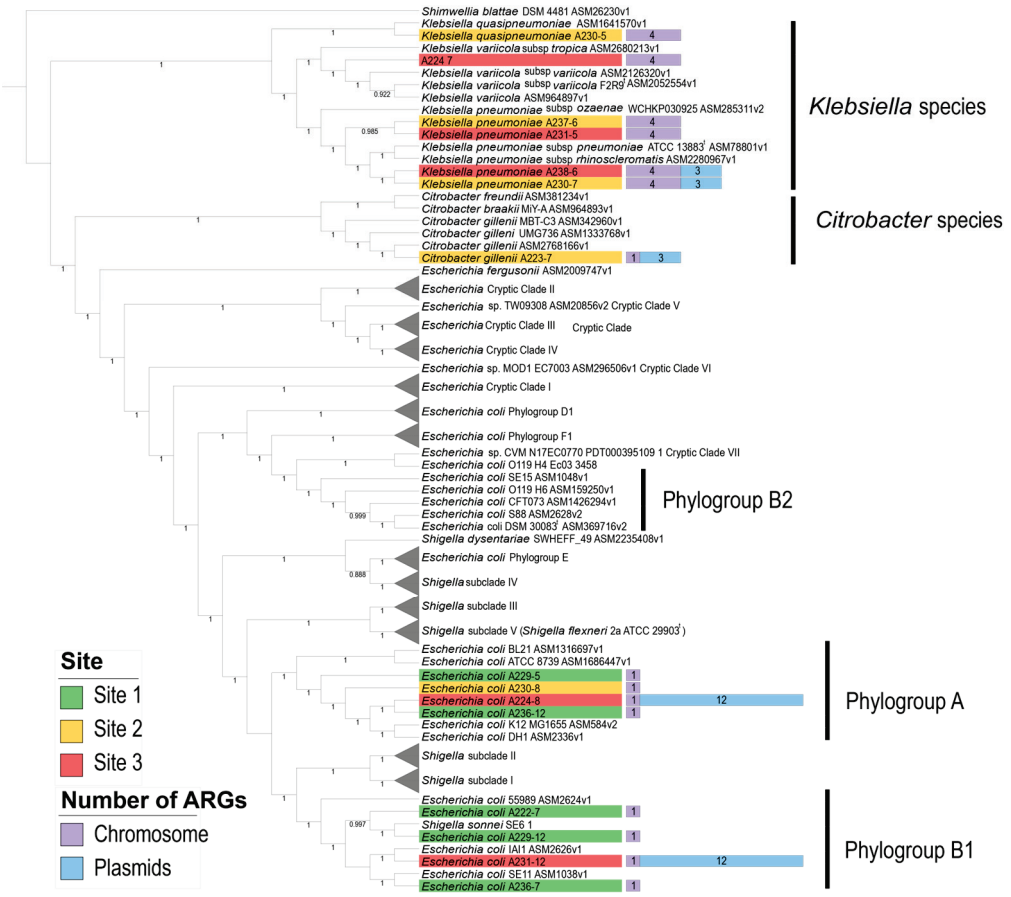

Figure 1. Phylogenomic tree representing the evolutionary relationships of the isolated bacteria and reference genomes. The tree was constructed using 908 single-copy core genes shared across all the genomes. Bootstrap values, based on 1000 resamplings, appear at the nodes. The height of the bars corresponds to the number of ARGs found in the chromosome (purple) and plasmids (blue), with the label colors indicating the sites where the bacteria were isolated. *Escherichia* and *Shigella* clades

unrelated to the isolates were collapsed to reduce the number of labels. The *E. coli* phylogroup B2 is highlighted due to the inclusion of the type strain *E. coli* DSM 30083^{t}. Branch lengths were omitted for improved visualization.

As previously classified, six isolates were assigned to the genus *Klebsiella*: isolate A224-7 is related to *Klebsiella variicola*, isolate A230-5 to *Klebsiella quasipneumoniae*, and isolates A237-6, A238-6, A230-7, and A231-5 to *Klebsiella pneumoniae*. Additionally, isolate A223-7 was confirmed to be related to *Citrobacter gillenii*, consistent with the GTDB tool's results. For the isolates classified as *E. coli*, we determined that they are grouped into two distinct phylogroups: phylogroup A, which includes isolates A224-8, A229-5, A230-8, and A236-12, and phylogroup B1, which contains isolates A236-7, A229-12, A222-7, and A231-12, along with the *Shigella sonnei* SE6-1 strain, which appears to have been misidentified as a *Shigella* strain [14].

2.2. Phenotypic Characterization of Antibiotic Resistance

Seven of the 15 evaluated isolates exhibited resistance to at least 1 of the 15 antibiotics evaluated (amikacin, gentamicin, ampicillin/sulbactam, ertapenem, meropenem, cephalothin, cefazolin, ceftazidime, ceftriaxone, cefepime, ciprofloxacin, norfloxacin, trimethoprim/sulfamethoxazole, fosfomycin, and nitrofurantoin) (Figure 2). None of the isolates from Site 1 (the least contaminated) displayed resistance to the tested antibiotics, whereas three isolates from Site 2 and four from Site 3 (the most contaminated) showed resistance to one or more antibiotics. Among the multidrug-resistant isolates (defined as resistance to three or more antibiotics) [15], *K. pneumoniae* A230-7 showed resistance to nitrofurantoin, trimethoprim/sulfamethoxazole, and an intermediate level of resistance to ampicillin/sulbactam. Meanwhile, *E. coli* A231-12 exhibited resistance to seven antibiotics—ampicillin/sulbactam, cefazolin, cefepime, ceftazidime, ceftriaxone, gentamicin, and trimethoprim/sulfamethoxazole—and was identified as an extended-spectrum beta-lactamase (ESBL) producer. Notably, five isolates presented resistance to nitrofurantoin, while three were resistant to trimethoprim/sulfamethoxazole.

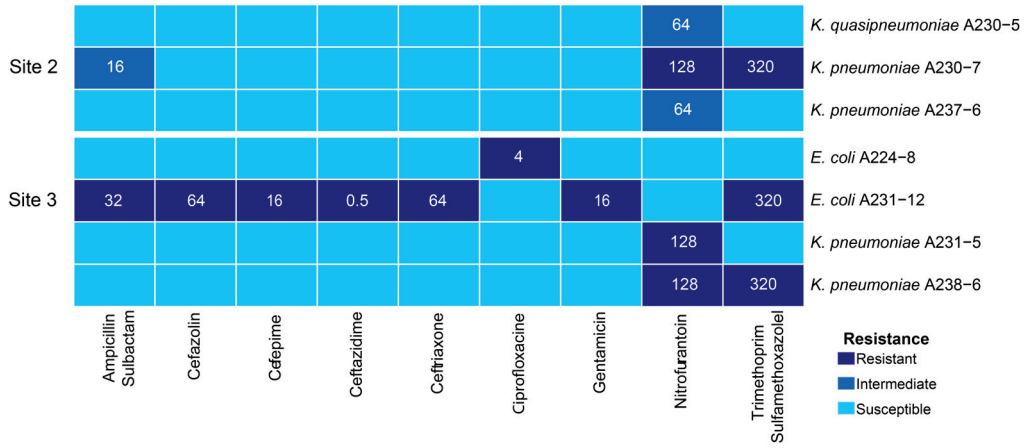

Figure 2. Antibiotic susceptibility testing of the evaluated isolates. Each box shows the minimum inhibitory concentration (MIC) obtained, and the colors represent the resistance phenotype: dark blue for resistant, blue for intermediate-resistant, and light blue for susceptible isolates. Resistance levels were assessed based on the MIC breakpoints for Enterobacterales as outlined in the 2020 CLSI guidelines [16].

2.3. Genotypic Characterization of Antibiotic Resistance

Across the 15 sequenced genomes, we identified 66 ARGs, 34 of which were unique (richness). Of these 66 ARGs, 33 (18 unique) were in bacterial chromosomes, while the remaining 33 ARGs (16 unique) were in plasmids. It was remarkable that no ARGs were detected in both the chromosome and plasmid contigs of the same isolate except for the

E. coli A224-8 isolate, which presented a plasmid sequence integrated into the bacterial chromosome. However, given that this might be a sequencing artifact, the sequence was classified as a plasmid. We provide all information regarding the ARGs identified by the ABRicate v1.0.0 software using the NCBI database in Supplementary Table S2.

At Site 1, we determined an abundance and richness of ARGs of 5 and 3, respectively. In contrast, Site 2 presented 20 ARGs, 19 of which were unique, while Site 3 showed the highest diversity values with a total of 41 ARGs, 29 of which were unique. Additional statistic values are presented in Table 2. As the most contaminated site, Site 3 contained the largest number of ARGs, followed by Site 2, with Site 1 having the lowest diversity of ARGs. We identified statistically significant differences in ARG distribution across the sites (Kruskal–Wallis, $\chi^2 = 9.95$; $p = 0.007$). However, post hoc Dunn tests showed significance only between Sites 1 and 3 ($Z = -3.130$, $p = 0.005$) but not between Sites 1 and 2 ($Z = -1.900$; $p = 0.086$) or between Sites 2 and 3 ($Z = -1.230$; $p = 0.219$). The isolates with the highest richness of ARGs were *E. coli* A231-12 (Site 3) with 13, *E. coli* A224-8 (Site 3) with 10 (11 after sequencing with PacBio), and *K. pneumoniae* isolates A238-6 (Site 3) and A203-7 (Site 2), both with 7 ARGs.

Table 2. Minimum, median, maximum, total abundance, and richness (unique ARGs or plasmids) values of ARGs and plasmids observed in genomes from the three sampling sites. The Kruskal–Wallis test revealed statistically significant differences in the number of ARGs between sites ($\chi^2 = 9.95$; $p = 0.007$) but not in the number of plasmids ($\chi^2 = 3.588$; $p = 0.166$). Only results from Illumina data were used in the comparisons.

Site	Statistic	ARGs	Plasmids
1	Minimum	1	1
	Median	1	2
	Maximum	1	4
	Abundance	4	11
	Richness	3	11
2	Minimum	1	1
	Median	4	2
	Maximum	7	6
	Abundance	20	16
	Richness	19	16
3	Minimum	4	2
	Median	7	4
	Maximum	13	7
	Abundance	41	23
	Richness	29	23

Concerning the differences in plasmid- or chromosomal-encoded ARGs, we identified 15 chromosomal ARGs associated with extended-spectrum beta-lactamases (ESBL) and 12 chromosomal ARGs from the *oqx* family, which encode RND efflux pumps linked to resistance against phenicol, quinolones, and nitrofurantoin [17,18]. Notably, streptomycin-resistance genes (10 ARGs) were observed exclusively in plasmids, encoding phosphotransferases and nucleotidyl transferases that modify ribosomes and allow evasion toaminoglycoside antibiotics. Additionally, we identified plasmid-encoded ARGs conferring resistance to sulfonamides (six ARGs) and tetracyclines (four ARGs) among others, as listed in Figure 3.

When analyzing point-mutation-associated resistance, we determined that *E. coli* A224-8 (Site 3) was the only one exhibiting a mutation in the *gyrA* gene (S83L), which confers resistance to ciprofloxacin. Additionally, mutations in the *nfsA* and *nfsB* genes, along with potential ARGs conferring nitrofurantoin resistance in *Klebsiella* isolates, are shown in Table 3.

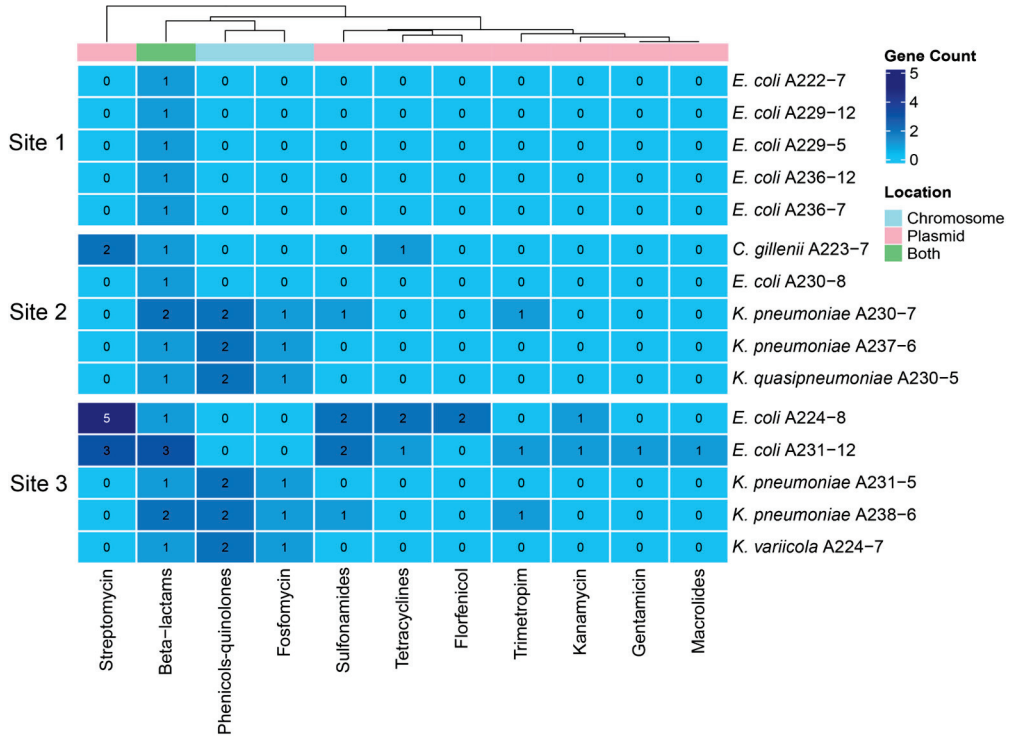

Figure 3. Potential phenotypes associated with ARGs identified in each genome analyzed. The heatmap color gradient represents the gene count of ARGs, while the column colors indicate the genomic location of the genes: light blue for chromosomal, pink for plasmid, and green for both chromosomal and plasmid locations.

Table 3. Mutations and *oqx* genes associated with nitrofurantoin resistance among isolates identified as *Klebsiella*. The genome of the susceptible reference strain *Klebsiella pneumoniae* ATCC 13883 (accession number JOOW01000000) was used for SNP detection.

Isolate (Site)	Phenotype	*nfsA* Mutations	*nfsB* Mutations	*oqx Genes*
K. pneumoniae A238-6 (3)	Resistant	E29A, G125W, G204S		*oqxB*, *oqxA*
K. pneumoniae A231-5 (3)	Resistant	Q195L		*oqxB11*, *oqxA5*
K. pneumoniae A237-6 (2)	Resistant	K222R		*oqxB25*, *oqxA6*
K. pneumoniae A230-7 (2)	Resistant	E29A, G125W, G204S		*oqxA*, *oqxB*
K. quasipneumoniae A230-5 (2)	Resistant	E29A, Q94E, Q147K, E191G, E194D	Q181K	*oqxA10*, *oqxB11*

2.4. Description of Plasmids with Antibiotic-Resistance Genes

In total, we detected 50 plasmids across the 15 sequenced genomes with the Illumina data (Supplementary Table S3). Site 3 presented the highest number of plasmids (23 in total); however, no statistically significant differences were observed between sites regarding plasmid numbers (Chi = 2.354; $p = 0.308$). Other statistical values associated with plasmid diversity are presented in Table 2. Interestingly, only 6 out of the 50 plasmids contained ARGs (Figure 4). All the plasmids were assigned to different incompatibility groups (when available) and exhibited variable sizes, except for plasmids pAB595-1 and pAB595-2, which were isolated from *K. pneumoniae* isolates at Sites 3 and 2, respectively. Both plasmids contained the same three ARGs (*blaTEM-1*, *dfrA26*, and *sul2*) but differed in size, with plasmid pAB595-2 being larger (7651 bp; Figure 4D) and containing additional coding sequences. Plasmids pAA998 and pAC802 were the only ones to possess an integron in their structure. However, while pAC802 contained an integron, it lacked an integrase gene and only carried three gene cassettes linked to antibiotic and biocide resistance (*sul1*, *aad5*, and *qacE*). In contrast, plasmid pAA998 presented a complete integron with three ARGs (*sul1*, *aadA2*,

and *drfA12*) alongside a biocide resistance gene (*qacE*). Additionally, plasmids pAC802, pAB190, pAB595-1, pAB595-2, and pAA998 contained insertion sequences (ISs) within their structures, with some located near ARGs. These IS elements are highlighted in graphical representations due to their potential roles in ARG mobilization and expression [19,20]. Other transposases that could not be identified as belonging to any specific IS family are labeled as a "Transposase" or "Putative Transposase (PT)".

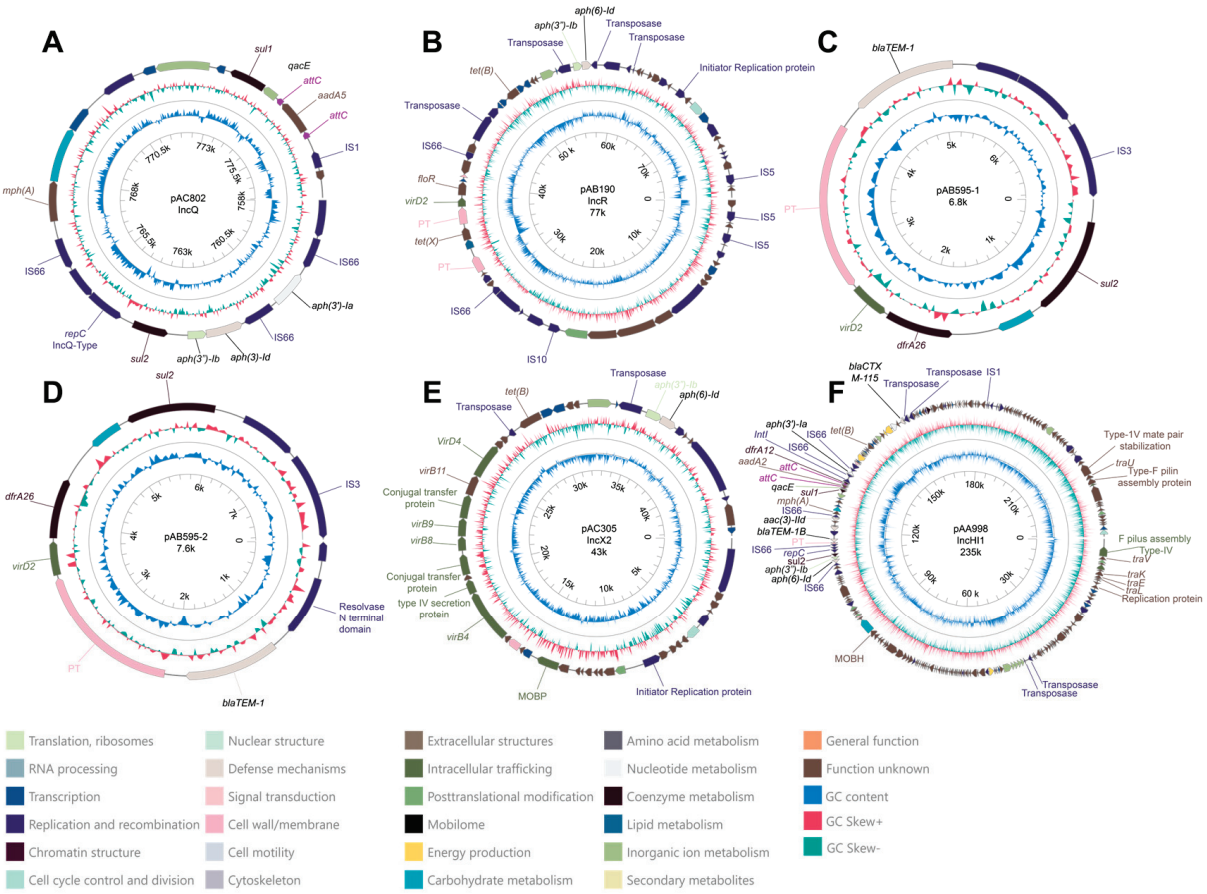

Figure 4. Graphical representation of plasmids containing ARGs, generated using the PlasmidScope web server. (**A**) Multi-drug-resistance region of plasmid pAC802 (*E. coli* A224-8), (**B**) plasmid pAB190 (*E. coli* A224-8), (**C**) plasmid pAB595-1 (*K. pneumoniae* A238-6), (**D**) plasmid pAB595-2 (*K. pneumoniae* A230-7), (**E**) plasmid pAC305 (*C. gillenii* A223-7), and (**F**) plasmid pAA998 (*E. coli* A231-12). Since plasmid pAC802 was found to be associated with a chromosomal contig after PacBio sequencing, we only present the multi-drug-resistance region integrated into the chromosome, which was linked to a plasmid sequence (from 758 to 777 kbp). In each graphical representation, all coding sequences (CDSs) identified by Prokka and Prodigal are displayed; however, only genes related to antibiotic resistance, biocide resistance, integrons, replication, insertion sequences (ISs), transposases, putative transposases (PTs), and other genes related with mobility functions are highlighted. Insertion sequence families were assigned based on MOB-suite results or annotated with the PlasmidScope web server. As *attC* sequences were not annotated with PlasmidScope, they were added manually based on genomic coordinates as indicated by IntegronFinder 2.0. Colors of CDSs in the plasmid maps are based on categorical functions assigned by eggNOG-mapper in PlasmidScope. Incompatibility groups (when available) and plasmid sizes were determined using the MOB-suite tool and are indicated in the graphics. To enhance visualization, all plasmid sequences are displayed in their circular form and gene names with light colors were changed to black. Additional details on these plasmids are provided in Supplementary Table S3.

2.4.1. Plasmid pAC082

This putative plasmid was initially identified by MOB-suite in the assembly of *E. coli* A224-8 (Site 3) using only Illumina data. However, after reconstructing the genome through hybrid assembly (PacBio and Illumina), the sequence of this plasmid was found to be integrated into the chromosomal contig of *E. coli* A224-8 (Supplementary Table S3). The plasmid carried seven ARGs (*aph(3′)-Ia*, *aph(6)-Id*, *aph(3′)-Ib*, *sul2*, *mph(A)*, *sul1*, and *aadA5*), along with an incomplete class 1 integron (lacking the integrase gene) and four insertion sequences (ISs). A replication sequence associated with the IncQ1 incompatibility group was also identified (Figure 4A). The incomplete integron contains two gene cassettes associated with antibiotic resistance (*sul1* and *aadA5*) and one linked to biocide resistance (*qacE*), as annotated by the IntegronFinder 2.0 software [20] (Supplementary Table S4). This plasmid lacks genes associated with horizontal transfer mechanisms or relaxase activity, classifying it as a non-mobilizable plasmid. The closest match observed in the database by the MOB-suite v3.1.8 software was plasmid pASL01 from *E. coli* (mash distance: 0.051; coverage: 57%; identity: 99.88%) with a size of 27 kb.

2.4.2. Plasmid pAB190

This plasmid was also observed in *E. coli* A224-8 and carried five ARGs (*tet(X)*, *tet(B)*, *aph(3″)-Ib*, *aph(6)-Id*, and *floR*), along with six ISs. Its replication sequence is associated with the IncR incompatibility group. Although genes coding for conjugative elements were not identified, a sequence corresponding to a MOBF-class relaxase was detected, indicating that the plasmid is likely mobilizable. The most similar plasmid in the databases was plasmid pEFER from *E. fergusonii* (mash distance: 0.017; coverage: 66%; identity: 99.46%) with an estimated size of 77 kb (Figure 4B).

2.4.3. Plasmids pAB595-1 and pAB595-2

Two plasmids were associated with the primary AB595 cluster according to the MOB-suite tool. They were present in *Klebsiella pneumoniae* A238-6 (Site 3, plasmid pAB595-1) and *Klebsiella pneumoniae* A230-7 (Site 2, plasmid pAB595-2), with approximate sizes of 6.8 kb and 7.6 kb, respectively (Figure 4C,D). Although MOB-suite classified both plasmids as non-mobilizable due to the absence of replicase, relaxase, or conjugative elements, additional analysis using the PlasmidScope server identified a VirD2-related gene within their sequences. The presence of VirD2 (relaxase), a component commonly involved in conjugative transfer, suggests that these plasmids may have potential mobility despite their initial classification. Both plasmid sequences contained three ARGs (bla_{TEM-1}, *sul2*, and *dfr26*), and an insertion sequence (IS) from the IS3 family. Both plasmids share the highest similarity with the *E. coli* plasmid RCS86_pI. For plasmid pAB595-1, we observed a mash distance of 0.036 with 47% coverage and 100% identity. Similarly, plasmid pAB595-2 exhibited a mash distance of 0.038 with 42% coverage and 100% identity.

2.4.4. Plasmid pAC305

This plasmid was identified in the *C. gillenii* isolate A223-5 (Site 2) and has an estimated size of 43 kb. Its replication sequence is associated with the IncX2 group, and it harbors three ARGs (*tet(B)*, *aph(3)-Ib*, and *aph(3)-Id*). The plasmid contains all the necessary genes for the conjugation process, classifying it as a conjugative plasmid. The closest match in the database is the R6K plasmid from *E. coli* (mash distance: 0.015; coverage: 76%; identity: 99.99%).

2.4.5. Description and Comparative Analysis of Plasmid pAA998

The plasmid pAA998, found in the *E. coli* A231-12 isolate (Site 3), contained 12 ARGs, the highest number of all the plasmids. Of these ARGs, three (*sul1*, *aadA2*, and *drfA12*) were located within a class 1 integron, along with a biocide-resistance gene (*qacE*) (Supplementary Table S4). This integron included the integrase gene, whose product is responsible for mobilization and structural rearrangements. The integron is situated alongside nine other

ARGs (*bla*$_{CTXM-115}$, *mph(A)*, *aac(3)-IId*, *tet(B)*, *aph(6)-Id*, *aph(3'')-Ib*, *aph(3)-Ia*, and *sul2*) within the multidrug-resistance region, spanning 110 kb to 160 kb (Figures 4F and 5). The plasmid is approximately 235 kb in size and carries two replicases from the IncH and IncQ1 groups (Supplementary Table S3). It also contains six insertion sequences (ISs), as well as all the necessary genes for conjugation, classifying it as a conjugative plasmid. The closest match in the database was plasmid pF8475 from *Salmonella enterica* (mash distance: 0.003, coverage: 89%, identity: 99.98%).

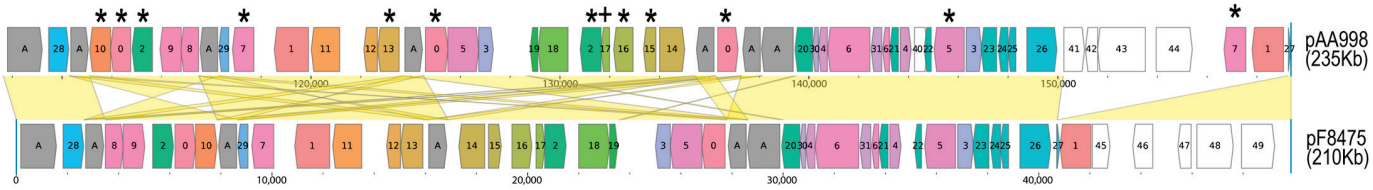

Figure 5. Comparative analysis of plasmid pAA998. Alignment of coding regions between plasmids pAA998 and pF8475, arranged based on their similarity in gene content (Jaccard index). Coding sequences (CDSs) that are unique to either plasmid are highlighted with a white background. CDSs associated with antibiotic-resistance genes are marked with an asterisk (*), while those related to quaternary-ammonium-compound resistance are indicated with a plus sign (+). For improved visualization, the section of plasmid pAA998 containing genes 32 to 39 (unique to pAA998) has been removed. Colors and numbers in the CDS boxes indicate associated functions, which are detailed further in Supplementary File S2.

Given the significance of IncH plasmids in ARG exchange within the Enterobacteriaceae family [21], we conducted further analyses to examine the genomic structure and relationships of pAA998. Specifically, we compared its complete structure with that of its closest relative matched in the MOB-suite database, plasmid pF8475, which is derived from an environmental isolate of *Salmonella enterica*. The alignment of the two plasmids is shown in Figure 5 While most coding sequences (CDSs) are conserved between the two plasmids, notable rearrangements such as inversions and translocations occur, even within the multidrug-resistance region. Full annotations of the CDSs based on COG and Pfam are provided in Supplementary File S2.

3. Discussion

In this work, we determined that as water quality in the river declined, the richness and complexity of ARGs in Enterobacterial plasmids increased. Human activities are strongly impacting plasmid dynamics and, therefore, also the dissemination of ARGs in natural environments. In this context, our results underscore the urgent need to expand antimicrobial-resistance surveillance to natural environments with both low and high human impact due to their significant implications for environmental and public health.

Seven isolates presented a resistance to at least 1 of the 15 antibiotics tested, with two classified as multidrug-resistant (resistant to three or more antibiotics) [15]. Of those seven, five showed resistance to nitrofurantoin, including those classified as *Klebsiella pneumoniae* (A231-5, A237-6, A230-7, and A238-6) and as *K. quasipneumoniae* (A230-5). *Klebsiella* isolates have been documented to frequently exhibit high resistance levels to nitrofurantoin, with resistance observed in 69.2% of evaluated isolates. This indicates a high prevalence of nitrofurantoin resistance in bacteria from this genus, which appears to be sustained in our isolates from a polluted river.

This antibiotic was introduced in 1953 as a first-line treatment for urinary tract infections and was widely used for two decades. Nonetheless, it was replaced mainly by trimethoprim/sulfamethoxazole and β-lactam antibiotics as these alternatives became more commonly employed [22]. However, the increasing resistance to trimethoprim/sulfamethoxazole and other antibiotics in clinical bacteria has led to a resurgence in its use in recent decades, which may be contributing to a rise in resistance to this antibiotic in environmental isolates [23,24].

The development of resistance to nitrofurantoin is primarily linked to mutations in the *nfsA* and *nfsB* genes and deletions in the *ribE* gene [25]. Likewise, resistance to this antibiotic has also been associated with the presence of efflux pumps of the RND family that are encoded by *oqx* genes mobilized on plasmids or inserted into the chromosome [22,26]. We observed multiple variants of the *oqx* genes in the chromosome of these isolates and different mutations in the *nfsA* gene (Table 3). This suggests that the E29A, G125W, G204S, Q195L, and K222R mutations associated with *oqx* genes could be involved in developing nitrofurantoin resistance in *Klebsiella* isolates. Notably, the high proportion of *Klebsiella* isolates exhibiting resistance to this antibiotic is noteworthy. Future studies should explore whether the environmental incidence of nitrofurantoin-resistant bacteria is associated with the high rates of prescription [27] and subsequent release of this antibiotic into the environment.

We also identified three isolates resistant to trimethoprim/sulfamethoxazole, a combination of antibiotics commonly used to treat urinary tract infections and various other infections [28]. One of these isolates (*K. pneumoniae* A230-7) was from Site 2, while the other two (*E. coli* A231-12 and *K. pneumoniae* A238-6) were obtained from Site 3. Furthermore, both trimethoprim and sulfamethoxazole were detected in water samples from Sites 2 and 3, with no presence in Site 1 (see Supplementary Table S5 for detected concentrations).

All the isolates resistant to trimethoprim and sulfamethoxazole contained the *dfrA* and *sul* genes on plasmids, which confer resistance to these respective antibiotics. Additionally, we observed the *sul1* and *dfrA12* genes in a class 1 integron (plasmid pAA998) in isolate *E. coli* A231-12. This finding aligns with other studies that consistently report a correlation between class 1 integrons and trimethoprim/sulfamethoxazole-resistance genes [29,30].

The presence of *sul* and *dfrA* genes, particularly within mobile genetic elements like plasmids and integrons in environmental isolates, may be linked to the presence of antibiotic residues in the aquatic environment, given that relevant concentrations of sulfamethoxazole and trimethoprim were detected only in Sites 2 and 3. Similar patterns have been observed in other studies, where aquatic sources that presented higher concentrations of sulfonamide residues (due to wastewater discharges) also exhibited a greater abundance of sulfonamide-resistant bacteria and *sul* genes [31,32].

In the same integron of plasmid pAA998, we detected the genes *qacE* and *aadA2*, which confer resistance to quaternary ammonium compounds (QACs) and streptomycin, respectively. This is notable, as the *qacE* gene was also found within the integron structure of *E. coli* isolate A224-8, another isolate from Site 3. Wastewater treatment plants commonly use QACs to reduce bacterial loads in wastewater [33]. Site 3, positioned downstream of a municipal wastewater treatment plant (see Supplementary Figure S1 and Section 4), likely receives these compounds, potentially explaining the presence of the *qacE* gene in these isolates. Detecting genes related to both QAC and antibiotic resistance within the same plasmids and integrons is highly relevant, as this co-localization may facilitate co-resistance in bacteria [34]. Therefore, future environmental monitoring of ARGs should include markers for these genes to better assess their co-existence and potential co-selection events.

Notably, *E. coli* A231-12, isolated from the most polluted site, showed resistance to seven antibiotics, including ampicillin/sulbactam and all the cephalosporins tested (Figure 2). It harbored two ARGs associated with beta-lactam antibiotic resistance; the $bla_{CTX\text{-}M\text{-}115}$ gene, which belongs to the class A extended-spectrum beta-lactamases, and the $bla_{TEM\text{-}1}$ gene, which encodes the class A broad-spectrum beta-lactamase TEM-1. According to the classification of Bush and Jacoby [35], the enzymes encoded by the gene families *CTX* and *TEM-1* are classified in the groups 2b and 2be, respectively. Group 2b confers a phenotype resistant to penicillin and some of the first-generation cephalosporins, while group 2be is resistant to an extended spectrum of cephalosporins. Both genes, $bla_{CTX\text{-}M\text{-}115}$ and $bla_{TEM\text{-}1}$, were found in the conjugative plasmid pAA998, and the $bla_{TEM\text{-}1}$ gene was also observed in the plasmids pAB595-1 and pAB595-2 (*K. pneumoniae* isolates A238-6 and A230-7, respectively) suggesting that these beta-lactamases might be spread to other bacteria in the environment.

In a developing country like Costa Rica, extended-spectrum beta-lactamase (ESBL)-producing bacteria have been documented in both clinical and environmental settings [36,37], with TEM-type ESBL producers consistently observed and CTX-type ESBL producers found less frequently [36]. Continuous monitoring of these bacteria is a core part of the country's National action plan to combat antimicrobial resistance, highlighting the importance of tracking ESBL-producing bacteria in natural and clinical settings [36].

The presence of genes encoding ESBLs in aquatic environments due to anthropogenic pollution, particularly sewage, has been widely studied in different regions; however, available published information is scarce for Latin America [37–39]. These genes pose a significant threat in hospital settings, where bacteria may carry them chromosomally or acquire them through plasmids, leading to multidrug resistance and complicating infection treatment. Detecting bacteria with these plasmids in community-accessible aquatic environments highlights the need for active ARG monitoring in natural settings, as they can serve as reservoirs of resistance genes relevant to treating infections in humans and animals. Thus, generating data on the prevalence of ESBL-positive isolates and identifying plasmid-borne $bla_{TEM\text{-}1}$ and bla_{CTX} genes in natural environments is crucial for ongoing monitoring efforts and risk assessment in Costa Rica and worldwide.

The plasmid pAA998, which harbored the higher number of ARGs, exhibited a conserved structure compared to its closest match in the MOB-suite database, plasmid pF8475; nevertheless, different genes are only present in pAA998 or in pF8475 (full annotations are listed in Supplementary File S2). Similarly, two ARGs present in plasmid pAA998 (*aph(3′)-Ia* and $bla_{CTX\text{-}M\text{-}115}$) are absent in pF8475, indicating that despite the strong genomic similarity between the two plasmids, their structures have undergone modifications. These changes are particularly evident in the multidrug-resistance region where various translocations and reversions are evident (Figure 5).

IncH plasmids are among the largest plasmids carrying antibiotic-resistance genes within the Enterobacteriaceae family [21], representing a potential risk as mobilizable elements that can acquire ARGs in the environment and transfer them to clinical settings. Both plasmids, pF8475 and pAA998, were found in environmental isolates of Enterobacteriaceae (*Salmonella enterica* and *Escherichia coli*, respectively) in two distanced countries (Czech Republic and Costa Rica). Although they share a high percentage of sequence identity, structural rearrangements and differences in their gene composition suggest that they are distinct plasmids with a common ancestral origin. Furthermore, the substantial presence of ARGs within these plasmids likely supports the fact that although the presence of ARGs in plasmids can be linked to their evolution under antibiotic pressure [40], the persistence of plasmids carrying ARGs in free-living bacteria is also highly frequent [40,41].

The mobility potential of plasmid pAA998 to other bacteria is notable, as the MOB-suite tool predicted it to be conjugative due to the presence of genes associated with the type IV secretion system (Figure 4F). Similarly, plasmid pAC305 exhibited conjugative elements in its structure, along with three antibiotic-resistance genes (Figure 4E). Otherwise, plasmid pAB190, which harbors five ARGs (Figure 4B), was classified as a mobilizable plasmid because it lacks conjugative elements but presented a relaxase gene. However, it was found in the *E. coli* isolate A224-8, which also contains other conjugative plasmids (Supplementary Table S3) that could facilitate the mobilization of pAB190 and its associated ARGs [42].

While plasmids pAB595-1, pAB595-2, and pAC802 were classified as non-mobilizable due to the absence of relaxase and conjugative elements in their sequences, this classification may be influenced by the MOB-suite limitations [43]. Notably, the presence of a VirD2-related gene in pAB595-1 and pAB595-2, as identified by PlasmidScope (which utilizes eggNOG-mapper), suggests a potential for mobility that might have been overlooked by MOB-suite. Reconstructing plasmid sequences from draft genomes is inherently challenging, and certain mobility-associated features may remain undetected. Nonetheless, our findings highlight the potential for highly contaminated environments to harbor plasmids with resistance genes that may be transferred to other relevant bacteria, such as

species within the Enterobacteriaceae family. However, further studies are needed to detect specific plasmid sequences using more sensitive techniques, such as qPCR, digital PCR, or metagenomic approaches, to understand these dynamics better.

Although no plasmid-borne ARGs were observed in Site 1, the presence of the *blaEC* gene in all five *E. coli* genomes isolated from this site, an area with relatively low human impact, represents a notable finding. The *blaEC* genes, also known as *ampC-EC* genes, are primarily chromosomally encoded and associated with *Escherichia coli* and *Shigella* strains [44,45]. Specifically, the *blaEC-15* gene is linked to *E. coli* phylogroup A, while the *blaEC-18* gene is associated with phylogroup B1 [45]. This aligns with our results, where isolate A229-5 from Site 1, which contains the *blaEC-15* gene, belongs to phylogroup A, whereas isolates A236-7, A229-12, and A222-7 from Site 1, as well as A231-12 from Site 3, harbor the *blaEC-18* gene and are classified into phylogroup B1.

However, none of the *E. coli* isolates from Site 1 exhibited phenotypic resistance to the antibiotics tested, even though the *blaEC-15* and *blaEC-18* genes have been reported as beta-lactamases with extended activity against cephalosporins [44,45]. It has been suggested that, despite the numerous variants of these genes, only some of them are expressed and confer a resistant phenotype to beta-lactam antibiotics. The effectiveness of these genes has been linked to the presence of nearby insertion sequence promoters [44] as well as their interactions with efflux pumps and low outer-membrane permeability [46]. Furthermore, the horizontal gene transfer of *ampC* genes has gained significance in recent years, as it has been associated with the clonal expansion of resistance strains [47], highlighting the need for continuous monitoring of these genes [48].

The detection of ARGs in environments with low human impact, such as Site 1, represents a novel and necessary strategy. These environments may serve as reservoirs of resistance genes that could later contribute to the broader dissemination of resistance traits. This also emphasizes the role of chromosomal ARGs, which can persist and potentially evolve within isolated bacterial populations, conferring resistance phenotypes. Thus, future studies in areas distant from human influence may further illuminate the origins and dynamics of chromosomally and plasmid-encoded ARGs, enhancing our understanding of how isolated reservoirs contribute to the global resistome.

Site 3 was chosen as a reference point in the Virilla River's contamination gradient, as it has been previously reported to have the highest pollution levels according to the BMWP-CR index (values between 17; classified as "very polluted") and the Dutch Quality Index (value of 11; classified as "severe") [49]. Additionally, it exhibited the lowest NSFWQI value obtained in this work (23; very poor quality; Supplementary Table S5). However, given the intensive effort required to assess microbiological pollution levels, all three sites may pose a potential risk to human health. Future studies should incorporate additional intermediate sites and expand the evaluation to cover a broader range of microbiological parameters and biological data, providing a more comprehensive understanding of this potential risk.

As mentioned above, the Virilla River is impacted by wastewater discharged from a treatment plant located between Site 2 and Site 3 (see Supplementary Figure S1 and Section 4). This discharge is likely a primary contributor to the elevated contamination levels observed at Site 3 and may be a significant factor in the presence of ARGs and multidrug-resistant bacteria, as wastewater treatment plants are known hotspots for the dissemination of antimicrobial-resistance traits [50]. Although our sample size is limited, discovering the highest number of ARGs and plasmids in isolates from Site 3 raises concerns about the potential selective pressure exerted by urban pollution (including sewage), which may enhance the transfer and distribution of plasmids carrying ARGs, as previously discussed. Similar patterns have been observed globally, such as in coastal regions of central Thailand, where urban pollution appears to drive the high prevalence of ARGs and mobile genetic elements in the aquatic environment [51]; likewise, in aquatic environments of the Bolivian Andes, a significant correlation was found between fecal contamination and an increase in ARGs as well as the presence of the *intl1* gene [52].

This relationship between urban contamination and the prevalence of ARGs is supported by experiments on microbial communities (microcosms) such as the one carried out by Aoife and Rima [53], where antibiotic-resistant bacteria proliferated more effectively in microcosms treated with wastewater than those treated only with river water; demonstrating that bacteria with ARGs that survive wastewater treatment can proliferate and persist in the aquatic environment after their release. Additionally, a study evidenced that in microcosms created from wastewater from a treatment plant in Scandinavia, conjugation rates were increased upon exposure to low concentrations of antibiotics and biocides [54]. These findings emphasize the importance of conducting further studies in various lotic water bodies, particularly those under strong urban influence.

Another noteworthy point is the isolation of multiple *E. coli* strains from all three sampling sites, each displaying distinct ARG profiles. In Figure 1, we highlight how isolates *E. coli* A224-8 and *E. coli* A231-12, both from Site 3, exhibit a significantly higher number of ARGs, due to the presence of plasmids, compared with other *E. coli* isolates from Sites 1 and 2, which belong to the same clades. This observation suggests that, despite sharing evolutionary origins within the same phylogroups, the acquisition of plasmid-borne ARGs occurs only in the isolates from Site 3, supporting the premise that the highly contaminated environment at Site 3 may facilitate the acquisition of new plasmids carrying resistance genes.

4. Materials and Methods

4.1. Sampling Sites

Field trips were conducted during the rainy season of 2021 to three locations along the Virilla River Watershed (Supplementary Figure S1). Site 1, near the river's source in Vásquez de Coronado (9°59′9″ N, 83°56′35″ W), shows minimal human impact and is situated at the highest elevation at an altitude of 2020 m. Site 2, located in Tibás (9°57′47″ N, 84°5′51″ W), is at an altitude of 1185 m and has a high population density with significant environmental transformation. Site 3 is located at the Brasil Dam in Mora (9°56′46″ N, 84°13′22″ W), where the Virilla River converges with the Uruca River. Positioned at an altitude of 720 m, this site is downstream of the "Los Tajos" treatment plant, which discharges wastewater directly into the Virilla River. Although it experiences moderate human impact, it recorded the lowest water quality among the sites. For the three sites, the National Sanitation Foundation Water Quality Index (NSFWQI) value was calculated [48], confirming that the surface water quality degraded during the rainy season of 2021 (poor quality for Sites 1 and 2 and very poor quality for Site 3) (Supplementary Table S5). Microbiological parameters of surface water samples collected during the rainy season (November 2021) were measured with the most-probable number technique, together with measures of conductivity, turbidity, color, and pH; other physical–chemical variables were analyzed in the field with a multiparameter probe (YSI, model 85 A, Yellow Springs, OH, USA) (Supplementary Table S5). Indices from the Costa Rica Ministry of Environment support these data and can be verified in the National Information System for Integrated Water Resource Management (SINIGIRH) [49].

4.2. Sample Processing and Bacterial Isolation

Surface water samples were collected on three separate days from the designated sites using sterile containers and processed on the same day. Initially, 250 mL of each sample was prefiltered through a sterile 80 μm pore glass fiber prefilter (Sartorius, Göttingen, Germany). Subsequently, each prefiltered sample was passed through a sterile 0.2 μm pore filter (Sartorius). The filters were then incubated at room temperature in 40 mL of Lauryl broth (Difco-BBL, Franklin Lakes, NJ, USA) for 24 h in aerobic conditions to promote the growth of fecal coliforms. After incubation, the Lauryl broth (BBL) was transferred to culture plates containing Levine (Oxoid, ThermoFisher™, Waltham, MA, USA) and MacConkey agar (Oxoid), where the colonies were incubated at 35 °C for 24 h. These media facilitate the differentiation of various morphotypes within the Enterobacteriaceae family. Colony-forming units from different sampling points were identified, and morphotypes

were transferred to new culture media to obtain pure cultures. These morphotypes were stored in duplicate 1.5 mL tubes containing 20% glycerol in Soy Tryptose Broth (Oxoid) after 24 h of growth and preserved at −80 °C.

4.3. Biochemical and Serological Analysis

Cryopreserved bacteria were inoculated onto MacConkey agar (Oxoid) and incubated for 24 h at 35 °C. After incubation, at least one lactose-positive morphotype from each sample was isolated and subcultured onto tryptic soy agar plates (Oxoid). Following another 24 h incubation at 35 °C, the API20E test (Biomerieux, Marcy-l'Étoile, France) was performed according to the manufacturer's instructions. Each bacterial isolate was identified based on its numerical profile using the apiweb™ v1.3.1 software. The Wellcolex *Shigella* color kit (ThermoFisher™, Waltham, MA, USA) was used for the detection and species identification of Enterobacteria genomically related to *Shigella* spp., following the manufacturer's instructions.

4.4. Chemical Analysis

Water samples were collected in 1 L amber glass containers and transported under appropriate conditions to the laboratory at the Center for Research in Marine Sciences and Limnology (CIMAR) at the University of Costa Rica for nutrient analysis (nitrite, ammonium, nitrate, phosphate, and silicate; Supplementary Table S5) [55,56].

4.5. Anthropogenic Contaminant Detection by Mass Spectrometry

Detection analyses of caffeine, terbutryn, and various antibiotics, including amoxicillin, ciprofloxacin, clarithromycin, doxycycline, enrofloxacin, erythromycin, lincomycin, oxytetracycline, penicillin V, sulfadiazine, sulfamethazine, sulfamethoxazole, sulfathiazole, tetracycline, tiamulin, trimethoprim, tylosin, chlortetracycline, levofloxacin, chlorhexidine, and moxifloxacin, in water samples were conducted at the Central American Institute for Studies on Toxic Substances (IRET) Laboratory at the National University of Costa Rica (UNA). The analyses utilized direct injection and LC-MS/MS measurements, employing an ACQUITY H-CLASS UPLC chromatographic system (Waters Corp., Milford, MA, USA) connected to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp., Wilmslow, Manchester, UK), which was equipped with an electrospray ionization interface operating in positive mode. Only detected contaminants are reported in Supplementary Table S5.

4.6. DNA Extraction and Sequencing

From the total collection of isolated bacteria (72 isolates), 15 were randomly selected for sequencing (5 from each sampling site). Genomic DNA was extracted using the DNeasy PowerSoil kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions, and samples were sequenced at Novogene using the Illumina NovaSeq platform (Hong Kong, China), yielding at least 1 GB of paired-end raw data per genome.

4.7. Quality Control of Raw Data

Raw data were processed with the BBduk function from the BBtools package [57] to remove remaining adapter sequences, and Trimmomatic [58] was employed to trim the first ten nucleotides due to uneven tetranucleotide frequencies. Sequences with average quality scores below 30 or lengths less than 100 bp were discarded.

4.8. Assembly and Quality Evaluation

Assembly was conducted using Spades v3.15.4 [59], with k-mer values of 21, 33, 55, 77, and 99. Contigs shorter than 1000 bp were removed, and assembly statistics were visualized with Quast [60]. The completeness and contamination of genomes were assessed with the checkm2 v1.0.2 software [61].

4.9. Taxonomic Identity and Phylogenomic Analysis

Taxonomic identity was determined using the Type (Strain) Genome Server (TYGS) [13]. For isolates not recognized by the TYGS tool, we employed the Genome Taxonomy Database (GTDB) and the neighbor-joining method to identify closely related genomes [62]. Additionally, we conducted a genome-wide phylogenomic analysis that included our genomes alongside 79 additional genomes downloaded from the GenBank database. Genomes were selected based on criteria such as their status as type strains, their representation of various phylogroups or subclades identified in previous studies [63–66], and reference genomes from NCBI. The selected genomes were imported into Anvi'o for coding sequence identification using Prodigal [67], followed by amino acid alignment with DIAMOND [68] and gene cluster creation using the MCL algorithm [69]. We then selected and concatenated the nucleotide sequences of all single-copy core genes (SCCGs) across all the genomes, which resulted in a dataset of 908 SCCGs. The nucleotide sequences of these genes were aligned using MAFFT v7.397 [70], and a maximum likelihood analysis was performed with FastTree v2.1.10 (GTR model with 1000 bootstrap replicates) [71]. The resulting phylogenomic tree was visualized and edited using iTOL [72].

4.10. Antibiotic Susceptibility Testing

Antibiotic-resistance profiles were determined by culturing isolates on LB agar for 24 h, followed by analysis with the VITEK2 system. The antibiotic susceptibility card VITEK® 2 AST-N401 (Biomerieux, Marcy-l'Étoile, France) included aminoglycosides (amikacin, gentamicin), β-lactams (ampicillin/sulbactam), carbapenems (ertapenem, meropenem), cephalosporins (cephalothin, cefazolin, ceftazidime, ceftriaxone, cefepime), quinolones (ciprofloxacin, norfloxacin), sulfonamides/diaminopyrimidines (trimethoprim/ sulfamethoxazole), and others such as fosfomycin and nitrofurantoin. Resistance levels were assessed based on the MIC breakpoints for Enterobacterales as outlined in the 2020 CLSI guidelines [16].

4.11. Identification of Antibiotic-Resistance Genes (ARGs) and Mobile Genetic Elements (MGEs)

Plasmids were reconstructed using MOB-suite v3.1.8 [73], which facilitated the separation of contigs associated with each plasmid from chromosomal sequences. Integrons were mapped utilizing Integron Finder 2.0 [74] to ascertain their genomic or plasmid contexts. Potentially reconstructed plasmids were annotated and visualized with the PlasmidScope v1.2 web server [75], incorporating coding sequences identified with Prokka v1.14.5 [76], and Prodigal v2.6.3 [67] and annotated with eggNOG-mapper v2 [77] but only highlighting the antimicrobial-resistance genes (ARGs), integrons, replicon sequences, insertion sequences (ISs) and other genes related to mobility.

Antimicrobial-resistance genes were identified using the ABRicate v0.9.8 tool [78], which employed the NCBI AMRfinderPlus database (updated to 1 November 2024) [79], applying a minimum coverage and similarity threshold of 80%. A Kruskal–Wallis test was conducted with a confidence level of 95% in R software (version 4.4.3) to evaluate significant differences in the abundance of ARGs and plasmids across sampling sites. Subsequently, a post hoc Dunn test was performed to determine specific sites exhibiting significant differences for each variable.

4.12. Search for Mutations Conferring an Antibiotic-Resistance Phenotype

All genomes were annotated using Prokka v1.14.6 [22], followed by manual searches for point mutations in specific resistance-related genes. The gyrA gene from susceptible *E. coli* K-12 substr. MG1655 (reference genome GCF_000005845.2) was used as a reference to compare amino acid sequences against the genome of isolates classified as *E. coli*. The same analysis was conducted for *nfsA* and *nfsB* genes associated with nitrofurantoin resistance in genomes of isolates classified as *Klebsiella*, comparing them against the reference strain *Klebsiella pneumoniae* ATCC 13883 (accession number JOOW01000000).

4.13. Sequencing of A224-8 and A231-12 Isolates Through PacBio Technology for Plasmid Confirmation

To confirm the presence of plasmids with ARGs, the genomes of two isolates were sequenced using PacBio technology. This approach aimed to improve the assembly quality and validate the plasmid structures, as plasmids often contain repetitive sequences exceeding 300 bp, which are challenging to assemble with Illumina data [80]. Samples were sent to Novogene for long-read sequencing, and hybrid assembly was performed with Spades v13.5.4. Subsequent analyses to annotate ARGs and plasmids employed the previously mentioned tools.

4.14. Genomic Comparison of Plasmid pAA998

The plasmid pAA998 belongs to the incompatibility group IncH and was identified in isolate A231-12, which harbors 12 antibiotic-resistance genes (ARGs). Given the importance of this incompatibility group in disseminating antibiotic resistance and the substantial number of ARGs present in its structure [21], we conducted a sequence comparison with the most closely related plasmid in the MOB-suite database, pF8475 (accession number KP899804). We annotated both plasmids using Anvi'o v7.1, employing the COG14 [81] and Pfam v32 [82] databases to achieve this. Alignments were generated and visualized following the PlasX-MobMess pipeline [83].

5. Conclusions

This study highlights the critical connection between urban pollution and the rise of antibiotic resistance in aquatic environments. Our findings reveal that both genotypic and phenotypic resistance levels were highest at more urbanized sites, with common resistance to nitrofurantoin, trimethoprim/sulfamethoxazole, and beta-lactams linked to the *sul*, *dfrA*, and *bla* genes present in mobile genetic elements, such as plasmids and class 1 integrons. Notably, an *E. coli isolate* from the most polluted site showed the highest ARG count on a single plasmid, classified within the IncH incompatibility group, which signals a strong potential for ARG transfer within Enterobacteriaceae. The observed increase in resistance complexity and abundance along the urban gradient underscores the need for antimicrobial-resistance surveillance within water quality monitoring to help mitigate the transfer of resistance traits between environmental and community settings.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13111089/s1, Supplementary Figure S1: Geographic map showing the location of the sampling sites (highlighted in red dots) and the wastewater treatment plant "Los Tajos" (yellow dot); Supplementary Figure S2: Tree inferred with FastME 2.1.6.1 from GBDP distances calculated for genome sequences obtained from the Type Strain Genome Server. The branch lengths are scaled in terms of the GBDP distance formula d5. The above branches are GBDP pseudo-bootstrap support values > 60% from 100 replications, with an average branch support of 34.8%. The tree was rooted at the midpoint; Supplementary Table S1: Accession numbers of the genomes used to reconstruct the phylogenomic tree illustrated in Figure 1; Supplementary Table S2: Complete annotation of antibiotic-resistance genes by the ABRicate tool employing the NCBI database; Supplementary Table S3: Plasmid annotation results obtained with MOB-suite software v3.1.8; Supplementary Table S4: Integron Finder annotation results for isolates A224-8 and A231-12; Supplementary Table S5: Physical–chemical and microbiological parameters used to calculate the NSFWQI of surface water samples during the rainy season (November 2021) in the Virilla River Watershed. Additionally, it includes anthropogenic contaminants measured by mass spectrometry. Supplementary File S1: Excel file containing all the supplementary tables; Supplementary File S2: Complete annotation of coding sequences found in plasmids pAA998 and pF8475 using the COG and Pfam databases.

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and editing, B.M.-G., K.B., C.R., K.R.-J. and M.A.-A.; visualization, B.M.-G.; supervision, M.A.-A. and K.R.-J.; project administration, K.B., C.R., K.R.-J. and M.A.-A.; funding acquisition, M.A.-A. and K.R.-J. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All generated data are available in the NCBI database under the Bioproject accession number PRJNA1164306. Illumina sequence reads can be retrieved with the accession numbers SRR30782601: SRR30782617. Other accession codes for additional data used are available in the supplementary tables. All codes employed in this study are available from the corresponding author upon request.

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Review

Environmental Antimicrobial Resistance: Implications for Food Safety and Public Health

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Abstract: Antimicrobial resistance (AMR) is a serious global health issue, aggravated by antibiotic overuse and misuse in human medicine, animal care, and agriculture. This study looks at the different mechanisms that drive AMR, such as environmental contamination, horizontal gene transfer, and selective pressure, as well as the severe implications of AMR for human and animal health. This study demonstrates the need for concerted efforts across the scientific, healthcare, agricultural, and policy sectors to control the emergence of AMR. Some crucial strategies discussed include developing antimicrobial stewardship (AMS) programs, encouraging targeted narrow-spectrum antibiotic use, and emphasizing the significance of strict regulatory frameworks and surveillance systems, like the Global Antimicrobial Resistance and Use Surveillance System (GLASS) and the Access, Watch, and Reserve (AWaRe) classification. This study also emphasizes the need for national and international action plans in combating AMR and promotes the One Health strategy, which unifies environmental, animal, and human health. This study concludes that preventing the spread of AMR and maintaining the effectiveness of antibiotics for future generations requires a comprehensive, multidisciplinary, and internationally coordinated strategy.

Keywords: antimicrobial resistance; One Health approach; antibiotic stewardship; surveillance systems; public health; animal husbandry; global action plan

1. Introduction

Antimicrobial resistance (AMR) is the capacity of microorganisms including bacteria, fungi, viruses and parasites to change over time and withstand the effects of antibiotics that were originally effective against them [1]. AMR has become an increasing and severe global health challenge. Microorganisms can gain this resistance through various processes, including spontaneous mutations in their genetic makeup; these mutations modify the antibiotics' target sites, thus making the antibiotics ineffective against the microorganism [2]. Additionally, microorganisms can acquire genes that are resistant to antibiotics from other organisms through horizontal gene transfer (HGT) processes including conjugation, transformation, and transduction [3]. AMR is exacerbated because the system works in a way that promotes the multiplication of microorganisms and increases their ability to withstand antimicrobial effects and the ability of resistant microorganisms to confer antimicrobial resistance to non-resistant microorganisms by the transfer of resistant genes.

The consequences of AMR are far-reaching and the treatment of infectious diseases has been severely hampered by AMR, prolonging illnesses and increasing healthcare costs. The rise in extensively drug-resistant (XDR) and multidrug-resistant (MDR) strains has made it increasingly difficult to treat common infectious diseases [4,5]. For example, the treatment of diseases like gonorrhea, tuberculosis, and other hospital-acquired infections has been made difficult by resistant bacteria strains [6,7]. The significant negative impact of AMR on healthcare means that more expensive and toxic pharmaceuticals are needed for treatments; it also means longer hospital stays for patients and demands for more intensive care. All these factors add up to the increasing overall costs of healthcare. For example, a study by the Rollins School of Public Health and Saint Louis University found that antibiotic resistance added USD 1383 per patient to treatment costs in the U.S., totaling around USD 2.2 billion nationally in 2014 [8]. AMR also has a significant impact on economies; it has been reported that in the absence of effective treatment interventions, AMR could reduce global gross domestic product (GDP) by 2–3.5% and force about 24 million people into extreme poverty globally by 2030 [9,10].

Furthermore, AMR is a factor in increased mortality. Infections from resistant strains have been reported to present with higher rates of morbidity and mortality, when compared to infections generated by non-resistant strains [11]. As of 2019, 1.27 million annual deaths globally were attributed to AMR; this number is expected to keep rising if the present trends continue [12]. The World Health Organization [13] has reported AMR in the top ten global public health threats to humanity, this highlights the crucial need for concerted global action to address AMR. Strong surveillance, careful administration and use of available antibiotics, and the development of new antimicrobials are very important to stay ahead in the continuous fight against AMR.

The study of AMR in the environment is very significant. The environment, including the soil, water, and food production systems, is a reservoir and conduit through which resistance genes are propagated [14,15]. Antibiotics and other antimicrobial compounds are regularly introduced into the environment through industrial processes, agricultural activities, and poor disposal of different pharmaceuticals [16,17]. These antimicrobial agents persist in the environment, exerting selective pressure on the microbial populations, in addition to encouraging the establishment and spread of resistant strains [18]. Furthermore, resistant bacteria and genes can spread from these environmental reservoirs to humans through different pathways, including food consumption, water use, and direct contact, posing considerable public health implications [19].

This paper aims to provide a comprehensive review of the environmental drivers of antimicrobial resistance (AMR), focusing on the mechanisms by which resistance genes spread in soil, aquatic systems, and food production environments. By critically analyzing the pathways through which AMR impacts food safety and public health, this paper highlights the complex interconnection between environmental contamination, human health, and agricultural practices. This study further evaluates the effectiveness of current surveillance systems, regulatory frameworks, and the One Health approach in mitigating AMR, while identifying gaps and proposing innovative strategies for reducing AMR at the global scale.

2. Development and Spread of AMR in Environmental Niches

2.1. Soil Environments

Antibiotics resistance genes (ARGs) are found in the complex and dynamic soil environment, which plays a major role in the emergence and spread of AMR. Agriculture, especially the widespread use of antibiotics in the cultivation of crops and livestock, is one of the major sources of antimicrobial compounds in the soil [20]. The use of animal waste as fertilizer introduces significant quantities of antimicrobials into soil ecosystems, influencing both microbial communities and their functional dynamics. This waste often contains residual antibiotics and other antimicrobial compounds that have not been entirely metabolized [21]. Antibiotics are commonly given to cattle for growth-promoting

and medicinal purposes, which causes them to build up in animal waste, which is commonly used as fertilizer [22]. By adding large amounts of antimicrobial substances to the soil, this creates a selection pressure that encourages the growth of resistant microorganisms, raising important considerations for environmental health and agricultural practices. Research has shown that soils treated with manure from livestock raised on antibiotics exhibit significant changes in microbial composition, particularly a heightened prevalence of antibiotic-resistant bacteria when compared to untreated soils [23]. This increase in resistance genes within soil microbial communities carries potential risks for the transfer of ARGs to both plant-associated and human-associated microbes. This underscores the importance of implementing stringent management practices regarding the application of animal waste.

The problem of resistance development in soil microbial communities is further exacerbated by the use of sewage sludge, with resistant bacteria from human waste and antibiotic residues [24]. Resistant bacteria and residual antibiotics can remain in soil for extended durations, facilitating opportunities for HGT and augmenting the reservoir of ARGs in agricultural settings. Consequently, soils that receive sewage sludge demonstrate increased resistance levels, which may impact resistance patterns in microbiomes associated with crops and potentially enter the human food chain.

The diversity of the mechanisms underlying the development of resistance in soil bacteria is indicative of the complexity of soil as an ecological niche. Resistance genes are spread among soil bacteria through HGT. The HGT process of transformation entails taking up free DNA from the surroundings, which could be made easier by DNA adhering to soil constituents like clay minerals [25]. Studies have demonstrated that plasmids adsorbed onto minerals, such as montmorillonite and kaolinite, can withstand degradation and are available for uptake by competent bacteria, hence facilitating the dissemination of resistance genes [26,27]. Conjugation, the direct transfer of genetic material between bacteria, is also affected by soil components. For example, at low concentrations, certain minerals, such as goethite, encourage the conjugative transfer of resistance genes by damaging cell membranes, increasing bacterial interactions and gene transfer rates [28,29]. In transduction, the exchange of bacterial genetic material is facilitated by bacteriophages—viruses that infect bacteria. During transduction, bacteriophages incorporate pieces of the host bacterial DNA, including AMR genes, and transfer these genes to new bacterial hosts when they attach and infect them. Transduction is relevant in soil ecosystems, where a diversity of bacteriophages thrive. The interactions between bacteriophages and bacteria promote the transfer of resistance genes in the soil, enhancing the spread of AMR and the genetic diversity of microbial communities [30].

In addition to HGT, AMR development is significantly influenced by soil bacteria's intrinsic resistance mechanisms. Soil bacteria inherently produce antibiotics as a competitive strategy. They have also developed a variety of resistance mechanisms to shield themselves from the antimicrobial substances produced by other microbes and their own antimicrobial compounds. A few of these methods are the enzymatic breakdown of antibiotics, the alteration of antibiotic targets, and efflux pumps, which remove antibiotics from cells [31]. Both naturally occurring and artificially produced antibiotics can select for bacteria that have these resistance characteristics in the soil environment, enriching the resistant populations. AMR also spreads quickly between different bacterial species and genera due to the high density and diversity of microbial communities in soil, which offer plenty of chances for the transfer of resistance genes.

The growth and dissemination of AMR can also be influenced by the physicochemical characteristics of soil, including pH, organic matter concentration, and the presence of metals and minerals [32]. Antibiotic bioavailability and environmental persistence may be impacted by interactions between soil components and antibiotics. To prolong their selective impact on bacterial communities, antibiotics can, for example, adsorb onto soil particles and be shielded from destruction. On the other hand, some elements of soil might decrease the effectiveness or inactivate antibiotics, which could result in exposure that

is sub-lethal, thus resulting in the emergence of resistance [33]. Furthermore, resistance gene expression can be altered by the interactions between bacteria and soil components. As an example, research has demonstrated that specific minerals found in soil can cause oxidative stress in bacteria, resulting in the SOS response (a network of genes in bacteria that addresses DNA damage), which is known to facilitate the horizontal transfer of resistance genes [25,34].

The ecological and public health ramifications of AMR within soil ecosystems are of considerable importance. A comprehensive understanding of AMR in soil is vital for both agricultural productivity and human health, as soil acts as a significant reservoir for ARGs that can transfer to pathogens affecting plants, animals, and humans. In agricultural contexts, the prevalence of ARGs in the soil may influence the microbial communities associated with crops, potentially leading to increased plant diseases or diminishing the effectiveness of biocontrol agents. From a public health perspective, there exists a risk that ARGs present in soil could be conveyed to human-associated pathogens, either through direct human contact with soil or via the consumption of crops cultivated in contaminated soils [35]. Furthermore, the presence of AMR in soil represents a threat to broader ecosystems, as ARGs can migrate into adjacent water bodies through runoff, thereby facilitating the dissemination of resistance within the environment and possibly affecting human populations through water supply systems [36]. Consequently, it is essential to address AMR in soil through the implementation of sustainable agricultural practices and effective waste management strategies to curtail the spread of resistance.

2.2. Aquatic Environments

Aquatic environments, which include lakes, rivers, oceans, and other bodies of water, are important in preserving ecological balance, promoting biodiversity, and providing resources for both animal and human populations. However, these natural environments are becoming increasingly contaminated because of anthropogenic activities involving the widespread use of antibiotics and their improper disposal. Municipal water ecosystems, notably those running through densely inhabited areas, act as major conduits for antibiotics into the environment. Antibiotic residues are accumulating in water bodies worldwide due to a variety of human activities, wastewater treatment facilities, agricultural runoff, and hospital system effluents (Figure 1) [37]. These pollutants persist in the environment, encouraging the emergence and spread of AMR and so fueling the increasing public health emergency. The implications of AMR in aquatic environments are substantial, as resistant pathogens have the potential to spread across international boundaries. This phenomenon has prompted global health initiatives, including the World Health Organization's Global Action Plan on AMR, to address the challenge. Consequently, addressing AMR in aquatic ecosystems is not only crucial for safeguarding marine and freshwater environments but also aligns with overarching health and environmental policies, emphasizing its critical role in the preservation of both ecological integrity and public health. These pollutants include antibiotics used in aquaculture, such as oxytetracycline, florfenicol, and ormetoprim [38,39].

Antibiotics are not fully metabolized by humans and animals and are released in the aquatic environment through feces and urine, thereby contaminating the aquatic environment. According to Shigei et al. [40], urban rivers and other bodies of water are further contaminated by the resulting pharmaceutical pollutants, including paracetamol, ofloxacin, carbamazepine etc., which are frequently present in wastewater treatment plant effluents. Additionally, surface runoff from farms is important in the introduction of antibiotics and other pollutants into aquatic systems. These settings accelerate the selection and spread of resistance traits among microbial communities due to the presence of antimicrobial residues, antimicrobial-resistant bacteria (ARB), and ARGs, posing significant threats to the environment and public health [41]. Antibiotic pollution in aquatic ecosystems can disrupt the ecological balance, affecting native species and vital ecosystem functions. The presence of antibiotic-resistant bacteria can alter these environments' microbiomes, negatively in-

fluencing nutrient cycling, primary production, and species survival, ultimately affecting biodiversity and ecosystem stability.

In aquatic environments, antibiotics and their residues, like those in soil, create selection pressure that favors the survival and growth of resistant bacteria. AMR is a global problem that is thought to have substantial reservoirs and transmission channels in certain ecosystems. Given that microorganisms are genetically adaptable, they may quickly adapt to the presence of antibiotics through processes like mutation, conjugation, transformation, and transduction [42]. This genetic plasticity drives the development of antimicrobial resistance in aquatic microbiomes. It is difficult to determine acceptable amounts of antibiotic residues in the environment because research indicates that even modest quantities of antibiotics in water might cause the selection of ARGs [43]. This problem is made worse because ARGs and ARBs cannot be completely removed from water by current water treatment processes, and this permits them to persist and spread across the environment [44,45]. Strategies to mitigate antibiotic pollution could involve advanced treatment techniques, such as membrane filtration and bioremediation, which aim to degrade antibiotics and ARGs more effectively. Additionally, optimizing antibiotic use in aquaculture and employing biosecurity measures can further reduce contamination in these environments.

ARG spread in aquatic environments is facilitated by different factors, including the direct discharge of untreated or poorly treated effluents from agricultural runoff, wastewater treatment plants, and the use of domestic wastewater for irrigation [46]. These processes deliver ARBs and ARGs to water bodies, from where they can be passed on to other organisms. This transfer happens via HGT, frequently resulting in the establishment of new, more resistant bacterial strains [47]. The prevalence of ARGs in aquatic settings highlights the complexities of the AMR problem, as these genes are difficult to degrade and can spread across multiple environments, including soil, water, sediments, and sewage [48].

Aquaculture, which makes a substantial contribution to global food production, is another important cause of antibiotic contamination in aquatic environments. The increasing number of ARGs in aquaculture systems is partly attributed to the use of antibiotics in the treatment and prevention of diseases in farmed fish [49]. Aquatic organisms can spread human pathogens through a variety of routes, such as eating contaminated fish or being exposed to the environment directly. Many of the antibiotics used in aquaculture—including tetracyclines, aminoglycosides, and macrolides—are vital to human medicine [50]. Numerous studies have reported on the indirect transfer of ARGs from aquatic habitats to human diseases. These findings underscore the significant risks to public health that arise from the use of antibiotics in aquaculture [45,51]. In response to these challenges, the implementation of sustainable aquaculture practices, including the use of probiotics, vaccination protocols, and enhanced husbandry techniques, may significantly diminish the dependence on antibiotics. This, in turn, could aid in curtailing the emergence and dissemination of antibiotic resistance.

Freshwater systems, such as rivers, lakes, and streams, are particularly susceptible to antibiotic contamination given their proximity to anthropogenic activities. These systems are continually inundated with antibiotics, ARBs, and ARGs from sources including wastewater treatment facilities, chemical production plants, and agricultural wastewater [52]. The presence of ARBs in these ecosystems has been reported even in remote and extreme locations, such as Eastern Siberia's permafrost and Antarctic freshwater lakes, implying that AMR spread is a global concern that goes beyond geographical boundaries [53].

Antibiotic pollution has a substantial effect on marine environments even though it differs from freshwater systems in the processes of AMR's development and transmission. ARBs from terrestrial sources are frequently carried by coastal runoff, which adds to the build-up of ARGs in marine environments [45]. The selection pressure for resistant bacteria is further increased by directly releasing antibiotic residues from operations like aquaculture into marine habitats. According to Miranda et al. [54], there is evidence that Chilean marine salmonid farms utilize a significant amount of antibiotics, which may be a contributing factor to the emergence of antibiotic-resistant marine bacteria. Given that

humans become exposed to marine ARGs through activities like swimming, fishing, and seafood consumption, the possibility of a bidirectional transfer of ARGs between human populations and marine environments cannot be ruled out [55].

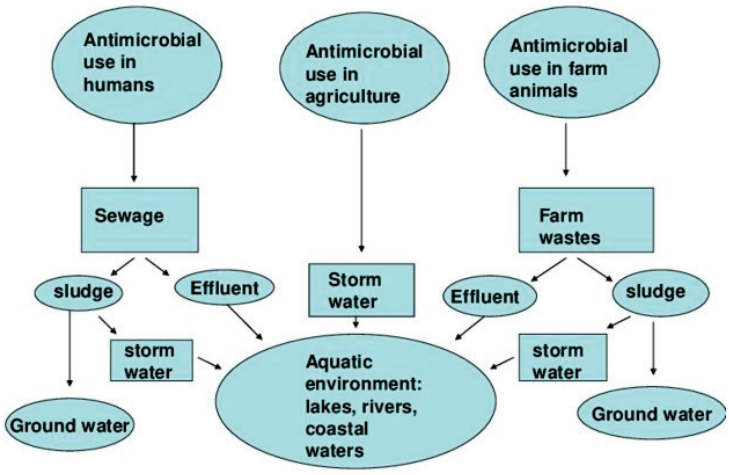

Figure 1. Pathways for the spread of antimicrobial residues and resistant bacteria in the aquatic environment [56].

2.3. Food Production Systems

The development of AMR and the application of antimicrobials in food production are closely linked. Subtherapeutic doses of antibiotics are frequently given to animals to enhance their growth and prevent diseases; this practice provides an ideal environment for the emergence of resistance strains. In the animal's gastrointestinal tract, these resistant bacteria can proliferate, persist, and can also pick up extra resistance genes through HGT, increasing their ability to withstand antibiotic treatments [57]. Cross-resistance emerges because the antibiotics used in human medicine are identical to those used in animals. As a result, these resistant bacteria represent a serious threat to public health when they infiltrate the environment or the food chain since they can result in diseases that are difficult to treat.

Similar to the emergence of AMR in the soil, one major factor contributing to AMR in food production systems is the extensive use of antibiotics in livestock husbandry and agriculture [58]. The magnitude of this problem is demonstrated by the fact that the amount of antibiotics employed in agriculture often exceeds the amount used in human treatment in several countries [59]. Introducing antibiotics to animal feed is a typical technique in intensive farming systems, where housing so many animals in close quarters increases the danger of disease transmission. The regular use of antibiotics in this way, however, is questionable since it contributes significantly to the development and spread of resistant bacteria that might infect humans. There are multiple routes via which resistance from animals can reach humans and promote AMR. Consuming animal products like meat, milk, or eggs that contain resistant bacteria is one of the primary routes [60]. These bacteria can survive cooking, especially if food is not properly cooked, and they can infect people with illnesses that are resistant to treatments. Direct interaction with animals or their natural environments is an important additional pathway, especially for those in the agricultural or animal husbandry businesses. In these environments, humans can come into close contact with animals or inhale dust particles, which can spread resistant bacteria [61].

Antimicrobial usage in food production is a complex topic that includes preventing and controlling the growth of bacteria in food items in addition to treating animal diseases. As already stated, antimicrobials are employed not just in livestock but also in aquaculture, agriculture, and other fields, which expands the application of AMR research. Antibiotics can be added to the surrounding water and cause the selection of bacteria that are resistant in aquatic environments. Antibiotics are used in aquaculture for the prevention and treatment of bacterial infections in fish. Similarly, using antimicrobials in crop production can

contaminate soil and water, which can lead to the spread of resistant bacteria. These actions have the combined effect of widely disseminating resistant bacteria throughout various ecosystems (Figure 2) which may eventually make their way into human populations through environmental exposure, water consumption, or food consumption [62].

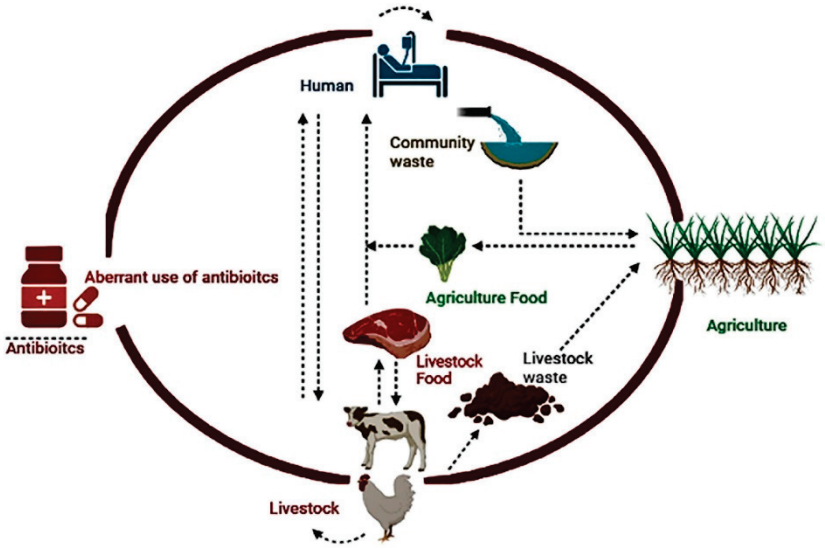

Figure 2. Pathways of antibiotics contamination of soil, water, and food and how they spread in animals and humans [63].

3. Implications of Environmental Antimicrobial Resistance on Food Safety

3.1. Transmission of Resistant Bacteria Through Food Chains

AMR bacteria and resistance genes can contaminate food in several ways which include cross-contamination during food handling, intentionally introducing bacteria such as probiotics or other bioconserving microorganisms which may carry resistance genes during food processing, and the use of antibiotics in agriculture [64]. Although bioconserving microorganisms which primarily are used to preserve and extend the shelf life of food products by inhibiting the growth of spoilage organisms and pathogens are of benefit to the food processing industry, they can pose a threat, especially in raw or minimally processed foods, which carry a higher risk because they can include live, resistant bacteria that, when consumed, might pass resistance genes to human bacteria [65]. Less processing can also keep stressed bacterial cells alive, which raises the possibility of resistance transfer even more.

3.1.1. Contamination During Food Processing and Handling

Food handlers are often linked to the spread of AMR bacteria as a result of cross-contamination from handling contaminated food or inadequate hygiene procedures [66]. This can lead to the dissemination of AMR bacteria across different food products, potentially reaching many consumers through various food channels which poses a significant threat to public health. Once introduced into the food supply, AMR bacteria can persist in the environment and spread through various means, including agricultural runoff and waste disposal, exacerbating the problem of AMR by contaminating additional food sources and contributing to the broader spread of resistance [67]. With the increase in demand for raw or minimally processed food, the chances for the proliferation of AMR bacteria have also increased as these foods, such as fresh fruits, vegetables and raw milk, may contain live, unstressed bacterial cells and are usually consumed without first going through any processing or preservation procedures [64]. This heightens the risk of AMR bacteria proliferation in the human population, making it more difficult to treat bacterial infections with

conventional antibiotics and potentially leading to increased health risks and challenges in managing resistant infections.

Usually, different food processing and preservation methods are applied to extend the shelf life of food or improve its taste or nutritional quality. However, these processes might affect bacterial flora differently, with some bacteria surviving these techniques without inhibited growth, while others may be inhibited, resulting in stressed or sub-lethally damaged cells [64,68]. Some of these bacteria that may survive these processes and continue to grow, may potentially include AMR bacteria, thereby contributing to the spread of resistance. Le Devendec et al. [69] evaluated the impact of heat treatment on AMR *E. coli* and found that they survived after a 60 °C incubation for 5 to 10 min while retaining their resistance genes. Although theoretically AMR genes surviving heat treatment and stomach acid could potentially transfer to other gut microbiota and contribute to AMR, there is currently no evidence in the literature showing that AMR genes from heat-treated or cooked food transfer to gut microbiota [70]. Non-thermal food processing methods like high pressure and ionizing radiation help to improve food safety by damaging microbial cell membranes without affecting nutritional value or taste; however, these techniques might release genetic material related to AMR, potentially transferring it to environments or bacteria that come into contact with the treated food, thereby exacerbating the spread of AMR within the food chain [66,71]. A study by Zarzecka et al. [68] examined how high-pressure processing affects antibiotic resistance in commercial starter culture strains. Results from the study found that high pressure reduced the expression of aminoglycoside resistance genes but increased the expression of tetracycline and chloramphenicol resistance genes, with variations depending on the pressure level applied, potentially leading to the spread of different resistance genes through the food chain.

3.1.2. Intentionally Introducing Microorganisms into Food as Supplementary Substances

The intentional introduction of microorganisms into food is a common practice in the food industry, usually aimed at enhancing food quality, safety, and nutritional value [72]. These microorganisms, often added as probiotics, bacteriophages, starter cultures, or biopreservatives, play crucial roles in fermentation, flavor development, and food preservation [64]. However, this practice can also contribute to the transmission of AMR within the food chain as these microorganisms may carry ARGs which can be transferred to other bacteria within the food chain through different mechanisms, such as horizontal gene transfer, conjugation, or selective pressure [64,73].

In various food processing contexts, supplementary substances such as growth promoters, preservatives, or additives can influence the microbial environment and potentially facilitate the transfer of AMR genes [74]. These substances might create conditions that either support the survival of resistant bacteria or promote their ability to exchange genetic material. For instance, substances like antibiotics used as preservatives or growth promoters in animal feed can exert selective pressure, allowing resistant bacteria to thrive and potentially transfer resistance genes through mechanisms like conjugation or horizontal gene transfer [75]. Similarly, certain preservatives or additives might affect the microbial community in food products in ways that enhance the survival of resistant strains, thus increasing the likelihood of gene transfer [74]. This was demonstrated in a study [76] where ampicillin resistance was transferred from a donor strain of *Salmonella* Typhimurium DT104 to a recipient strain of *Escherichia coli* K12. Similarly, Toomey et al. [77] demonstrated that lactic acid bacteria (LAB) such as *Enterococcus faecalis* and *Lactococcus lactis* can transfer resistance to pathogenic strains like *Listeria*, *Salmonella*, *Staphylococcus aureus*, and *E. coli* in fermented milk, emphasizing LAB's potential role as a source of resistance determinants. This suggests that while LAB is traditionally considered beneficial, its potential contribution to the spread of AMR poses a serious risk to public health, as resistant strains of pathogenic bacteria could emerge and proliferate, which threatens the safety of food products.

Most LAB colonize the intestines briefly, but probiotics can attach to intestinal epithelial cells, allowing them to colonize longer, thereby increasing the risk of horizontal

gene transfer of ARGs from these LAB to other gut microbiota, including pathogenic bacteria [78]. LAB, including species from *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus*, are widely used as probiotics and starter cultures due to their established safety [79]. However, despite their safety record, numerous *Lactobacillus* species have been reported to possess antibiotic resistance, both intrinsic and acquired, to various antibiotics such as aminoglycosides, tetracycline, and erythromycin [80–82]. Foods containing probiotics are widely consumed, and microorganisms present in large numbers in food or human intestines are more likely to transfer AMR genes. This risk is particularly relevant for probiotics and biopreservatives added in substantial amounts to food products, as these microorganisms can harbor and potentially transfer resistance genes to other bacteria in the gut [79]. Additionally, the starter cultures used in fermentation processes, which often proliferate extensively, can act as reservoirs for AMR genes, further amplifying the potential for gene transfer within the food chain [80]. The widespread consumption of such foods could inadvertently contribute to the dissemination of resistance genes, posing challenges for public health. Similarly, bacteriophages have been implicated as environmental reservoirs of resistance genes, although they can be employed to render foodborne pathogens and spoilage organisms inactive [64]. In their study, Colomer-Lluch et al. [83] investigated the occurrence of three β-lactam ARGs (*blaTEM*, *blaCTX-M9*, and *mecA*) in bacteriophage DNA from environmental water samples. Using quantitative PCR, these genes were found in all tested samples, with high gene copy numbers correlating with fecal pollution levels, except for *mecA*, which was more abundant in river water, suggesting that bacteriophages can act as reservoirs of AMR genes, facilitating the spread of resistance within the food chain through environmental contamination.

3.2. Risk Assessment of Resistant Bacteria in Food Products

Several health implications are associated with the presence of AMR bacteria in food products. According to estimates from the Centres for Disease Control and Prevention (CDC), over 35,000 people die from AMR-related infections every year in the United States alone, where at least 2.8 million individuals contract infections [84]. Infections resistant to antibiotics are also linked to higher rates of complications and mortality, longer hospital stays, and higher healthcare expenses. The prevalence of AMR bacteria in food products poses a significant public health risk, necessitating comprehensive risk assessment to mitigate potential outbreaks and protect consumer health [85]. Public health organizations, including the CDC, Food and Drug Administration (FDA), and World Health Organization WHO, are integral to the monitoring and intervention strategies related to AMR. Their initiatives encompass the surveillance of AMR trends, the formulation of guidelines aimed at reducing antibiotic usage in agricultural practices, and the encouragement of research focused on the pathways of AMR transmission. These agencies are pivotal in the orchestration of risk assessment frameworks that facilitate the monitoring of AMR throughout the food supply chain, thereby enabling the implementation of effective preventative measures.

For dairy cattle, there is limited information on the risk of AMR transmission to humans, but the main concern is the development of resistance in livestock gut bacteria and the potential transfer of AMR genes to zoonotic pathogens that can infect humans [86]. Several studies have investigated the role of food as an important vehicle in the transmission of resistant bacteria to the digestive tract of consumers. For instance, in a cross-sectional study by Beyene et al. [87], involving 193 samples from dairy farms and abattoirs, *Staphylococcus* species were isolated from 47.7% of the samples, with a higher prevalence in dairy farms (50%) compared to abattoirs (46.3%). Among the isolates, 95.3% exhibited resistance to penicillin-G, while 88.4% showed resistance to nalidixic acid. Notably, significant multidrug resistance was observed, particularly in *S. aureus*, where 73.3% were resistant to vancomycin, and all isolates displayed resistance to more than three drugs. A similar study conducted in Japan by Hiroi et al. [88] found that *Campylobacter coli* had a higher resistance compared to *Campylobacter jejuni* in retail meats and food-animal feces. Additionally, methicillin-resistant *Staphylococcus aureus* (MRSA) was identified in 3% of meat samples,

with no vancomycin-resistant enterococci detected. *S. aureus* showed high resistance to ampicillin and tetracycline, and identical resistance patterns were observed in *C. jejuni* from both a hospital patient and chicken samples, suggesting a potential link between the meat and the infections.

As meat consumption has risen, so has the demand for meat products due to their desirable sensory properties and efficient use of carcass parts unsuitable for fresh consumption [89]. The pork industry, in particular, has expanded significantly due to pork's high-quality, low-cost protein, even though pigs have become major reservoirs of AMR due to the misuse of antibiotics in their production process for the growth promotion or prophylactic treatment of diseases [90]. Bouchami et al. [91] carried out a study to determine if the pork production chain is a source of MRSA ST398 for human colonization and infection by analyzing samples from live pigs, meat, the environment, and slaughterhouse workers. They found that MRSA ST398, which is multidrug-resistant and carries genes for biocide resistance and enterotoxins, was present along the entire pork production chain and in workers, with evidence of cross-transmission between live pigs, meat, and workers. Similarly, Cao et al. [92] used samples from 21 volunteers as well as pig and poultry carcasses to confirm the hypothesis that foodborne bacteria transfer AMR genes to the human gut microbiota. They found that AMR genes that were resistant to several antimicrobial classes, including vancomycin, tetracycline, and macrolides, were present in both humans and food animals. To address this growing threat, a comprehensive risk assessment and mitigation approach is essential. Risk mitigation strategies play a pivotal role in controlling the spread of AMR within the meat industry. Adopting best practices necessitates the implementation of more stringent regulations regarding antibiotic usage, restricting its application solely to therapeutic purposes. Moreover, the enforcement of residue testing is vital to ensure compliance with these regulations. The establishment of comprehensive biosecurity measures, including the isolation of infected animals and the enhancement of hygiene standards within slaughterhouses, is critical for minimizing the risk of cross-contamination. Furthermore, augmented training for workers on safe handling and processing practices can significantly mitigate transmission risks, thereby protecting both employees and consumers alike. Surveillance and monitoring should be prioritized to track AMR bacteria throughout the meat production chain. Regular sampling and genetic analysis can help identify specific resistance genes and their potential for transfer between animals, food products, and humans; this is crucial for understanding the scope of the problem and implementing targeted interventions. Additionally, regulations must be enforced to limit the use of antibiotics in the meat production process, coupled with improved biosecurity measures and hygiene practices in slaughterhouses and processing plants to minimize the risk of cross-contamination. Public health interventions should focus on educating both workers and consumers about the risks associated with AMR and promoting safe handling, cooking, and consumption practices.

Outbreaks of Foodborne Illnesses Caused by Resistant Bacteria, Europe as a Case Study

The 2011 foodborne disease outbreak in Central Europe, particularly Germany, due to a highly resistant hybrid strain of O104:H4 STEC from contaminated sprouts serves as an important example of the threat AMR poses to the food and health sectors. This outbreak resulted in nearly 4000 infections, with over 900 cases of hemolytic uremic syndrome (HUS) and 54 deaths [93]. The strain was notable for its combination of genetic traits from Enteroaggregative *Escherichia coli* (EAEC) and its ability to produce Shiga toxin (*stx2a*), despite lacking other traditional STEC virulence markers like *eae* and *hlyA* [94]. Crucially, the strain also carried ARGs $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1}}$ on plasmids, which contributed to the difficulty in treating infections. The presence of these resistance genes heightened the severity of the outbreak, complicating treatment options and exacerbating the public health crisis [95]. This highlighted the need for increased vigilance regarding AMR spread from food.

In 2018–2019, the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) jointly analyzed data from 28 EU Member States

on AMR in zoonotic bacteria including *Salmonella* and *Campylobacter*, as well as indicator bacteria such as *Escherichia coli* and MRSA from humans, animals, and food [96]. In 2018, high levels of resistance were reported among human *Salmonella* isolates, with sulfonamides (30.5%), tetracyclines (28.8%), and ampicillin (25.9%) showing significant resistance. Resistance varied widely by serovar and country, with monophasic *S.* Typhimurium and *S.* Kentucky showing extremely high resistance, while *S.* Enteritidis showed lower resistance overall. Notable variations included higher resistance to ampicillin in Italy for *S.* Infantis and lower resistance in Malta for monophasic *S.* Typhimurium. Gentamicin resistance was generally low except for in *S.* Kentucky, and chloramphenicol resistance was low overall but moderate in *S.* Typhimurium. *Salmonella* spps. from human cases in 2019 are highly resistant to ampicillin, sulfonamides, and tetracyclines but have low resistance to third-generation cephalosporins like cefotaxime (1.8%) and ceftazimide (1.2%) [97]. These high resistance levels, especially to critical antibiotics like sulfonamides and tetracyclines, complicate treatment for patients infected with these resistant strains of *Salmonella* and other bacteria, leading to longer illness durations, increased healthcare costs, and higher morbidity and mortality rates. Following this trend, a more concerted global effort is needed to avert the "antibiotics catastrophe" that the world may face in the coming decades.

4. Public Health Impact and Human Health Risks of AMR

4.1. Global Landscape of AMR

The global landscape of AMR is one of increasing incidence and the burden of multidrug-resistant infections that are spreading out of control. AMR is the cause of 4.95 million deaths worldwide, with 1.27 million deaths directly related to resistant pathogens (including *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*), which are among the most alarming, according to the WHO priorities [98]. These organisms are resistant to many types of antibiotics such as fluoroquinolones or β-lactams, which are considered the mainstay of treatment. Since their resistance occurs more frequently and easily than that of other antibiotics, they pose an even greater public health threat in low- and middle-income countries (LMICs), where healthcare infrastructure is weaker. For instance, mortality rates are dramatically increased, and treatment is more complicated, in regions with a high prevalence of infections such as sub-Saharan Africa and South Asia [99].

Reducing the burden of AMR involves infection prevention, vaccination, and encouraging the use of appropriate antibiotics, and centers on high-burden regions. The spread of resistant bacteria from contaminated wastewater is another environmental culprit. The economic impact is immense, with AMR-related health expenditures and productivity losses exceeding USD 55 billion in the US [67]. AMR is a global problem, and reducing its burden requires coordinated efforts across the globe in increased surveillance, new drug development, and public health interventions to prevent this growing threat from worsening.

Table 1 provides an overview of the global landscape of AMR, summarizing key studies, statistics, and initiatives across various aspects of AMR. It includes details on the global burden and mortality caused by drug-resistant infections, highlighting the critical pathogens involved, such as *E. coli*, *S. aureus* and *K. pneumoniae*. The table also explores the impact of resistance to specific antibiotics, regional disparities in AMR burden, and the challenges faced in different healthcare settings and populations.

Table 1. Studies on human health risks associated with AMR infections.

Aspect of AMR	Summary of Studies	Key References
Antibiotic Use in Healthcare and Agriculture	Antibiotic consumption remains high globally, with variations across regions. In Europe, community consumption varies widely (11.3 to 31.9 DDD per 1000 inhabitants per day), while hospital use is substantial (2.0 DDD per 1000 inhabitants). In the US, outpatient use is prevalent (258 million courses annually).	[100]

Table 1. *Cont.*

Aspect of AMR	Summary of Studies	Key References
Factors Contributing to Resistance	Overuse and inappropriate use of antibiotics in both community and hospital settings drive resistance. Misconceptions and self-medication practices also contribute significantly to the problem, particularly in developing countries.	[101]
Case Studies: S. *aureus*, NTS, *K. pneumoniae*	Specific pathogens like MRSA, NTS, and ESBL-producing *K. pneumoniae* show high resistance rates globally, affecting treatment efficacy and healthcare outcomes.	[102]
Global Burden and Mortality	Drug-resistant infections in 2019 contributed to an estimated 4.95 million deaths globally, with 1.27 million directly attributable to AMR. AMR ranks as a leading global health concern, third in causes of death following ischemic heart disease and stroke in a hypothetical scenario without infections.	[103]
Environmental Spread of Resistance	Environmental factors, such as wastewater contamination, contribute to the spread of antibiotic-resistant bacteria, highlighting the need for environmental stewardship and surveillance.	[104,105]
Public Health and Economic Impact	Antibiotic-resistant infections in the US result in over 2 million illnesses and 23,000 deaths annually, with associated costs exceeding USD 55 billion, including direct healthcare expenses and lost productivity. In Europe, resistance contributes to 25,000 deaths annually, with estimated costs exceeding 1.5 billion euros.	[106]
Tuberculosis and Drug Resistance	MDR-TB and XDR-TB pose significant challenges to global TB control efforts, with high mortality rates and limited treatment options. The development of resistance is linked to treatment non-compliance and inadequate drug regimens.	[107]
Regional Disparities in AMR Burden	Low- and middle-income countries (LMICs), particularly in sub-Saharan Africa and South Asia, face higher AMR-related mortality rates. Factors include critical infections, inadequate healthcare infrastructure, and inappropriate antibiotic use.	[108]
Study for Monitoring AMR Trends (SMART)	Surveillance data are available through reports in scientific and medical journals, as well as freely available on hosts' websites (e.g., EARS-net).	[12]
Impact on Specific Populations and Settings	AMR significantly affects vulnerable populations (e.g., cancer patients, transplant recipients, neonates) and specialized settings (e.g., neonatal intensive care units, surgical wards), leading to increased morbidity and mortality.	[109]
Impact of Resistance to Specific Antibiotics	Resistance to fluoroquinolones and β-lactam antibiotics is prominent across various pathogens, contributing significantly to mortality rates and treatment challenges.	[110]
Challenges and Strategies	Intervention strategies include infection prevention, vaccination, reducing unnecessary antibiotic exposure, promoting appropriate antibiotic use, and developing new antibiotics. Crucial for regions with high AMR burdens and limited healthcare resources.	[111]
Pathogens Contributing to AMR	Six major pathogens (*E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *A. baumannii*, *P. aeruginosa*) are significant contributors to AMR burden. These pathogens are prioritized by WHO due to their resistance profiles and global impact.	[112]
UK Government Actions on AMR	The UK government has implemented a 20-year vision to control and contain AMR by 2040, supported by 5-year national action plans. Achievements include reduced antibiotic use in food-producing animals, improved surveillance systems, and new payment schemes for antibiotics on the NHS.	[113]

4.2. Public Health Impact of AMR

The public health impact of AMR is profound, affecting not only individual patients but also healthcare systems and societies at large.

4.2.1. Increased Morbidity and Mortality

AMR significantly contributes to higher morbidity and mortality rates worldwide. Infections caused by resistant pathogens are more difficult to treat, often requiring longer hospital stays and more intensive care. Pneumonia, tuberculosis, and sepsis—diseases that were once easily treated with antibiotics—now often result in death as bacteria grow increasingly resistant to drugs [114]. Over time, this threatens to reverse much of the progress medicine has made over the past century, turning even ordinary infections into potentially serious conditions. In the absence of antibiotics, even a minor infection could kill us, highlighting the need for new antimicrobial strategies worldwide.

4.2.2. Complications in Medical Procedures

AMR complicates a wide range of medical procedures and treatments. Modern medicine relies heavily on effective antibiotics for surgeries, cancer therapy, organ transplants, and the care of premature infants. The success of these procedures is threatened by the growing prevalence of resistant infections. For instance, patients undergoing chemotherapy or major surgery are at higher risk of infection, and without effective antibiotics, their chances of recovery diminish significantly [115].

4.2.3. Economic Burden

The economic impact of AMR is substantial. We have earlier established that treating resistant infections are more expensive due to the need for more complex and prolonged treatment regimens, including the use of newer, more expensive antibiotics. Extended hospital stays also increase healthcare costs for both patients and health systems. For instance, resistant infections in U.S. hospitals in 2014 have been associated with an additional USD 1383–1488 in treatment costs per infection, and AMR-related hospitalizations are often twice as long as those for non-resistant infections [8]. This cost amounted to a national treatment cost of approximately USD 2.2 billion in that year. The World Bank predicts that by 2050, the global economy could lose up to USD 100 trillion due to AMR [115]. In the United States alone, AMR adds an estimated USD 20 billion in direct healthcare costs annually, with additional costs due to lost productivity as high as USD 35 billion [116]. Developing countries, where healthcare resources are already limited, face even greater challenges in managing the economic burden of AMR.

4.2.4. Impact on Global Health Security

AMR poses a significant threat to global health security. The spread of resistant pathogens knows no borders, making it a global issue that requires coordinated international action. Outbreaks of resistant infections can lead to public health emergencies, as seen with multidrug-resistant tuberculosis (MDR-TB) and MRSA [117]. The emergence of "superbugs" that are resistant to multiple antibiotics further complicates the global health landscape. These pathogens can spread rapidly within communities and across countries, necessitating robust surveillance and response mechanisms to detect and contain outbreaks [118].

4.2.5. Reduced Effectiveness of Antibiotics

The overuse and misuse of antibiotics in humans, animals, and agriculture have accelerated the development of resistance. As a result, common antibiotics are losing their effectiveness, leading to a situation where even minor infections could become untreatable. This "post-antibiotic era" could turn back the clock on medical advancements, making routine infections deadly once again [119].

4.2.6. Impact on Vulnerable Populations

Certain populations are more vulnerable to the effects of AMR, including the elderly, young children, and immunocompromised individuals. These groups are more likely to require antibiotics and are at higher risk for resistant infections. In developing countries, where access to healthcare and antibiotics is often limited, the impact of AMR can be even more devastating, leading to higher mortality and morbidity rates [117].

4.3. Human Health Risks of AMR

Infections Caused by Resistant Bacteria and Challenges in Treatment and Increased Healthcare Costs

AMR can occur in various settings, including hospitals and communities, and through the consumption of contaminated food. Resistant bacteria can lead to severe illnesses such as urinary tract infections, respiratory infections, and bloodstream infections, among others. The presence of AMR in the environment ensures that these bacteria can persist and spread, increasing the likelihood of human exposure and causing subsequent infection [110].

Resistant bacteria, such as MRSA, ESBL-producing *E. coli*, and carbapenem-resistant *Pseudomonas aeruginosa*, complicate treatment efforts by limiting the effectiveness of standard antibiotics and often requiring the use of more expensive and less readily available alternatives. For example, respiratory infections caused by penicillin-resistant *Streptococcus pneumoniae* and multidrug-resistant *Mycobacterium tuberculosis* necessitate higher doses, longer treatment durations, and the use of second-line drugs, which are often more toxic and less effective [101].

The economic impact of treating infections caused by resistant bacteria is substantial. In addition to the need for more expensive medications and prolonged hospital stays, interventions such as intensive care and surgical procedures are also causes of increased hospital costs. For example, the treatment of MDR-TB and XDR-TB involves the long-term use of costly second-line antibiotics, significantly driving up overall healthcare expenses. Similarly, managing bloodstream infections caused by carbapenem-resistant *Klebsiella pneumoniae* requires extended ICU stays and high-cost antibiotics, contributing to increased treatment costs [106].

Table 2 outlines various infections caused by antibiotic-resistant bacteria, detailing the pathogens involved, types of resistance, and associated challenges and impacts. Respiratory infections, such as those caused by *Streptococcus pneumoniae*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*, show resistance to penicillin, multiple drugs, and carbapenems [120]. These infections result in increased morbidity and mortality, with limited treatment options and high costs. Urinary tract infections (UTIs) caused by pathogens like *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterococcus faecalis* exhibit resistance to β-lactamase, carbapenems, and vancomycin, leading to high recurrence rates and complications, particularly in healthcare settings and vulnerable populations [120].

Skin and soft tissue infections, often due to *Staphylococcus aureus* (including MRSA), *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, are challenging to treat and have high transmission rates, increasing treatment costs [121]. Gastrointestinal infections from *Salmonella* spp., *Clostridium difficile*, *Shigella* spp., and *Campylobacter jejuni* show resistance to fluoroquinolones, multiple drugs, and macrolides, causing severe diarrhea, colitis, and significant healthcare burdens [122]. Bloodstream infections, involving pathogens like *Enterococcus faecium*, *Klebsiella pneumoniae*, *Staphylococcus aureus* (including MRSA), and *Acinetobacter baumannii*, present high mortality rates and prolonged hospital stays due to vancomycin, carbapenem, methicillin, and multidrug resistance [120].

Sexually transmitted infections (STIs) caused by *Neisseria gonorrhoeae* and *Treponema pallidum* demonstrate growing resistance to cephalosporins, azithromycin, and penicillin, which limit treatment options and poses public health risks. Hospital-acquired infections, with pathogens like *Pseudomonas aeruginosa*, *Enterobacter* spp., *Acinetobacter baumannii*, and *Escherichia coli*, are difficult to control and lead to extended hospital stays and increased

healthcare costs due to carbapenem and multidrug resistance. Gastrointestinal infections from *Helicobacter pylori* and *Vibrio cholerae* exhibit resistance to clarithromycin and tetracycline, complicating treatment and increasing morbidity [123].

Central nervous system infections caused by *Neisseria meningitidis* and *Streptococcus pneumoniae* are resistant to penicillin and ciprofloxacin, resulting in high fatality rates and complex treatments. Lastly, bone and joint infections, typically due to *Staphylococcus aureus* (including MRSA) and *Pseudomonas aeruginosa*, require prolonged treatment and have high recurrence rates due to methicillin and carbapenem resistance. Overall, antibiotic-resistant infections across these categories significantly challenge healthcare systems, increase morbidity and mortality, and necessitate urgent action to mitigate their impacts [124].

Table 2. Infections caused by resistant bacteria, challenges in treatment, and increased healthcare costs.

Infection Category	Common Resistant Bacteria	Notable Antibiotic Resistance Types	Impact and Challenges
Respiratory Infections	*Streptococcus pneumonia*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa*	Penicillin resistance, MDR-TB, XDR-TB, and β-lactam resistance, and carbapenem resistance	Increased morbidity and mortality, limited treatment options, and high treatment costs
Urinary Tract Infections	*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterococcus faecalis*	ESBL-producing strains, carbapenem resistance, and vancomycin resistance	High recurrence rates, complicated infections in healthcare settings, and difficult management in vulnerable populations
Skin and Soft Tissue Infections	*Staphylococcus aureus (MRSA)*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*	Methicillin resistance, carbapenem resistance, and multidrug resistance	Difficult to treat, high transmission rates in community and hospital settings, and increased treatment costs
Gastrointestinal Infections	*Salmonella* spp., *Clostridium difficile*, *Shigella* spp., and *Campylobacter jejuni*	Fluoroquinolone resistance, multidrug resistance, and macrolide resistance	Severe diarrhea and colitis, high relapse rates, and increased healthcare burden
Bloodstream Infections	*Enterococcus faecium*, *Klebsiella pneumoniae*, *Staphylococcus aureus (MRSA)*, and *Acinetobacter baumannii*	Vancomycin resistance, carbapenem resistance, methicillin resistance, and multidrug resistance	High mortality rates, challenges in infection control, and prolonged hospital stays
Sexually Transmitted Infections	*Neisseria gonorrhoeae* and *Treponema pallidum*	Resistance to cephalosporins, resistance to azithromycin, and resistance to penicillin	Increasing prevalence, limited treatment options, and public health implications
Hospital-acquired Infections	*Pseudomonas aeruginosa*, *Enterobacter* spp., *Acinetobacter baumannii*, and *Escherichia coli*	Carbapenem resistance, multidrug resistance, and ESBL-producing strains	Prolonged hospital stays, increased healthcare costs, and difficult to control outbreaks
Gastrointestinal Infections	*Helicobacter pylori* and *Vibrio cholera*	Clarithromycin resistance and tetracycline resistance	Complicated treatment and increased morbidity
Central Nervous System Infections	*Neisseria meningitidis* and *Streptococcus pneumonia*	Penicillin resistance and ciprofloxacin resistance	High fatality rates and complicated treatment
Bone and Joint Infections	*Staphylococcus aureus (MRSA)* and *Pseudomonas aeruginosa*	Methicillin resistance and carbapenem resistance	Prolonged treatment and high recurrence rates

Sources: [101,120,124].

4.4. Public Health Policies Addressing AMR

Public health policies and regulations play a pivotal role in controlling and mitigating the impact of AMR. These policies may include guidelines for the prudent use of antibiotics in both human and veterinary medicine, regulations on the discharge of pharmaceuticals

and other contaminants into the environment, and standards for infection prevention and control in healthcare settings. Public health regulations also support the development and implementation of national and international action plans to combat AMR [125]. These plans often emphasize the importance of a One Health approach, which recognizes the interconnectedness of human, animal, and environmental health in addressing the AMR challenge.

4.4.1. Antimicrobial Stewardship

- Regulations often include guidelines for the appropriate use of antibiotics to prevent overuse and misuse, which are primary drivers of resistance. This includes promoting the prescription of antibiotics only when necessary and ensuring the correct dosages and duration of treatments [126,127].
- Stewardship programs are implemented in healthcare settings to educate healthcare providers and patients about the responsible use of antimicrobials [128].

4.4.2. Infection Prevention and Control

- Policies emphasize the importance of infection prevention and control measures in healthcare facilities, such as hygiene practices, sterilization procedures, and isolation protocols for patients with resistant infections [115,129].
- Vaccination programs are also promoted to reduce the incidence of infections that might otherwise require antibiotic treatment [130].

4.4.3. Research and Development

- Public health regulations support research into new antibiotics, alternative treatments, and rapid diagnostic tools. This is critical for staying ahead of evolving resistant strains and ensuring that effective treatments remain available [131].
- Incentives for pharmaceutical companies to develop new antimicrobials and support for public-private partnerships are often part of these strategies [126].

4.4.4. Education and Awareness

- Increasing awareness about AMR among healthcare professionals, policymakers, and the public is a key component of most AMR policies. Educational campaigns aim to inform about the dangers of AMR and the importance of prudent antimicrobial use [114,119].
- Training programs for healthcare providers ensure that they are equipped with the knowledge and tools to combat AMR effectively [128].

4.4.5. Surveillance, Monitoring, and Reporting Systems

Surveillance and reporting systems for AMR are essential tools in the global fight against antibiotic-resistant infections. These systems provide crucial data on the prevalence, spread, and trends of resistant pathogens, enabling timely and informed public health responses. For instance, data collected through these systems have directly influenced policy decisions, such as the implementation of targeted antibiotic stewardship programs and the allocation of resources for infection control measures [106]. The WHO has established the Global Antimicrobial Resistance and Use Surveillance System (GLASS), which standardizes data collection and reporting across participating countries. The GLASS monitors resistance in human and veterinary pathogens, allowing for a comprehensive view of AMR trends and facilitating global comparisons [103]. In Europe, the European Antimicrobial Resistance Surveillance Network (EARS-Net) plays a pivotal role. It collects, analyses, and disseminates data on AMR from clinical samples across member countries. Looking forward, improvements in AMR surveillance could include enhanced international collaboration, such as joint data-sharing initiatives, and advancements in technology, like the integration of artificial intelligence for real-time data analysis. This network helps identify emerging resistance patterns and informs public health strategies at both national and international

levels [102]. In the United States, the National Antimicrobial Resistance Monitoring System (NARMS) tracks changes in the susceptibility of intestinal bacteria found in ill people, retail meats, and food animals. This tripartite system, involving the CDC, FDA, and United States Department of Agriculture (USDA), provides critical information for risk assessments and policy decisions aimed at curbing AMR [132].

The Study for Monitoring Antimicrobial Resistance Trends (SMART) is another significant initiative. The SMART collects data through scientific and medical journals and public databases like EARS-Net. It focuses on temporal and spatial trends in AMR, offering valuable insights into local, national, and international resistance dynamics [12]. These surveillance systems are supported by diagnostic advancements, such as the development of commercial laboratory kits for phenotypic and molecular testing. These kits enable the rapid and accurate detection of resistant bacteria and resistance markers, enhancing the efficiency of surveillance efforts [108]. Public health campaigns, such as World Antimicrobial Awareness Week (WAAW), further bolster these efforts by raising awareness and promoting best practices in antibiotic use. These campaigns have been shown to significantly increase public awareness and encourage responsible antibiotic use, evidenced by measurable outcomes such as reduced antibiotic prescriptions in participating regions. Initiatives like the British Society for Antimicrobial Chemotherapy's "Stop Superbugs" campaign and the CDC's "Combating Antimicrobial Resistance" portfolio provide resources and guidance to support public awareness and health literacy [106].

Effective AMR policies prioritize robust surveillance systems to monitor the prevalence and spread of antimicrobial-resistant infections. This data collection helps in identifying trends, outbreaks, and high-risk areas, enabling timely and targeted interventions [84,117]. Monitoring systems are also essential for tracking antimicrobial use in both human and veterinary medicine, ensuring that usage patterns do not contribute to the development of resistance [133].

4.4.6. Regulatory Frameworks

- Governments and international bodies establish regulatory frameworks to control the sale and distribution of antibiotics. This includes restricting over-the-counter sales of antibiotics and enforcing prescription-only policies [133,134].
- Regulations also encompass the agricultural sector, limiting the use of antibiotics in livestock and promoting the use of alternatives to enhance animal health and productivity without contributing to resistance [135].

4.5. Importance of AMR Policies and Regulations

4.5.1. Protecting Public Health

By curbing the spread of resistant infections, these policies help maintain the efficacy of existing antibiotics, ensuring they remain effective for treating infections [114]. They reduce the burden of healthcare-associated infections, lowering morbidity and mortality rates [126].

4.5.2. Promoting Sustainable Healthcare

Effective AMR policies contribute to the sustainability of healthcare systems by preventing the escalation of treatment costs associated with resistant infections [115,127]. They support the development of new antimicrobial agents and treatments, ensuring a continuous pipeline of effective therapeutic options [136].

4.5.3. Enhancing Global Health Security

Coordinated international efforts to combat AMR enhance global health security by preventing the cross-border spread of resistant pathogens [114,118]. These efforts support the resilience of healthcare systems worldwide, improving their ability to respond to AMR-related challenges and emergencies [133].

4.5.4. Encouraging Responsible Practices

Policies and regulations encourage responsible antibiotic use in both human and veterinary medicine, preserving the effectiveness of these vital drugs for future generations [137]. They promote best practices in infection control and prevention, reducing the incidence and spread of infections [129].

Global and national policies have been established globally to combat AMR through a diverse range of strategies, underscoring the critical importance of responsible antibiotic utilization, vigilant surveillance, comprehensive research, and effective infection control measures. Prominent international frameworks include the WHO Global Action Plan [114], which delineates a holistic approach to address AMR through enhanced awareness, robust surveillance systems, and the promotion of responsible antibiotic practices. Similarly, the European Union's Action Plan on AMR [133] adopts a "One Health" paradigm to enhance surveillance and research initiatives throughout Europe.

At the national level, various countries have crafted specific action plans to mitigate AMR. For instance, the United States National Action Plan for Combating Antibiotic-Resistant Bacteria [127] prioritizes surveillance, stewardship, and research efforts within its healthcare system. Australia's Antimicrobial Resistance Strategy (2015–2019) similarly emphasizes the necessity of infection control and stewardship [128].

Regional initiatives further bolster these efforts, as demonstrated by Canada's Pan-Canadian Framework [138] and Japan's National Action Plan [134], each tailored to address AMR through innovative surveillance and targeted measures. Additionally, collaborative endeavors such as the Global Antibiotic Research and Development Partnership [131] and the Global AMR Research and Development Hub [139] are dedicated to advancing the development of novel treatments for resistant infections on a global platform.

Countries as diverse as China [137], India [130], South Africa [135], and Thailand [140] have formulated national action plans that align with the WHO framework, implementing strategies meticulously tailored to their unique health infrastructures. The WHO AWaRe Classification [141] further supports the promotion of judicious antibiotic use worldwide by categorizing antibiotics in a manner that optimizes stewardship practices. Collectively, these policies signify a concerted international endeavor to alleviate AMR through integrated strategies that span the human, animal, and environmental health sectors.

5. Combatting AMR Through Innovations in Antibiotic Development and Strategic Enhancements

5.1. Mechanisms of Resistance

A fundamental aspect of developing new antibiotics is understanding the mechanisms by which bacteria resist current treatments. Bacterial resistance arises through several sophisticated mechanisms: enzymes that break down drugs, mutations that alter drug targets, efflux pumps that expel drugs from cells, and the formation of biofilms that shield bacteria from antibiotics [142,143]. To design effective new antibiotics, it is crucial to identify bacterial targets that are essential for the bacteria's survival and less prone to resistance. This helps in targeting vital components of bacterial physiology while avoiding mechanisms that are likely to evolve resistance. Advances in genomics and proteomics have made it possible to uncover these targets with greater precision, laying the groundwork for novel antibiotic development [144]. The phenomenon of bacterial resistance to antibiotics is a multifaceted issue, characterized by several strategies that bacteria employ to evade the effects of these drugs. Understanding these mechanisms is vital when developing new antibiotics or improving existing treatments.

One of the primary ways bacteria resist antibiotics is through modifying the structure of antibiotic molecules. Bacteria can produce specific enzymes that chemically alter an antibiotic, thereby diminishing its ability to function effectively. For instance, aminoglycosides, which target bacterial ribosomes, often fall victim to such modifications. Enzymes like acetyltransferases, adenyltransferases, and phosphotransferases attach chemical groups to the antibiotic, changing its structure and preventing it from binding to its target, thus

reducing its antibacterial potency [145]. Another significant resistance mechanism is limiting the penetration of antibiotics into bacterial cells. This is particularly prevalent in Gram-negative bacteria, which can alter their outer membrane to decrease their permeability [146]. By reducing the expression of porins—channels that allow antibiotics to enter the cell—bacteria can effectively prevent drugs like β-lactams and fluoroquinolones from reaching their targets within the cell, thereby reducing their efficacy [147].

Bacteria also utilize efflux pumps to resist antibiotics. These pumps are proteins embedded in the bacterial cell membrane that actively expel antibiotics from the cell, keeping the intracellular concentration of the drug low enough to avoid lethal effects [148]. Efflux pumps can be either highly specific to certain antibiotics or broad-spectrum, affecting multiple drug classes. This mechanism is a common cause of resistance to drugs like tetracyclines, macrolides, and fluoroquinolones, and the genes responsible for these pumps are often found on mobile genetic elements or integrated into the bacterial genome [149]. In addition to these strategies, bacteria can interfere with antibiotic action by altering or protecting their target sites. For example, mutations in the bacterial ribosomal RNA or associated proteins can prevent antibiotics from binding effectively, thereby neutralizing their intended effects [150]. Some bacteria produce proteins that shield these target sites from antibiotics. This protection mechanism is observed with tetracycline resistance proteins like Tet(M) and Tet(O), which dislodge the antibiotic from its binding site on the ribosome [151]. Similar protective mechanisms have been noted for resistance against fluoroquinolones and fusidic acid [151].

Mutations in the target sites of antibiotics are another common resistance strategy. These genetic changes can alter the structure of the target site, making it less susceptible to antibiotic binding. A well-known example is resistance to rifampin, a critical drug for tuberculosis treatment, which arises from mutations in the gene encoding RNA polymerase [152]. These mutations change the drug's binding site, reducing its ability to inhibit the bacterial enzyme, and further contributing to bacterial resistance. Some bacteria acquire enzymes that chemically modify their target sites, reducing the binding affinity of antibiotics. For example, the methylation of ribosomal RNA by enzymes encoded by the erm genes results in resistance to macrolides [153]. Additionally, the chloramphenicol–florfenicol resistance (*cfr*) gene confers resistance to multiple antibiotic classes by methylating the antibiotic binding site on the bacterial ribosome [154].

Finally, bacteria can resist antibiotics by replacing or bypassing the target sites. In some cases, bacteria evolve alternative target sites that perform the same function but are not affected by the antibiotic. MRSA is a classic example, as it has acquired an alternative penicillin-binding protein that is not inhibited by methicillin [155]. Bacteria can also bypass the metabolic pathways that antibiotics target, as seen in resistance to trimethoprim-sulfamethoxazole, where overproduction of the enzyme dihydropteroic acid synthase allows the bacteria to survive despite the drug's presence [156].

5.2. Design and Synthesis of New Antibiotics Against Resistant Microorganisms

In the face of the growing crisis of antibiotic resistance, the design and synthesis of new antibiotics have become a critical imperative for modern medicine [157]. As bacteria evolve and develop resistance to existing drugs, the need for novel antibiotics capable of overcoming these resistance mechanisms has never been more urgent [158]. The increasing challenge of antibiotic resistance, worsened by misuse, has driven the need for new antimicrobial agents. The World Health Organization highlights the urgent need for new antibiotics to target high-risk bacteria with limited treatment options [159]. Despite the critical need for new antibiotics, the past few decades have seen a stark decline in novel drug discoveries. This stagnation is partly due to pharmaceutical companies withdrawing from antibiotic research, driven by low financial returns, high costs, and the rapid development of resistance. High-throughput screening (HTS), a prevalent method for drug discovery, has proven ineffective in finding new antibiotics, with many large-scale efforts failing to yield viable candidates [160].

The creation of new antibiotics is essential in addressing the challenge of drug-resistant bacteria. Notable examples include Teixobactin, identified in 2015, which combats Gram-positive bacteria by disrupting their cell walls, reducing the likelihood of resistance. Lefamulin, approved in 2019, is used to treat drug-resistant Streptococcus pneumoniae by blocking protein synthesis. Zoliflodacin, currently being researched for resistant gonorrhea, interferes with DNA replication. Cefiderocol, a siderophore cephalosporin, is particularly effective against drug-resistant Gram-negative bacteria, including those resistant to carbapenems, by disrupting cell walls and enhancing iron uptake. These innovative drugs provide alternative treatments for persistent infections [161]. Although developing new antimicrobial agents is crucial in addressing AMR, the scientific community seems to lag in the rapid evolution of MDR bacteria. While discovering entirely new drugs is a logical strategy, the time and resources required are substantial [162].

One promising area is the rational design of antibiotics, which involves the use of computational models. Computational methods are increasingly essential for developing new antibacterial agents by modelling how drug modifications can enhance their effectiveness or combat resistance. In-silico modelling uses computer simulations to explore drug interactions and predict new candidates, such as antimicrobial peptides (AMPs) and natural compounds with potential antibacterial properties [163]. Fragment-Based Drug Design (FBDD) starts with small molecular fragments to create potent inhibitors for various targets [164]. For instance, β-lactam antibiotics are often rendered ineffective by enzymes like New Delhi Metallo-β-lactamase-1 (NDM-1), making it essential to develop new inhibitors. FBDD was used to identify potential NDM-1 inhibitors through the virtual screening of over 700,000 compounds, fragmenting the hits, and then testing them with NMR. The method identified 37 promising fragments, leading to the synthesis of Indenone 89, which effectively inhibited NDM-1 with a Ki value of 4 μM [165].

Furthermore, the exploration of novel antibiotic classes, such as AMPs, has gained attention. AMPs are small, diverse proteins either naturally found in various organisms or synthetically produced, known for their ability to fight bacteria, viruses, and fungi and modulate the immune system. They hold significant potential in tackling MDR bacteria due to their strong, fast-acting, and low-resistance nature [166]. Key examples include the human cathelicidin peptide LL-37 and colistin-based derivatives, both demonstrating effective antimicrobial properties [167]. However, AMPs face challenges such as their susceptibility to degradation in the body, thereby affecting their stability. Innovations like selectively targeted AMPs (STAMPs) are being developed to improve their targeting and effectiveness; however, further research is necessary to fully utilize them [168].

Another cutting-edge approach is the synthesis of hybrid antibiotics that combine the pharmacophores of two different antimicrobial agents. Recent efforts in antibiotic development emphasize hybrid antibiotics, Antimicrobial Peptide-Based Combinatorial Treatments (AMP-based combinations), and Metal Nanoparticle-Based Combinatorial Treatments (MNP-based therapies). Hybrid antibiotics aim to combat resistance by targeting multiple bacterial pathways simultaneously. Combining AMPs with antibiotics enhances their effectiveness and allows for lower dosages. Likewise, MNPs, particularly those based on silver, have been found to significantly boost the efficacy of existing antibiotics, especially against resistant bacteria. These combined approaches offer promising strategies for creating new antibacterial treatments to address the growing issue of antibiotic resistance [169]. For example, fluoroquinolones, a type of quinolone derivative, have become the most frequently prescribed antibiotics for treating a wide range of bacterial infections. Combining the quinolone structure with other antibacterial elements allows these hybrid compounds to target multiple drug mechanisms, helping to counteract resistance. As a result, quinolone hybrids serve as valuable models in the fight against drug-resistant pathogens [170].

5.3. Strategies to Enhance Efficacy and Reduce Resistance

Enhancing the efficacy of existing antibiotics and reducing the potential for resistance development are essential components of the fight against AMR. Several strategies have been proposed and are being actively investigated. These approaches include modifying the chemical structure of current antibiotics, employing combination therapies, utilizing drug-adjuvant pairings, developing new antibiotic derivatives, and exploring alternative treatments beyond traditional antibiotics.

One effective method to combat antibiotic resistance is by altering the chemical structure of existing antibiotics to enhance their potency against resistant bacteria. A prime example of this is the modification of vancomycin, which has been a reliable treatment against Gram-positive bacteria for nearly 60 years [171]. However, the emergence of resistance, such as in Vancomycin-Resistant Enterococcus (VRE), prompted researchers to modify vancomycin's structure. These modifications have not only increased its effectiveness by 6000 times against resistant strains but also introduced new mechanisms of bacterial destruction, illustrating the power of chemical alterations in overcoming resistance [172].

Another essential tactic is the use of combination antibiotic therapies, which involves the simultaneous application of multiple antibiotics to reinstate bacterial susceptibility and enhance treatment success. This strategy can function through various mechanisms, such as targeting different bacterial pathways or attacking the same bacterial component through different mechanisms [173]. For instance, combination therapy has been successfully used in treating diseases like HIV/AIDS, malaria, and tuberculosis, significantly improving treatment outcomes. Additionally, sequential dose regimens, where antibiotics are alternated over time, can increase bacterial sensitivity. However, this approach requires careful monitoring to prevent adverse drug interactions that might reduce efficacy or cause toxicity [174].

The combination of drugs with adjuvants—molecules that enhance antibiotic activity—is another promising approach. Adjuvants themselves do not kill bacteria but can significantly boost the effectiveness of antibiotics when used together [175]. A well-known example is Augmentin®, a combination of amoxicillin and clavulanic acid, where clavulanic acid inhibits enzymes that would otherwise render amoxicillin ineffective. This combination has successfully delayed the development of resistance, and ongoing research aims to discover new adjuvants, such as avibactam, which has shown effectiveness against resistant bacteria like Pseudomonas aeruginosa [176].

In addition to these strategies, the development of new antibiotic derivatives, particularly aminoglycosides, plays a crucial role in addressing resistance. Aminoglycosides, including streptomycin, have been effective against a wide range of bacteria, but resistance due to aminoglycoside-modifying enzymes (AMEs) has become a significant problem [177]. To combat this, new aminoglycosides like plazomicin have been developed. Plazomicin has shown superior activity against MDR Gram-negative bacteria and MRSA, highlighting the importance of creating new antibiotics to tackle resistance [178].

6. Environmental and Ecological Perspectives

6.1. Environmental Reservoirs of Resistant Genes

The acquisition and transmission of AMR is primarily facilitated by certain reservoirs and hotspots of ARGs and ARB. As a result, these favorable environments hasten the process of AMR development and effectively support its dissemination. Recent reports have implicated wastewater and sludge from clinical settings, such as hospital sewage discharges, wastewater treatment plants (WWTP), pharmaceutical industries, and animal production sites in increasing the likelihood of human exposure through direct inhalation, ingestion, or consumption of contaminated food, or water, posing significant public health risks [179,180].

Clinical settings have a relatively high level of antibiotic consumption which has been impacted by the COVID-19 pandemic and are considered AMR hotspots. Ruzsa et al. [181] reported a 397% increase in the consumption of azithromycin between 2019

and 2020 in Hungary, with an expected high level of antibiotic effluents. Furthermore, hospital effluents are rich in patient-derived microorganisms that readily acquire ARGs and pose further danger [182]. Chng et al. [183] studied the colonization patterns of resistomes and the genomic characterization of AMR cassettes in a tertiary-care hospital. Their findings indicated persistence of MDR bacteria for extended periods which aim to opportunistically infect patients. A significant portion of the antibiotics within therapeutic doses are released into the environment through the improper disposal of medical and pharmaceutical important waste products, which facilitates the entry of antibiotic residues into the soil and groundwater. Siri et al. [184] investigated the prevalence of MDR bacteria in hospital wastewater and its receiving waters. The results from their analysis of untreated wastewater, aeration tanks, sedimentation tanks, effluent after disinfection, upstream canals, and downstream canals indicated a relatively high level of resistance after treatment, with an overall MDR rate of 33.3%. Hospital wastewater is an important wastewater hotspot for ARGs, and recent research has demonstrated that the patterns of ARGs in wastewater resemble those found in clinical settings [185]. Wastewaters are usually subjected to treatment plants in most areas; however, this has proven insufficient in the complete elimination of ARB and ARGs and have been implicated in the dissemination of antibiotic parent compounds and metabolites into receiving waters [186,187].

High concentrations of ARGs have been reported in livestock and poultry farm sites, which further intensifies the levels ARGs in surface water and soil [188]. A recent meta-analysis study of 460 studies showed that animal production sites are reservoirs of ARGs with therapeutic potential [189]. Additionally, slaughterhouses have been reported as a major hotspot for the development, progression, and transmission of AMR pathogens [190]. The quantification of ARGs and mobile genetic elements (MGEs) in slaughterhouse wastes in Al-Dewanyiah province, Iraq indicated that the waste from livestock serves as a potential hotspot for AMR in the environment [191]. Furthermore, manure from antibiotic-consuming animals, irrigation water, and soil in animal production sites are considered hotspots due to the high consumption of antibiotics in these areas. A study on the antibiotic resistance profile of *E. coli* isolates from lettuce, poultry manure, irrigation water, and soil in Ghana indicated high levels of resistance among isolates. Subsequently, microorganisms associated with farm animals develop AMR through constant exposure to antimicrobial residues and further spread of resistance through the transfer of ARGs.

Recent studies have reported high levels of ARGs in the soil. Gekenidis et al. [192] reported the contribution of tap water and surface water on overhead-irrigated chive plants from planting to harvest. Findings showed that water quality affects the quality of irrigated produce, as high levels of ARGs were detected in both water sources. Similarly, Kampouris et al. [193] reported a correlation between the relative abundance of ARGs and treated wastewater irrigation intensity which decreased during irrigation breaks and showed a high spread of ARGs during treated wastewater irrigation, relative to freshwater irrigation. These reservoirs and hotspots act not only as incubators for the emergence of AMR but also as transmission routes for the transfer of ARB and ARGs. Certain ecological factors influence the persistence and spread of AMR in the environment, which affects the amount, composition, and durability of ARB and ARGs in the environment.

6.1.1. Climate

High temperatures have been linked with increased levels of AMR persistence. A recent study on AMR in river biofilms explored the effect of temperature on naturally occurring resistomes in the river. The results showed that higher temperatures increased the abundance of naturally occurring ARGs [194]. Again, Van Eldijk et al. [195] reported that environmental factors such as a small change in temperature can affect the mutation rate towards antibiotic resistance. Extending the scale and depth of monitoring, Li et al. [196] estimated that every 1 °C increase in average ambient temperature was associated with a 2.71% increase in the prevalence of *E. coli* resistance to ceftriaxone and cefotaxime, but

persuasively concluded that, in addition to temperature levels, socioeconomic factors play supportive roles in this positive correlation.

Precipitation relates to a high level of relative humidity, which is concomitant with increments in ARGs abundances. Rainfall promotes conditions conducive for the horizontal transfer of ARGs [197]. Molecular evidence from a study conducted by Zahra et al. [198] on the prediction and interpretation of the prevalence of ARGs in beaches affected by urban wastewater discharge showed that water temperature, precipitation, and tide are relevant predictors of the abundance of ARGs at beaches. Overall, reports on the frequencies of ARGs during winter and summer indicated a significant influence of seasonality on the abundance of ARGs [199].

These results indicate a significant positive correlation between climatic factors and the occurrence of ARGs and ARB, which aligns with reports on the effect of sunlight on the abundance of ARGs and ARB. Azuma and Hayashi [200] reported that the irradiation associated with exposure to sunlight was found to inactivate a large group of target ARB after 5 h of exposure. Upon exposure to sunlight, certain antibiotics may deplete, which includes improperly disposed antibiotics or constituents of wastewater that contain metabolites which may cause mutations and a rise of AMR in the surrounding microbiome [201]. However, environmental factors that result in the death and decomposition of bacteria allow the release and spread of ARGs. Additionally, ARGs in the atmosphere are affected by wind speed, wind direction, and the height of the mixing layer, which play a significant role in their persistence and transport [202].

6.1.2. Habitats

ARB and ARGs move through habitats and must overcome resilient barriers to develop niches and thrive. The abundance of ARGs across different habitats is based on varying habitat determinants. Consequently, different habitats pose different strategic barrier methods. Mostly, autochthonous communities in certain habitats with higher diversity, richness, and evenness may develop diversity-based resilience against ARG migration, which could have a significant impact on the likelihood of ARGs being horizontally transferred to the endemic microbiota [203]. Han et al. [204] assessed the behavior of various ARGs in paddy soil during the rice growth period. Results showed that the abundance of ARGs in non-flooded soil exceeded that of flooded soil during rice growth (decreased by 33.4%). Furthermore, the study demonstrated an effective reduction in the proliferation and dissemination of most ARGs in paddy fields via the dry–wet alternation in soil, indicating that a short break of dryness may reduce the incidence of ARGs transmission. Xu et al. [34] analyzed 291,870 records on the abundance of 290 ARGs from five types of habitats between 2013 and 2020. A statistically significant difference was observed in the persistence of ARGs in different habitats, with the sediment habitat accruing 43.64% and 32.40% of multidrug and aminoglycoside resistance genes, respectively. Additionally, a study of the ARGs in the surface water, sediment, and biofilm in a wastewater-impacted river in southern Germany showed that biofilm samples presented a relatively higher abundance of ARGs [205]. In another study, a notable pattern of resistome coalescence was observed within similar habitat types among the five habitats evaluated for ARGs [206].

Soil structure, high altitude, and water stratification have also been reported to significantly influence the presence, transport, and persistence of ARGs within an environment. A recent metagenomic analysis showed that conservation tillage, which decreases soil disturbance and improves its physical and chemical properties relative to conventional tillage practices, showed a decreased abundance of ARGs [207]. Consequently, soil structure and porosity determine the movement of pollutants, which alters the persistence of ARGs in the soil. Relative to altitude, high-altitude lakes and low-altitude lakes in Mountain Sangguniang in the Eastern Tibetan Plateau were evaluated for the abundance of ARGs; an abundance of ARGs was observed with high-altitude lakes, indicating atmosphere-mediated organic pollutant enrichment driven by the Indian monsoon due to elevation [208].

6.1.3. Environmental Contaminants

Environmental pollutants have a discernible role in the rapid development of resistance in pathogens. Heavy metals may occur as a result of anthropogenic wastes and are inherent constituents of the earth's crust that have significantly been linked with the persistence of ARGs and ARB. Komijani et al. [209] reported ARG abundance in lakes and wetlands in Iran and a strong correlation between the concentration of heavy metals, such as vanadium, with that of antibiotics. Similarly, Edet et al. [210] reported a high level of co-existence between MDR bacteria and heavy metals present in dumpsites. The coexistence between antibiotics and heavy metals creates a synergistic relationship that amplifies the abundance of ARGs in the environment by protein expression [49]. Pesticides and herbicide deposits in soils have also been implicated in the increasing levels of ARGs in soil. Liao et al. [211] showed that glyphosate, glufosinate, and dicamba used as herbicides increased cell membrane permeability and frequency in conjugation of MGEs, which facilitates an ease in the uptake of ARGs among bacteria. On the same scale, co-contamination of topramezone and polymyxin reportedly resulted in elevated levels of MGEs and ARGs [212], and fungicides have been shown to alter the microflora within the gut of soil animals and enrich ARGs [213]. Additionally, molecular evidence shows that soils contaminated with carbon-rich compounds and microplastics affect the persistence of ARGs and ARB. Maurya et al. [214] reported the abundance of ARGs related to tetracycline, sulfonamides, aminoglycosides, ampicillin, and fluoroquinolone resistance in polyaromatic hydrocarbon (PAH)-contaminated environments. Biochar serves as an enabler of ARG transfer [215]. Microplastics serve as a colonization surface for most resistomes in river microbiomes. These microenvironments seemingly absorb toxic pollutants and further assist the transport of ARGs [216,217] and promotes environmental selection pressure [218].

6.2. Impact of Human Activities

Anthropogenic activities act as a significant driver in the increasing burden of AMR. These activities include the excessive and indiscriminate consumption of antibiotics, inappropriate disposal of antimicrobials, prophylactic use of antibiotics in food production, pollution, and poor waste management, which are disproportionately higher in low- and middle-income countries and allow the seepage of ARGs into the soil and surface and ground waters, facilitating their transmission through the food-supply chain and posing serious public health risks [219].

The prophylactic use of antibiotics in agriculture has raised serious concerns in recent times due to the increased purchase of antibiotics from local stores to feed animals without clinical signs of diseases. In the 1950s, the rising demand for food put pressure on the food industry, resulting in the use of antibiotics in food animals to boost production for economic gains. Although this practice was banned in some countries in the 2000s, farmers continue to use antibiotics indiscriminately for prophylactic purposes and as growth promoters to meet the high demand for agricultural products and profit goals. This ongoing use has resulted in the presence of resistomes in river reservoirs of resistance. In various regions of Africa, the use of antibiotics in livestock has been frequently reported. A recent study in Kenya reported that 92.7% of 175 cattle farms primarily used antibiotics for prophylactic purposes [220]. Similar trends have been reported across most low- and middle-income countries [221–223]. Evidence from current research suggests that more antibiotics are administered by farmers, majorly as prophylaxis, than for any other reason [223–225]. The continuous use of antibiotics in sub-therapeutic doses as growth promoters, feed enhancers, and prophylaxis has been reported as a significant contributor to the increasing levels of AMR [226]. Again, to increase the shelf life of animal products, antibiotics are being added directly to raw milk by vendors In Ethiopia [227]. Studies have further traced the 'silent pandemic' to the limited awareness and degree of misunderstanding about the problem of indiscriminate antibiotic use, AMR, and its consequences on health and economy among farmers [228,229]. ARGs and ARB extend into the broader environment through fertilizers made from manure, contaminating waterways and food produce that

facilitate direct contact with humans [230]. Trends observed by Smith et al. [231] in an assessment of livestock farms in England show trends of AMR across the farms did not have corresponding trends in their antibiotic use, thereby suggesting the role of external factors in the persistence of AMR, which may involve the presence of environmental ARGs present in farm sites majorly because of direct human activities.

The combination of poverty, poor regulations, and lack of knowledge has significantly led to a high rate of dissemination and development of AMR. The misuse and overuse of antimicrobials, for example, by stockpiling leftovers for future use, transferring prescriptions, and the circulation of counterfeit drugs by individuals and manufacturing companies with the wrong ingredients and incorrect dosages, are worsened by poor hygiene and pollution. This allows AMR to thrive, especially in low- and middle-income countries. The improper disposal of waste containing therapeutic and sub-therapeutic doses of non-metabolized antibiotics and their residues is a major driver in the development and transmission of AMR in the environment. Antibiotics are indiscriminately consumed in high quantities, with most of them undergoing only partial metabolism within the human body. The unmetabolized parts are then excreted into the environment, thereby contributing significantly to antibiotic pollution. Poor sanitation and hygiene have been reported in different studies as a key driver of AMR caused by human activities. For example, in areas with poor hygiene practices, synanthropic filth flies serve as a vehicle for the localized transport of ARB and associated ARGs from human feces [232]. In certain areas, humans use a single water source for defecating, fishing, bathing, and carrying out domestic chores. In 2022, the WHO estimated that at least 1.7 billion people used a drinking water source contaminated with feces, which may cause illness and indirectly affect antimicrobial consumption, as well as transmit ARB and ARGs [233]. The improper release of leftover spent containers and packages or expired products into the environment contributes to the generation of waste that contains ARB or can lead to the development of AMR [234]. Likewise, significant quantities of solid waste such as over-the-counter antibiotics, prescription-only-antibiotics, contaminated gloves, masks, syringes, dusters, rags, mops, needles, etc., and liquid wastes, including wastewater and chemicals generated from hospitals, households, and pharmaceutical manufacturing industries, cause selective pressure on the bacteria present in that environment, which results in the development of various resistance mechanisms that act as defense strategies against antibiotics, enabling them to survive in antibiotic-loaded environments and trigger the development of AMR, which poses risks to food safety and public health [235].

7. Integrated Strategies to Combat AMR

7.1. Multidisciplinary Approaches

Containing and controlling AMR requires coordinated collaboration across various disciplines, including scientists (such as microbiologists, chemists, biochemists, and biologists) and public health experts, and sectors such as healthcare, agriculture, finance, trade, education, and NGOs at both national and international levels. Combating AMR requires a multidisciplinary collaborative approach involving contributions from the biological and social sciences to address it. To fully understand the AMR problem, one must consider social disciplines like economics and politics. Although the link between these fields and AMR might seem unlikely, decisions such as the choice of antibiotics are often based on cost-effectiveness, which is an economic consideration [236]. National public health bodies typically set treatment guidelines, resulting in a uniform national strategy where the same antibiotic is used across an entire region, thereby providing the ideal environment for the emergence of AMR when treatment regimens are not followed as it has been highlighted that using a single medication within a population encourages the development of AMR when the medication is abused [237]. To tackle this issue, public health experts and scientists can collaboratively implement informed prescription practices by tailoring antibiotics to individual patients, thereby reducing the selection pressure on a particular antibiotic and minimizing resistance development. Primarily, doctors and veterinarians

often employ broad-spectrum antimicrobials due to functional limitations in quickly and accurately diagnosing infectious diseases, their causative organisms, and the susceptibility profile of pathogens [238]. Scientists and public health experts can collaborate to tackle this by developing targeted therapies, designing combination treatments using narrow-spectrum antibiotics, regulating the availability of antimicrobials as over-the-counter drugs (especially in developing countries), and establishing guidelines to ensure precise antibiotic use [239]. This can be achieved by conducting joint research, sharing data, and creating educational campaigns to promote responsible antibiotic prescription and usage, ultimately reducing AMR.

7.2. Development of Comprehensive AMR Mitigation Strategies

The continuous increase in the global human population has led to an increased demand for high-quality animal products, leading to the use of animal growth promoters in farm animals to sustain production and keep up with demand [240]. However, the widespread assumption is that the use of antibiotics in food animals for growth promotion and disease treatment and prevention is linked to the global spread of AMR [241]. When animals are continuously exposed to these growth promoters, bacteria in their bodies can evolve resistant genes to the antimicrobial agents in these growth promoters through mechanisms such as horizontal gene transfer or selective pressure [242]. There exists a potential for these resistant bacteria or their genes to be transferred to humans through the consumption of these animal products or water contaminated by animal wastes from the farm environment [59], leading to the development of AMR in humans and the potential compromise of the efficacy of antimicrobial therapy in both veterinary and human medicine [240]. As a result, it is critical to control the use of antibiotics for the stimulation of growth in animals, manage the use of antibiotics that are clinically significant, and optimize the dosage and duration of current antibiotic therapies [59]. Veterinary prescription guidelines should be mandatory, particularly for antibiotics used to treat infectious diseases in both humans and animals. Developing protocols to verify the prudent administration of antibiotics, setting goals for monitoring advancements, and formulating approaches for employing veterinary antibiotics are very crucial [66]. In the United States of America, for instance, the use of antibiotics as growth promoters in agriculture has been regulated by the FDA by classifying the two categories of antibiotic usage in their veterinary feed directive (VFD) regulations as therapeutic and non-therapeutic [243]. These regulations change how certain over-the-counter antimicrobial drugs are classified and ban their use in animal production, while also restricting the use of VFD drugs in animal feed to those prescribed by a licensed veterinarian based on a VFD order [244]. This regulation implies that some over-the-counter antimicrobial drugs such as tetracyclines like oxytetracycline and chlortetracycline were reclassified as VFD drugs, requiring veterinary supervision for their use according to a VFD order. The FDA also mandated that antibiotics in animal feed be clearly labelled for their specific approved uses, such as growth promotion or disease treatment, and ensured compliance through inspections and enforcement [244]. These regulations aim to balance the need for effective disease management and growth promotion in livestock to reduce the risk of antibiotic resistance affecting both animals and humans.

As demonstrated by Mir et al. [245], antibiotic usage in food animals may not be the only primary source of antibiotic resistance in grazing beef cattle operations. The study found that cefotaxime-resistant bacteria were present in 4.5% to 30% of cattle across seven herds that had never been treated with cefotaxime, suggesting that AMR microorganisms can colonize beef cattle without antibiotic exposure, likely originating from environmental sources. To mitigate the rise of AMR in grazing beef cattle operations, strategies should include enhancing biosecurity measures to prevent environmental contamination, monitoring water and soil quality for resistant bacteria [241], promoting proper manure management, and implementing rotational grazing to reduce pathogen build-up [241,246]. These strategies underline the significance of a One Health approach—a collaborative, multi-sectoral

strategy for addressing AMR that emphasizes the interconnection of human, animal, and environmental health. This strategy intends to strengthen surveillance, antimicrobial stewardship, and education and awareness across every sector to successfully combat the spread of AMR. The One Health concept aims to provide a holistic response to AMR concerns by combining efforts from human healthcare, animal medicine, and environmental science, eventually protecting public health, ensuring food safety, and conserving effective infection treatment options.

Antimicrobial stewardship (AMS) is a coordinated strategy aimed at optimizing the use of antimicrobial medications to enhance patient outcomes, reduce resistance, lower antibiotic costs, and minimize adverse effects. This approach is essential in the fight against AMR [247]. This can be achieved through several key strategies. First, evidence-based guidelines for the appropriate use of antimicrobials should be developed and implemented [238]. Second, protocols for diagnosing infections and selecting the appropriate antimicrobial therapy need to be established [247]. Additionally, ongoing education and training for healthcare providers regarding proper antimicrobial use and resistance prevention are essential. It is also important to educate patients about the significance of completing prescribed courses and the risks associated with misuse [238]. Moreover, ensuring that antimicrobials are prescribed at the correct dose and duration will help maximize efficacy and minimize resistance. Finally, when appropriate, favoring narrow-spectrum antimicrobials over broad-spectrum ones can effectively target specific pathogens while reducing collateral damage to the microbiome [248]. These could encompass public health campaigns, hospital-based programs, National Action Plans (NAPs), and the Global Action Plan (GAP), including awareness initiatives like World Antimicrobial Awareness Week (WAAW) [249,250]. The GAP, for instance, highlights the importance of improving infection prevention and control measures through effective sanitation, vaccination programs, and stringent hygiene practices in healthcare settings, which aids in preventing the spread of infections and reducing the need for antibiotics [238]. For example, the success of hand hygiene campaigns in hospitals has been linked to reduced rates of healthcare-associated infections and, consequently, lower demand for antibiotics [251].

Hospital-based AMS programs can aid in managing the prudent use of antimicrobials to combat AMR, tailoring approaches to local resources and contexts, focusing on prescribing guidelines, monitoring use, and educating healthcare workers [252]. Outpatient AMS programs similarly promote appropriate antibiotic use in clinics and doctors' offices through the education of patients and healthcare workers, and monitoring of prescription patterns [247]. For most bacterial illnesses, the typical course of antibiotic therapy is usually too lengthy and is not supported by evidence. Reducing the duration of typical antimicrobial regimens is therefore a straightforward and secure way to lower the use of antibiotics [253]. Recent infectious diseases pharmacotherapy has focused on defining the shortest effective therapy duration for various infections. In a study by Teshome et al. [254], over 7000 ICU patients were evaluated, and it was found that each additional day of antipseudomonal beta-lactam therapy increased the risk of new beta-lactam resistance, highlighting the importance of minimizing therapy duration to reduce AMR.

Surveillance, which is the systematic monitoring and analysis of the occurrence and spread of AMR pathogens, is a key priority of the GAP on AMR, aiding countries in collecting data on AMR and antimicrobial use to enhance patient outcomes, inform policy, and recommend interventions [114]. AMR surveillance typically involves collecting data on infections and antimicrobial use from clinical settings and laboratories, performing resistance testing on bacterial isolates, and analyzing trends and patterns in resistance [255]. These data are reported in national and regional summaries and used to provide feedback to healthcare practitioners. Surveillance systems, including local, national, and international, help coordinate data collection and inform public health interventions and infection control measures to address AMR [238]. Comprehensive surveillance of antimicrobial use is essential to prevent antibiotic-resistant bacteria and monitor AMR, helping to assess the problem's scale, track new and spreading resistances, and determine trends and outbreak

causes [247]. Surveillance reports have shown that between 2000 and 2015, global antimicrobial use expressed in defined daily doses (DDD) increased by 65% (21.1–34.8 billion DDDs), and if unregulated, this could rise to 42 billion DDD by 2030 [256]. Effective surveillance systems are crucial for monitoring AMR across animals, humans, agriculture, and the environment, helping to identify the presence of AMR in specific populations, including food-producing animals like chickens, pigs, cattle, and goats, as well as their products [257,258]. The large-scale use or misuse of antibiotics in food-producing animals and humans may promote the development of AMR, necessitating surveillance programs [114,257].

Various antimicrobial surveillance programs have been instituted over the years. An example is the GLASS, which was created in 2015 to encourage the monitoring of AMR in bacteria that cause common infections in humans and food-producing animals, as well as the development of strategies such as collecting and analyzing data from various sources, including epidemiological, population-level, clinical settings, and laboratories, to monitor resistance patterns and guide interventions [114]. Additionally, the FAO prioritizes monitoring AMR in healthy food-producing animals to ensure food safety, recommending the surveillance of indicator microorganisms like *E. coli* and *Enterococcus* spp., helping to identify resistance patterns in bacteria [115]. These strategies help detect emerging resistance trends and inform measures to mitigate the impact of AMR on both human health and food safety. In addition, efficient laboratory practices are essential for effective AMR surveillance, as they involve detecting, isolating, and monitoring resistant pathogens. Laboratories also perform antimicrobial susceptibility testing and use advanced molecular techniques, such as PCR and whole-genome sequencing, to understand the genetic basis of AMR [259]. These practices are integral to strategies for mitigating AMR, as they provide critical data needed to identify resistance patterns, inform treatment decisions, and develop targeted interventions to control the spread of resistant infections.

7.3. Policy and Regulatory Frameworks

To guarantee that everyone has access to the continuous, successful treatment and prevention of infectious diseases with quality-assured, safe, and effective medicines—while using antimicrobials responsibly—the World Health Assembly's "Global Action Plan (GAP)" outlined five strategic objectives: to improve the awareness and understanding of AMR; to strengthen knowledge through surveillance and research; to reduce the incidence of infection; to optimize the use of antimicrobial agents; and to ensure sustainable investment in countering AMR strategies to be implemented within the next five to ten years [114]. In 2019, the WHO, the International Federation of Pharmaceutical Manufacturers and Associations, the European Investment Bank, and the Wellcome Trust launched the AMR Action Fund, a nearly USD 1 billion initiative aimed at advancing promising antibiotics through the later stages of clinical trials and developing 2–4 new antibiotics by 2030. This fund aims to address unmet medical needs, advance medical science, slow resistance emergence, and support appropriate patient access by creating favorable market conditions for sustainable investment in the antibiotic pipeline [260]. So far, the fund has supported biotech companies in navigating the complex and costly process of bringing new antibiotics to market [261]. However, while this fund is a significant step forward, it is not a complete solution; substantial government support is still needed to implement long-term market reforms and incentives, as the current economic model for antibiotic development is inadequate, discouraging investment due to low potential returns.

Many countries have developed National Action Plans (NAPs) on AMR, aligned with the GAP objectives, to monitor and address antimicrobial resistance using a "One Health" approach [249]. For instance, The United Kingdom's current NAP, which is based on its 2013–2018 strategy, was created in consultation with a wide range of stakeholders from a variety of sectors, and is linked to international frameworks and plans for action aimed at addressing AMR both domestically and internationally, with the ultimate goal of ensuring the realization of the 20-year vision on AMR; enabling the effective containment and management of resistance escalation [261]. To achieve this goal, the UK government has

taken steps such as implementing antimicrobial stewardship initiatives across the National Health Service to ensure that antibiotics are prescribed appropriately and only when necessary, including regular training for healthcare professionals and developing guidelines for the prescription of antibiotics [262]. The UK government also established surveillance systems such as the English Surveillance Program for Antimicrobial Utilisation and Resistance (ESPAUR), which monitors antibiotic use and resistance patterns [263]. In 2014, the Canadian government introduced a federal framework involving a One Health approach to combat AMR through coordinated efforts from agencies like the Health Canada (HC), Public Health Agency of Canada (PHAC), Agriculture and Agri-Food Canada (AAFC), the Canadian Food Inspection Agency (CFIA), the Canadian Institutes of Health Research (CIHR), Industry Canada (IC), and the National Research Council (NRC) to prevent, limit, and control the emergence and spread of AMR in humans, animals, and the environment through optimized treatment practices, enhanced surveillance of antimicrobial use and resistance, and prevention of AMR transmission [264]. Through agencies such as the Canadian Antimicrobial Resistance Surveillance System (CARSS) and Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), the Canadian government has been able to collect and analyze AMR and antimicrobial use data from various sources, including humans, animals, and food; and specifically track AMR trends in bacteria across these sources [265]. Building on the 2015–2019 strategy, the Australian government has unveiled the National Antimicrobial Resistance Strategy: 2020 and beyond, a 20-year action plan that synchronizes with the GAP of the WHO to tackle AMR concerns both nationally and internationally through actions such as improving infection prevention and control practices in healthcare settings and enhancing antimicrobial stewardship in both humans and animals [266]. However, when compared with the intervention tactics of developed countries, sub-Saharan African countries are not as equipped for AMR because they lack real-time surveillance, monitoring, and regulatory standards [267]. A study by Iwu and Patrick [268] showed that GAP implementation in the WHO African region is generally inadequate, with Ethiopia, Kenya, and Zambia performing relatively better, but most countries scoring poorly, which may be due to economic, sociological, technological, industrial, and ecological challenges.

In 2017, the WHO created the Access, Watch, and Reserve (AWaRe) classification to guide more sustainable and rational antimicrobial use, aiming to reduce AMR's development and spread [269]. The AWaRe classification is a framework designed to guide the appropriate use of antibiotics based on specific conditions and recommended drugs, informing policy and practice in AMS programs to optimize antimicrobial use [247]. This framework categorizes antimicrobials based on their range of activity and their likelihood to contribute to AMR [270]. Narrow-spectrum antimicrobials that are effective against a particular class of pathogens and less likely to cause resistance are found in the "Access" group. Considering how important these antimicrobials are for treating a variety of common diseases, they should also be readily available, reasonably priced, and of high quality [270]. Broader-spectrum antibiotics with a higher potential for resistance are included in the "Watch" group [270]. Because overuse and misuse of these antimicrobials offer serious hazards for the formation and spread of AMR, their usage should be continuously regulated. Finally, the "Reserve" group consists of last-resort antibiotics that, if all other treatments are ineffective, should be administered judiciously and sparingly for serious infections [270]. However, while the AWaRe classification system has made significant strides in promoting the rational use of antibiotics and mitigating AMR, challenges remain in its effective implementation. Considering that the framework helps in setting guidelines, its major success depends on consistent adherence to these guidelines across healthcare settings. Issues such as the availability of essential antibiotics, varying levels of regulation and enforcement, and the need for continuous education and monitoring pose ongoing challenges [247]. Improving access to appropriate antibiotics, ensuring their quality and affordability, and strengthening stewardship practices are crucial for enhancing the impact of the AWaRe classification and combating AMR more effectively.

8. Conclusions

Addressing AMR is a multifaceted and challenging issue, necessitating a concerted, multidisciplinary approach that involves collaboration from the scientific, public health, agricultural, and policy sectors. The growing concern of AMR, fueled by factors including the abuse of antibiotics in human medicine and animal husbandry, over-reliance on broad-spectrum antibiotics, and inadequate regulatory framework implementation, highlights the critical need for comprehensive preventive and therapeutic strategies. Integrated approaches, such as the development of targeted therapies, improved antimicrobial stewardship, and effective surveillance systems, are crucial in preventing the spread of AMR.

It is imperative that all stakeholders commit to immediate actions that prioritize antibiotic stewardship and responsible use across all sectors. The effective implementation of national and international action plans guided by a One Health approach is crucial for promoting responsible antibiotic use, enhancing infection prevention and control practices, and maintaining sustained investment in antibiotic research and development. Future research should focus on identifying novel antimicrobial agents, optimizing the use of existing antibiotics, and exploring alternative therapies, such as phage therapy and immunotherapies, to combat resistant strains. Furthermore, research should focus on the identification of sustainable agricultural practices aimed at minimizing antibiotic utilization. This includes the enhancement of animal husbandry techniques, the adoption of integrated pest management strategies, and the implementation of crop rotation systems designed to decrease reliance on chemical inputs. Additionally, investigating the efficacy of probiotics and alternative treatments to antibiotics in livestock management holds promise for significantly reducing the risks associated with AMR. Moreover, fostering collaboration among researchers, agricultural practitioners, and policymakers is essential in the formulation and dissemination of best practices within the realm of sustainable agriculture that prioritize both animal welfare and environmental health. Achieving success in the reduction or mitigation of AMR will necessitate a multifaceted approach that combines robust legislative frameworks, public awareness campaigns, and ongoing training for healthcare professionals and agriculturalists. The successful reduction or mitigation of AMR involves a combination of solid legislative frameworks, public awareness initiatives, and continuous training for healthcare professionals.

The Global Action Plan and efforts such as the AMR Action Fund represent important steps forward, but long-term solutions will require sustained governmental backing and international collaboration. Collective global action must include the establishment of robust monitoring systems for antibiotic use and resistance patterns, coupled with public health campaigns that inform communities about the consequences of antibiotic misuse. The challenges posed by AMR are enormous, but with concentrated efforts and a dedication to innovation and collaboration, it is possible to ensure antibiotic efficacy for future generations while avoiding the life-threatening consequences of untreatable infections.

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Comprehensive Study of Antibiotic Resistance in *Enterococcus* spp.: Comparison of Influents and Effluents of Wastewater Treatment Plants

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Abstract: Background/Objectives: The spread of antibiotic resistance, particularly through *Enterococcus* spp., in wastewater treatment plants (WWTPs) poses significant public health risks. Given that research on antibiotic-resistant enterococci and their antibiotic-resistance genes in aquatic environments is limited, we evaluated the role of *Enterococcus* spp. in WWTPs by comparing the antibiotic resistance rates, gene prevalence, biofilm formation, and residual antibiotics in the influent and effluent using culture-based methods. **Methods**: In 2022, influent and effluent samples were collected from 11 WWTPs in South Korea. Overall, 804 *Enterococcus* strains were isolated, and their resistance to 16 antibiotics was assessed using the microdilution method. **Results**: High resistance to tetracycline, ciprofloxacin, kanamycin, and erythromycin was observed. However, no significant differences in the overall resistance rates and biofilm formation were observed between the influent and effluent. Rates of resistance to ampicillin, ciprofloxacin, and gentamicin, as well as the prevalence of the *tetM* and *qnrS* genes, increased in the effluent, whereas resistance rates to chloramphenicol, florfenicol, erythromycin, and tylosin tartrate, along with the prevalence of the *optrA* gene, decreased. *E. faecium*, *E. hirae*, and *E. faecalis* were the dominant species, with *E. faecalis* exhibiting the highest resistance. **Conclusions**: Our results suggest that WWTPs do not effectively reduce the rates of resistant *Enterococcus* spp., indicating the need for continuous monitoring and improvement of the treatment process to mitigate the environmental release of antibiotic-resistant bacteria.

Keywords: *Enterococcus* spp.; antibiotic resistance; biofilm; antibiotic resistance gene; wastewater treatment plant

1. Introduction

Antibiotic resistance is an escalating global health crisis driven by the emergence of multidrug-resistant (MDR) bacteria, which pose a significant threat to public health [1]. The excessive use of antibiotics to treat infections applies selective pressure on bacteria, fostering the emergence and persistence of resistant strains. This raises concerns regarding their transmission and survival in various environments [2,3]. The increasing prevalence of antibiotic resistance not only undermines the effectiveness of existing treatments but also complicates efforts to manage and control infections, particularly in healthcare settings, leading to an increase in healthcare-associated infections caused by these hard-to-treat pathogens [4]. Moreover, the impact of antibiotic resistance extends beyond human health and affects agriculture, animal husbandry, and environmental ecosystems [5]. Contamination from pharmaceutical manufacturing, agricultural runoff, and inadequate wastewater treatment fosters the persistence and spread of resistant bacteria in natural water bodies and soils, further compounding this problem [6,7].

Enterococcus spp., recognized as opportunistic pathogens, have garnered interest for their ability to acquire and disseminate antibiotic-resistance genes (ARGs). These bacteria are responsible for severe human infections including urinary tract infections, endocarditis, skin infections, and bacteremia [8,9]. *E. faecalis* and *E. faecium*, which constitute over 80% of *Enterococcus* spp. isolates, are major contributors to nosocomial infections, ranking third and fourth globally, after *Staphylococcus aureus* and *Pseudomonas aeruginosa* [10]. *E. faecalis* accounts for 85–90% of these infections, whereas *E. faecium* is responsible for 5–10% of infections [11,12]. Both species are demonstrating a global increase in the prevalence of antibiotic-resistant strains, as reported by Polish and European health agencies [13]. Beyond their clinical significance, enterococci serve as indicators for monitoring fecal contamination in water due to their abundance in the human intestine and their resilience to environmental conditions [14]. However, research on antibiotic-resistant enterococci and their ARGs in aquatic environments remains limited, which is crucial for understanding their spread [15].

Aquatic environments, particularly wastewater treatment plants (WWTPs), serve as critical reservoirs and transmission pathways for antibiotic-resistant bacteria (ARB), facilitating their movement between human and animal populations [16]. WWTPs have been identified as hotspots for the spread of ARB and ARGs, integrating antibiotics from various sources such as households, hospitals, and agriculture [15,17]. These facilities are substantial sources of antibiotic residues, ARB, and ARGs, which are continuously released into the environment, leading to the contamination of surface water, groundwater, and agricultural soil [18,19]. The presence of pharmaceuticals in wastewater exacerbates antibiotic resistance [20]. The urban water cycle, which involves water use and sewage discharge, transfers resistance between the environment and humans [21]. The environmental conditions in WWTPs facilitate bacterial survival and horizontal gene transfer of antibiotic resistance, thereby promoting the spread of ARB across aquatic and terrestrial ecosystems [22].

Enterococcus spp. are key members of the microbial communities in WWTPs and are associated with pollution from human and animal waste. MDR *Enterococcus* strains are commonly found in these facilities [23]. They exhibit greater environmental persistence than *Escherichia coli* and are resilient to various environmental stressors [24,25]. Their antibiotic resistance allows them to survive and spread in hospital environments, with transmission occurring from wastewater from domestic, industrial, veterinary, and hospital sources to municipal WWTPs [26]. Enterococci serve as reservoirs for ARGs aided by their conjugative plasmids and transposons, which can transfer resistance to other bacteria [27]. The release of ARB into water bodies can contribute to the spread of antibiotic resistance among native bacterial populations [15].

The widespread presence of *Enterococcus* spp. in water sources poses a health risk to individuals who come into contact with these environments. Therefore, continuous monitoring of enterococci in aquatic environments and identification of hotspots of MDR *Enterococcus* spp. are essential strategies to protect public health. This study aimed to evaluate how *Enterococcus* spp. in WWTPs, which are known hotspots for ARB, contribute to the spread of antibiotic resistance. We compared antibiotic resistance rates, ARG prevalence, biofilm formation, and residual antibiotic levels between the influent and effluent at WWTPs, in addition to resistance rates and gene prevalence among different *Enterococcus* spp. isolated from WWTPs.

2. Results

2.1. Identification of Enterococcus spp.

Overall, 804 *Enterococcus* isolates were identified from 11 WWTPs, with 479 and 325 isolates characterized from the influents and effluents, respectively. The distribution of *Enterococcus* spp. in the influent and effluent is presented in Figure 1. Four *Enterococcus* spp. were identified in the influent, with *E. faecium* being the most dominant at 280 isolates (58.5%). This was followed by *E. hirae* with 178 isolates (37.2%), *E. faecalis* with 20 isolates

(4.2%), and *E. durans* with one isolate (0.2%). Further, seven *Enterococcus* spp. were identified in the effluent, with *E. faecium* being the most dominant, comprising 216 isolates (66.5%). This was followed by *E. hirae* with 78 isolates (24%), *E. faecalis* with 24 isolates (7.4%), *E. gallinarum* with three isolates (0.9%), and *E. casseliflavus* with two isolates (0.6%). Additionally, one isolate each of *E. thailandicus* and *E. canintestini* were identified. The proportions of *E. faecium* and *E. faecalis* increased in the effluent from 58.5% to 66.5% and 4.2% to 7.4%, respectively, compared with those in the influent, whereas the proportion of *E. hirae* decreased from 37.2% to 24.0%.

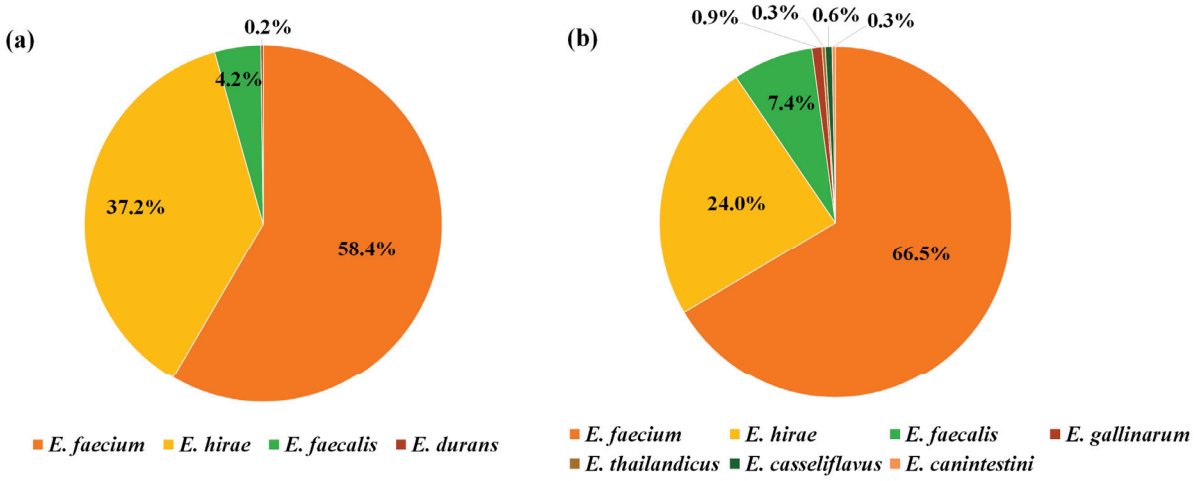

Figure 1. Identification of *Enterococcus* spp. isolated from the wastewater treatment plants (WWTPs). (**a**) Species composition in the influent. (**b**) Species composition in the effluent.

2.2. Antibiotic Resistance Phenotype

Antibiotic resistance of the *Enterococcus* isolates was assessed using 16 different antibiotics. The overall resistance rate of the influent isolates was 59.9%. Additionally, 20.9% of the isolates were MDR (resistant to three or more classes of antibiotics). The overall resistance rate of the effluent isolates was slightly higher (62.8%). The proportion of MDR strains in the effluents was 18.2%. The overall antibiotic resistance and MDR rates were not significantly different between the influents and effluents (Table 1).

Table 1. Comparison of antibiotic resistance rates in wastewater before and after passing through WWTPs and antibiotic resistance rates among different *Enterococcus* spp.

	WWTPs		**Three Major *Enterococcus* spp.**		
	Influents	**Effluents**	***E. faecium***	***E. faecalis***	***E. hirae***
Antibiotic resistance rate (resistant to at least one antibiotic)	59.9% (287/479)	62.8% (204/325)	62% (308/496)	100% (44/44)	52% (134/256)
Multidrug resistance rate (resistant to three or more classes of antibiotics)	20.9% (100/479)	18.2% (59/325)	20% (99/496)	43.2% (19/44)	15.2% (39/256)

A comparison of the antibiotic resistance rates between the influents and effluents is shown in Figure 2. In the influents, the highest resistance rates were observed in the following order: tetracycline (36.3%), erythromycin (22.8%), ciprofloxacin (21.7%), and kanamycin (21.7%). Conversely, the highest resistance rates observed in the effluents were against tetracycline (35.7%), ciprofloxacin (31.1%), kanamycin (17.2%), and erythromycin (15.1%). Ampicillin, ciprofloxacin, and gentamicin showed a statistically significant increase in resistance rates in the effluents compared with that in the influents, whereas chloramphenicol, florfenicol, erythromycin, and tylosin tartrate showed a decrease. Specifically, ampicillin resistance increased from 1.0% to 4.9%, ciprofloxacin from 21.7% to 31.1%, and gentamycin

from 3.8% to 8.6%. In contrast, chloramphenicol resistance decreased from 6.3% to 2.8%, florfenicol from 5.4% to 1.2%, erythromycin from 22.8% to 15.1%, and tylosin tartrate from 16.7% to 11.4%. The resistance rates to other antibiotics were similar between the influents and effluents.

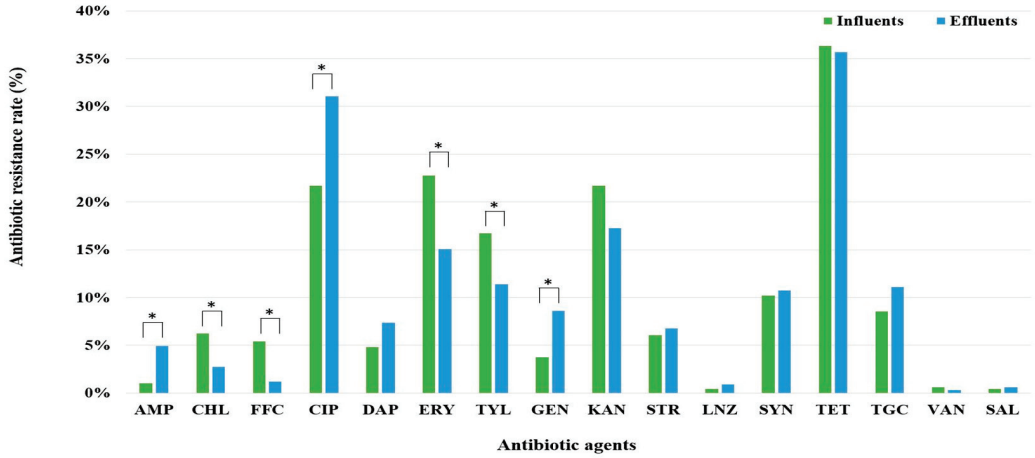

Figure 2. Comparison of resistance rates to 16 antibiotics between *Enterococcus* isolates from influents and effluents of WWTPs. AMP, Ampicillin; CHL, Chloramphenicol; FFC, Florfenicol; CIP, Ciprofloxacin; DAP, Daptomycin; ERY, Erythromycin; TYL, Tylosin tartrate; GEN, Gentamicin; KAN, Kanamycin; STR, Streptomycin; LNZ, Linezolid; SYN, Quinupristin/dalfopristin; TET, Tetracycline; TGC, Tigecycline; VAN, Vancomycin; SAL, Salinomycin. Significant differences between groups are marked with an asterisk (*).

We examined the antibiotic resistance patterns of three major *Enterococcus* spp., *E. faecium*, *E. hirae*, and *E. faecalis*, isolated from the influents and effluents of the WWTPs (Table 1, Figure 3). Although *E. faecalis* had the lowest number of strains among the three tested species, it exhibited the highest overall antibiotic resistance rate (Table 1). Notably, *E. faecalis* exhibited 100% resistance. Furthermore, 43.2% of the strains were MDR. In contrast, *E. faecium* exhibited resistance and MDR rates of 62% and 20%, respectively, whereas *E. hirae* exhibited resistance and MDR rates of 52% and 15%, respectively.

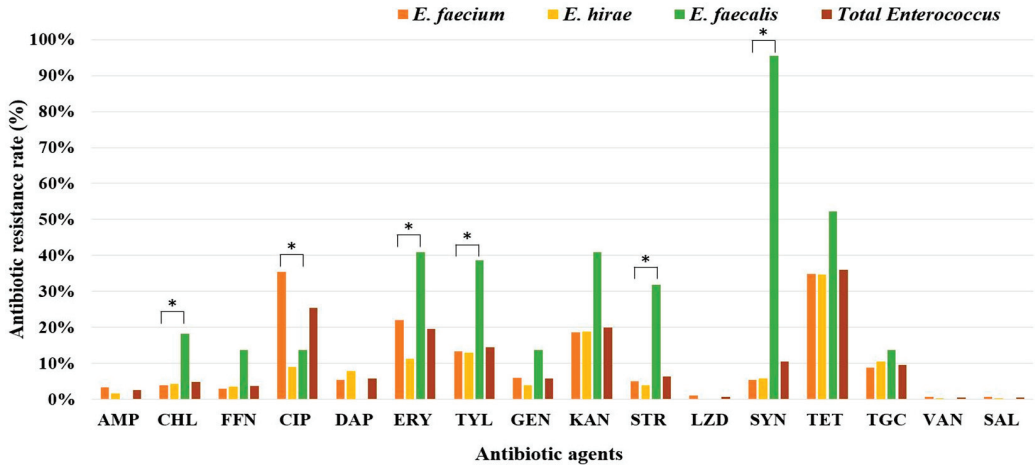

Figure 3. Comparison of resistance rates to 16 antibiotics among the three major *Enterococcus* spp. (*E. faecium*, *E. hirae*, and *E. faecalis*) isolated from the WTTPs. Significant differences between groups are marked with an asterisk (*).

The analysis of resistance to 16 different antibiotics revealed that *E. faecalis* generally exhibited higher resistance rates than the overall resistance rates of *Enterococcus* spp. (Figure 3). Notably, the rate of resistance to quinupristin/dalfopristin was the highest, at

95.5%. Other antibiotics against which *E. faecalis* showed significant resistance included tetracycline, kanamycin, erythromycin, tylosin tartrate, streptomycin, chloramphenicol, and florfenicol. In contrast, *E. faecium*, which constituted a majority of the *Enterococcus* isolates, demonstrated a high resistance rate to ciprofloxacin. Further chi-square analysis revealed statistically significant differences in resistance to six antibiotics (chloramphenicol, ciprofloxacin, erythromycin, tylosin tartrate, streptomycin, and quinupristin/dalfopristin) among the different *Enterococcus* spp.

2.3. Antibiotic Resistance Genes

The presence of ARGs was investigated in 491 of the 804 antibiotic-resistant isolates. Among the 17 ARGs tested, only eight (*tetM*, *tetL*, *ermA*, *ermB*, *qnrS*, *optrA*, *poxtA*, and *vanA*) were identified (Figure 4). In comparison to influent water, the genes *tetM*, *ermB*, and *qnrS* showed an increased prevalence in *Enterococcus* strains isolated from effluent water, whereas the prevalence of the remaining five genes either decreased or remained at comparable levels. The retention rate of *tetM* increased significantly from 38.0% to 50.5%, that of *qnrS* from 3.1% to 9.8%, and that of *optrA* decreased from 7.7% to 2.5%.

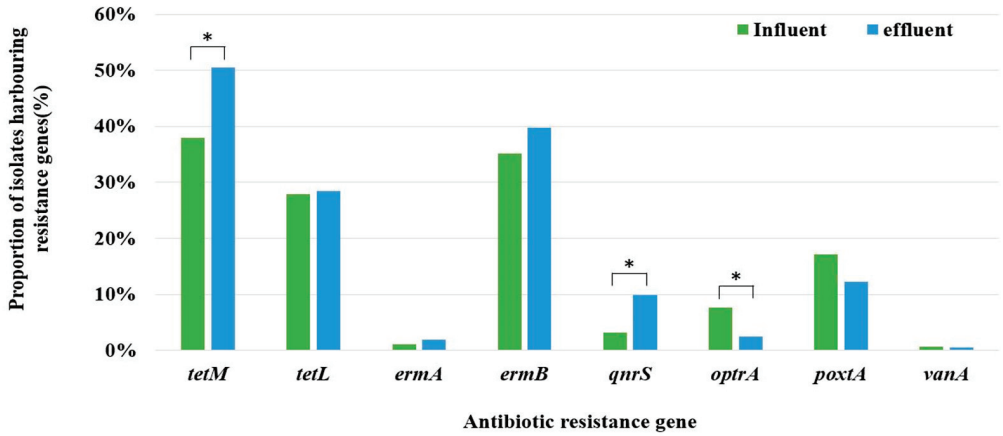

Figure 4. Comparison of the prevalence of antibiotic-resistance genes (ARGs) identified in *Enterococcus* spp. isolated from the influents and effluents of the WTTPs. Significant differences between groups are marked with an asterisk (*).

Chi-square analysis of the relationship between antibiotic resistance phenotypes and genotypes revealed a statistically significant association between tetracycline resistance and *tetM* and *tetL*. Additionally, a statistically significant relationship was observed between tylosin tartrate resistance and *ermA* and *ermB*.

Analysis of the prevalence of ARGs in the three main *Enterococcus* spp. (*E. faecium*, *E. hirae*, and *E. faecalis*) revealed that *E. faecalis* had the highest overall rate of ARGs (Figure 5). In particular, *tetM*, *tetL*, *ermA*, *ermB,* and *optrA* were found at higher rates in *E. faecalis* than the overall average. Conversely, *poxtA* was found to have a higher prevalence in *E. faecium*.

2.4. Evaluation of Biofilm Formation Capability

The comparison of biofilm formation rates between the influents and effluents revealed no significant increase in biofilm formation after passing through the WWTP, with overall formation rates of 23.6% and 20.6%, respectively (Table 2). The evaluation of biofilm formation by the influent isolates indicated weak formation by 18.2%, moderate formation by 5%, and strong formation by 0.4% of isolates. In contrast, weak, moderate, and strong formations were observed in 10.8%, 8%, and 1.8% of the effluent isolates, respectively. A minor increase in the number of strains showing strong formation was noted; however, this difference was not significant.

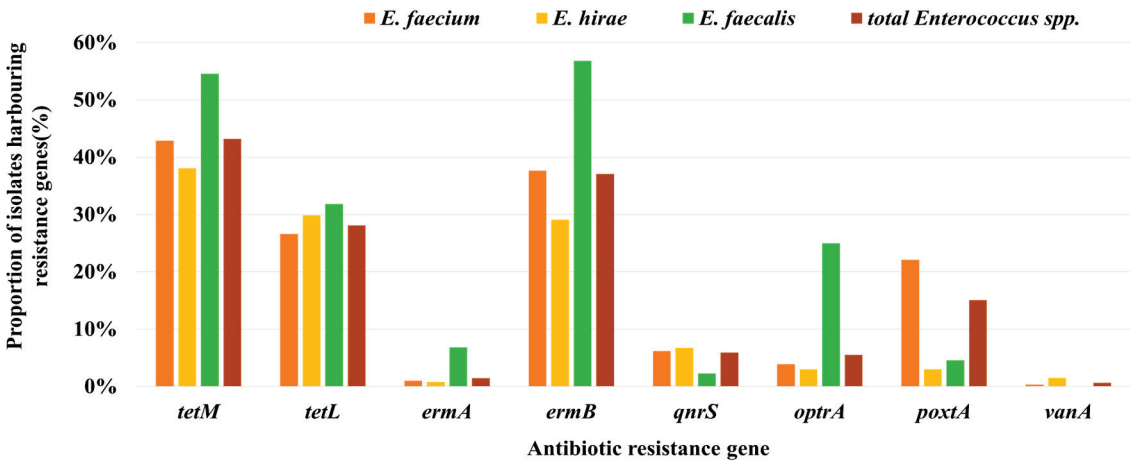

Figure 5. Comparison of the prevalence of ARGs among the three major *Enterococcus* spp. (*E. faecium*, *E. hirae*, and *E. faecalis*) isolated from the WTTPs.

Table 2. Comparison of biofilm formation abilities of *Enterococcus* isolates in the influents and effluents of the WWTPs.

	None	Weak	Moderate	Strong
Influents	76.4% (366/479)	18.2% (87/479)	5.0% (24/479)	0.4% (2/479)
Effluents	79.4% (258/325)	10.8% (35/325)	8.0% (26/325)	1.8% (6/325)

2.5. Measurement of Residual Antibiotics

Measurement of residual antibiotic concentrations for 26 types of antibiotics in influents revealed detectable residues of amoxicillin (0.003 μg/L), ceftazidime (0.01 μg/L), chloramphenicol (0.023 μg/L), ciprofloxacin (0.188 μg/L), sulfamethoxazole (0.064 μg/L), tetracycline (0.076 μg/L), and trimethoprim (0.038 μg/L) (Figure 6). In effluents, residues of ceftazidime (0.008 μg/L), ciprofloxacin (0.133 μg/L), sulfamethoxazole (0.03 μg/L), tetracycline (0.009 μg/L), trimethoprim (0.018 μg/L), and tylosin (0.004 μg/L) were detected. In particular, ciprofloxacin concentrations were notably high in both the influent and effluent water. Overall, the concentrations were observed to decrease in the effluents compared with the influents.

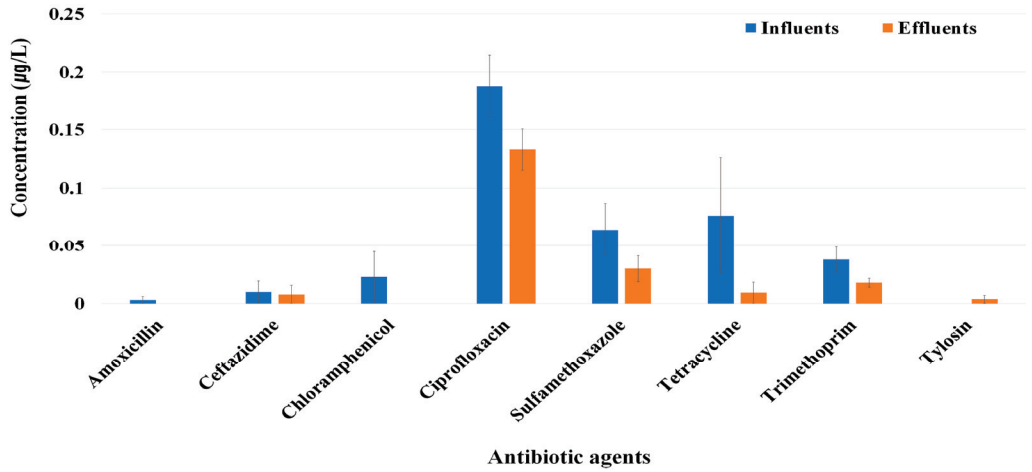

Figure 6. Comparison of residual antibiotic concentrations detected in influents and effluents from the WWTPs.

3. Discussion

In this study, we conducted a comparative analysis of the antibiotic resistance rates, ARGs, and biofilm formation abilities of *Enterococcus* strains isolated from the influent and effluent of WWTPs. The analysis revealed that despite the acknowledgment of WWTPs as hotspots for ARB and ARG dissemination, the differences between the influent (59.9%) and effluent (62.8%) were not statistically significant. These results indicate a consistent level of antibiotic resistance and the presence of MDR *Enterococcus* strains throughout the wastewater treatment process, suggesting that ARB are not adequately controlled during treatment.

In recent years, a few studies have compared the resistance rates between influents and effluents using culture-based methods. Costa et al. [22] also reported no differences in antibiotic resistance rates between inflows and outflows. The MDR rate of *Enterococcus* spp. in both the influent and effluent of the WWTP was approximately 20%, which was lower than the 68% MDR rate reported for *Enterococcus* spp. in WWTPs in a recent study [26]. This may be due to the lower inclusion of hospital-derived *Enterococcus* strains compared with those in other studies. Additionally, this study revealed that although the overall antibiotic resistance rates remained relatively unchanged before and after wastewater treatment, variations in resistance rates to specific antibiotics were noted. Resistance rates to ampicillin, ciprofloxacin, gentamycin, and tigecycline increased slightly after treatment, whereas those to chloramphenicol, florfenicol, erythromycin, and tylosin tartrate decreased. These findings suggest that while the wastewater treatment process effectively reduces resistance to certain antibiotics, it may inadvertently contribute to increased resistance to others.

Based on the isolation of *Enterococcus* spp. from the influents and effluents, *E. faecium* was identified as the dominant species, followed by *E. hirae* and *E. faecalis*. Most studies that have focused on WWTPs revealed *E. faecium* to be the dominant species, whereas the proportions of *E. hirae* and *E. faecalis* varied. Typically, *E. faecalis* is more prevalent than *E. hirae* [13,19]; however, instances in which *E. hirae* was more dominant than *E. faecalis* have been reported [14,28]. In an urban watershed, *E. mundtii* was recently found to be the most dominant species at 32% [29] and in hospital wastewater, *E. faecalis* was the second most dominant species after *E. faecium* [30]. *E. faecalis* is predominantly found in hospital-derived samples, whereas *E. hirae* is more common in livestock manure samples [11,24]. *E. faecium* and *E. faecalis* are primarily associated with the human environment, whereas *E. hirae* is frequently isolated from cattle feces and related wastewater [24]. The higher ratio of *E. hirae* than of *E. faecalis* in this study is likely because four of the 11 WWTPs were associated with livestock manure treatment facilities.

Additionally, we identified variations in the resistance patterns among different *Enterococcus* spp., with some species exhibiting higher levels of resistance to certain classes of antibiotics. In particular, *E. faecalis* had an antibiotic resistance rate of 100%, which was significantly higher than those of *E. faecium* and *E. hirae*. This is likely attributable to the high quinupristin/dalfopristin resistance rate of 95.5% observed in *E. faecalis*, which is believed to result from intrinsic resistance, as demonstrated in previous studies [25]. Its MDR rate was also more than twice that of the other two species. However, recent studies have reported that notwithstanding variations among antibiotics, *E. faecium* generally exhibits higher antibiotic resistance rates than *E. faecalis* [11,29,31]. In particular, for *Enterococcus* strains isolated from hospitals, the MDR rate of *E. faecium* was 75.6%, which was significantly higher than the 21.9% observed for *E. faecalis* [32]. In addition, *E. faecium* showed higher rates of resistance to ampicillin and aminoglycoside antibiotics such as gentamicin and streptomycin than *E. faecalis*. However, in the present study, among the antibiotics with statistically significant differences in resistance rates among *Enterococcus* spp., *E. faecalis* exhibited higher rates of resistance to chloramphenicol, erythromycin, tylosin tartrate, streptomycin, and quinupristin/dalfopristin, whereas *E. faecium* showed a higher rate of resistance to ciprofloxacin. Although the ampicillin antibiotic resistance rate of hospital-derived *E. faecium* is known to exceed 90%, this study, focusing on *E. faecium* isolated from WWTPs, found the rate to be less than 5%. This result aligns closely

with the findings of the National Antibiotic Usage and Resistance Monitoring Reports (2022–2023) [33,34], which reported an ampicillin resistance rate of *E. faecium* isolated from livestock feces at approximately 5.5–8.8%. This indicates that antibiotic resistance patterns vary according to the *Enterococcus* spp. and strain of origin, highlighting the need for species-level identification and targeted approaches to better understand the dynamics and public health implications.

Recently, WWTPs have been identified as key areas for the spread of vancomycin-resistant enterococci [19], leading to increased research in this area. However, *Enterococcus* strains isolated from clinical samples exhibit high susceptibility to linezolid and vancomycin [11,35], a pattern that has also been observed in strains isolated from WWTPs. Although some studies have reported vancomycin resistance rates as high as 62% in *Enterococcus* strains isolated from WWTPs [26], in most cases, including our study, the vancomycin resistance rate in WWTPs was less than 1% [22,28]. Recent studies have indicated that vancomycin-resistant *Enterococcus* spp. rates range from less than 1% in some settings to as high as 55% in others [36]. In addition, a meta-analysis found an average vancomycin resistance rate of approximately 4.3% across multiple studies, indicating significant heterogeneity depending on geographical location and clinical setting [37]. This variation is influenced by factors such as antibiotic usage patterns, infection control practices, and local microbial ecology.

As the incidence of antibiotic resistance in *E. faecalis* increases worldwide, underscoring its role as a potential opportunistic pathogen, there is an urgent need to develop alternative treatments and new antibiotic agents to effectively combat resistant strains [38]. Recent meta-analysis results indicated that daptomycin and tigecycline have potential as treatment options for *E. faecalis* owing to their low resistance rates in Europe and Australia [37]. Based on the results of this study, the usual antibiotics for treatment of *E. faecalis* infection such as ampicillin remain susceptible.

The most frequently detected ARGs were *tetM* and *tetL*, which are associated with tetracycline resistance, and *ermB*, which is associated with macrolide resistance. Regardless of the strain origin, *tetM* and *ermB* are commonly observed in *Enterococcus* spp. isolated from various sources [10,24]. Notably, these genes were most frequently detected in *E. faecalis* compared with other species, which is consistent with previous research results [15]. The plasmid-mediated quinolone resistance gene, *qnrS*, was significantly more prevalent in the effluent than in the influent. *qnrS* facilitates the spread of resistance among bacteria in various environments, including WWTPs [39]. In addition, some studies have indicated that this ARG is often not fully eliminated from WWTPs, leading to higher concentrations in effluents [40]. Among the genes associated with oxazolidinone resistance, *optrA* was detected at high frequencies in *E. faecalis*, whereas *poxtA* was predominantly detected in *E. faecium*. In a previous study conducted in China, *E. faecalis* isolated from poultry and pig fecal samples showed a higher detection rate for *optA* than other species [10].

Sub-inhibitory concentrations of antibiotics in wastewater can promote the growth of resistant bacteria, facilitate gene transfer, and enhance biofilm formation in wastewater treatment tanks or pipelines [18,22]. Elevated levels of antibiotics and other pharmaceuticals in the environment create favorable conditions for the selection of antibiotic resistance and serve as significant hotspots for the horizontal gene transfer of ARGs, promoting the evolution of resistance [2]. In this study, ciprofloxacin was detected at higher concentrations than the other residual antibiotics in both the influent and effluent of the 11 WWTPs. Additionally, the ciprofloxacin resistance rate was significantly higher in the effluent than in the influent. Although the residual ciprofloxacin concentration was not high, continuous exposure to WWTPs may have contributed to the increase in antibiotic resistance rates in enterococci [41]. In the case of ampicillin and gentamicin, despite an increase in antibiotic resistance rates after passing through the WWTP, no residual antibiotics were detected. This suggests that the presence of ARB in wastewater does not necessarily indicate the presence of the corresponding antibiotics [42]. Possible reasons for the presence of resistant bacteria, even in the absence of detectable residual antibiotics, include prior exposure to antibiotics

before entering the WWTP, horizontal gene transfer of ARGs, and the development of resistance mechanisms due to environmental stress factors such as high concentrations of organic matter within the WWTP.

Biofilm formation, a significant virulence factor in enterococci, varies globally and is influenced by the species, host, and environmental conditions. It not only increases antibiotic resistance but also poses a risk factor for the spread of hospital-acquired infections. Biofilm-forming bacteria are responsible for more than 65% of nosocomial infections and 80% of bacterial infections, with *Enterococcus* biofilm production being a key pathogenic trait, particularly in urology [43,44]. However, the biofilm formation rate of *Enterococcus* spp. isolated from WWTPs was relatively low at approximately 20%, with most strains forming only weak biofilms. Although recent research has shown that vancomycin-resistant strains exhibit greater biofilm formation than non-resistant strains [9], none of the vancomycin-resistant strains formed biofilms. In previous studies, bacteria in biofilms formed in effluents exhibited higher resistance to all tested antibiotics than those found in influent biofilms [45]. In contrast, in the present study, we found that strong biofilm formation did not consistently correlate with higher resistance rates to multiple antibiotics.

The final effluent discharged into the environment contains MDR *Enterococcus* spp., posing a potential health risk to receiving aquatic systems, as these bacteria may be transmitted to humans and animals exposed to the contaminated water [2]. The antibiotic resistance profiles of *Enterococcus* spp. were found to be very similar when comparing samples from a WWTP with those collected at a site located 500 m downstream of the WWTP [13]. The results of this study showed no difference in antibiotic resistance rates between the influent and effluent, indicating that ARB were not completely eliminated during wastewater treatment. As we examined the influent and effluent of the combination of 11 WWTPs, it was only possible to determine the overall trend of how antibiotic resistance rates changed as they passed through the WWTPs. However, because each WWTP employs different water treatment processes, the efficiency of antibiotic resistance removal likely varies. Therefore, future research is necessary to explore how antibiotic resistance changes based on the specific treatment methods employed by individual WWTPs. Furthermore, because surveillance studies are crucial for identifying shifts in the resistance patterns of key pathogens [11], continued research on major pathogens other than *Enterococcus* spp. in WWTPs, which serve as the primary route to the natural environment, is essential.

4. Materials and Methods

4.1. Isolation and Identification of Enterococcus spp.

In 2022, influent and effluent samples were collected from 11 WWTPs in South Korea. After thorough homogenization, 100 μL of each sample was inoculated onto kanamycin esculin azide agar (MB Cell, Seoul, Republic of Korea) to obtain pure strains of *Enterococcus* spp. The plates were then incubated at 36 °C for 24 h, and grayish-black colonies with black halos were selected. These colonies were transferred to *Enterococcus*-selective agar (Sigma-Aldrich, Saint Louis, MO, USA) for further isolation and incubated at 36 °C for 24 h. Pink and dark red colonies were identified. For species confirmation, the selected colonies were transferred to tryptic soy agar (BD Difco™, Franklin Lakes, NJ, USA), incubated, and analyzed using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) system (Bruker Daltonics, Billerica, MA, USA), which provided the final identification of *Enterococcus* spp. [46].

4.2. Antibiotic Susceptibility Testing

The antibiotic susceptibility of the *Enterococcus* isolates was assessed using the microdilution method, a widely adopted approach for evaluating phenotypic resistance. Bacterial suspensions were standardized to a 0.5 McFarland turbidity and inoculated into a commercially available 96-well microtiter plate (KRVP2F, Daejeon, Republic of Korea), which contained 16 antibiotic agents and is commonly utilized in the livestock industry. After a 24 h incubation at 35 °C, the minimum inhibitory concentration (MIC) was deter-

mined either by visual inspection or using an automated reader. The automated reader streamlined the interpretation of the results by accurately detecting microbial growth and ensuring consistent data recording. MIC breakpoints for the 16 antibiotic agents were interpreted according to the established guidelines of the Clinical Laboratory Standard Institute [47], the European Committee on Antimicrobial Susceptibility Testing [48], and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme [49]. The proportion of antibiotic-resistant strains was calculated as the number of strains with MIC values exceeding the respective breakpoint for each antibiotic divided by the total number of isolates tested. The concentration range of each antibiotic tested and the breakpoints for the resistance readings are shown in Supplementary Table S1.

4.3. Identification of Antibiotic Resistance Genes

Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocol. To detect the presence of ARGs, polymerase chain reaction (PCR) testing was conducted targeting specific genes. The genes investigated included tetracycline resistance genes (*tetA*, *tetB*, *tetM*, and *tetL*), macrolide resistance genes (*ermA*, *ermB*, and *ermC*), fluoroquinolone resistance genes (*qnrA*, *qnrB*, *qnrS*, and *qepA*), the phenicol resistance gene (*catA*), oxazolidinone resistance genes (*optrA* and *poxtA*), and glycopeptide resistance genes (*vanA*, *vanB*, and *vanHM*) [50–52]. The primer sequences and PCR conditions are presented in Supplementary Table S2. Additionally, plasmids were generated and used as positive controls, utilizing bacterial strains confirmed to be positive for each respective ARG.

4.4. Analysis of Biofilms

Biofilm formation assays were carried out following established protocols [51–53], with minor adjustments. Bacterial cultures were grown on Mueller–Hinton agar and standardized to a density equivalent of 0.5 McFarland units in distilled water. A 10-µL aliquot of each bacterial suspension was diluted 1:20 in 190 µL Luria–Bertani broth in 96-well plates. The plates were then incubated at 37 °C for 24 or 48 h. After incubation, non-adherent bacteria were removed by washing the wells three times with distilled water. The adherent cells were fixed by adding 200 µL methanol per well. After air-drying the plates, the remaining biofilms were stained with 0.1% crystal violet solution for 20 min. The wells were rinsed thoroughly with distilled water and left to dry. To quantify biofilm formation, 200 µL ethanol was added to each well to resuspend the crystal violet bound to the cells, and the optical density (OD) was measured at 550 nm. The biofilm formation levels were categorized as negative, weak, moderate, or strong based on the OD readings. The cutoff value (ODc) was calculated as the mean OD of the negative control plus three standard deviations. All experiments were conducted in triplicate, and the average results were recorded. Following a previously described method [51,53,54] with minor modifications, OD values from the negative control, measured at 595 nm, were used to determine the ODc. Biofilm production was classified as follows: OD < ODc for non-biofilm producers; ODc < OD < 2× ODc for weak producers; 2× ODc < OD < 4× ODc for moderate producers; and OD > 4× ODc for strong producers.

4.5. Detection of Antibiotic Residues

A total of 26 antibiotics were examined for residual presence. The antibiotics tested were amoxicillin, ampicillin, cefepime, cefoxitin, ceftazidime, ceftiofur, chloramphenicol, ciprofloxacin, clavulanic acid, erythromycin, florfenicol, linezolid, meropenem, nalidixic acid, salinomycin, sulfamethoxazole, sulfisoxazole, tetracycline, tigecycline, trimethoprim, tylosin, vancomycin, kanamycin, streptomycin, gentamicin, and colistin. To analyze antibiotic residues in the wastewater samples, 500 mL of each sample was first filtered through a 0.2-µm polyvinylidene difluoride membrane filter. Next, 900 µL of the filtered solution was transferred into amber autosampler vials, followed by the addition of 100 µL of 1% acetic acid solution, 40 mg/mL ethylenediaminetetraacetic acid disodium salt dihydrate, and 10

μL of 10 ng/mL isotopically labeled internal standards. The pretreated sample (200 μL) was subsequently analyzed using high-performance liquid chromatography combined with tandem mass spectrometry [55,56]. The findings on the presence and average concentrations of antibiotic residues from across the 11 WWTPs are reported in the Results Section.

4.6. Statistical Analysis

A chi-square test was conducted using R software (version 4.3.0) to assess the statistical significance of differences in antibiotic resistance rates between the influent and effluent and evaluate the association between the antibiotic resistance phenotype and corresponding ARGs. All statistical analyses were performed at a significance level of $p < 0.05$.

5. Conclusions

In conclusion, this study demonstrated that the antibiotic resistance rate of *Enterococcus* spp. did not significantly change after treatment in WWTPs and the failure to fully eliminate these resistant bacteria allows them to be discharged into aquatic environments, posing ongoing public health risks. These findings underscore the need for continuous monitoring and improvement of WWTP processes to enhance the removal of antibiotic-resistant bacteria. The detection of high resistance in species such as *E. faecium*, *E. hirae*, and *E. faecalis* further emphasizes the need to identify and mitigate hotspots of multidrug-resistant bacteria. Given the variability in the treatment efficiency across different WWTPs, future studies should focus on the effects of specific treatment methods on antibiotic resistance. Furthermore, broader surveillance of major pathogens beyond *Enterococcus* spp. is critical, because WWTPs are key conduits for these bacteria in the natural environment. A comprehensive approach that includes prudent antibiotic use, development of new antimicrobial agents, robust infection control, and global surveillance is essential to combat the spread of antibiotic resistance and safeguard public health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13111072/s1, Table S1: MIC breakpoints for antibiotic susceptibility testing, Table S2: List of primers and PCR conditions used in this study.

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