antibiotics

Special Issue Reprint

Antibiotics and Environment

Research and Development toward the One Health Approach

Edited by
Takashi Azuma

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Antibiotics and Environment – Research and Development toward the One Health Approach

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Guest Editor

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About the Editor

Takashi Azuma

Takashi Azuma is a Junior Associate Professor at the Graduate School of Pharmaceutical Sciences, Osaka Medical and Pharmaceutical University, Japan. He received his Ph.D. in Engineering from Kyoto University. His studies are focused on the occurrence and environmental fate of antimicrobials and antimicrobial-resistant bacteria (AMRB) in water environments, the investigation and risk assessment of hospital effluent, and the development of advanced water treatment systems to assess effective water management in river basins.

Preface

Antimicrobial resistance (AMR) has emerged as one of the most pressing global health and environmental challenges. The rapid spread of antimicrobial-resistant bacteria threatens the continued effectiveness of antimicrobial therapies and undermines efforts to control infectious diseases. Recognizing its global impact, the World Health Organization (WHO) has identified AMR as a critical issue requiring urgent and coordinated international action. Beyond clinical settings, community-acquired infections associated with asymptomatic carriers, as well as the dissemination of resistant strains originating from livestock, aquaculture, and other production systems, have become increasingly important concerns. Addressing these complex challenges requires a comprehensive understanding of the global dynamics of antimicrobial resistance and the implementation of a One Health approach that integrates human, animal, and environmental perspectives.

Environmental compartments play a crucial role in the emergence, persistence, and dissemination of antimicrobial-resistant bacteria and antimicrobial compounds. These contaminants originate from diverse human and animal activities and occur simultaneously within interconnected environmental systems. Therefore, clarifying their occurrence, environmental fate, and associated ecological and human health risks is essential for developing effective mitigation strategies and achieving sustainable coexistence between modern society and environmental integrity.

This Reprint focuses on antimicrobials and antimicrobial-resistant bacteria in diverse environmental settings, including rivers, lakes, coastal waters, water treatment systems, wastewater treatment plants, and livestock-related environments. This Reprint supports original research on occurrence, environmental dynamics, risk assessment, and treatment technologies, as well as review articles that synthesize current knowledge, introduce innovative analytical approaches, and propose future strategies for AMR mitigation based on the One Health framework.

Takashi Azuma
Guest Editor

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Article

Electron Beam Irradiation for Efficient Antibiotic Degradation in Aqueous Solutions

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Abstract

Background: Recently, extensive use of antibiotics has increased the amount of antibiotic residues in the natural water environment. **Methods:** This study presents an experimental investigation into the degradation of penicillins, tetracyclines, streptomycin and chloramphenicol in aqueous solutions when exposed to 1 MeV accelerated electrons with doses of 0.1, 1, 3 and 7 kGy using HPLC-HRMS analysis. **Results:** It was found that electron beam irradiation with a dose of 7 kGy ensures 98–99% removal of antibiotics, with the initial concentrations ranging from 15 mg/L to 30 mg/L depending on the class of antibiotic. The mathematical model proposed in the study, which estimates the dose dependencies of the relative concentrations of antibiotics and their degradation products in aqueous solutions, reveals different decomposition rates of antibiotics of different classes due to the different radiosensitivities of antibiotics. It has been found that tetracycline has a considerably higher radiation–chemical yield compared to the other antibiotics when exposed to accelerated electrons. **Conclusions:** Using density functional theory in combination with the mathematical model, we have developed a novel approach to establishing a quantitative irradiation marker of antibiotic degradation as a result of irradiation, which involves finding the degradation product whose formation requires a minimum number of ionization events. Using such an approach, it is possible to establish the extent of antibiotic degradation in water after irradiation with different doses and find the optimal irradiation doses for industrial water treatment.

Keywords: water irradiation; electron beam irradiation (EBI); antibiotics; high-performance liquid chromatography–mass spectrometry; degradation products; DFT calculation

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1. Introduction

Recently, extensive use of antibiotics in medicine, agriculture and livestock farming has increased the amount of antibiotic residues in aquatic environments, which has raised serious concerns in the scientific community [1,2]. Recent reports have estimated that antibiotics consumed worldwide in 2018 alone amounted to 40 billion defined daily doses (DDDs), which is a 46% increase since 2000 [3]. Moreover, global antibiotic consumption is projected to be 200% higher in 2030 than in 2015, with the greatest growth in low- and middle-income countries [4]. Most of the antibiotics consumed by humans or animals are not fully metabolized in their bodies and are excreted into the environment through feces and the urinary tract. The increasing overutilization of antibiotics in various sectors is becoming a major contributor to antibiotic pollution in the natural environment since antibiotics linger in the environment as they are not easily biodegradable [5–8].

As regulators and environmental agencies increasingly recognize the importance and urgency of antibiotic accumulation in the natural environment, developing effective methods to remove antibiotics from water has become a priority. Antibiotic residues are partially eliminated in wastewater treatment plants, but after entering water bodies and wastewater, the remaining metabolites can contribute to the development of drug-resistant bacteria, which pose a serious threat to human health [9–11]. Biological processes, which are widely used these days, such as conventional activated sludge wastewater treatment, effectively remove pollutants from wastewater using microorganisms. However, these methods are not suitable for removing pharmaceuticals, pathogens and antibiotic-resistant genes (ARGs), which are discharged into the environment along with secondary effluents [12]. Various treatment methods, including physical and chemical types, have been studied and applied to remove antibiotics from various media to minimize their impact on the environment, including adsorption, coagulation, membrane separation, intense oxidation, etc. [13–15]. In recent years, advanced oxidation methods (AOPs) have been increasingly studied and applied to remove antibiotics due to several advantages, such as high reaction rate, high efficiency and productivity [16,17]. UV and photocatalysis, Fenton oxidation, ozone oxidation and peroxide catalytic oxidation, among others, inevitably produce intermediates or byproducts of antibiotics that are more stable and toxic than the parent compounds, so they are limited in terms of degradation efficiency [18].

Compared with traditional processing technologies, electron beam irradiation is more efficient since it obviates the need to use other reagents in the processing and does not cause secondary pollution. Moreover, this highly automated and fast-speed technology allows the irradiation of large volumes of substances while ensuring complete degradation of toxic and persistent organic pollutants [19,20], which makes electron beam irradiation universally applicable in a vast number of areas, ranging from medicine to the food industry. E-beam irradiation can be particularly effective for the treatment of liquid substances due to the dual action of direct ionization by primary accelerated electrons and the indirect action of irradiation of organic pollutants through water radiolysis products [21]. $OH\bullet$ and $H\bullet$ radicals, hydrated electrons e_{aq}^{-}, hydrogen H_2, hydrogen peroxide H_2O_2 and hydroxonium ions H_3O^+ formed as a result of water radiolysis break chemical bonds of organic pollutants, resulting in their partial or complete decomposition, which is manifested by the release of CO_2 and H_2O [8]. Considering the adaptability of electron beam accelerators to different volumes of effluents due to their varying operating modes, electron beam accelerators can be integrated both in small- and large-scale water treatment facilities.

Recent studies on the effect of gamma irradiation on antibiotics of different classes [21–25] attest to the partial or complete decomposition of antibiotics in aqueous solutions. However, there is no clear understanding of the reaction mechanisms and degradation pathways of antibiotics of different classes in water. Considering the current trend to switch from the gamma sources ^{60}Co and ^{137}Cs to electron accelerators in industrial irradiation facilities, it is important to investigate the efficiency of accelerated electrons for the degradation of antibiotics in aqueous solutions and the mechanisms behind the chemical transformation of antibiotics into antibiotic degradation products.

The object of the study is to explore the impact of e-beam irradiation on a wide range of doses on antibiotics commonly used in medicine, agriculture and livestock farming. In the experiment, tetracycline, doxycycline, benzylpenicillin, amoxicillin, ampicillin, streptomycin and chloramphenicol were diluted in aqueous solutions representing water matrices contaminated with antibiotics. For the identification and quantitative assessment of degradation products of antibiotics, the study used high-performance liquid chromatography combined with high-resolution mass spectrometry (HPLC-HRMS), which is the most promising method for the determination of antibiotics in various matrices and the identification of antibiotic degradation products [26,27]. Another essential goal is to find reliable markers of antibiotic degradation, which would be the basis for an integrated approach to estimating the extent of antibiotic degradation and quantitative assessment of electron beam irradiation efficiency for the treatment of sewage and natural water polluted with antibiotics. Density functional theory (DFT) calculations and mathematical modeling were applied to gain insight into the reaction mechanisms and transformations of antibiotics into the identified degradation products in order to determine the quantitative markers of antibiotic degradation as a result of e-beam irradiation.

2. Results and Discussion

2.1. The Impact of Accelerated Electrons on Antibiotics in Aqueous Solutions

Immediately after e-beam irradiation, solutions of individual antibiotics were analyzed by HPLC-HRMS to measure the relative concentration of antibiotics and their degradation products. The conditions for recording the mass chromatograms of each antibiotic are presented in Table 1.

Table 1. Chromatography–mass spectrometric characteristics of antibiotic identification in aqueous solutions (standard deviation of peak areas is not more than 20%, n = 3).

Antibiotic	*m*/*z*	Polarity	Retention Time (RT), min	Peak Area (0 kGy), arb. unit.
Amoxicillin (Figure 1A)	366.1110	$[M+H]^+$	4.45	3.7×10^8
Penicillin G (Figure 1B)	335.1056	$[M+H]^+$	9.62	9.7×10^8
Ampicillin (Figure 1C)	350.1163	$[M+H]^+$	6.00	2.5×10^9
Tetracycline (Figure 1D)	445.1592	$[M+H]^+$	6.68	1.9×10^9
Doxycycline (Figure 1E)	445.1594	$[M+H]^+$	7.70	4.5×10^9
Streptomycin (Figure 1F)	582.2725	$[M+H]^+$	0.86	7.4×10^7
Chloramphenicol (Figure 1G)	321.0050	$[M+H]^-$	8.73	2.4×10^9

Figure 1A–G show the chromatograms of non-irradiated (0 kGy) and irradiated (0.1, 1, 3 and 7 kGy) solutions of seven antibiotics and their corresponding retention times. Table 2 represents data on the removal of antibiotics from aqueous solutions under the action of e-beam irradiation. It has been found that irradiation with a dose of 7 kGy can eliminate benzylpenicillin and streptomycin from water, while negligible traces of tetracycline, doxycycline and ampicillin can still be detected. Table 2 also shows that amoxicillin, benzylpenicillin (penicillin G) and ampicillin, belonging to the same class, are the most susceptible to irradiation as they are destroyed, on average, by 20–21% at the minimum dose of 0.1 kGy, while the other antibiotics are decomposed, on average, by not more than 10% at the same dose, with chloramphenicol remaining the most resistant. A two-factor ANOVA analysis was used to assess whether the average value of antibiotic removal varies depending on the irradiation dose and the type of antibiotic. The ANOVA analysis shows that the differences in the average removal values for all antibiotics are significant, with a significance level of $p = 0.05$, which can also be seen in Table 2. Additionally, the Tukey's range test was performed for each pair of antibiotics, which reveals statistically insignificant differences in the degradation of benzylpenicillin, amoxicillin and ampicillin belonging to the penicillin class of antibiotics.

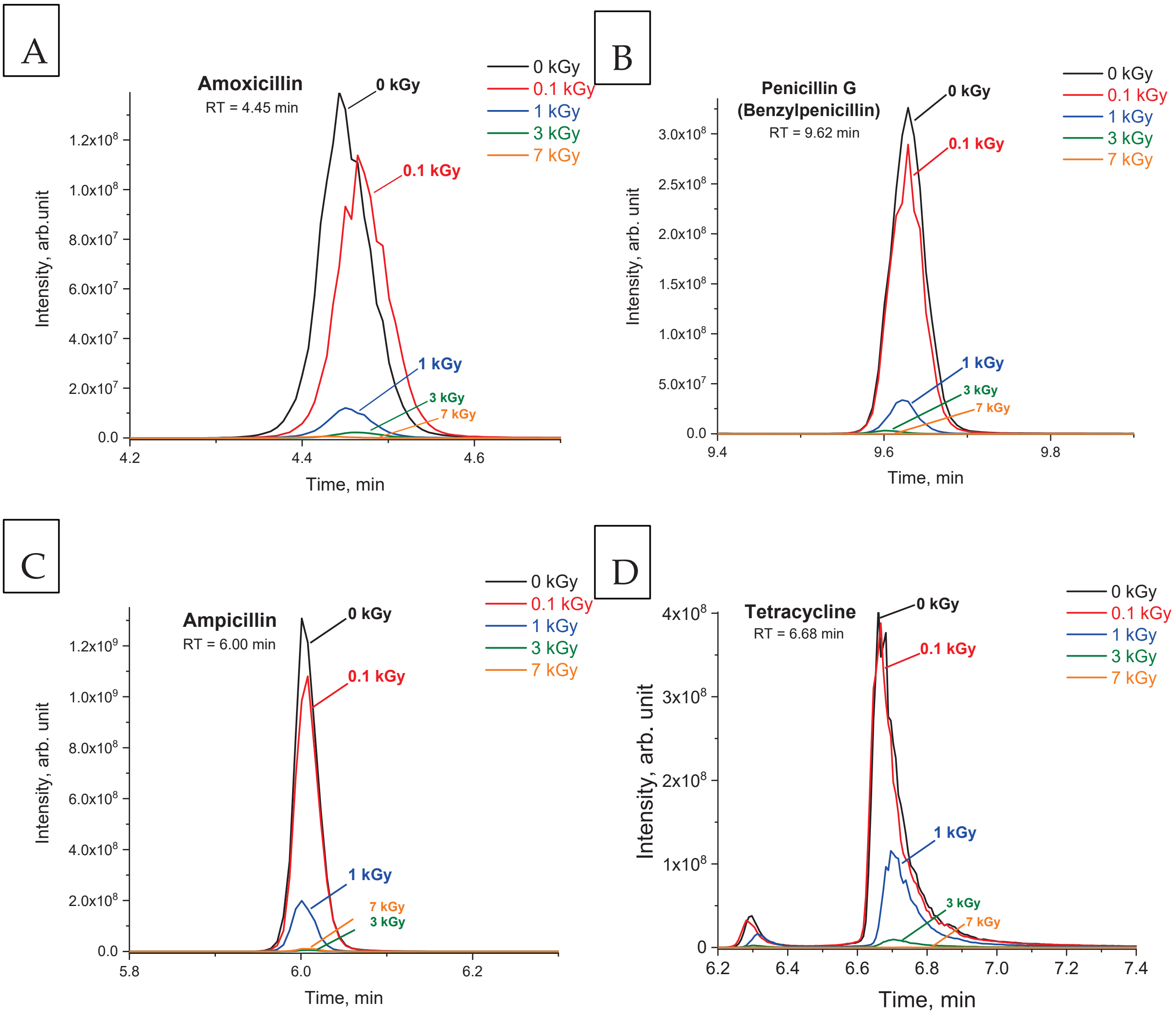


Figure 1. *Cont.*

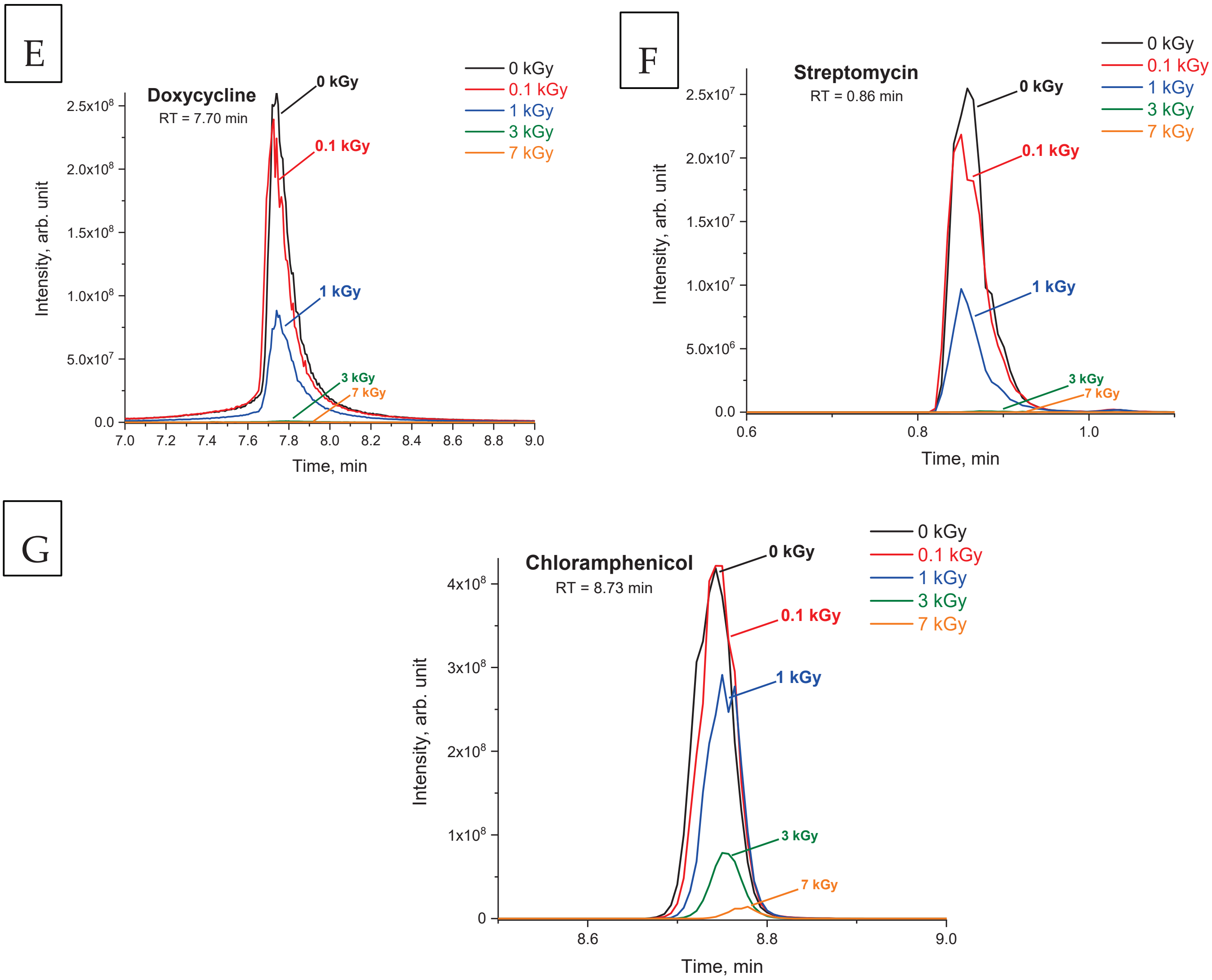


Figure 1. Chromatograms of solutions of amoxicillin (**A**), benzylpenicillin (penicillin G) (**B**), ampicillin (**C**), tetracycline (**D**), doxycycline (**E**), streptomycin (**F**) and chloramphenicol (**G**), non-irradiated (black line) and irradiated with doses of 0.1 (red), 1 (blue), 3 (green) and 7 (orange) kGy.

Table 2. Removal of antibiotics from aqueous solutions (in %) depending on irradiation dose (n = 3, p = 0.95). [1] Not detected.

Antibiotic	**Removal, %**				
	0 kGy	**0.1 kGy**	**1 kGy**	**3 kGy**	**7 kGy**
Amoxicillin (Figure 1A)	0	21.5 ± 3.1	88.4 ± 8.6	97.8 ± 0.8	98.9 ± 0.4
Penicillin G (Figure 1B)	0	20.8 ± 4.7	92.6 ± 3.3	99.3 ± 0.6	100 [1]
Ampicillin (Figure 1C)	0	20.1 ± 9.9	91.2 ± 18.4	99.4 ± 1.9	99.6 ± 1.0
Tetracycline (Figure 1D)	0	6.2 ± 2.4	65.3 ± 5.5	97.9 ± 3.3	99.9 ± 0.1
Doxycycline (Figure 1E)	0	8.6 ± 1.8	65.2 ± 14.8	99.4 ± 0.8	99.9 ± 0.1
Streptomycin (Figure 1F)	0	10.1 ± 2.5	61.6 ± 14.4	99.8 ± 0.3	100 [1]
Chloramphenicol (Figure 1G)	0	2.4 ± 0.5	35.1 ± 8.1	82.8 ± 3.8	98.8 ± 3.4

Figure 2A–E show chromatograms of the degradation products of amoxicillin, benzylpenicillin, ampicillin, tetracycline and streptomycin identified after e-beam irradiation of antibiotic solutions. During the experiment, doxycycline and chloramphenicol degradation products were not detected, which can be explained by the insufficient sensitivity of the suggested method.

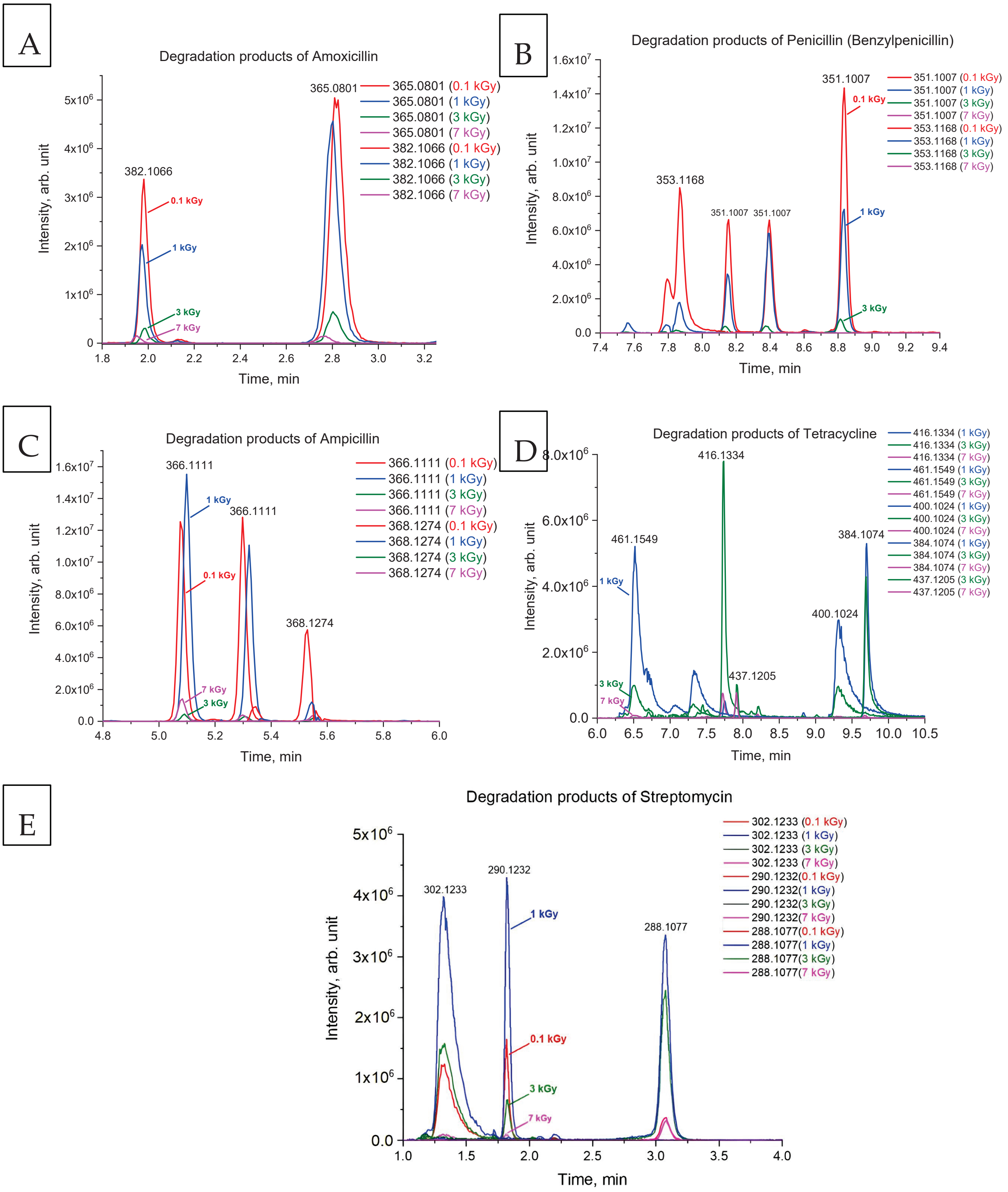


Figure 2. Chromatograms of the degradation products of amoxicillin (**A**), benzylpenicillin (penicillin G) (**B**), ampicillin (**C**), tetracycline (**D**) and streptomycin (**E**).

Chromatograms 2A and 2B show that the content of degradation products of amoxicillin and benzylpenicillin at the dose of 0.1 kGy reached its maximum. With a further increase in the irradiation dose up to 3 kGy, the degradation products of amoxicillin and benzylpenicillin decreased markedly, and the maximum dose of 7 kGy destroyed the degradation products almost completely, with only a negligible amount traceable. The high radiosensitivity of penicillins can be associated with the presence of a strained beta-lactam ring in their structure [28], which makes them more reactive to water radiolysis species. Other antibiotics demonstrated similar dose behavior involving an increase in the concentration, with a further decrease and with an increase in the irradiation dose. It should be noted that no degradation products of tetracycline were detected at the dose of 0.1 kGy, four degradation products were identified at the dose of 1 kGy, and one degradation product was detected only at 3 kGy. However, the degradation products of other antibiotics, except for doxycycline and chloramphenicol, were found in the aqueous solutions irradiated with the minimum dose of 0.1 kGy. Table 2 shows that chloramphenicol is the most radioresistant to e-beam irradiation since its concentration decreased by under 3% after irradiation with 0.1 kGy, which indicates a low susceptibility to attacks by reactive oxygen species.

Thus, electron beam irradiation with the dose of 7 kGy ensures 98–99% removal of all classes of antibiotics, with the initial concentrations ranging from 15 mg/L to 30 mg/L depending on the antibiotic type. The comparison of the degradation of tetracycline after e-beam irradiation with other common methods of antibiotics removal has shown that while photo-Fenton processing ensures a 94.2% removal of tetracycline from model solutions [29], and a sequencing-batch membrane bioreactor used for swine wastewater treatment ensures 90% removal of tetracycline antibiotics [30], e-beam irradiation with the dose of 7 kGy achieved at least 99.9% elimination in experimental conditions. With a 99% removal rate, e-beam irradiation can be compared with ozonation in terms of its efficiency [31], as ozonation has been reported to achieve 98% removal of oxytetracycline from aquaculture effluents. Although the adsorption methods are simple and inexpensive compared to other methods, they require subsequent treatment of the adsorbent to remove antibiotics from pores, and some amount of antibiotics can enter the watercourse, which makes such methods less sustainable [32,33]. Using photocatalytic degradation technology allows for the decomposition of tetracycline to carbon dioxide and water by over 90% [34]. However, catalysts involved in photocatalytic degradation are specific for a particular antibiotic, which makes it necessary to select the optimal combination of catalysts for each case of effluent water treatment. E-beam irradiation, on the contrary, allows the desired result to be achieved irrespective of the type and combination of antibiotics and without any considerable adjustments while ensuring bacterial purification of water. Considering that e-beam irradiation ensures the removal of antibiotics of all classes and combinations, it can be regarded as a more reliable and scalable water treatment method.

2.2. A Mathematical Model Describing the Dependency of the Concentrations of Antibiotics and Degradation Products on Irradiation Dose

As can be seen from the chromatograms (Figure 2A–E), after irradiation of the antibiotic solutions, the initial concentration of antibiotics decreased to negligible values, while the content of their degradation products increased, reaching its maximum at 0.1–3 kGy depending on the class of antibiotics. Further increase in the irradiation dose to 7 kGy led to nearly complete elimination of antibiotics and their degradation products (Figure 3). The non-linear effect of the irradiation dose on the rate of decomposition of each antibiotic and the rate at which their degradation products are accumulated are described using mathematical modeling.

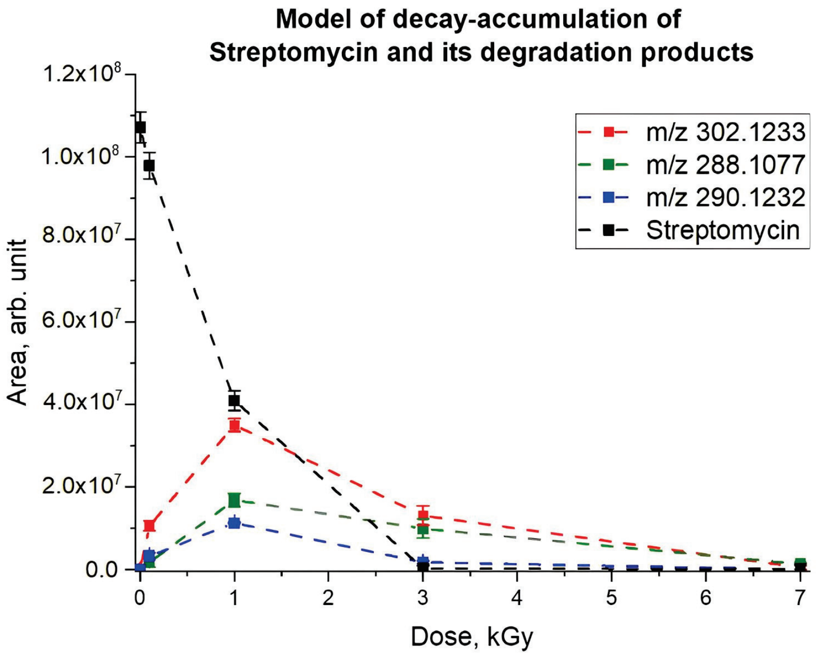


Figure 3. Dependencies of the absolute areas of the chromatographic peaks of streptomycin (black line) and its degradation products (ions with *m*/*z* values of 302.1233, 288.1077 and 290.1232 are shown in red, green and blue lines, respectively) on the radiation dose.

The exponential decrease in antibiotic concentration with increasing dose can be described by the following differential equation:

$$\begin{cases} \frac{dC_a}{dD} = -\alpha C_a \\ C_a(0) = C_0 \end{cases}, \tag{1}$$

where α (Gy^{-1}) is the decomposition rate of antibiotic molecules per unit of the absorbed dose, C_0 (rel.un.) is the concentration of the original antibiotic in the non-irradiated solution, and C_a (rel.un.) is the concentration of antibiotic in the solution irradiated with the dose D. The solution of the equation is represented as:

$$C_a(D_0) = C_0 e^{-\alpha\ D_0}. \tag{2}$$

A significant decrease in the concentration of antibiotic degradation products observed, following an initial increase in the higher irradiation dose, is a sign of two competing processes: the accumulation of the degradation product due to the decomposition of the initial antibiotic and the decomposition of the degradation product itself under the action of accelerated electrons.

The differential equation describing the change in the concentration of any degradation product with increasing irradiation dose can be expressed as follows:

$$\frac{dC_p}{dD} = -\beta C_p + kC_a, \tag{3}$$

where β (Gy^{-1}) is the decomposition rate of the degradation product, C_p (rel.un.) is the concentration of the degradation product in aqueous solution, and k (Gy^{-1}) is a coefficient depending on the decomposition rate of initial antibiotic and ratio of initial antibiotic molecules converted into a specific degradation product. When Equation (2) is incorporated into Equation (3), the latter takes the following form:

$$\frac{dC_p}{dD} = -\beta C_p + kC_0 e^{-\alpha D}. \tag{4}$$

The solution of the inhomogeneous differential Equation (4) is represented as:

$$C_p = \left(\frac{kC_0 e^{D(-\alpha+\beta)}}{\beta - \alpha} + P \right) e^{-\beta D}, \tag{5}$$

where P (rel. un.) is the integration constant.

Since different degradation products of antibiotics were identified at different irradiation doses, let us determine the dose D_0 as the threshold dose for the formation of a specific degradation product. This means that when the aqueous solution is irradiated with doses ranging from 0 to D_0, the concentration of the degradation product C_p is equal to 0. Taking into account the initial condition $C_p(D_0) = 0$, we find the constant P:

$$P = \frac{kC_0 e^{D_0(-\alpha+\beta)}}{\alpha - \beta}. \tag{6}$$

Then, the solution of Equation (4) takes the following form:

$$C_p = \frac{kC_0}{\beta - \alpha} \left(e^{D(-\alpha+\beta)} - e^{D_0(-\alpha+\beta)} \right) e^{-\beta D}. \tag{7}$$

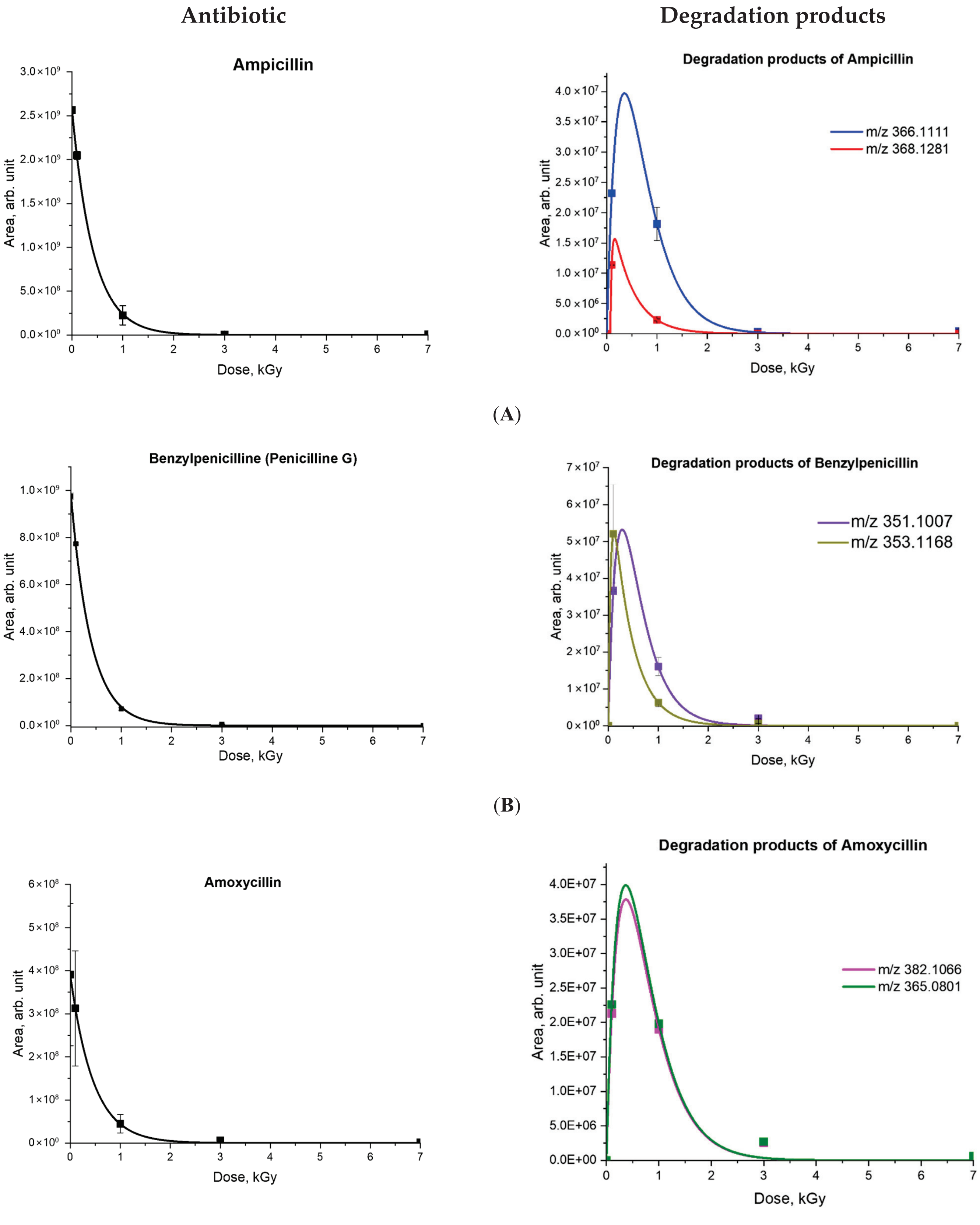
Solution (8) can be represented using the Heaviside function:

$$C_p = H(D - D_0) \frac{kC_0}{\beta - \alpha} \left(e^{D(-\alpha+\beta)} - e^{D_0(-\alpha+\beta)} \right) e^{-\beta D}. \tag{8}$$

Since the degradation products of streptomycin, amoxicillin, ampicillin and benzylpenicillin (Figure 2A–C,E) were identified at the dose of 0.1 kGy, dose D_0 for these classes of antibiotic is equal to 0, and the solution of Equation (4) is represented as follows:

$$C_p = \frac{kC_0}{\beta - \alpha} \left(e^{D(-\alpha+\beta)} - 1 \right) e^{-\beta D}. \tag{9}$$

Figure 4 shows the experimental dependencies of the absolute peak areas of the initial antibiotics and their degradation products on the irradiation dose and the dependencies calculated using Formulas (8) and (9). As can be seen, approximation dependencies adequately describe the experimental data, revealing the decomposition of original antibiotics and accumulation and decomposition of degradation products as a result of e-beam irradiation. Table 3 shows the values of the coefficients C_0, α, β, D_0 and k, calculated using Equations (1) and (4), for experimental data on the relative concentrations of antibiotics irradiated with accelerated electrons and the detected degradation products. While benzylpenicillin has a higher decomposition rate compared to other antibiotics studied, doxycycline showed the lowest rate of decomposition as a result of irradiation (Table 3). A low margin of error in the approximation coefficients represented in Table 3 attests to the adequacy of the suggested model.

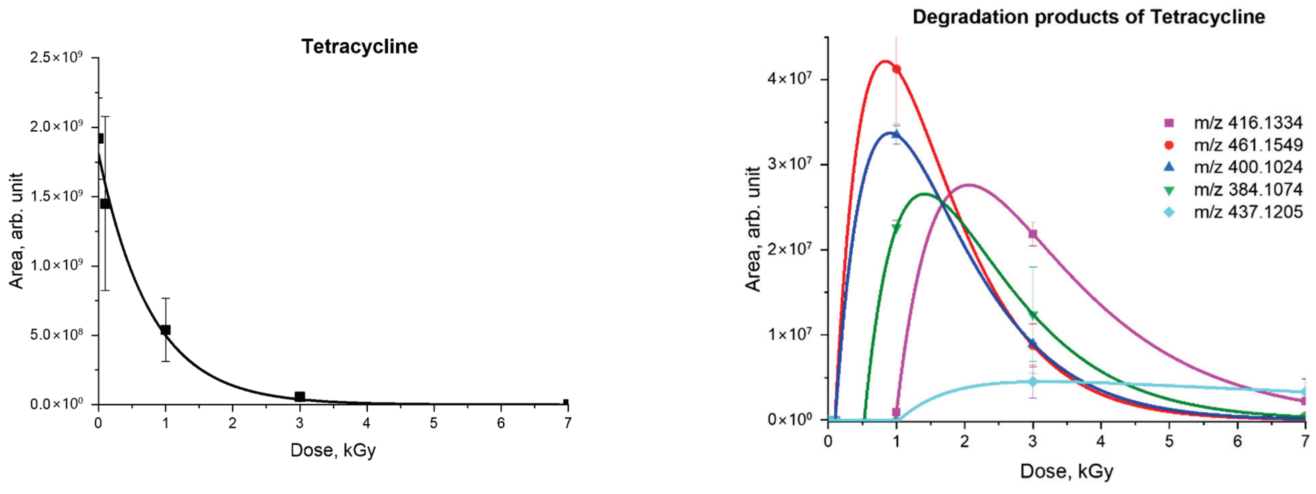


(**A**)

(**B**)

(**C**)

Figure 4. *Cont.*



(**D**)

Figure 4. Dependencies of the absolute peak areas of the initial antibiotics and their degradation products on the irradiation dose for ampicillin (**A**), benzylpenicillin (**B**), amoxicillin (**C**) and tetracycline (**D**).

Table 3. Approximation coefficients describing the transformations of antibiotics and their degradation products.

Antibiotic	C_0	α	R_{corr}	Degradation Products	β	D_0	k
Tetracycline	100	1.28 ± 0.16	0.99	461.1549	0.65 ± 0.08	0.98 ± 0.12	0.14 ± 0.02
				400.1024	1.44 ± 0.18	0.09 ± 0.01	0.10 ± 0.01
				384.1074	1.20 ± 0.15	0.09 ± 0.01	0.07 ± 0.01
				416.1334	0.98 ± 0.12	0.53 ± 0.07	0.09 ± 0.01
				437.1205	0.10 ± 0.01	1.04 ± 0.13	0.015 ± 0.002
Ampicillin	100	2.38 ± 0.24	0.98	366.1111	3.38 ± 0.34	2.33 ± 0.24	0.12 ± 0.01
				368.1274	32.12 ± 3.25	0.07 ± 0.01	0.28 ± 0.03
Amoxicillin	100	2.18 ± 0.84	0.99	365.0801	3.29 ± 1.27	6.01 ± 2.32	0.72 ± 0.26
				382.1066	3.34 ± 1.30	3.08 ± 1.19	0.76 ± 0.29
Benzylpenicillin	100	2.51 ± 0.12	0.99	351.1007	4.97 ± 0.23	3.86 ± 0.18	0.54 ± 0.02
				353.1168	22.77 ± 1.05	0.0022 ± 0.0001	1.60 ± 0.07
Streptomycin	100	1.02 ± 0.50	0.99	302.1233	1.12 ± 0.56	2.39 ± 1.19	0.96 ± 0.48
				290.1232	3.28 ± 1.65	0.05 ± 0.03	0.74 + 0.37
				288.1077	0.68 ± 0.34	0.05 ± 0.03	0.38 ± 0.19
Doxycycline	100	0.56 ± 0.04	0.89	Not detected			
Chloramphenicol	100	1.49 ± 0.08	0.98	Not detected			

The model suggested above allows the determination of the doses at which not only the initial antibiotics contained in water but also their degradation products are removed from the water. On the other hand, factoring in the concentrations of antibiotic degradation products in the water irradiated with different doses in the mathematical model makes it possible to determine the initial level of the water contamination with antibiotics.

2.3. *Antibiotic Degradation Pathways in Water Under E-Beam Irradiation Using Tetracycline as an Example*

Table 4 shows 15 degradation products (DPs) of tetracycline, amoxicillin, ampicillin, benzylpenicillin and streptomycin identified in aqueous solutions after e-beam irradiation; however, of all of them, tetracycline decomposed into the largest variety of different degradation products as revealed at different irradiation doses. Therefore, further in this article, we focus on possible e-beam irradiation-induced mechanisms of tetracycline decomposition.

Table 4. Degradation products of antibiotics identified in antibiotic aqueous solutions after e-beam irradiation.

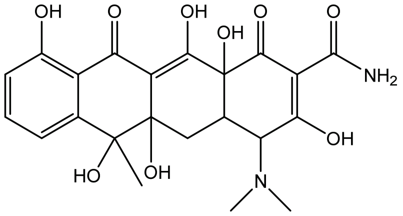
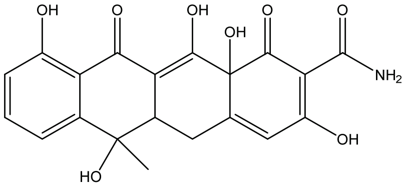
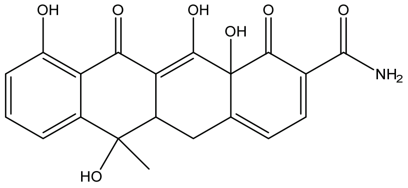
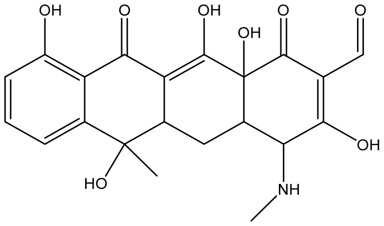
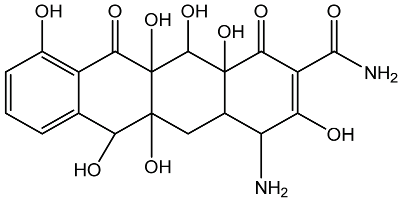
Antibiotic	Name and Molecular Formula $[M+H]^+$	Retention Time, min	Dose, kGy	Accurate Mass $[M+H]^+$	Mass Accuracy, ppm	Structure
Tetracycline	DP-TC-460 $C_{22}H_{25}N_2O_9$	6.51 и 7.31	1	461.1549	−1.31	
	DP-TC-399 $C_{20}H_{18}NO_8$	9.33	1	400.1024	−0.77	
	DP-TC-383 $C_{20}H_{18}NO_7$	9.68	1	384.1074	−0.37	
	DP-TC-415 $C_{21}H_{22}NO_8$	7.74	1	416.1334	−1.44	
	DP-TC-436 $C_{19}H_{21}N_2O_{10}$	7.92	3	437.1205	0.31	

Table 4. *Cont.*

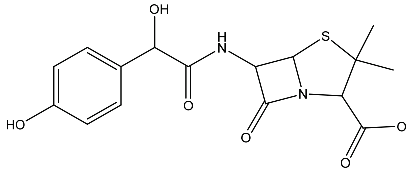
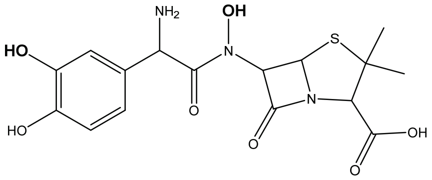
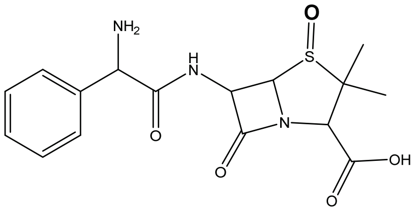
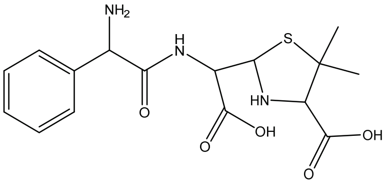
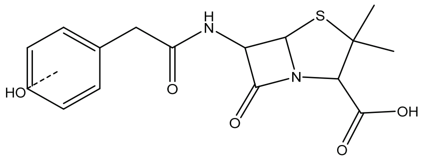
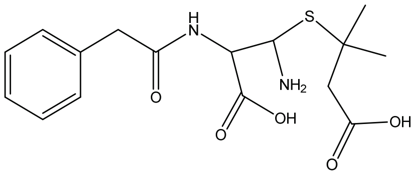
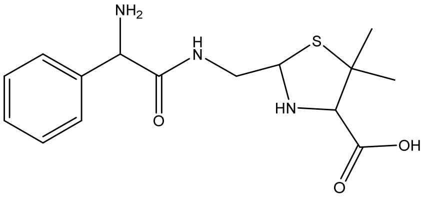
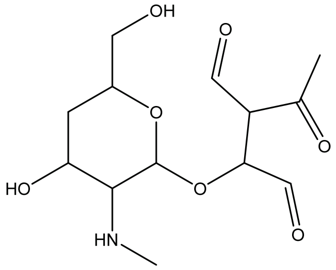
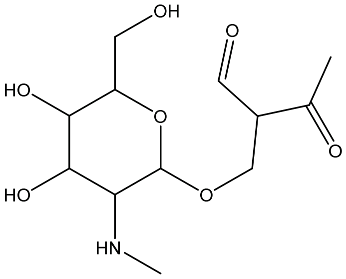
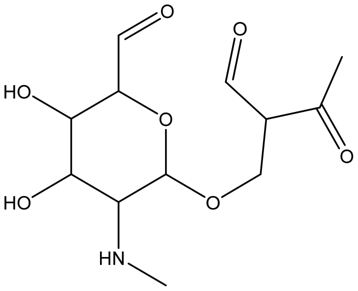
Antibiotic	Name and Molecular Formula $[M+H]^+$	Retention Time, min	Dose, kGy	Accurate Mass $[M+H]^+$	Mass Accuracy, ppm	Structure
Amoxicillin	DP-AMO-364 $C_{16}H_{17}N_2SO_6$	2.81	0.1	365.0801	0.07	
	DP-AMO-381 $C_{16}H_{20}N_3SO_6$	1.97	0.1	382.1066	−0.33	
Ampicillin	DP-AMP-365 $C_{16}H_{20}N_3SO_5$	5.12	0.1	366.1111	−0.37	
	DP-AMP-367 $C_{16}H_{22}N_3SO_5$	5.60	0.1	368.1274	0.82	
Benzylpenicillin	DP-PENG-350 $C_{16}H_{19}N_2SO_5$	8.16; 8.40; 8.83	0.1	351.1007	−0.34	
	DP-PENG-352 $C_{16}H_{21}N_2SO_5$	7.83	0.1	353.1168	0.54	
	DP-PENG-308 $C_{15}H_{21}N_2SO_3$	7.83	0.1	309.1276	−0.2	

Table 4. *Cont.*

Antibiotic	Name and Molecular Formula [M+H]$^+$	Retention Time, min	Dose, kGy	Accurate Mass [M+H]$^+$	Mass Accuracy, ppm	Structure
Streptomycin	DP-STR-301 $C_{13}H_{20}O_7N$	1.31	0.1	302.1233	−0.56	
	DP-STR-289 $C_{12}H_{20}O_7N$	1.82	0.1	290.1232	−0.79	
	DP-STR-287 $C_{12}H_{18}O_7N$	3.07	0.1	288.1077	−0.18	

Since irradiation of the tetracycline solution had a higher radiation–chemical yield compared to other antibiotics, the dataset of tetracycline degradation products was analyzed on the software package PRIRODA04, using the density functional method (DFT) to make theoretical assumptions regarding the formation of degradation products from the original tetracycline. The DFT method, factoring in thermodynamic calculations of radiation-induced chemical reactions involving antibiotics, provides a molecular-level explanation of the reasons why certain antibiotic degradation products occur. This knowledge is critical for designing an effective water irradiation methodology for eliminating antibiotic residual ecotoxicity.

As can be seen from Figure 1D, the initial tetracycline molecule shows a pronounced chromatographic peak at 6.68 min with the m/z value of 445.1592 (the TIC chromatogram and ESI mass spectra of tetracycline are shown in Figure S1 in Supplementary Materials). At a dose of 0.1 kGy, the intensity and peak area of tetracycline decreased by 6.2%, and no degradation products were observed. At a dose of 1 kGy, the degradation products with m/z values of 461.1549 (DP-TC-460), 400.1024 (DP-TC-399), 384.1074 (DP-TC-383) and 416.1334 (DP-TC-415) appeared (Figure 4D). When exposed to 3 kGy, the degradation product with an m/z value of 437.1205 (DP-TC-436) peaked in the mass chromatogram.

The degradation products identified in water occur as a result of different radiation-induced reactions, and the most probable degradation pathways for tetracycline are shown in Figure 5. The analysis of the structures and the type of degradation products suggests that the main degradation pathways of tetracycline are demethylation, deaminomethylation and dehydroxylation, as well as hydroxylation, occurring without disturbing the ring structure of the original molecule.

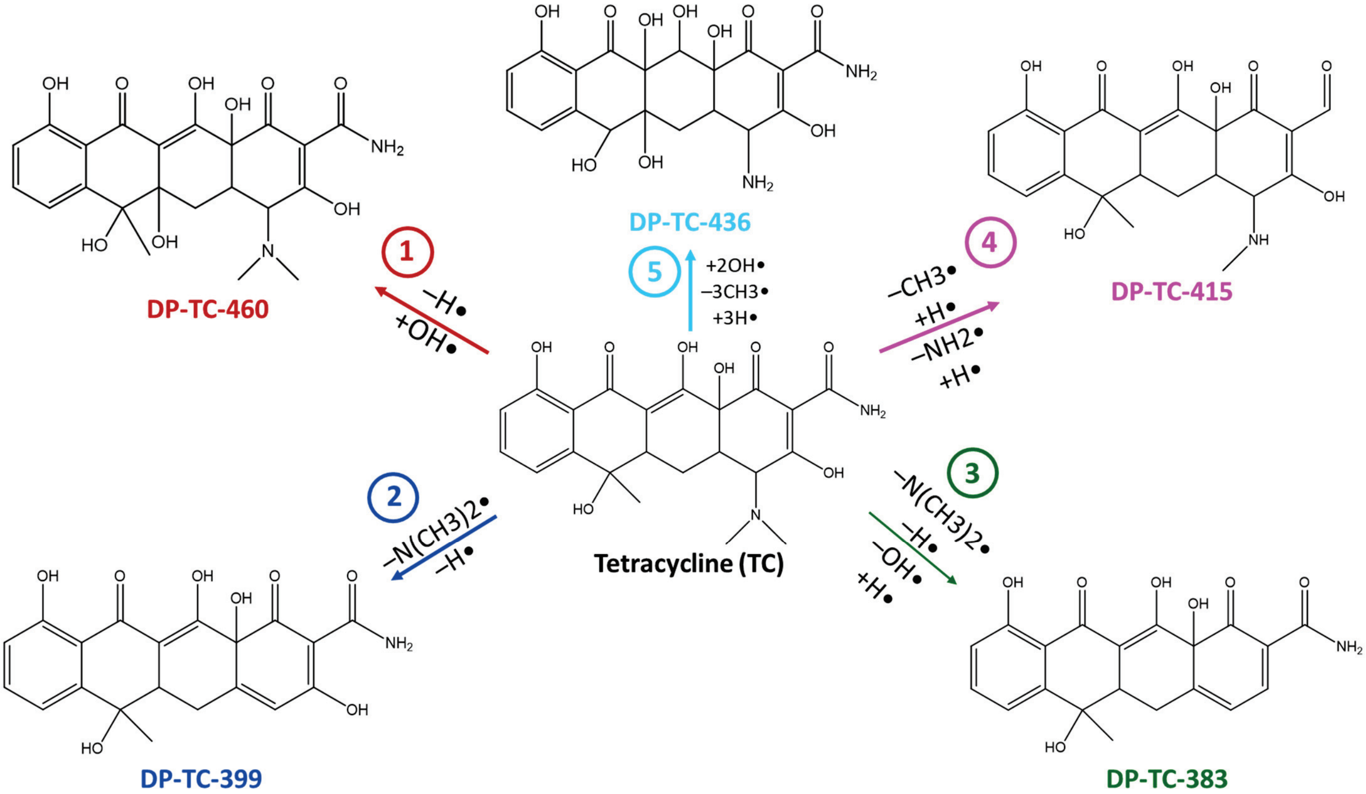


Figure 5. The most probable pathways of tetracycline degradation under the action of accelerated electrons.

As can be seen from Figure 4D, the concentration of the product DP-TC-460 in the water irradiated with 1 kGy was higher than the concentrations of the other degradation products DP-TC-399, DP-TC-383 and DP-TC-415 identified at this dose. It can be assumed that the product DP-TC-460 is a hydroxylated product of tetracycline with a substituted hydroxyl radical OH• instead of the H atom (Figure 6). Hydroxyl radical OH•, formed as a result of water radiolysis, attacks the tetracycline molecule, and the molecular structure of tetracycline can provide at least seven theoretically possible positions of the OH group (seven isomers). The criterion for the formation of the products can be the energy of breaking the C-H bond at various carbon atoms: C4, C5, C6, C9, C14, C15 and C19. The energy of C-H bond-breaking ΔE^I and Gibbs energy ΔG^I, revealing the nature of the chemical reaction, are calculated using the following equations:

$$\text{Tetracycline (TC)} \rightarrow \text{Radical (Cn)}\bullet + \text{H}\bullet \tag{10}$$

$$\Delta E^I = E(\text{Radical(Cn)}\ \bullet) + E(\text{H}\bullet) - E(\text{TC}) \tag{11}$$

$$\Delta G^I = \Delta E^I - [G(\text{Radical(Cn)}\ \bullet) + G(\text{H}\bullet)] - G(\text{TC})], \tag{12}$$

where ΔE^I, kcal/mol is the total energy of compounds, and ΔG^I, kcal/mol is Gibbs energy.

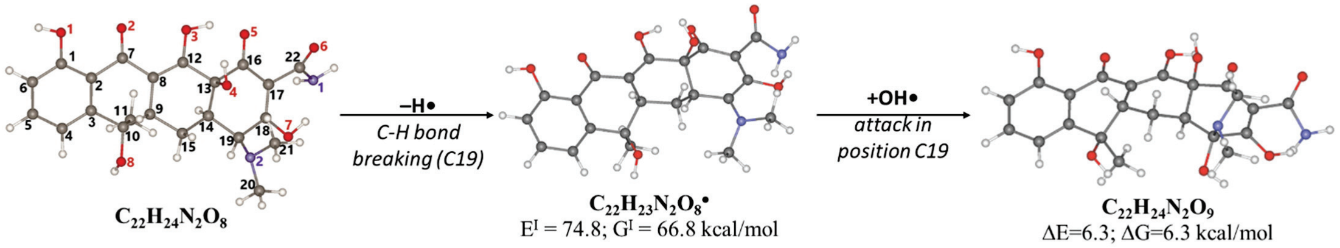


Figure 6. The optimized structures of tetracycline and its degradation product with an m/z value of 461.1549 (DP-TC-460) and energies of the formation of the intermediate and final product. Carbon atoms are grey, hydrogen atoms are white, oxygen atoms are red, and nitrogen atoms are violet.

According to Table 5, the bond dissociation energies at carbon atoms C4, C5, C6, C9, C14, C15 and C19 show that the hydrogen atom at position 19 is prone to dissociation due to a low bond energy, which leads to the formation of DP-TC-460 upon attack by the hydroxyl radical OH• at position 19. The energies of the remaining isomers of the hydroxylated product DP-TC-460 are given in Table S2 in Supplementary Materials. The energy of the isomer with the hydroxyl position at C19 is 6.3 kcal/mol higher compared to that with the hydroxyl position at C5, which suggests that this configuration does not yield any energy gain. It can be assumed that the breaking of the C-H bond plays a major role in the formation of the primary radical (Cn)•, since the dissociation energy of hydrogen from C19 is 39 kcal/mol lower than for C5. Thus, the DFT method shows that DP-TC-460 is formed due to two consecutive events: direct ionization manifested in the breaking of the C-H bond and the interaction of OH• with the primary radical (Cn)•.

Table 5. Total energy E^I and Gibbs energy G^I for tetracycline molecules in different positions of carbon atoms (Figure 6).

	C_4•	C_5•	C_6•	C_9•	C_{15}•	C_{14}•	C_{19}•
E^I, kcal/mol	113.5	115.2	115.5	77.5	99.7	94.7	74.8
G^I, kcal/mol	105.4	105.8	106.6	69.4	90.9	85.2	66.8

A slightly lower concentration of the degradation product DP-TC-399 with m/z 400.1024 compared to DP-TC-460 was identified in water irradiated with 1 kGy (Figure 7). It can be assumed that the tetracycline molecule transforms into the degradation product DP-TC-399 by losing C_2H_8N, which results in the loss of the dimethylamine group. DFT calculations show that the dimethylamine group breaks off from the C19 position, and the dissociation requires the energy of 38.4 kcal/mol. The second step in the formation of DP-TC-399 is the dissociation of the hydrogen radical H•, with 37.8 kcal/mol expended in the process. Thus, the decomposition of the tetracycline molecule with the formation of the degradation product DP-TC-399 involves two direct ionizations, resulting in the breaking of the bond C19-N$(CH_3)_2$ and dissociation of the hydrogen radical H•.

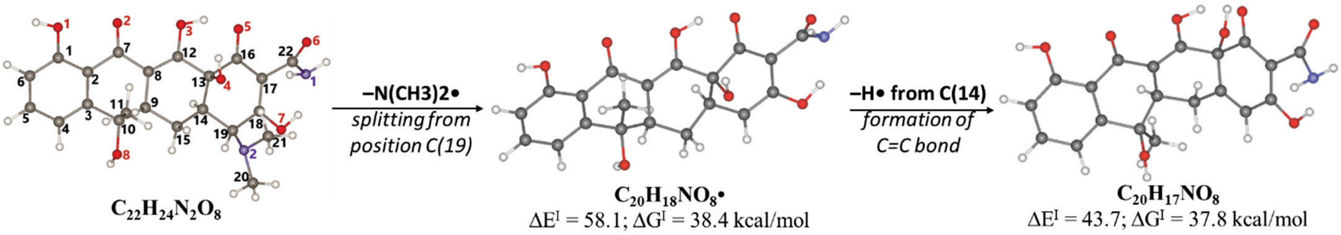


Figure 7. The dissociation of the dimethylamino group from position C(19) to form DP-TC-399 with an m/z value of 400.1024. Carbon atoms are grey, hydrogen atoms are white, oxygen atoms are red, and nitrogen atoms are violet.

The degradation product DP-TC-383 is formed from DP-TC-399 by the elimination of the hydroxyl group, presumably at C18, and the subsequent addition of the hydrogen radical H• (Figure 8). Then, the dimethylamino group is eliminated from position C19 and the hydrogen radical H• from position C14. The total energy gain of this process is 72.8 kcal/mol (see Figure S37 in Supplementary Materials). It should be noted that the degradation products DP-TC-460, DP-TC-399 and DP-TC-383 were found in tetracycline solution irradiated with ultraviolet, which attests to the consistency of our findings [35].

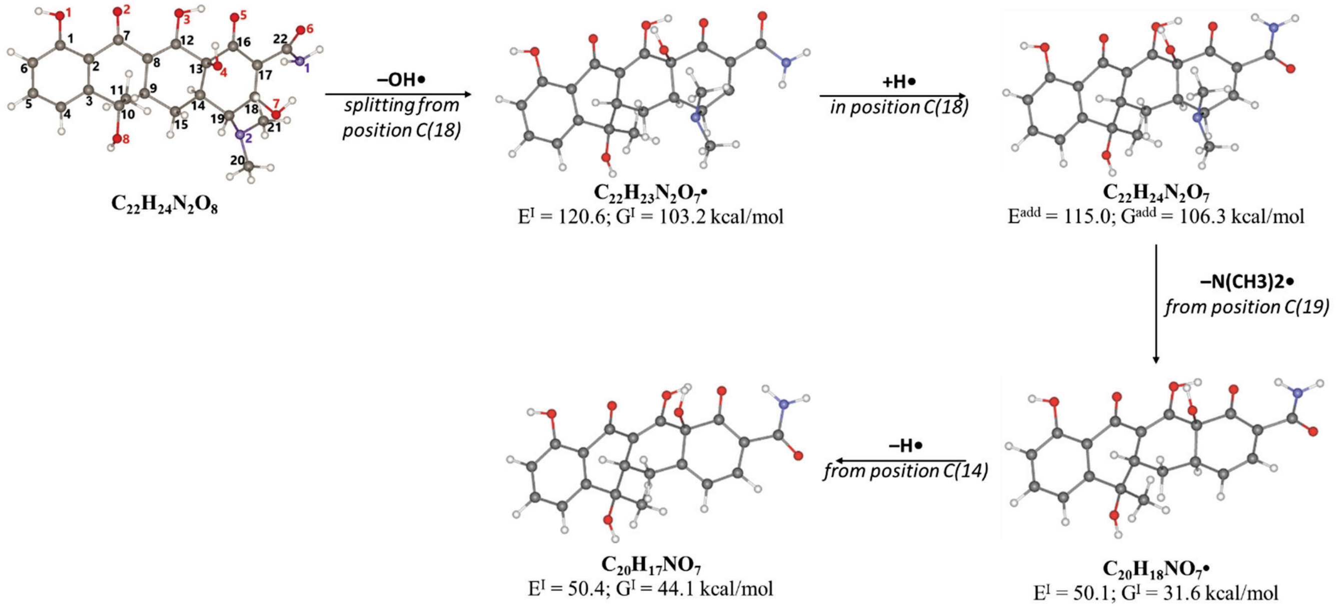


Figure 8. Formation of product DP-TC-383 with *m*/*z* value of 384.1074. Carbon atoms are grey, hydrogen atoms are white, oxygen atoms are red, and nitrogen atoms are violet.

The product DP-TC-415 is obtained by the successive elimination of one methyl radical from the nitrogen atom in the dimethylamino group and the amino group from C22 (Figure 9). This process occurs with a decrease in energy by 22.2 kcal/mol compared to the original tetracycline molecule (see Figure S38 in Supplementary Materials). Thus, the decomposition of the tetracycline molecule with the formation of the degradation product DP-TC-415 involves three direct ionization events and indirect ionization by hydrogen radical H•. This means that the degradation product DP-TC-415 requires a higher irradiation dose of 3 kGy to form from the original tetracycline molecule as opposed to the aforementioned degradation products, which require 1 kGy to be easily detectable in water.

As can be seen from Figure 4D, the concentration of the degradation product DP-TC-436 is considerably lower compared to other products, and it was found at doses above 3 kGy. Thus, it can be assumed that the formation of DP-TC-436 is a complex process requiring a greater number of ionization events. DFT calculations confirm that DP-TC-436 can be formed as a result of the addition of two hydroxyl radicals OH• at positions C8 and C9, followed by the elimination of the dimethylamino group $N(CH_3)_2$• from C19 and the addition of the amino radical NH_2• at C19, with a further elimination of the methyl radical CH_3• from C10 (Figure 10). This process occurs with a decrease in energy by 68.3 kcal/mol compared to the initial tetracycline molecule (see Figure S39 in Supplementary Materials).

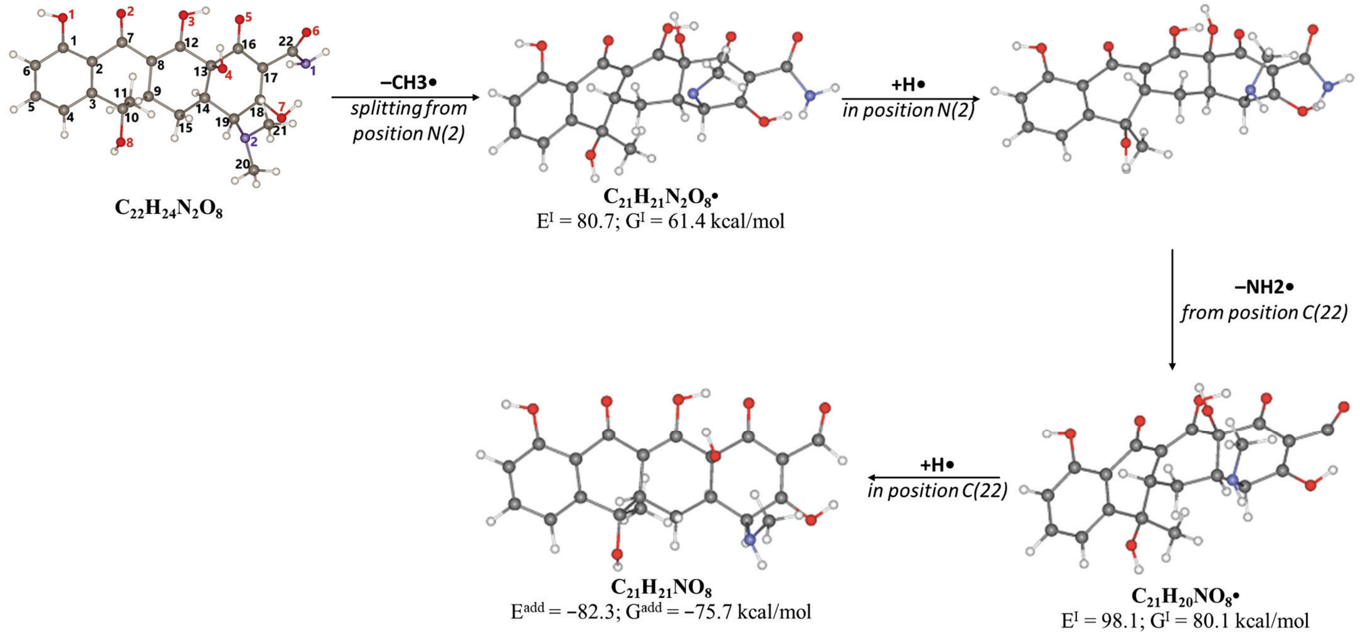


Figure 9. The formation of degradation product DP-TC-415 with an m/z value of 416.1334. Carbon atoms are grey, hydrogen atoms are white, oxygen atoms are red, and nitrogen atoms are violet.

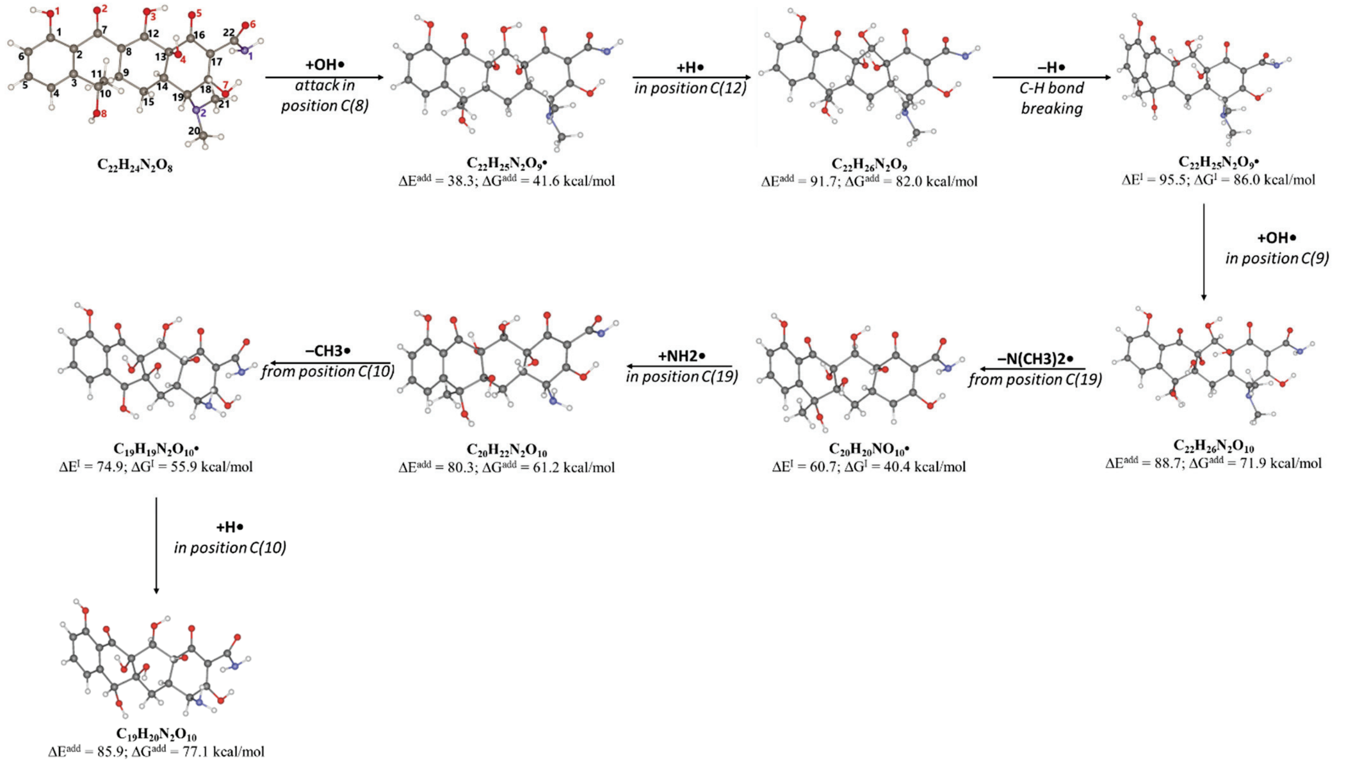


Figure 10. The formation of degradation product DP-TC-436 with an m/z value of 437.1205. Carbon atoms are grey, hydrogen atoms are white, oxygen atoms are red, and nitrogen atoms are violet.

Considering the complexity of possible radiation-induced mechanisms of antibiotic degradation in aqueous solutions calculated using the DFT model, resulting in the formation of degradation products, it can be concluded that the degradation products occurring at low doses are easily formed since they require fewer ionization events involving direct ionization by primary electrons and ionization through water radiolysis species compared to other degradation products. Considering that the mathematical model describing the dose-dependencies of the concentrations of antibiotic and degradation products yields more accurate dependencies when it processes the data on the degradation products with shorter formation pathways, such degradation products can be effectively used as markers of the degradation of initial antibiotics. Knowing the concentration of antibiotic degradation markers, the concentration of the initial antibiotic in a water source can be reconstructed to estimate the extent of contamination of the natural environment with antibiotics.

3. Materials and Methods

3.1. Research Stages

To study the impact of accelerated electrons with different doses on the degradation of antibiotics, aqueous solutions of antibiotics (tetracycline (TC), doxycycline (DOX), ampicillin (AMP), amoxicillin (AMO), benzylpenicillin (PENG), streptomycin (STR) and chloramphenicol (CAP)) were prepared for further irradiation and HPLC-HRMS analysis. The methodology consists of four main steps, including sample preparation, irradiation, dosimetry control and HPLC-HRMS analysis of irradiated and non-irradiated samples (Figure 11). Solutions of each class of antibiotics were distributed into six 0.5 mL Eppendorf tubes, with three iterations for each irradiation dose (Step 1), and then irradiated with doses of 0.1, 1, 3 and 7 kGy (Step 2). The total number of samples was 210, with 30 for each class of antibiotics. Fricke dosimetry was used to estimate the dose absorbed by the samples during irradiation. The uniformity of absorbed dose distribution was calculated using Geant 4 computer simulation (Step 3). After irradiation, the samples were analyzed to study the transformation of the original antibiotics and obtain the structure of their degradation products (Step 4). Density functional method (DFT) calculations were applied to understand the degradation mechanisms of the antibiotics (Step 5).

3.2. Reagents, Equipment, Reference Materials and Sample Preparation for Analysis

Reagents

The following reagents were used for the experiment: 98% formic acid (33015–500 ML, ACS reagent puriss. p.a., Sigma-Aldrich, St. Louis, MO, USA); acetonitrile (AC03292500, HPLC-grade, Scharlau, Barcelona, Spain); methanol (EVA-MEM-2.5, for HPLC, Eva Science, Saint Petersburg, Russia); deionized water purified in a Milli-Q system (Millipore, Temecula, CA, USA). Automatic pipettes (5–50 µL, 10–100 µL, 20–200 µL and 100–1000 µL), with a limit of measurement error of no more than ± 5% (Labmate, Chicago, IL, USA), were used to obtain an accurate aliquot. Weighing of accurate reagent suspensions was carried out on the analytical scales 'Vibra' with an accuracy of 0.0001 g (Japan).

Equipment

To analyze the degradation of antibiotics in aqueous solutions and quantify the antibiotic content as a function of exposure dose, we used an Ultimate 3000 RSLC liquid chromatograph (Dionex, Germering, Germany) with an automatic sample introduction system and an Orbitrap Fusion Lumos high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) containing an electrospray ionization source. Chromatographic separation was performed on an Acclaim RS LC HPLC column (150 mm × 2.1 mm, sorbent grain diameter 2.2 µm) manufactured by Dionex (Sunnyvale, CA, USA). A Security Guard C18 precolumn (Phenomenex, Torrance, CA, USA) was installed in the system to extend the lifetime of the chromatographic column. Chromatograms were recorded using Analyst 2005 (AB Sciex MSD, Concord, ON, Canada) and Xcalibur version 1.5 software packages. Also, for preliminary experiments to study the separation of antibiotics and optimize their detection using a mass spectrometer, an HPLC–MS system consisting of an Expec L-Chrom MS HPLC system (Expec, Hangzhou, China) was used.

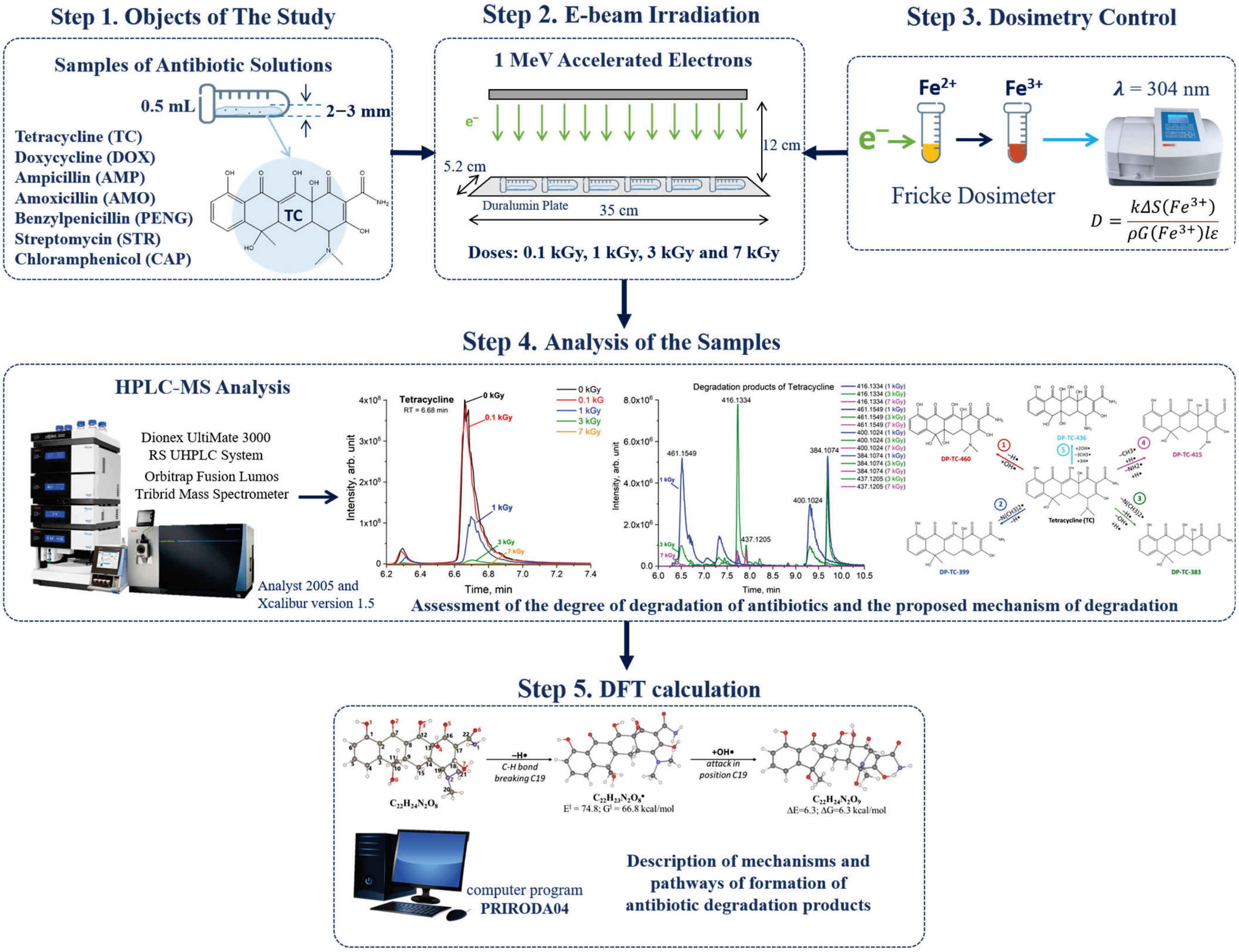


Figure 11. Research stages.

Sample Preparation

The objects of the study were aqueous solutions of standard samples of seven antibiotics, which are widely used by humans in medical practice: tetracycline (TC)—tetracycline hydrochloride, T3383-25G, Sigma-Aldrich; doxycycline (DOX)—doxycycline hyclate D9891, Sigma; amoxicillin (AMO)—amoxicillin A8523, Sigma-Aldrich; ampicillin (AMP)—ampicillin sodium salt A9518-5G, Sigma-Aldrich; benzylpenicillin (PENG)—penicillin G sodium salt 13752, Sigma; streptomycin (STR)—streptomycin sulfate salt, S6501-5G, Sigma-Aldrich; and chloramphenicol (CAP)—chloramphenicol, C0378, Sigma-Aldrich. The purity of the standards used was above 99%. The physical and chemical properties and structures of the antibiotics are summarized in Table S1 in Supplementary Materials. Stock solutions of antibiotics with concentrations of 850–1000 mg/L were prepared by dissolving suspensions of each antibiotic in an exact volume of solvent, water or methanol, depending on the solubility of the antibiotics.

The concentrations of antibiotics in aqueous solutions prepared for irradiation were TC—21.3 mg/L, DOX—21 mg/L, PENG—18.2 mg/L, AMP—18.3 mg/L, AMO—19.8 mg/L, CAP—15.2 mg/L and STR—29.3 mg/L. All solutions were placed in 2 mL Eppendorf-type plastic microcentrifuge tubes, with 0.5 mL of solution in each Eppendorf tube (OAO RZP, Rybinsk, Russia) for subsequent irradiation. For each antibiotic, 30 Eppendorf tubes were prepared, with three iterations for each radiation dose.

3.3. E-Beam Irradiation of Aqueous Solutions

The aqueous solutions containing antibiotics were irradiated with accelerated electrons generated by a continuous electron accelerator UELR-1-25-T001 (Skobeltsyn Research Institute of Nuclear Physics, Moscow State University, Moscow, Russia) with a maximum beam energy of 1 MeV (Figure 2A). The beam current varied from 360 to 2633.3 nA to reduce the exposure time when the samples were irradiated with high doses. For each irradiation session, 6 Eppendorf tubes containing antibiotic solutions were placed on a duralumin plate according to the irradiation method described in [36]. During each irradiation session, to control the dose absorbed by the samples, we measured the charge absorbed by the duralumin plate. The margin of error in the charge absorbed by the plate, measured using ADC (LLC 'Production Association OVEN', Moscow, Russia), did not exceed 2%. To ensure uniform dose distribution over the entire volume of the samples due to the low penetration depth of 1 MeV electrons, the height of the solution was no more than 2 mm. Based on Geant 4 computer simulation [36], it was found that the dose uniformity in aqueous solutions when irradiated with 1 MeV electrons was 0.6. A wide dose range from 0.1 kGy to 7 kGy was selected in order to cover the dose range commonly used in water and food irradiation. The ambient temperature during each irradiation session was 20 °C.

3.4. Dosimetry Control

A ferrous sulfate Fricke dosimeter was used to measure the doses absorbed by the samples. A total of 0.5 mL of $FeSO_4$ solution was placed in 2 mL Eppendorf tubes similar to the experimental ones and irradiated under the same conditions as the antibiotic solutions. The exposure time of the solution was recorded during each session. As a result of the radiolysis of water under the action of free radicals reacting with $FeSO_4$ solution, Fe^{2+} ions are oxidized to Fe^{3+} ions, which leads to a change in the optical density of the solution. The concentration of Fe^{3+} ions was estimated by comparing the optical densities of irradiated and non-irradiated samples using a spectrophotometer UV-3000 (TM Ecoview, Moscow, Russia) at a wavelength of 304 nm [37].

Based on the change in optical density, which depends on the transition of Fe^{2+} to Fe^{3+} at irradiation with different doses, the following formula was used to find the doses absorbed by the solutions:

$$D = \frac{k\Delta S\left(Fe^{3+}\right)}{\rho G\left(Fe^{3+}\right) l\varepsilon}, \tag{13}$$

where $k = 9.65 \times 10^6$ is the dimensionless coefficient, ΔS is the optical density of the Fricke solution, $\rho = 1.024$ g/cm^3 is the density of the solution, $G(Fe^{3+}) = 15.\ 6$ ion/100 eV is the radiation–chemical yield when exposed to accelerated electrons with the energy up to 10 MeV, $l = 1$ cm is the optical path, and $\varepsilon = 2160$ l/(mol•cm) is the extinction coefficient of Fe^{3+} ions [38].

Table 6 contains the exposure time, beam current, the charge absorbed by the duralumin plate and the dose for twelve irradiation sessions. The margin of error in each irradiation parameter was no more than 5%. The samples were irradiated with doses of 0.1 kGy, 1 kGy, 3 kGy and 7 kGy.

Table 6. Irradiation parameters.

Session	Irradiation Time (avg.), s	Charge (avg.), nC	Dose (avg.), Gy
1–3	32.3	5375	103.4
4–6	122.3	52,356.7	1007
7–9	73.7	157,733.3	3033.3
10–12	150	365,666.7	7032.1

3.5. HPLC-MS Analysis

Immediately after irradiation, the antibiotic solutions were subjected to HPLC-MS analysis. Two 0.5 mL antibiotic solutions from each Eppendorf tube irradiated with the same dose were placed in 1.5 mL chromatographic vials. The electrospray ionization source was used in the mode of registration of positively charged or negatively charged ions. The resolution of the mass analyzer was not less than 30,000 rel.un., and the error in determining m/z values did not exceed 5 ppm. Chromatographic separation was carried out in the gradient elution mode. The mobile phase A was 0.1% formic acid, and the mobile phase B was acetonitrile. All the conditions of HPLC-MS analysis are summarized in Table A1 in Appendix A.

3.6. Density Functional Theory (DFT) Calculation

To calculate the most probable transformation pathways of antibiotics to the identified degradation products, DFT calculations were used.

The geometries of molecules were fully optimized by means of density functional theory (DFT) calculations. We used first-principles PBE functionals [39]. The full electron basis set L1 was used, where L1 stands for double set size. The numbers of contracted and primitive functions used in L1 are, respectively, {2,1}/{6,2} for H and {3,2,1}/{10,7,3} for C, N and O [40]. Stationary points on the potential energy surface (PES) were identified by analyzing Hessians. The thermodynamic functions (Gibbs energies, G) at 298.15 K were calculated using an approximation of a restricted rotator and harmonic oscillator. All calculations were performed using a personal computer with the use of the PRIRODA04 program written by Laikov [41].

4. Conclusions

The study focuses on determining the doses of electron beam irradiation at which seven antibiotics commonly used in medicine—ampicillin, amoxicillin, benzylpenicillin, streptomycin, tetracycline, doxycycline and chloramphenicol—are removed from water. It has been proven that 1 MeV electron beam irradiation with a dose of 7 kGy ensures 98–99% removal of antibiotics, with the initial concentrations ranging from 15 mg/L to 30 mg/L depending on the antibiotic type. It should be noted that the antibiotics selected for the study have different radiosensitivities. While in the case of ampicillin, amoxicillin and benzylpenicillin, their degradation products were completely eliminated from the water after irradiation with 7 kGy, the degradation products of streptomycin and tetracycline were still present in negligible amounts in the water after irradiation with the same dose. A mathematical model used in the study, describing the monotonous decline of antibiotics and non-monotonous behavior of antibiotic degradation products depending on the irradiation dose, determined that the decomposition rate of antibiotics of the penicillin class is, on average, two times higher than that of streptomycin and tetracycline. In contrast, chloramphenicol with an initial concentration of 15.15 mg/L had the highest resistance to e-beam irradiation, and even the highest dose of 7 kGy could not completely eliminate it from the water, and at the same time, its decomposition rate was the lowest.

It has been found that different degradation products of antibiotics are detected at different irradiation doses. Density functional theory calculations have proven that the presence of the threshold dose at which specific degradation products are found is determined by the number of ionization events triggering the chemical transformation of antibiotic molecules. The degradation products requiring only one or two direct or indirect ionization events can serve as potential markers of antibiotic degradation since the mathematical model proposed in the study allows the dose dependencies of the initial antibiotics to be reconstructed with high accuracy.

Future studies will validate the application of electron beam irradiation for the treatment of wastewater and foods, assess the toxicological safety of degradation products, and optimize electron beam irradiation efficiency. The radiation-induced mechanisms of antibiotic degradation, confirmed by DFT calculations, open the way to more effective methods of radiation–chemical purification of water that may contain antibiotics and other drugs. Our further research will aim at gaining a deeper understanding of the combined action of ozonation and electron beam irradiation to enhance the efficiency of the removal of antibiotics and degradation products when treating water sources or biological substances. Further, we intend to study the impact of water quality parameters on antibiotic degradation as a result of irradiation to understand how varying water parameters can affect the outcome of irradiation efficiency. Such studies will accelerate the practical deployment of electron beam irradiation to ensure water and food safety in response to the challenges faced by the natural environment due to the overuse and incorrect disposal of antibiotics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics14080833/s1. Figure S1: TIC chromatogram of Tetracycline, retention time(RT) 6.68 min; Figure S2: ESI mass spectra of Tetracycline in the positive ion mode; Figure S3: MS2 spectra of Tetracycline molecular ion m/z 445.1592; Figure S4: MS2 spectra of DP-TC-460 with m/z 461.1549; RT = 6.51 min; detected at a dose of 1 kGy; Figure S5: MS2 spectra of DP-TC-399 with m/z 400.1024; RT = 9.33 min; detected at a dose of 1 kGy; Figure S6: MS2 spectra of DP-TC-383 with m/z 384.1074; RT = 9.68 min; detected at a dose of 1 kGy; Figure S7: MS2 spectra of DP-TC-415 with m/z 416.1334; RT = 7.74 min; detected at a dose of 1 kGy; Figure S8: MS2 spectra of DP-TC-436 with m/z 437.1205; RT = 7.92 min; detected at a dose of 3 kGy; Figure S9: TIC chromatogram of Amoxicillin, retention time 4.45 min; Figure S10: ESI mass spectra of Amoxicillin in the positive ion mode; Figure S11: MS2 spectra of Amoxicillin molecular ion m/z 366.1110; Figure S12: MS2 spectra of DP-AMO-364 with m/z 365.0801; RT = 2.81 min; detected at a dose of 0.1 kGy; Figure S13: MS2 spectra of DP-AMO-381 with m/z 382.1066; RT = 1.97 min; detected at a dose of 0.1 kGy; Figure S14: TIC chromatogram of Ampicillin, retention time 6.00 min; Figure S15: ESI mass spectra of Ampicillin in the positive ion mode; Figure S16: MS2 spectra of Ampicillin molecular ion m/z 350.1163; Figure S17: MS2 spectra of DP-AMP-365 with m/z 366.1111; RT = 5.12 min; detected at a dose of 0.1 kGy; Figure S18: MS2 spectra of DP-AMP-367 with m/z 368.1274; RT = 5.60 min; detected at a dose of 0.1 kGy; Figure S19: TIC chromatogram of Benzylpenicillin, retention time 9.62 min; Figure S20: ESI mass spectra of Benzylpenicillin in the positive ion mode; Figure S21: MS2 spectra of Benzylpenicillin molecular ion m/z 335.1056; Figure S22: MS2 spectra of DP-PENG-350 with m/z 351.1007; RT = 8.16; 8.40; 8.83 min; detected at a dose of 0.1 kGy; Figure S23: MS2 spectra of DP-PENG-352 with m/z 353.1168; RT = 7.83 min; detected at a dose of 0.1 kGy; Figure S24: MS2 spectra of DP-PENG-308 with m/z 309.1276; RT = 7.83 min; detected at a dose of 0.1 kGy; Figure S25: TIC chromatogram of Streptomycin, retention time 0.86 min; Figure S26: ESI mass spectra of Streptomycin in the positive ion mode; Figure S27: MS2 spectra of Streptomycin molecular ion m/z 582.2725; Figure S28: MS2 spectra of DP-STR-301 with m/z 302.1233; RT = 1.31 min; detected at a dose of 0.1 kGy; Figure S29: MS2 spectra of DP-STR-289 with m/z 290.1232; RT = 1.82 min; detected at a dose of 0.1 kGy; Figure S30: MS2 spectra of DP-STR-287 with m/z 288.1077; RT = 3.07 min; detected at a dose of 0.1 kGy; Figure S31: TIC chromatogram of Doxycycline, retention time 7.70 min; Figure S32: ESI mass spectra of Doxycycline in the positive ion mode; Figure S33: MS2 spectra of Doxycycline molecular ion m/z 445.1594; Figure S34: TIC chromatogram of Chloramphenicol, retention time 8.73 min; Figure S35: ESI mass spectra of Chloramphenicol in the negative ion mode; Figure S36: MS2 spectra of Chloramphenicol molecular ion m/z 321.0050; Figure S37: Dependence of free energy on the structure of the compound for DP-TC-383; Figure S38: Dependence of free energy on the structure of the compound for DP-TC-415; Figure S39. Dependence of free energy on the structure of the compound for DP-TC-436; Table S1: Physical and chemical properties of antibiotics; Table S2: Hydroxylated decomposition products resulting from the cleavage of the C-H bond and the addition of an OH-group. Reference [42] is cited in the supplementary materials.

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Abbreviations

The following abbreviations are used in this manuscript:

HPLC-HRMS	**High-performance liquid chromatography combined with high-resolution mass spectrometry**
DFT	Density functional theory
TC	Tetracycline
DOX	Doxycycline
AMP	Ampicillin
AMO	Amoxicillin
PENG	Penicillin G (Benzylpenicillin)
STR	Streptomycin
CAP	Chloramphenicol
DPs	Degradation products

Appendix A

Table A1. HPLC-MS analysis conditions.

HPLC-MS Parameters	Value
HPLC	
Column	Thermo RS LC HPLC (150 mm × 2.1 mm, 2.2 μm particle size)
Mobile phases	Mobile phase *A*: 0.1% formic acid in water Mobile phase *B*: 100% acetonitrile
Flow rate	0.3 mL/min
Column temperature	35 °C
Injection volume	20 μL
Gradient elution mode	0–2 min—5% B; 2–15 min—95% B; 15–18 min—95% B; 18–19 min—5% B; 19–24 min—5% B.
MS	
Ionization method	Electrospray ionization (ESI)
Ion generation mode	Both polarities (+ and −)
Ion source temperature	325 °C
Drying gas pressure	344.7 kPa
Nebulizing gas pressure	69 kPa
Capillary voltage	+3500; −2500 V
Mass scanning range	100–1000 m/z

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antibiotics

Article

Antimicrobial Use and Antimicrobial Resistance in Food-Producing Animals: Cross-Sectional Study on Knowledge, Attitudes, and Practices Among Veterinarians and Operators of Establishments in the Republic of Cyprus

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Abstract: Background: Current evidence suggests that more than half of all antimicrobials are used in the sector of food-producing animals, thus constituting a major risk factor for development and spread of antimicrobial resistance (AMR). **Methods**: This cross-sectional study assessed the knowledge, attitudes, and practices regarding antimicrobial resistance (AMR) and antimicrobial use (AMU) among veterinarians (n = 26) working with food-producing animals and operators (n = 165) of establishments that keep food-producing animals, across all districts of Cyprus between October and December 2024. **Results**: Most veterinarians demonstrated sufficient knowledge regarding AMR and AMU; however, certain knowledge gaps were identified. There was a general trend toward desired responses aimed at reducing AMU. Despite this, the level of responses advocating for restrictions on the use of specific priority antimicrobials and broad-spectrum antibacterials was unsatisfactory. Over half of veterinarians prescribed Category B substances. Furthermore, there was no significant association between the use of "restricted" antibiotics and veterinarians' level of knowledge nor between antibiotic use and the practice of microbiological culture and susceptibility testing. Among operators, positive attitudes were significant predictors of implementing of good practices. Additionally, operators who had contracts with a veterinarians were more likely to follow good practices. **Conclusions**: There is a need for further education on AMR in the veterinary sector in the Republic of Cyprus. Raising awareness among animal producers, is also crucial, along with enforcing a policy on antimicrobial use. Comprehensive governance involving all stakeholders must be implemented to address AMR more effectively.

Keywords: antimicrobial resistance; antibiotic use; surveillance; food-producing animals; veterinarians; operators; one health

1. Introduction

Antimicrobial resistance (AMR) is recognized globally as one of the most serious public health challenges. In 2019, AMR was identified by the World Health Organization (WHO) as one of the most serious global threats to public health, and in July 2022, it was identified by

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the European Commission as one of the three major threats to public health in the European Union (EU) [1]. AMR poses a cross-border threat to public health, animal health, plant health, and the environment. Infections due to resistant bacteria account for 700,000 deaths globally every year and could cumulate to 10 million by 2050 if no constant efforts to tackle AMR are implemented [2,3]. The estimates from the European Union/European Economic Area (EU/EEA) indicate that every year, more than 670,000 infections are due to resistant bacteria, and approximately 33,000 people die as a direct consequence. [4]. The health burden of antimicrobial resistance is caused by 40% of infections with bacteria resistant to critically important antibiotics [5]. According to the Organization for Economic Co-operation and Development (OECD), the rates of AMR infections will continue to rise in the EU/EEA, with a significant impact on the budget of healthcare systems [5]. Without immediate effective action, the annual cost of AMR is expected to increase to EUR 1.1 billion between 2015 and 2050 in all EU and EEC countries [5].

The four main sectors involved in the spread of AMR are the human medicine sector in the community and in the hospital settings, animal production, agriculture, and the environment [6]. According to The State of the World's Antibiotics 2015, two thirds of all antibiotics produced worldwide each year (65,000 tons out of 100,000 tons) are used in livestock farming [7]. Similarly, the global AMU in bovine animals, poultry, and porcine animals was estimated at 93,309 tons in 2017, and another study showed that the same animal groups, along with ovine animals, consumed 99,502 tons of antibiotics in 2020 and was predicted, based on those trends, to increase to 107,472 tons by 2030 [8]. It was also estimated that worldwide, the antimicrobial use in food-producing animals exceeds the antimicrobial use in human health [9]. The sixth report of WOAH, which includes the AMU reported by 109 participant countries for 2018, showed that 69,455 tons of antimicrobials were used in food animals in 2018. WOAH estimates that the adjusted total amount could be 76,704 tons [10]. According to the thirteenth ESVAC report which presents data on the sales of veterinary antibiotic agents from 31 European countries in 2022, overall aggregated sales of antibiotic veterinary medicinal products were 73.9 mg/PCU.

The transmission of antibiotic-resistant bacteria and genes from animals to humans has been well documented in the literature [11–13]. The transmission of AMR to establishments keeping food-producing animals has been studied across various species including [14,15] pigs, cattle [16], and arthropods [17–19]. The extent of AMR transmission between animals and humans is a topic of great interest, with significant implications for both public and animal health [20–22]. Notably, EFSA's 2021 scientific opinion on the role of the environment in the emergence and spread of antimicrobial resistance through the food chain provides insight into the role of environmental factors in the spread of AMR in plant-based food production in the EU, terrestrial animals (poultry, cattle, and pigs), and aquaculture [23].

The development and spread of antimicrobial resistance is extensively seen by organizations like the European Food Safety Authority, the World Health Organization, and the Lancet Infectious Diseases Commission as a consequence of the use and overuse of antibiotics in both human and veterinary medicine. Scientific research led to a consensus among many scientists that, for certain bacterial infections, such as *Campylobacter* spp. and *Salmonella* spp., the use of antibiotics in animal farms is the primary cause of resistance to human infections. The emergence of resistance to critically important antibiotics in *Escherichia coli* and *Salmonella* spp. is a significant issue, resulting from the inappropriate use of these antibiotics in both human and veterinary medicine. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains that affect humans are also becoming an increasing concern, due to the widespread use of certain antibiotics in food-producing animals [24]. The prudent use of antimicrobials along with high standards of infection prevention and control in both animals and humans, are crucial components in tackling the issue of antimicrobial resistance. Understanding the knowledge, attitudes, and practices (KAP) of veterinarians as well as those of operators of establishments keeping food-producing animals is crucial for developing effective interventions. [25]. Insufficient knowledge and awareness of antimicrobial use (AMU) and AMR have been reported as contributing factors to the inappropriate prescribing and dispensing of antibiotics. [26,27]. These groups are therefore considered key stakeholders, central to the efforts required to mitigate the emerging threat of AMR globally.

According to the thirteenth ESVAC report, Cyprus has the highest sales at 254.7 mg/PCU. Although overall sales in 2022 decreased by 14.1% in comparison to 2021 (from 296.5 mg/PCU to 254.7 mg/PCU) they are still at a high level. In 2022, the proportion of veterinary antimicrobials belonging to AMEG categories B was at 3.3%. The overall sales of the antibiotic classes of EMA AMEG Category B, i.e., third- and fourth-generation cephalosporins, fluoroquinolones, other quinolones and polymyxins among 31 countries, from <0.01 to 0.47 mg/PCU, <0.01 to 12.6 mg/PCU, 0 to 0.75 mg/PCU, and 0 to 10.2 mg/PCU, accounting for 0.17%, 2.8%, 0.16%, and 2.8% of total aggregated sales, respectively [28]. This report highlights certain classes of antimicrobials included in Category B of the categorization made by the EMA's Antimicrobial Advice ad hoc Expert Group (AMEG) in 2019 [29]. The World Health Organization (WHO) categorization of antimicrobials (6th revision) [30] is taken into consideration, as well as the necessity for the respective antimicrobials in veterinary medicine and the probability of transferring antimicrobial resistance from animals to humans. Category B of the AMEG categorization includes those veterinary antibiotics from which the risk to public health is estimated to be higher than from other classes of antibiotics; fluoroquinolones, other quinolones, third- and fourth-generation cephalosporins, and polymyxins are included in this category. Third- and fourth-generation cephalosporins, and fluoroquinolones are considered by the WHO as 'highest-priority critically important antimicrobials' (HPCIAs) in human medicine. These groups have also been categorized as Veterinary Critically Important Antimicrobial Agents in the WOAH list of antimicrobials of veterinary importance [31].

According to the most recent Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA VI report) published in 2024, in 2021, the total antimicrobial consumption (AMC) was assessed at 125.0 mg/kg of biomass for humans (28 EU/EEA countries, range 44.3–160.1). In Cyprus, the total AMC was assessed at 139.9 of biomass for humans and 296.5 mg/kg of biomass for food-producing animals. The total AMC was assessed at 92.6 mg/kg of biomass for food-producing animals (29 EU/EEA countries, range 2.5–296.5). In 2021, the EU/EEA population-weighted mean consumption of fluoroquinolones and other quinolones was 6.3 mg/kg estimated biomass in humans and 2.9 mg/kg of estimated biomass in food-producing animals. The EU/EEA population-weighted mean consumption of fluoroquinolones and other quinolones was 6.3 mg/kg estimated biomass in humans and 2.9 mg/kg of estimated biomass in food-producing animals. The relevant mean for polymyxins was 0.7 mg/kg and 2.5 mg/kg, respectively. There was a statistically significant association between the consumption of fluoroquinolones in food-producing animals and fluoroquinolone resistance in *E. coli* from food-producing animals. The same results were observed for colistin for the time periods 2018–2019 and 2019–2020 [32].

Evidence recorded to describe antimicrobial resistance levels in the annual reports of the European Union on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals, and food, shows very high resistance to fluoroquinolones (ciprofloxacin) among *Salmonella* isolates from broilers (50%) in Cyprus [32,33]. Multidrug resistance was observed at very to extremely high levels among *Salmonella* spp., *Campylobacter coli* and *Campylobacter jejuni*, and indicator *Escherichia coli* recovered from food-producing animals. Resistance to ciprofloxacin ranged from high to extremely high in *C. jejuni*, *C. coli* isolates, and *E. coli* commensal from food-producing animals. A statistically significant increase in resistance to ciprofloxacin was noted in *E. coli* isolates from broilers between 2014 and 2022. Additionally, resistance to erythromycin was observed at very high levels in *C. coli* from pigs.

To this end, evaluating the knowledge, attitudes, and practices (KAP) of veterinarians and operators of food-producing animal establishments is important for a better understanding the drivers of antimicrobial resistance in the animal health sector and providing evidence for the development of effective interventions.

2. Results

2.1. Socio-Demographic Characteristics of Respondents

Overall, all veterinarians (n = 26) working in the food-producing animal sector and 165 (approximately 50% of the total) operators of establishments keeping food-producing animals participated in the current study. The veterinarian's socio-demographic characteristics are presented in Table 1.

Table 1. Socio-demographic characteristics of veterinarians participating in the study.

Variables		**N (%)**
Gender	Female	6 (23.1)
	Male	20 (76.9)
Age (years)	23–30	3 (11.5)
	31–45	14 (53.8)
	46–60	7 (26.9)
	61 or more	2 (7.7)
Educational level	Degree in veterinary medicine	17 (65.4)
	Master's degree or doctorate	9 (34.6)
Area of residence	Rural	12 (46.2)
	Urban	14 (53.8)
Training on the use of antimicrobials		26 (100.0)
Percentage of veterinarians working at a veterinary pharmaceutical company		9 (34.6)

The relevant characteristics of operators of establishments keeping food-producing animals, are presented in Table 2. The distribution of the studied animal population in each district is included in Table 3.

Table 2. Socio-demographic characteristics of operators participating in the study.

Variables		**N (%)**
Gender	Female	20 (12.1)
	Male	145 (87.9)
Age (years)	18–30	17 (10.3)
	31–45	52 (31.5)
	46–60	43 (26.1)
	61 or more	53 (32.1)

Table 2. *Cont.*

Variables		N (%)
Educational level	Elementary school graduate	22 (13.3)
	High school graduate	85 (52.5)
	Undergraduate degree	47 (28.5)
	Master's degree or doctorate	11 (6.7)
Area of residence	Rural	108 (65.5)
	Urban	57 (34.5)
District where establishments are located	Nicosia	64 (38.8)
	Larnaca	42 (25.5)
	Limassol	16 (9.7)
	Ammochostos	14 (8.5)
	Paphos	29 (17.6)
Types of establishments	Bovine animal establishments	32 (19.4)
	Ovine and caprine animal establishments	103 (62.4)
	Porcine animal establishments	16 (9.7)
	Poultry establishments	14 (8.5)
Professional experience (years)	<4	12 (7.3)
	5–8	15 (9.1)
	9–12	16 (9.7)
	>13	122 (73.9)
Main employment/occupation	Livestock farming	148 (89.7)
	Other	17 (10.3)

Table 3. Distribution of food-producing animal establishments which constituted the population studied.

	Types of Farms			
District	**Number of Bovine Establishments n (%)**	**Number of Ovine and Caprine Establishments n (%)**	**Number of Porcine Establishments n (%)**	**Number of Poultry Establishments n (%)**
Nicosia	14 (43.8)	32 (31.1)	8 (50.0)	10 (71.4)
Larnaca	9 (28.1)	25 (24.3)	7 (43.8)	1 (7.1)
Limassol	2 (6.3)	14 (13.6)	0 (0.0)	0 (0.0)
Ammochostos	2 (6.3)	10 (9.7)	0 (0.0)	2 (14.3)
Paphos	5 (15.6)	22 (21.4)	1 (6.3)	1 (7.0)
Total	32 (100.0)	103 (100.0)	16 (100.0)	14 (100.0)

2.2. *Knowledge, Attitudes, and Practices of Veterinarians on AMR and AMU*

Generally, the submitted responses indicate a high level of knowledge (Table 4 and Figure 1), with more than 82% of answers being correct. The majority of veterinarians working with food-producing animals reported being aware of the severity of antimicrobial resistance.

Table 4. The proportion of veterinarians who correctly/positively responded to knowledge, attitudes, and practices questions on AMR and AMU.

	Knowledge Factors	**N (%)**
V_B1	Do you know what antimicrobial resistance is?	26 (100.0)
V_B2	Are you aware of the World Health Organization's (WHO) categorization of antimicrobials?	22 (84.6)
V_B3	Are you aware the categorization of veterinary antimicrobials by the World Organization for Animal Health (WOAH)?	22 (84.6)
V_B4	Are you aware of the updated criteria for the categorization of antibiotics used in veterinary medicine as established by an expert group (AMEG) of the European Medicines Agency (EMA)?	14 (53.8)
V_B5	Do you consider antimicrobial resistance to be a serious public health threat?	24 (92.3)
V_B6	Do you believe that the use of antibiotics in animals is a major contributor to the development of resistance to bacterial infections in humans?	21 (80.8)
V_B7	Are you aware of information about methicillin-resistant *Staphylococcus aureus* (MRSA)?	18 (69.2)
V_B8	Do you believe that the use of expired antimicrobials contributes to the development of antimicrobial resistance?	17 (65.4)
V_B9	Do you know what antimicrobial stewardship is?	10 (38.5)
V_B10	Are you familiar with the recommendations outlined in the Council Recommendation (2023) on strengthening EU action to combat antimicrobial resistance within the context of the One Health approach?	12 (46.2)
V_B11	Do you believe that antimicrobials are effective in treating both bacterial and viral infections?	18 (69.2)
V_B12	Do you believe there is an ongoing misuse of antibiotics in the veterinary sector?	21 (80.8)
V_B13	Do you think that antibiotic residues in food contribute to the development of antimicrobial resistance in humans?	22 (84.6)
	Attitude Factors	**N (%)**
V_C1	The potential contribution of veterinary antimicrobial medicinal products to the development of resistance in humans is concerning.	18 (69.2)
V_C2	I support the policy of reducing veterinary antibiotic consumption by 50% by 2030, compared to 2019 levels.	19 (73.1)
V_C3	My goal is to minimize the use of antibiotics as much as possible within the context exercise of my profession.	23 (88.5)
V_C4	Reducing veterinary antibiotic use by 50% will lead to negative effects on animal health and welfare.	12 (46.2)
V_C5	I have become more aware of the need to impose restrictions to the use of antibiotics in recent years.	19 (73.1)
V_C6	Do you think that using two or more classes of antibiotics in combination is always more effective for infection control?	14 (53.8)
V_C7	Do you believe that a thorough examination of animals is necessary before prescribing or administrating an antibiotic?	26 (100.0)
V_C8	Do you think that broad-spectrum antibiotics are better than narrow-spectrum antibiotics, even when narrow spectrum drugs are available and suitable?	15 (57.7)
V_C9	Do you believe priority antibiotics should be restricted to human use only?	13 (50.0)
V_C10	Do you think that improved livestock practices and the implementation of biosecurity measures can help reduce antibiotic consumption?	26 (100.0)
	Practices Factors	**N (%)**
V_D1	Do you use microbiological culture and susceptibility testing to select the appropriate antibiotics for treatment?	8 (30.8)
V_D2	Do you check the expiry date of antibiotics before using them?	26 (100.0)
V_D3	Do you advise farmers on the withdrawal period for antimicrobial medicines?	26 (1000)

Table 4. *Cont.*

	Practices Factors	N (%)
V_D4	Do you provide advice to farmers on how to use antimicrobial medicines over the phone?	10 (38.5)
V_D5	Do you often prescribe more than one antimicrobial in a single prescription?	16 (61.5)
V_D6	Do you advise farmers to complete the full course of antimicrobials that you prescribe?	26 (100.0)
V_D7	Are you using alternatives to antimicrobials in your practice?	24 (92.3)
V_D8	Do you advise farmers on appropriate vaccination strategies to help reduce the use of antimicrobials?	26 (100.0)

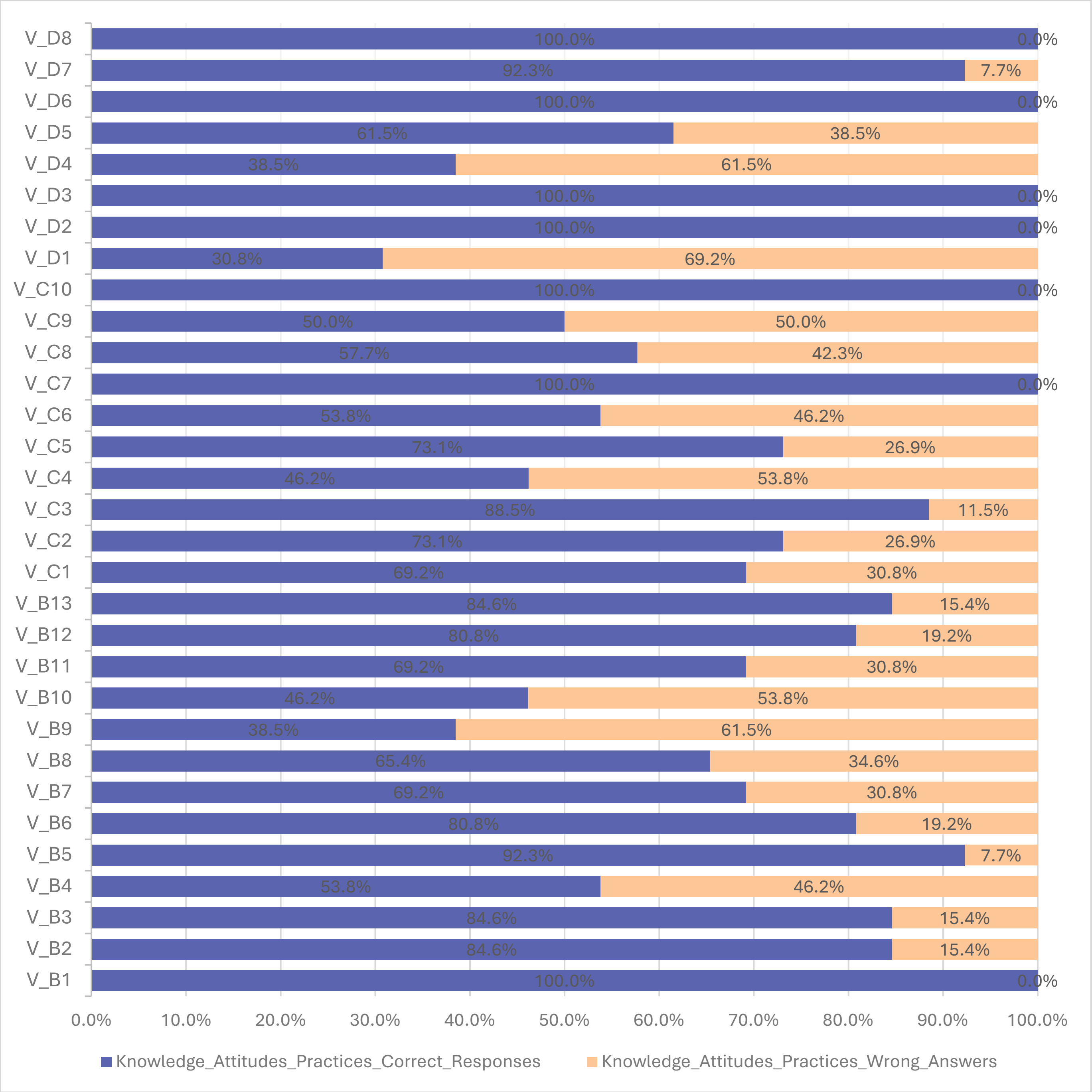

Figure 1. The proportion of veterinarians who correctly or incorrectly responded to knowledge, attitudes, and practices questions on AMR and AMU.

Regarding the need to restrict the use of specific classes of antibiotics (HPCIAs; critically important for human medicine) as well as broad-spectrum antibiotics, there was a lack of positive attitudes, with a target response rate of only 57.7%. Additionally, only half of the participants agreed that critically important antibiotics should be restricted to human use only. Fewer than half of the participants responded negatively to the question of whether reducing the use of veterinary antibiotics by 50% would have negative effects on animal health and welfare (46.2%, n = 12). Similarly, the proportion of respondents who answered negatively to the question asking "if using two or more classes of antibiotics in combination is always better for infection control" was also low (53.8%, n = 14).

Interestingly, 61.5% of all respondents frequently prescribed more than one antimicrobial in a single prescription. Additionally, more than half of the veterinarians (57.7%, n = 15) prescribe Category B antibiotics (polymyxins, quinolones, fluoroquinolones), which are considered "restricted" according to the European Medicines Agency (EMA) categorization. On the other hand, 69.2% of veterinarians involved with food-producing animals prescribe Category D antibiotics (such as penicillins and tetracyclines), and 50% of them prescribe Category C antibiotics.

To initially investigate whether there is an association between the knowledge of the updated criteria for classifying antibiotics by the EMA and the use of "restricted" antibiotics (Class B), the chi-squared statistical test was applied. The results indicated no statistically significant association between the level of knowledge on antibiotic categorization criteria and the use of Class B antibiotics. This observation suggests that although there is knowledge, attitudes may influence practices that are not aligned with recommended guidelines.

The number of respondents using microbiological culture and susceptibility testing to select the appropriate antibiotics for treatment was low (30.8%, n = 8). When investigating the dependence between the use of microbiological culture and susceptibility testing and the use of Class B antibiotics, no statistically significant association was found between the relevant variables. This suggests that Class B antibiotics are often prescribed without prior susceptibility testing. As for Class D antibiotics (considered for "prudent use"), they are commonly used alongside Category B and Category C antibiotics, with only two veterinarians prescribing only Class D antibiotics.

2.3. Association Between Factors Related to Knowledge, Attitudes, and Practices

Table 5 presents the cross-tabulation of the use of "restricted" antibiotics in food-producing animals and various variables related to KAP. No statistically significant association was observed between the tested variables.

Table 5. Cross-tabulations: the variable "use of class B antibiotics" and variables related to knowledge, attitudes, and practices among veterinarians.

		Use of Class B Antibiotics		***p*-Value**	**Odds Ratio for (No/Yes)**	**95% Confidence Interval**	
		No	**Yes**			**Lower Bound**	**Upper Bound**
Veterinary Pharmaceutical companies	No	6 (75.0%)	11 (73.3%)	0.931	1.09	0.15	7.80
	Yes	2 (25.5%)	4 (26.7%)				
Knowledge—EMA classification	No	4 (50.0%)	6 (40.0%)	0.645	1.50	0.27	8.45
	Yes	4 (50.0%)	9 (60.0%)				
Knowledge—Antimicrobial stewardship	No	6 (75.0%)	10 (66.7%)	0.676	1.50	0.22	10.30
	Yes	2 (25.0%)	5 (33.3%)				

Table 5. *Cont.*

		Use of Class B Antibiotics		*p*-Value	Odds Ratio for (No/Yes)	95% Confidence Interval	
		No	**Yes**			**Lower Bound**	**Upper Bound**
Knowledge—Council Recommendations (2023)	No	3 (37.5%)	10 (66.7%)	0.179	0.30	0.05	1.80
	Yes	5 (62.5%)	5 (33.3%)				
Attitude—Reducing by half the use of veterinary antibiotics	Yes	1 (12.5%)	4 (26.7%)	0.433	0.39	0.04	4.28
	No	7 (87.5%)	11 (73.3%)				
Attitude—Use of two or more classes of antibiotics in combination	Yes	4 (50.0%)	6 (40.0%)	0.645	1.50	0.27	8.45
	No	4 (50.0%)	9 (60.0%)				
Attitude—Broad-spectrum antibiotics vs. narrow spectrum	Yes	3 (37.5%)	6 (40.0%)	0.673	0.69	0.12	3.96
	No	5 (62.5%)	9 (60.0%)				
Attitude—Restrict priority antibiotics to human use only	No	4 (50.0%)	6 (40.0%)	0.645	1.50	0.27	8.45
	Yes	4 (50.0%)	9 (60.0%)				

2.4. Knowledge, Attitudes, and Practices of Operators of Establishment Keeping Food-Producing Animals on AMR and AMU

Based on responses, participants' knowledge of AMR and the use of antimicrobials is generally moderate (Table 6 and Figure 2). It was also observed that a high percentage of operators (82.4%, n = 136) reported that antimicrobials are effective in treating both bacterial and viral infections, while 17.6% of all respondents gave the correct answer "No".

Table 6. Proportion of operators of establishments keeping food-producing animals who correctly or incorrectly responded to knowledge, attitudes, and practices questions on AMR and AMU.

	Knowledge Factors	**N (%)**
O_B1	Do you know who is authorized to issue a prescription?	159 (96.4)
O_B2	Do you know what antimicrobial medicines are?	147 (89.1)
O_B3	Do you know what antimicrobial residues are?	126 (76.4)
O_B4	Do you know what the withdrawal period for antimicrobial medicine is?	150 (90.0)
O_B5	Do you know what antimicrobial resistance is?	119 (72.1)
O_B6	Did you know that consuming food of animal origin before the withdrawn period can contribute to the development of antimicrobial resistance in humans?	116 (70.3)
O_B7	Can the development of antimicrobial resistance be reduced by avoiding the excessive use of antimicrobials in animal production?	128 (77.6)
O_B8	Are you aware of specific antimicrobials that target particular diseases?	119 (72.1)
O_B9	Do you think antimicrobials can transfer to humans through the consumption of animal products?	120 (72.7)
O_B10	Do you think antimicrobials are effective in treating both bacterial and viral infections?	29 (17.6)
O_B11	Do you think antimicrobials can have side effects?	124 (75.2)
O_B12	Do you think that all antimicrobials produce the same therapeutic effect on animal diseases?	111 (67.3)
O_B13	Do you think zoonotic agents in animals can develop resistance to antimicrobials?	114 (69.1)
O_B14	Do you think antimicrobial resistance in animals a significant concern for public health?	139 (84.0)
	Attitude Factors	**N (%)**
O_C1	Do you think that antimicrobials used accidentally could contribute to antimicrobial resistance?	97 (58.8)
O_C2	Do you think insufficient dosage can contribute to antibiotic resistance?	101 (61.2)
O_C3	Do you think that limiting the use of antimicrobials could cause more harm than benefits?	54 (32.7)

Table 6. *Cont.*

	Attitude Factors	**N (%)**
O_C4	Do you think antimicrobials should be stored in restricted areas and accessed only by specific staff when needed?	15 (9.1)
O_C5	Do you reserve antimicrobials for later use?	150 (90.9)
O_C6	Do you use any alternatives to antibiotics in animal feed such as probiotic, organic acids, or others?	91 (55.2)
O_C7	Would you use fewer antimicrobials if you knew that accidental use could hinder recovery in the future?	141 (85.5)
	Practice Factors	**N (%)**
O_D1	Do you have an agreement with a veterinarian to monitor your establishment?	120 (72.7)
O_D2	If you have answered "Yes" to question D1, does the veterinarian monitoring your establishment work for a veterinary pharmaceutical company?	97 (58.8)
O_D3	If you have answered "No" to question D1, do you receive advice from veterinarians working in veterinary pharmaceutical companies?	70 (42.4)
O_D4	Have you used antimicrobial medicines in your animals on your own initiative?	106 (64.2)
O_D5	Do you reserve antimicrobials for later use?	62 (37.6)
O_D6	Do you follow recommendations for the use of antimicrobials from non-veterinarians?	145 (87.9)
O_D7	Check the expiry date of antimicrobial medicines before purchasing them?	149 (90.3)
O_D8	Do you adhere to the antimicrobial withdrawal period?	160 (97.0)
O_D9	Do you increase the dose and frequency of antimicrobials when there are no signs of recovery?	128 (77.6)
O_D10	Do you stop administering antimicrobials when there is evidence of improved animal health?	59 (35.8)
O_D11	Are prescriptions issued in accordance with the instructions of the veterinarian who issued them?	159 (96.0)
O_D12	Is there a responsible person in your establishment to administer medicines?	142 (86.1)
O_D13	Do you apply biosecurity measures on your farm?	99 (60.0)
O_D14	If your answer to question D15 is "Yes", what biosecurity measures do you apply to your establishment (including situations describing actions related to biosecurity)?	50 (30.3)

The positive attitude corresponded to a correct response rate of more than 63%, while the negative attitude was associated with less than 63%. When asked whether participants would use fewer antimicrobials if they knew that accidental use could prevent recovery in the future, a high percentage responded positively (85.5%, n = 141). Additionally, 61.2% of all producers (n = 110) believed that "insufficient dosing can contribute to antibiotic resistance", while 58.8% (n = 97) agreed that "randomly used antimicrobials could contribute to antimicrobial resistance". A general trend was observed where most of the operators believed that antimicrobials should be stored in restricted areas and accessed only by specific staff when needed, though only 9.1% of respondents answered this correctly. Similarly, less than half of respondents (32.7%, n = 54) agreed with the statement that "limiting the use of antimicrobials can cause more harm than benefits", indicating a negative attitude. This information is detailed in Table 6 and Figure 2.

Importantly, a significant proportion (72.7%, n = 120) of food-producing animal establishment operators reported having an agreement with a veterinarian to monitor their establishments. Additionally, 87.90% of respondents indicated that they did not receive recommendations on the antimicrobial from non-veterinarians, and almost all (96.4%, n = 159), stated that prescriptions are made according to the veterinarian's instructions. However, 64.2% reported interruptions in antimicrobial treatment, while 22.4% of respondents mentioned increasing the dose and frequency of antimicrobials when there was no evidence of improvement in animal health. Furthermore, some operators (36.80%, n = 59) reported using antimicrobials in their animals on their own initiative. When asked if they reserved antimicrobials for later use, only 37.60% answered correctly.

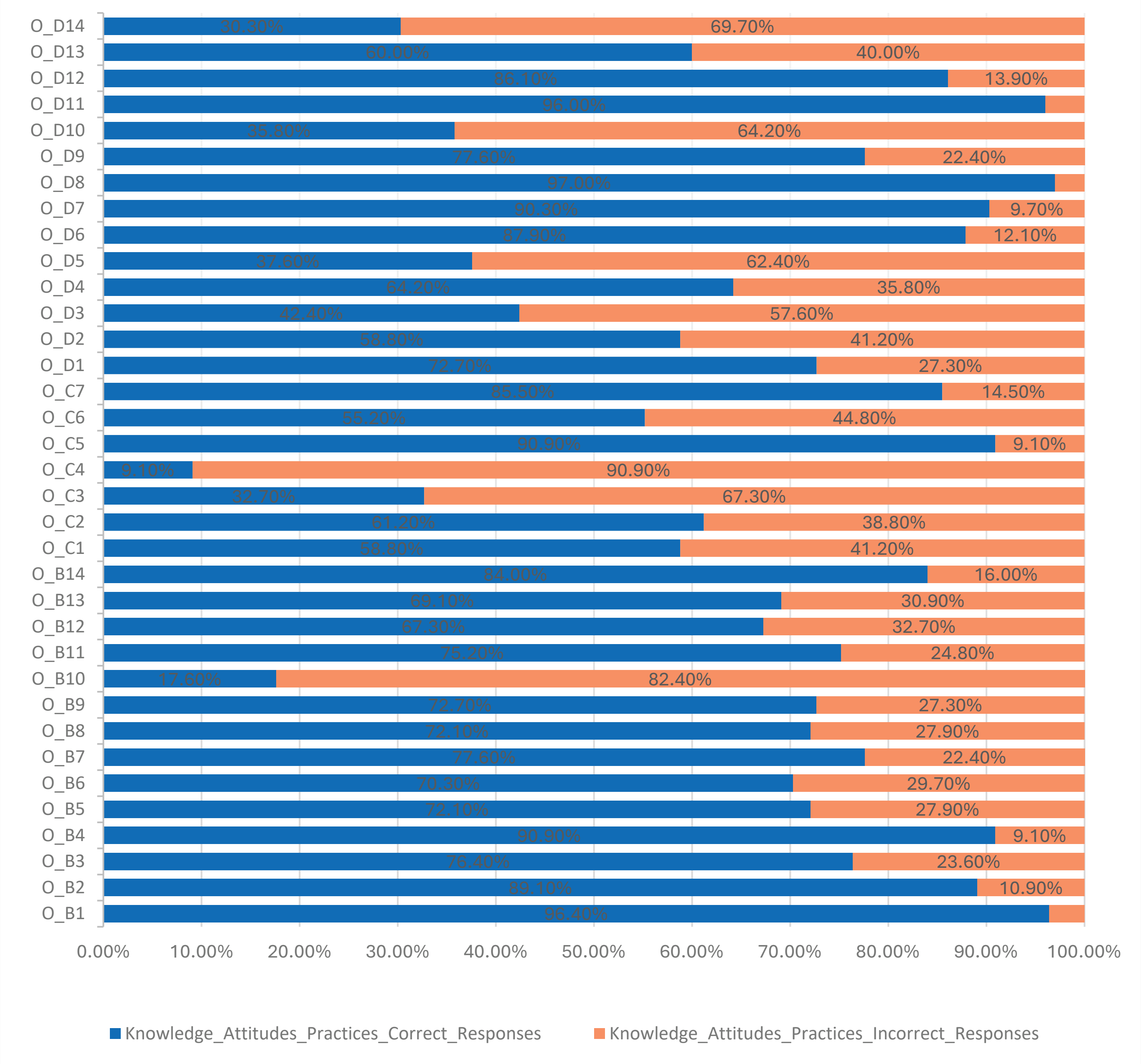

Figure 2. Proportion of operators of establishments keeping food-producing animals who correctly or incorrectly responded to knowledge, attitudes, and practices questions on AMR and AMU.

Regarding the implementation of biosecurity measures, 60.00% of participants reported applying biosecurity measures in their establishments. However, a smaller proportion (30.30%, n = 50) demonstrated knowledge of what biosecurity measures entail, as indicated by their responses to the following question. The remaining 29.70%, of the 60.00%, either did not answer the question "mention biosecurity measures you apply to your holding" or provided incorrect answers.

2.5. Association Between Factors Related to Knowledge, Attitudes, and Practices for Operators of Establishments

According to the results of a logistic regression of adjusted odds ratios for certain categorical attitude variables in relation to the adoption of good practice on farms (Table 7), such as using alternatives to antibiotics, responders who answered the attitude questions correctly were 2.44 times more likely (OR = 2.44, 95% CI: 1.19, 5.00) and 2.33 times more likely (OR = 2.33; 95% CI: 1.12, 4.76) to implement good practices like using alternatives compared to those who did not answer the attitude questions correctly. Furthermore, operators who have an agreement with a veterinarian to monitor their establishments were 2.56 times more likely (OR = 2.56, 95% CI: 1.22, 5.56) to implement good practices such as using alternatives to antibiotics compared to operators who do not have a contract with a veterinarian for monitoring their establishments.

Table 7. Logistic regression analysis of attitude parameters associated with good practices (use of alternatives to antibiotics).

Variables	Adjusted Odds Ratio	95% Confidence Interval	
		Lower	Upper
Conclusion of an agreement with a veterinarian monitoring the establishment	2.56	1.22	5.56
Randomly used antimicrobials could contribute to antimicrobial resistance	0.95	0.47	1.96
Do you think insufficient dosage can contribute to antibiotic resistance	2.44	1.19	5.00
Do you think that limiting antimicrobials may cause more harm than benefits	2.33	1.12	4.76

The logistic regression (Table 8) shows that positive attitude factors are important predictors for implementing good practices. Operators who answered the attitude questions correctly, as described in Table 8, are 2.33 times more likely (OR = 2.33, 95% CI: 1.03, 5.26) and 3.13 times more likely (OR = 3.13; 95% CI: 1.47, 6.67) to apply good practices such as implementing biosecurity measures compared to operators who did not respond correctly to the attitude questions.

Table 8. Logistic regression analysis of attitude parameters associated with good practices (implementation of biosecurity measures).

Variables	Adjusted Odds Ratio	95% Confidence Interval	
		Lower	Upper
Conclusion of an agreement with a veterinarian monitoring the holding	1.43	3.45	5.92
Randomly used antimicrobials could contribute to antimicrobial resistance	1.85	0.82	4.17
Do you think insufficient dosage can contribute to antibiotic resistance	2.33	1.03	5.26
Do you think that limiting antimicrobials may cause more harm than benefits	3.13	1.47	6.67

Table 9 shows the cross-tabulation of the use of a “contract with a veterinarian who monitors the establishment” and several variables related to attitudes and practices. The data suggest that the likelihood of a negative attitude among operators without a contract with a veterinarian is 2.89 times greater (95% CI, 1.43–5.85) compared to those who have a contract with a veterinarian. Additionally, the likelihood that good practices are not adopted by operators without a contract with a veterinarian is 3.02 times greater (95% CI, 1.48–6.17) compared to those who have a contract with a veterinarian.

Table 9. Cross-tabulation between the variable "contract with a veterinarian who monitors the establishment" and other attitude variables using the chi-square test to estimate the level of significance (*p*-value) in each case.

		D1—Contract with a Veterinarian Who Monitors the Establishment		***p*-Value**	**Odds Ratio for (No/Yes)**	**95% Confidence Interval**	
		No	**Yes**			**Lower**	**Upper**
Implementation of biosecurity measures	No	36 (80.0%)	79 (65.8%)	0.078	2.08	0.91	4.72
	Yes	9 (20.0%)	41 (34.2%)				
Attitude—Random AMU contributes to AMR	No	27 (60.0%)	41 (34.2%)	0.003	2.89	1.43	5.85
	Yes	18 (40.0%)	79 (65.8%)				
Attitude—Insufficient dosage can contribute to AMR	No	22 (48.9%)	42 (35.0%)	0.103	1.78	0.89	3.56
	Yes	23 (51.1%)	78 (65.5%)				
Attitude—Limiting antimicrobials may cause more harm than benefits	No	35 (77.8%)	76 (63.3%)	0.078	2.03	0.92	4.49
	Yes	10 (22.2%)	44 (36.7%)				
Use any alternatives to antibiotics in feed	No	29 (64.4%)	45 (37.5%)	0.002	3.02	1.48	6.17
	Yes	16 (35.6%)	75 (62.5%)				

2.6. Association Between Socio-Demographic Characteristics of Operators of Food-Producing Animal Establishments with Knowledge, Attitudes, and Practices

The results in Table 10 show that the educational level of responders was positively associated with both their level of knowledge ($p = 0.025$), and attitudes ($p = 0.032$). However, no statistically significant association was found between the educational level and the level of practices. Regarding the animal species and farm districts, there was significant heterogeneity in the number of responders in each category. This variability may have impacted the results, as a statistically significant association was observed between the district and levels of attitudes, between the district and levels of knowledge, and between animal species and the levels of knowledge and attitudes. This could potentially lead to inaccuracies in generalizing the findings to the broader population.

Table 10. Association between socio-demographic characteristics of food-producing animal establishment operators with knowledge, attitudes, and practices using the Kruskal–Wallis test.

		Knowledge		**Attitudes**		**Practices**	
Socio-Demographics		**Median (IQR)**	***p*-Value**	**Median (IQR)**	***p*-Value**	**Median (IQR)**	***p*-Value**
Age (years)	18–30	0.71 (0.29)	0.646	0.71 (0.43)	0.371	0.75 (0.31)	0.196
	31–45	0.79 (0.27)		0.57 (0.28)		0.66 (0.19)	
	46–60	0.79 (0.36)		0.57 (0.28)		0.69 (0.25)	
	>61	0.86 (0.29)		0.57 (0.28)		0.75 (0.18)	

Table 10. *Cont.*

		Knowledge		Attitudes		Practices	
Socio-Demographics		**Median (IQR)**	***p*-Value**	**Median (IQR)**	***p*-Value**	**Median (IQR)**	***p*-Value**
Educational level	Elementary school graduate	0.61 (0.43)	0.025	0.43 (0.14)	0.032	0.75 (0.31)	0.328
	Middle school graduate	0.71 (0.36)		0.43 (0.28)		0.72 (0.12)	
	High school graduate	0.86 (0.29)		0.57 (0.28)		0.69 (0.18)	
	Highest education	0.79 (0.29)		0.57 (0.28)		0.69 (0.19)	
	Master's degree and doctorate	0.93 (0.07)		0.71 (0.29)		0.75 (0.12)	
District where the establishment is located	Nicosia	0.79 (0.29)	0.010	0.57 (0.28)	0.008	0.75 (0.19)	0.135
	Larnaca	0.86 (0.22)		0.57 (0.14)		0.69 (0.20)	
	Limassol	0.75 (0.22)		0.43 (0.14)		0.75 (0.18)	
	Ammochostos	0.75 (0.45)		0.43 (0.14)		0.63 (0.21)	
	Paphos	0.71 (0.40)		0.57 (0.35)		0.63 (0.19)	
Target species	Bovine animals	0.86 (0.36)	0.000	0.57 (0.28)	0.000	0.69 (0.19)	0.057
	Caprine and ovine animals	0.71 (0.29)		0.57 (0.14)		0.69 (0.19)	
	Porcine animals	0.90 (0.14)		0.86 (0.26)		0.78 (0.24)	
	Poultry	0.86 (0.16)		0.71 (0.43)		0.72 (0.15)	
Professional experience (years)	<4	0.68 (0.45)	0.070	0.36 (0.39)	0.075	0.60 (0.30)	0.178
	5–8	0.71 (0.43)		0.43 (0.42)		0.75 (0.19)	
	9–12	0.79 (0.41)		0.57 (0.28)		0.69 (0.19)	
	>13	0.86 (0.29)		0.57 (0.28)		0.69 (0.18)	

3. Discussion

Antimicrobial resistance and the inappropriate use of antimicrobials are global challenges, and this issue is also evident in Cyprus [28]. Reducing AMR in both public and animal health necessitates a coordinated effort from all stakeholders, including operators of food-producing animal establishments and veterinarians. Gaining insights into the knowledge and awareness levels regarding antimicrobial resistance among veterinarians and individuals in rural areas is crucial. This information plays a vital role in the context of the One Health approach, which aims to address and reduce the growing threat of AMR.

This study is the first of its kind conducted in the Republic of Cyprus to assess the KAP of veterinarians and operators of food-producing animals establishments concerning AMR and AMU. The findings revealed that veterinarians generally exhibited a high level of knowledge, with most reporting an understanding of the severity of AMR. However, there was a noticeable gap in knowledge regarding the concept of antimicrobial stewardship. Antimicrobial stewardship is a critical component of a multisectoral strategy to combat AMR, involving a series of actions designed to promote the responsible and prudent use of antibiotics [34]. This highlights the need for further awareness and education on AMR and its management in veterinary practice [35].

All respondents agreed that improved husbandry practices and biosecurity measures can effectively reduce antibiotic consumption. This attitude was supported by a statistically significant positive association between the implementation of certain good practices by operators and the establishment of a contract with a veterinarian for monitoring purposes. Other studies have similarly shown that veterinarians have a substantial influence on the attitude of the livestock farmers [36–39], reinforcing the conclusion that continuous training of veterinarians on the severity of AMR and its connection to antimicrobial use is crucial. Furthermore, fostering communication between operators of food-producing animal establishments and veterinarians is vital for promoting responsible antimicrobial use [40].

Several veterinarians reported that they often prescribe more than one antimicrobial in a single prescription. Additionally, more than half of the veterinarians prescribe Category B antibiotics (polymyxins, quinolones, fluoroquinolones) which are classified as 'restricted' according to the European Medicines Agency (EMA) categorization [41]. These antibiotics are also considered as the highest-priority critically important antimicrobials (HPCIAs) by the World Health Organization (WHO) [42].

No statistically significant association was found between the knowledge of antibiotic categorization criteria. and the practices of antibiotic use. This suggests that despite existing knowledge, attitudes can still lead to unacceptable practices. Similar findings have highlighted the issue of prescribing critically important antimicrobials as the first line of treatment across Europe [43–45]. However, according to the latest 2022 report from EMA, between 2011 and 2022, sales of third- and fourth-generation cephalosporins decreased by 49.0%, sales of fluoroquinolones by 24.7%, sales of other quinolones by 89.7%, and sales of polymyxins by 81.0%. The inappropriate prescription of antibiotics by veterinarians could be a significant contributor to the development of antimicrobial resistance, as observed in previous studies [46,47].

The use of microbiological culture and susceptibility testing to select the appropriate antibiotics during treatment is not a common practice. A significant negative association was observed between the use of susceptibility testing and the use of Class B antibiotics. This suggests that Category B antibiotics are often prescribed without prior microbiological cultures and susceptibility testing. These findings contradict those of other researchers [48], who reported that the majority of veterinarians used the susceptibility testing to select appropriate antimicrobials. Sensitivity testing is an important tool in the veterinary field for selecting the most suitable antimicrobial product to treat bacterial diseases in animals. For Class D antibiotics ("prudent use"), they are often used alongside Category B and Category C antibiotics, while only 2 of the 26 veterinarians in our study used only Class D antibiotics. A study involving 25 European countries highlighted that the most critical factors influencing veterinarians' selection of antibiotics in animal treatment are antibiotic susceptibility test results, their own experience, the risk of developing AMR, and easy administration [48]. This observation may suggest a need to improve access to services that provide susceptibility testing.

The responses gathered from operators of food-producing animal establishments suggest that participants' knowledge on AMR and the use of antimicrobials is moderate. The majority of responsible farm operators of food-producing animals, based on their declarations, are aware of what antimicrobials are, the withdrawal times for antimicrobials, and the severity of antimicrobial resistance. However, according to other studies, although producers are aware that antibiotic misuse is linked to antibiotic resistance, they do not view it as a major problem. They are also not particularly concerned about the consequences of the reckless use of antimicrobial medicines and the impact of antimicrobial resistance on both animal and public health [49,50]. Less than half of the respondents believe that "accidentally used antimicrobials could contribute to antimicrobial resistance," reflecting a lack of sufficient knowledge and negative attitudes toward the issue.

Approximately two-thirds of farm operators of food-producing animals have an agreement with a veterinarian who monitors their farm. Additionally, the majority of respondents reported not receiving recommendations for the use of antimicrobials from non-veterinarians, and almost all indicated that prescriptions are made according to the instructions of the veterinarian issuing them. However, there is evidence of the potential misuse of antimicrobial in the surveyed establishments, including practices such as maintaining doses of antimicrobial medicines for future use, interrupting the administration of antimicrobials, and increasing the dose and frequency of antimicrobials when there is no evidence of an improvement in animal health. Additionally, some operators reported using antibiotics without consulting a veterinarian. Parameters that could lead to such practices include inadequate knowledge on prudent use of antimicrobials, and the role of veterinarians in observing the establishments [40]. The results also show that the educational level of responders was positively linked to the level of knowledge, and the level of attitudes. The findings of several studies are consistent with this study's results [27,51].

Good practices are among the most effective methods for preventing antibiotic misuse and overuse, thus helping to reduce AMR [52–57]. The current study emphasized the adoption of good practices by several participants, including the use of alternatives to antibiotics, which demonstrated a statistically significant positive association with attitude parameters. The study also highlighted that positive attitudes are key predictors for the implementation of good practices. Operators who answered attitude-related questions incorrectly were less likely to adopt good practices, such as the implementation biosecurity measures, compared to those who responded correctly. Factors that may influence the adoption of good practices, like biosecurity measures, include collaboration with veterinarians as a source of information, the operators' experience, availability of time, and the cost of necessary investments. As observed in the current study, operators that have an agreement with a veterinarian to monitor their establishments are more likely to implement good practices such as the use of alternatives to antibiotics in relation to operators who do not have a contract with a veterinarian for monitoring their establishments. These findings agree with those of Rayner et al. (2019), who stated that operators who have regular twice-a-year or annual visits from their vet may have active, beneficial flock health plans in place including measures to prevent disease and reduce the use of antibiotics [58]. Regarding the use of probiotics, operators must be persuaded of the benefits of approaches that reduce antibiotic usage while ensuring safe and effective health outcomes through probiotic use. According to a relevant study on the benefits, costs, and considerations of using antibiotic alternatives in food-producing animals, further research is necessary to confirm that probiotics are viable long-term alternatives for operators in place of antibiotics [59].

Limitations of the Study

One of the main limitations of this study is the response rate of operators in food-producing animals, which was 48%. This limits the ability to explore potential variations in characteristics across different participant categories. As a result, there may be a systematic non-response bias, as those who chose not to participate likely have different characteristics than those who did. However, compared to similar studies, this rate is considered relatively high [60,61]. Similarly, despite capturing the total number of veterinarians in the sector, their small sample size may lead to potential overestimation of findings related to their responses. Additionally there was heterogeneity in the number of participants from different districts and an unequal distribution of operators of establishments across different animal species. Since the sampling of the studied population was not random but rather based on convenience, this contributed to the uneven distribution across different data categories. This approach may have led to an overestimation of positive findings, as it likely included a higher proportion of individuals with a positive attitude toward antimicrobial resistance and antimicrobial use.

Misinterpretation and potential ambiguity of questions could also introduce systematic information bias. To minimize the risk, closed questions were used. However, closed questions may be prone to a systematic error related to social desirability bias, where participants may answer in the most socially acceptable way, especially given the sensitive nature of the prudent use of antimicrobials. This could result in responses that reflect what is perceived as the "correct" or socially approved answer rather that the participants' true beliefs or practices.

4. Materials and Methods

4.1. Ethical Approval

This study was approved by the Cyprus National Bioethics Committee (CNBC 2024.01.299). Participant consent was obtained electronically from those who received the questionnaires via email, and via paper forms from those who were given printed questionnaires. The consent statement was included within the questionnaire itself.

4.2. Place and Period of the Study

The study was conducted across all districts of Republic of Cyprus (Nicosia, Limassol, Famagusta, Larnaca, and Paphos) between October and December 2024, using an online questionnaire. The study population consisted of operators of bovine, ovine, caprine, and porcine establishments, as well as operators of poultry establishments and registered veterinarians listed in the Veterinary Registry.

4.3. Sampling Method

For the selection of operators of food-producing animal establishments, a non-proportional stratified random sampling method (with respect to animal species) and proportional stratified random sampling method (with respect to different districts) were employed. Regarding the selection of registered veterinarians from the Cypriot Veterinary Register who are employed in the field of productive animals, data from the Pancyprian Veterinary Association indicated that there are approximately twenty-six (n = 26) such veterinarians and they constituted the target population of the study. Regarding operators, operators of bovine, caprine, and porcine animal establishments registered in the Animal Identification and Registration System of the Veterinary Services were eligible for inclusion in the study. Additionally, operators of poultry establishments who are registered in the central register maintained by the Veterinary Services were also eligible.

Due to reduced responses from the food-producing animal sector, convenience sampling was employed. The sample size for operators was determined to be 341 using the Raosoft electronic program (http://www.raosoft.com/samplesize.html) (accessed on 11 October 2024). This sample size was calculated based on a 50% response distribution, a 5% margin of error, and a 95% confidence interval. The 50% response rate was assumed because the actual response rate was unknown as there were no similar previously published studies from Cyprus to reference.

4.4. Questionnaire Development

Following a comprehensive literature review of comparable studies [49,62–66], two district questionnaires were developed: one for veterinarians working in the field of food-producing animals, and another for operators of food-producing animal establishments. The reliability of internal consistency was estimated using Cronbach's alpha, calculated as follows:

$$a = \frac{k}{k-1}\left(1 - \frac{\sum_{i=1}^{k} \sigma_\iota}{\sigma_{total}^2}\right)$$

where k is the number of questions in the scale, σ_ι^2 represents the variation in each question, and σ_{total}^2, is the overall variation in the sum of all questions' scores. Cronbach's alpha was calculated to be 0.78 for knowledge, attitudes, and practices questions in the questionnaire for operators of food-producing animal establishments and at 0.69 for the questionnaire for veterinarians. The Cronbach's alpha values of 0.69 and 0.78 are generally acceptable. The lower value in the range is slightly below the ideal threshold of 0.7.

The questionnaire includes a declaration of consent to participate in the study and is divided into four sections, predominantly consisting of closed-type questions. The first section addresses the socio-demographic characteristics of the participants. The second section focuses on gathering information related to knowledge of antimicrobial resistance and the use of antimicrobials. The third section is designed to assess respondents' attitudes toward antimicrobial use and antimicrobial resistance. The fourth section examines the practices of animal producers concerning the topics being studied. Both negative and positive elements were included for each topic.

A score of "1" was assigned for each correct answer, while a score of "0" was given for incorrect or doubtful responses. Based on previous studies [67,68], a high level of knowledge was associated with a correct response rate above 82%, a moderate level with a correct response rate between 55 and 82%, and a low level of knowledge with a rate below 55%. Regarding good practices, the corresponding percentages were above 58%, between 35 and 58%, and below 35%. A positive attitude was defined by a correctness rate of more than 63%, while a negative attitude was associated with a correctness rate of less than 63%.

4.5. Method of Data Collection

Data were collected using Google Forms (retrieved 20 October 2024, from https://forms.google.com.

The link was sent via e-mail. For operators of animal establishments, printed questionnaires were also provided as an alternative method of data collection.

4.6. Statistical Analysis and Data Processing

The data were analyzed using the software GNU PSPP version 2.0.1-gff8d3d. The data collection involved two main categories of responses: 'yes', and 'no' or 'don't know', concerning various knowledge, attitudes, and practices related to antimicrobial use and antimicrobial resistance. For the correct/positive or correct/negative responses, the value "1" was assigned, while for the incorrect/negative or incorrect/positive answers, or if the response was "don't know", the value "0" was given.

Initially, descriptive statistical methods were applied, using absolute frequencies (n) and relative frequencies (%) to evaluate demographic characteristics, as well as the level of knowledge, attitudes, and practices related to antimicrobial use and antimicrobial resistance.

To assess the influence of various factors of attitudes, knowledge, and practices on certain good practices (e.g., knowledge and implementation of biosecurity measures and the use of alternatives to antibiotics), a list of all potential independent variables was first compiled, and relevant tables of relevance (cross-tabulations) were created. The statistical test χ^2 was applied with an alpha level of significance set at 0.05 for all inferential statistics. In the next step, multiple logistic regression models were created, including variables for which the probability of falsely rejecting the null hypothesis (i.e., the p-value) was less than $\alpha = 0.05$.

Multivariable logistic regression was used, with responses related to attitudes as independent variables and a dichotomous assessment of the implementation of good practices as the dependent variable. The multivariable logistic regression models were used to estimate the adjusted odds ratio for the variables under examination, such as "agreement with a veterinarian", and other attitude-related factors described with detail in Tables 7 and 8. The results were expressed as odds ratios (ORs) with 95% confidence intervals (95% CIs), and a p-value of <0.05 was used as the threshold for statistical significance.

To explore the association between socio-demographic characteristics and knowledge, attitudes, and practices, the non-parametric Kruskal–Wallis test was employed.

5. Conclusions

It has emerged that the role of the veterinarian is crucial in encouraging producers to adopt good practices. Additionally, the role of veterinarians is essential in changing the behavior of stakeholders involved in veterinary practices. Changes in the attitudes and practices of veterinarians in Cyprus regarding antimicrobial use are essential. This can be achieved by further promoting continuous education and the dissemination of information within the veterinary sector about the use of antimicrobial medicinal products and AMR at both undergraduate and postgraduate levels. Additionally, promoting antimicrobial stewardship is crucial to educate and support veterinarians following adopting evidence-based practices for prescribing and administering the highest-priority critically important antimicrobials (HPCIAs). For operators of food-producing animal establishments, it is important to note that positive attitudes were predictors of good practices. However, further efforts are needed to strengthen collaboration between operators and veterinarians to achieve higher rates of good practices. Programs must be designed to raise awareness about the risks of antimicrobial resistance and the importance of responsible antibiotic use should be expanded. Operators of animal establishments should understand the broader impact of AMR on public health, animal health, and the environment.

Although improving the awareness and understanding of AMR is one of the primary objectives of the Cyprus AMR National Action Plan, the results of the current study suggest that there is still significant progress to be made. There is an urgent need to enhance the awareness and understanding of AMR through effective communication, education, and training, involving all stakeholders and facilitating behavioral change interventions. Previous research has shown that experience gained through appropriate training, access to published literature, and the availability of treatment guidelines play a crucial role in changing veterinarians' prescribing behavior [69]. There is a need to establish evidence-based guidelines for prescribing antimicrobials, particularly for "restricted" and "highest-priority" antibiotics, which should emphasize the importance of microbiological cultures and susceptibility tests before prescribing antimicrobials. Veterinarians should be encouraged or required to use diagnostic tools. Financial or logistical support could be provided to make these tests more accessible to veterinary practices.

There is a strong need for policies that enforce the restricted use of the highest-priority antibiotics. These measures should be coupled with more robust governance to ensure that both veterinarians and farm operators adhere to best practices in antimicrobial use. Furthermore, continuous training and information dissemination should be prioritized in the veterinary sector to keep up with evolving guidelines and resistance patterns.

Governments, veterinary associations, and industry groups could launch communication campaigns targeting farm operators, focusing on the importance of following veterinarian advice and reducing antimicrobial use where possible. Veterinary associations, governments, and research institutions should work together to develop and implement policies, guidelines, and educational campaigns that promote the responsible use of antimicrobials in food-producing animals.

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Article

Listeria monocytogenes in Fruits and Vegetables: Antimicrobial Resistance, Biofilm, and Genomic Insights

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Abstract: Background/Objectives: *Listeria monocytogenes* is a foodborne pathogen that can infect both humans and animals and cause noninvasive gastrointestinal listeriosis or invasive listeriosis. The objectives of this study were to determine the genetic diversity of *L. monocytogenes*; the genes associated with its resistance to antibiotics, benzalkonium chloride (BC), and cadmium chloride ($CdCl_2$); and its biofilm formation. Methods: A total of 132 fresh fruits (44 samples) and vegetables (88 samples) were selected for this study. The genetic diversity of the isolates and the genes associated with their antibiotic resistance were determined using PCR amplification; meanwhile, their levels of susceptibility to antibiotics were determined using the agar diffusion method. Their levels of resistance to BC and $CdCl_2$ were determined using the minimum inhibitory concentration method, and their capacity for biofilm formation was evaluated using the crystal violet staining method. Results: A total of 17 *L. monocytogenes* strains were collected: 12.8% (17/132) from fresh fruits and vegetables in this study. The isolates of *L. monocytogenes* belonged to phylogenetic groups I.1 (29.4% (5/17); serotype 1/2a) and II.2 (70.5% (12/17); serotype 1/2b); strains containing *Listeria* pathogenicity islands (LIPIs) were also identified at prevalence rates of 100% for LIPI-1 and LIPI-2 (17/17), 29.4% for LIPI-3 (5/17), and 11.7% for LIPI-4 (2/17). The antibiotic susceptibility tests showed that the *L. monocytogenes* isolates exhibited six different multiresistant patterns, with multiple antibiotic resistance (MAR) index of $\geq$0.46 (70.5%; 12/17); additionally, the genes *Ide, tetM,* and *msrA*, associated with efflux pump Lde, tetracycline, and ciprofloxacin resistance, were detected at 52.9% (9/17), 29.4% (5/17), and 17.6% (3/17), respectively. The phenotypic tests showed that 58.8% (10/17) of cadmium-resistant *L. monocytogenes* isolates had a co-resistance of 23.5% (4/17) to BC. Finally, all strains of *L. monocytogenes* exhibited moderate biofilm production. Conclusions: The results of this study contribute to our understanding of the persistence and genetic diversity of *L. monocytogenes* strains isolated from fresh fruits and vegetables; in addition, their resistance to $CdCl_2$, which is correlated with co-resistance to BC disinfectant, is helpful for the food industry.

Keywords: *Listeria monocytogenes*; virulence factors; antimicrobial resistance; benzalkonium chloride; biofilms

1. Introduction

Listeria monocytogenes is a facultative intracellular pathogen, widely distributed in nature, that causes noninvasive gastrointestinal listeriosis or invasive listeriosis. The clinical manifestations of invasive listeriosis include septicemia, encephalitis, endocarditis, meningitis, abortions, and fetal death, while noninvasive gastrointestinal listeriosis may be asymptomatic or include only flu-like symptoms or febrile gastroenteritis syndrome [1,2]. Invasive listeriosis affects high-risk groups, including populations such as elderly adults (>65 years), pregnant people, newborns, immunocompromised people, and patients with cancer or diabetes [3,4]. The Centers for Disease Control and Prevention (CDC) estimates that there are approximately 1600 cases of listeriosis, causing 260 deaths, annually [3]. In the European Union (EU), there are approximately 2500 cases of invasive listeriosis in humans annually, and it is the most serious cause of foodborne disease, with high rates of hospitalization and death; additionally, the zoonosis of this pathogen in 2022 increased by 15.9% compared to 2021, with 2738 cases [4].

L. monocytogenes can be spread through the agricultural environment, such as soil and irrigation water, as these sources may contaminate fresh produce, including fruits and vegetables at various stages of production and processing [5–7], thereby creating a public health problem and economic losses for the food industry.

In fact, the Interagency Food Safety Analytics Collaboration (IFSAC) reports that 76% of foodborne *L. monocytogenes* illnesses (2016–2020) in the United States were associated with three categories in particular: dairy products (37.1%), fruits (24.8%), and row vegetable crops (14.1%) [8]. The United States Food and Drug Administration Recalls, Market Withdrawals, & Safety Alerts publication reported recalls of fruits and vegetables in 2023 due to the presence of this pathogen in organic green kiwifruit, organic frozen pineapple, and frozen fruit blends containing organic frozen pineapple, kale, spinach, collard green products, and mung bean sprouts [9]. Furthermore, Food Standards Australia–New Zealand (FSANZ), during the period from 2019 to 2023, reported 83 food recalls associated with contamination by foodborne pathogens, of which *L. monocytogenes* represented 36% (30 recalls), followed by *Salmonella* spp. (33%; 27 recalls) and *Escherichia coli* (22%; 18 recalls); the recalls included several food categories, such as fruits and vegetables, dairy products, meats and processed meats, etc. [10].

Additionally, the prevalence of resistant *L. monocytogenes* isolates in food and the environment has been associated with the use of antibiotics in medicine, veterinary medicine, and agricultural production systems, as well as with practices such as treating soils with manure or growth promoters and the misuse of therapeutic treatments for veterinary purposes [11–13]. The increase in multiresistant pathogens is a significant public health problem, a situation that becomes increasingly severe as these pathogens spread globally and acquire new resistance mechanisms until there are no alternative therapies for their control [14,15]. In fact, the World Health Organization estimates that bacterial resistance will cause 10 million deaths by 2025 [16]. Moreover, the persistence of *L. monocytogenes* is linked to the following further issues: (i) resistance to antimicrobials or sanitizing treatments; (ii) the ability of cells to form biofilm on equipment or in the surrounding environment; (iii) the survival of strains under various food preservation conditions or environmental stresses; and (iv) the inability to remove cells from niches in the food environment [17–20]. Therefore, the objectives of the present study were to determine the following: (i) the genetic diversity of *L. monocytogenes* isolated from fresh fruits and vegetables; (ii) the genes associated with antibiotic- and multidrug-resistant strains of *L. monocytogenes*; and (iii) the resistance of some strains to benzalkonium chloride (BC) and cadmium chloride ($CdCl_2$) and their biofilm formation capacity.

2. Results

2.1. Genomic Profile of L. monocytogenes Sublineages and Virulence Genes

In total, 17 *L. monocytogenes* isolates were obtained from the 17 positive samples (12.8%: 1.5% cilantro, 2.2% broccoli, 3.7% lettuce, and 5.3% Hass avocados), while 115 (86.9%) out of 132 samples were negative (qualitative detection (limit of detection < 1 CFU per analytical unit)) for *L. monocytogenes*. The isolates of *L. monocytogenes* belonged to phylogenetic groups I.1 (29.4% (5/17); serotype 1/2a) and II.2 (70.5% (12/17); serotype 1/2b). Additionally, pathogenicity islands were detected in *L. monocytogenes*, including LIPI-1 and LIPI-2 (100%; 17/17), LIPI-3 (29.4%; 5/17), and LIPI-4 (11.7%; 2/17); among the isolates, the following pathogenicity islands were found: LIPI-1 + LIPI-2 (70.5% (12/17)), LIPI-1 + LIPI-2 + LIPI-3 (17.6% (3/17)), and LIPI-1 + LIPI-2 + LIPI-3 + LIPI-4 (11.7% (2/17)) (Table 1). The *prfA* and *actA* genes were detected in 100% (17/17) of the isolates.

Table 1. Genetic analysis and antibiotic resistance of *L. monocytogenes* isolates from fruits and vegetables.

Strain No.	Sample	Genetic Determinants of Virulence	Phylogenetic Group	Serotype	$CdCl_2$	CPF	Antimicrobial Resistance Genes	Antibiotic Resistance Pattern	MAR Index
Lm-11	Cilantro	LIPI-1 + LIPI-2	II.2	1/2b	R	S	---	PE-CF-AM-CFX-DC	0.38
Lm-14	Broccoli	LIPI-1 + LIPI-2	II.2	1/2b	R	I	*Ide*	PE-CF-AM-CFX-DC	0.38
Lm-13	Lettuce	LIPI-1 + LIPI-2	II.2	1/2b	R	S	*msrA* + *tetM*	PE-CF-AM-CFX-DC-TE	0.46
Lm-17	Lettuce	LIPI-1 + LIPI-2	II.2	1/2b	R	I	*Ide*	PE-CF-AM-CFX-DC	0.38
Lm-18	Cilantro	LIPI-1 + LIPI-2	II.2	1/2b	R	I	*Ide*	PE-CF-AM-CFX-DC-CLM	0.46
Lm-42	Hass avocados	LIPI-1 + LIPI-2	II.2	1/2b	S	S	*tetM*	PE-CF-AM-CFX-DC-CLM-TE	0.53
Lm-43	Hass avocados	LIPI-1 + LIPI-2	II.2	1/2b	S	S	---	PE-CF-AM-CFX-DC-TE	0.46
Lm-68	Hass avocados	LIPI-1 + LIPI-2	II.2	1/2b	S	I	*Ide* + *msrA*	PE-CF-AM-CFX-DC	0.38
Lm-136	Hass avocados	LIPI-1 + LIPI-2	II.2	1/2b	S	I	*Ide*	PE-CF-AM-CFX-DC-CLM	0.46
Lm-133	Hass avocados	LIPI-1 + LIPI-2	II.2	1/2b	S	S	---	PE-CF-AM-CFX-DC-CLM	0.46
Lm-147	Hass avocados	LIPI-1 + LIPI-2	II.2	1/2b	S	R	*Ide* + *tetM*	PE-CF-AM-CFX-DC-CPF-CLM-TE	0.61
Lm-138	Hass avocados	LIPI-1 + LIPI-2	II.2	1/2b	R	I	*Ide*	PE-CF-AM-CFX-DC-CLM	0.46
Lm-19	Lettuce	LIPI-1 + LIPI-2 + LIPI-3	I.1	1/2a	S	S	*tetM*	PE-CF-AM-CFX-DC-TE	0.46
Lm-24	Lettuce	LIPI-1 + LIPI-2 + LIPI-3	I.1	1/2a	R	S	---	PE-CF-AM-CFX-DC-CLM	0.46
Lm-15	Broccoli	LIPI-1 + LIPI-2 + LIPI-3	I.1	1/2a	R	S	*msrA*	PE-CF-AM-CFX-DC-CLM	0.46
Lm-27	Lettuce	LIPI-1 + LIPI-2 + LIPI-3 + LIPI-4	I.1	1/2a	R	R	*Ide* + *tetM*	PE-CF-AM-CFX-DC-CPF-CLM-TE	0.61
Lm-41	Broccoli	LIPI-1 + LIPI-2 + LIPI-3 + LIPI-4	I.1	1/2a	R	I	*Ide*	AM-CFX-DC	0.23

LIPI-1, isolates harboring virulence genes *prfA, hly, plcA, plcB, mpl,* and *actA*; LIPI-2, isolates harboring virulence genes *inlA, inlB, inlC,* and *inlJ;* LIPI-3, isolates harboring virulence genes *llsA, llsG, llsH, llsX, llsB, llsY, llsD,* and *llsP;* LIPI-4, isolates harboring virulence genes *licC, licB, licA*, and *glva*; I, intermediate resistance to ciprofloxacin; R, resistance to ciprofloxacin or $CdCl_2$; S, susceptible to ciprofloxacin or $CdCl_2$; CPF, ciprofloxacin; AM, ampicillin; CLM, clindamycin; CF, cephalothin; CFX, cefotaxime; CL, chloramphenicol; GE, gentamicin; E, erythromycin; TE, tetracycline; PE, penicillin; DC, dicloxacillin. MAR, multiple antibiotic resistance.

2.2. Antimicrobial Resistance Gene Profiling

A total of 17 isolates were screened for the ciprofloxacin resistance gene *Ide* (52.9%; 9/17), of which 41.1% (7/17) showed intermediate phenotypic resistance, and 11.7% (2/17) showed resistance to ciprofloxacin. The tetracycline resistance gene *tetM* was detected in 29.4% (5/17), whereas five isolates showed phenotypic resistance, and the macrolide resistance gene *msrA* was detected in 17.6% (3/17). However, neither the macrolide resistance gene *ermA* nor the chloramphenicol resistance gene *cat* was detected (Table 1).

2.3. Antimicrobials, Sanitizing, Cadmium, and Biofilm

L. monocytogenes was found to be susceptible to ciprofloxacin (47%; 8/17), tetracycline (70.5%; 12/17), gentamicin (94.1%; 16/17), erythromycin (94.1%; 16/17), vancomycin (100%; 17/17), chloramphenicol (100%; 17/17), and trimethoprim–sulfamethoxazole (100%; 17/17), according to Table 2; however, the isolates were resistant to first- and third-generation β-lactams (penicillin, ampicillin, dicloxacillin, cephalothin, and cefotaxime) (94.1–100%; 16–17/17), clindamycin (52.9%; 9/17), tetracycline (29.4%; 5/17), and ciprofloxacin (11.7%; 2/17). Among the tested *L. monocytogenes* isolates, six different multiresistant patterns were observed, with the most common being PE-CF-AM-CFX-DC (23.5%; 4/17) and PE-CF-AM-CFX-DC-CLM (35.2%; 6/17). The MAR index for *L. monocytogenes* isolates ranged from 0.23 to 0.61, with 70.5% (12/17) presenting a MAR index of $\geq$0.46 (Table 1). Additionally, intermediate clindamycin and ciprofloxacin resistance was present in 41.1% (7/17) (Table 2). In addition, the MICs of 76.4% (13/17) of the isolates was between 0.7 and 3.1 μg/mL BC, while the criterion for resistance to BC is CMI $\geq$ 6 μg/mL, at least twice the MIC for the predominant number of *L. monocytogenes* strains (MIC = 3.1 μg/mL) (Table 3). The MICs of 41.1% (7/17) of the isolates were <70 μg/mL $CdCl_2$, and 58.8% (10/17) were cadmium-resistant *L. monocytogenes* strains (MIC $\geq$ 70 μg/mL). Finally, all *L. monocytogenes* were classified as moderate biofilm producers (Table 3).

Table 2. Antimicrobial susceptibility test for *L. monocytogenes*.

Antimicrobial Class According to the WHO		Antibiotic [1]	No. (%) of *L. monocytogenes* Strains		
			Resistant	**Susceptible**	**Intermediate**
Highly important	Phenicols	CL		100	
	Cephalosporines (1st generation)	CF	94.1		5.8
	Lincosamides	CLM	52.9	5.8	41.1
	Sulfonamides	SXT		100	
	Cyclic peptides	TE	29.4	70.5	
Critically important	Macrolides	E		94.1	5.8
	Aminoglycosides	GE		94.1	5.8
	Fluoroquinolones	CPF	11.7	47	41.1
	Cephalosporines (3rd generation)	CFX	100		
	Glycopeptides	VA		100	
	β-Lactams	DC	100		
		AM	100		
		PE	94.1	5.8	

[1] AM, ampicillin; CLM, clindamycin; CF, cephalothin; CFX, cefotaxime; CPF, ciprofloxacin; CL, chloramphenicol; GE, gentamicin; E, erythromycin; TE, tetracycline; VA, vancomycin; SXT, trimethoprim–sulfamethoxazole; PE, penicillin; DC, dicloxacillin.

Table 3. Minimum inhibitory concentration values of BC and $CdCl_2$ in *L. monocytogenes* strains in relation to biofilm formation.

Strain No.	Phylogenetic Group	Serotype	MIC (μg/mL)	BC	$CdCl_2$	Biofilm Formation (Microtiter Plate Assays)
Lm-11	II.2	1/2b	6.2	■	■	Moderate biofilm
Lm-14	II.2	1/2b	6.2	■	■	Moderate biofilm

Table 3. *Cont.*

Strain No.	Phylogenetic Group	Serotype	MIC (μg/mL)	BC	$CdCl_2$	Biofilm Formation (Microtiter Plate Assays)
Lm-13	II.2	1/2b	6.2	■	■	Moderate biofilm
Lm-17	II.2	1/2b	6.2	■	■	Moderate biofilm
Lm-18	II.2	1/2b	3.1	□	■	Moderate biofilm
Lm-27	I.1	1/2a	3.1	□	■	Moderate biofilm
Lm-42	II.2	1/2b	3.1	□	□	Moderate biofilm
Lm-43	II.2	1/2b	3.1	□	□	Moderate biofilm
Lm-68	II.2	1/2b	3.1	□	□	Moderate biofilm
Lm-133	II.2	1/2b	3.1	□	□	Moderate biofilm
Lm-136	II.2	1/2b	3.1	□	□	Moderate biofilm
Lm-138	II.2	1/2b	1.5	□	■	Moderate biofilm
Lm-41	I.1	1/2a	0.7	□	■	Moderate biofilm
Lm-147	II.2	1/2b	0.7	□	□	Moderate biofilm
Lm-19	I.1	1/2a	0.7	□	□	Moderate biofilm
Lm-24	I.1	1/2a	1.5	□	■	Moderate biofilm
Lm-15	I.1	1/2a	1.5	□	■	Moderate biofilm

Black box indicate resistance to BC (CMI $\geq$ 6 μg/mL) or $CdCl_2$ (CMI $\geq$ 70 μg/mL).

3. Discussion

Listeria monocytogenes is a foodborne pathogen that causes invasive or noninvasive listeriosis in humans; the severity of the pathogenesis is associated with several factors, including risk groups and hazard characterization. In the present study, 12.8% of fresh fruits and vegetables tested were contaminated with *L. monocytogenes* strains belonging to serotypes 1/2a (29.4%) and 1/2b (70.5%). Several authors [5,7,21,22] have reported similar prevalence percentages for serotypes 1/2a–3a (33–65%) and 1/2b-3b-7 (50–79.6%); however, Maćkiw et al. [22] and Chen et al. [23] identified a lower prevalence for serotypes 1/2a–3a (10.8%) and 1/2b (2%) among the isolated *L. monocytogenes* found in ready-to-eat (RTE) foods, fruits, and fresh and frozen vegetables. Likewise, Kayode and Okoh [7] did not detect serotype 1/2a, but 1/2b (79.61%) and 4b (8.7%) were detected among *L. monocytogenes* strains found in fruit and vegetable samples. Indeed, serotypes 1/2a, 1/2b, 1/2c, and 4b are responsible for 95% of human listeriosis cases and have been frequently isolated from food products and patients [5,24,25], suggesting that the diversity of *L. monocytogenes* serotype prevalence may be related to geographic regions, monitoring procedures, methodologies for detecting of *L. monocytogenes* in various food categories (fruits, vegetable row crops, dairy, pork, chicken, beef, etc.), specific characteristics of the food (fresh or frozen), or sources of contamination that interact during food production and distribution.

In this investigation, we detected the presence of *L. monocytogenes* pathogenicity islands (LIPIs) in order to assess the potential risks that *L. monocytogenes* may pose to public health. All strains of *L. monocytogenes* isolated from fresh fruits and vegetables were found to have *Listeria* pathogenicity island 1 (LIPI-1; *prfA, hly, plcA, plcB, mpl,* and *actA*), which encodes virulence factors that promote the growth and spread of *L. monocytogenes*. Once inside the host cell, the phagocytic vacuole is lysed by listeriolysin O (LLO), which is a pore-forming toxin encoded by the *hly* gene that mediates the lysis of bacterial cells in the host cytoplasm and enhances its cytolytic action through phosphatidylinositol-PLC and phosphatidylcholine-PLC, which mediate pathogen escape from single- and double-membrane-bound vacuoles. ActA plays a role in facilitating the motility of the bacterial cell to the host cell's cytoplasm, and the actin cytoskeleton is hijacked to favor cell-to-cell spread [26–28]. The *prfA* gene, which encodes the PrfA regulatory protein that controls the expression of the pathogenicity determinants of *L. monocytogenes*, was also identified in this study [29]. Our results are similar to those reported by several other researchers [5,7,30,31], showing that *prfA* (100%), *mpl* (92–100%), *plcA* (92–100%), *plcB* (100%), *hly* (100%), and *actA* (84–100%) were detected in the *L. monocytogenes* strains isolated from fruits and fresh and frozen vegetables, as well as agricultural environments such as irrigation water and agricultural soil. *Listeria* pathogenicity island 2 (LIPI-2; *inlA*, *inlB*, *inlC*, and *inlJ*) was detected in all the *L. monocytogenes* strains identified in this study, which is in agreement with several prior investigations detecting the genes *inlA* (74.1–100%), *inlB* (81.5–100%), *inlC* (70.6–100%), and *inlJ* (66.7–100%), which encode a set of internalins that play roles in the adhesion and invasion of *L. monocytogenes* cells to the host cells, as well as *L. monocytogenes* dissemination [5,7,22,29–31]. InlA adheres to and invades intestinal epithelial cells that express the E-cadherin receptor, thereby facilitating intestinal barrier crossing; additionally, InlC and InlJ are involved in the postintestinal dissemination of *L. monocytogenes* infection [6,22,32].

Other islands detected in this study were *Listeria* pathogenicity island 3 (LIPI-3), in 29.4%, and *Listeria* pathogenicity island 4 (LIPI-4), in 11.7% of the samples. LIPI-3 encodes listeriolysin S (LLS), a bacteriocin with hemolytic and cytotoxic factors that contributes to polymorphonuclear neutrophil survival as well as to alteration in the gut microbiota [22,30,33], while LIPI-4 is involved in the infection of the host's neuronal and placental tissues, in addition to conferring potential hypervirulent strains [34,35].

Additionally, all *L. monocytogenes* isolates detected in this study exhibited antibiotic resistance, with penicillin, ampicillin, dicloxacillin, cephalothin, cefotaxime, clindamycin, tetracycline, and ciprofloxacin resistance being the most frequently encountered. Several studies have reported varying prevalences of antimicrobial resistance to penicillin (2.5–100%), ampicillin (50–100%), gentamicin (20–40%), STX (30%), erythromycin (23.5–100%), tetracycline (90–100%), chloramphenicol (20–70%), cefotaxime (80–100%), clindamycin (57.5–100%), cephalothin (50–100%), and ciprofloxacin (35.2–40%) in *Listeria* spp., and particularly in *L. monocytogenes* isolates from different categories of food and food processing environments [31,36–38]. The intermediate phenotypic resistance to clindamycin (41%), ciprofloxacin (41%), erythromycin (5.8%), and gentamicin (5.8%) demonstrated in the *L. monocytogenes* isolates in this study is also in agreement with the findings of other investigators [37–39], who reported the prevalences of intermediate resistance to clindamycin (30%), ciprofloxacin (5–64.7%), gentamicin (2.5%), and tetracycline (2–5.8%) in *L. monocytogenes.* The overprescription of antibiotics in clinical practice, the use of antibiotics in animal production, inadequate veterinary treatment for preventing animal disease, and the migration and accumulation of veterinary antibiotic residues in agricultural soils and irrigation water may contribute to the antimicrobial resistance and heterogeneity in the prevalence levels of the patterns observed in *L. monocytogenes* isolates [11,13,38,40].

The results of this study demonstrate that *L. monocytogenes* strains exhibit six different multiresistant patterns, with a MAR index of $\geq$0.46 (70.5%), indicating a higher risk of the source having been exposed to antibiotics, as a MAR index of $\geq$0.2 suggests the intensive use of antibiotics in the region and a high risk of promoting antibiotic resistance [41,42]. Iwu and Okoh [31], as well as Maurice Bilung et al. [36], reported similar MAR index values (0.31–0.85) for multidrug-resistant *Listeria* spp. and for *L. monocytogenes* isolates from irrigation water and agricultural soil (0.2–1), suggesting a high-risk source that is constantly exposed to antibiotics that are used to prevent or treat animal disease and promote animal growth. Agricultural activities such as the use of fertilizers containing antibiotic residues increase antibiotic resistance as well as the prevalence of antibiotic-resistant strains in the soil and in the water used for plant irrigation [12,43].

Furthermore, *L. monocytogenes* has been found to develop resistance to several antibiotics, including tetracycline, ciprofloxacin, erythromycin, clindamycin, penicillin, and ampicillin, through the acquisition of genetic elements such as conjugative transposons and self-transferable or mobilizable plasmids [13,28,44]; therefore, the detection of *Ide*, *tetM*, and *msrA* in the *L. monocytogenes* isolates in our study may be related to resistance mechanisms such as efflux pump Lde and transposon Tn916 harboring *tetM*, which confer resistance to ciprofloxacin and tetracycline [44,45], which is in accordance with the detection of *Ide, tetM*, and *msrA* in *Listeria* spp. and *L. monocytogenes* isolates from slaughtering and processing environments, both food-related and clinical [7,15,39,46]. Although we did not detect the presence of *cat* or *erm*A genes among our *L. monocytogenes* isolates, *cat* (100%) and *ermA* (16.9%) have already been detected among *Listeria* spp. in food and processing environments [15,46]. In fact, these mechanisms of resistance affect the treatment of human listeriosis with regard to drugs such as (*i*) first-line ampicillin or penicillin G in combination with an aminoglycoside (gentamicin) and (*ii*) second-line trimethoprim in combination with a sulfonamide, such as sulfamethoxazole-co-trimoxazole, as well as erythromycin, tetracycline, and vancomycin [28,45].

This investigation suggests that the high prevalence of resistance and intermediate resistance in *L. monocytogenes* isolates may be due to the inadequate use of antimicrobial agents in veterinary medicine, the extensive use of animal foodstuffs, agricultural production systems, or the intrinsic resistance of *L. monocytogenes* to cephalosporins and fluoroquinolones, which is associated with the lack or low affinity of the enzyme that catalyzes the final step of cell wall synthesis [28,45]. However, the prevalence of antibiotic resistance reported in different countries is influenced by the health policies related to comprehensive antimicrobial management and the determination of antimicrobial breakpoints specific to veterinary medicine, particularly regarding the methods for antimicrobial susceptibility testing for bacterial pathogens of animal origin and zoonotic bacteria that can affect humans [39,47].

Moreover, *L. monocytogenes* has demonstrated resistance to nonessential toxic metals, including arsenic and cadmium [48]. In this study, 58.8% of the *L. monocytogenes* isolates were resistant to $CdCl_2$ (MIC ≥ 70 μg/mL); this was a particularly common occurrence among the isolates of serotypes 1/2a and 1/2b, and similar findings have been reported by other researchers [30,49–51] regarding the prevalence of cadmium resistance (63–90%) in serotypes 1/2a and 1/2b of *L. monocytogenes* isolated from food and the environment. The presence of heavy metal residues in the environment is related to anthropogenic sources, including the industrial sector, as well as agricultural practices such as using phosphate fertilizers, which represent significant sources of cadmium in agricultural soil, water, and food [52], thus increasing the survival potential of *L. monocytogenes* by inducing the acquisition of mobile genetic elements of heavy metal resistance determinants in diverse environmental niches. Likewise, Zhang et al. [51] argued that cadmium exerts long-term selective pressure, allowing *L. monocytogenes* to develop tolerance.

On the other hand, QACs are used in the food industry during disinfection processes to control, reduce, and inactivate foodborne pathogens [53–56]; however, the prevalence of QAC resistance, particularly to BC, has been detected in *L. monocytogenes* isolated from food and processing plant environments and is associated with cadmium [57–60]. Our research showed that four *L. monocytogenes* isolates (23.5%) were resistant to BC, and ten isolates (58.8%) were resistant to cadmium with co-resistance to BC and cadmium (23.5%). Ratani et al. [61] isolated strains that showed 14% resistant to BC and 57% to Cd, while Xu et al. [50] detected 16.7% resistant to BC and Cd in *L. monocytogenes*; however, the cadmium- and BC-resistant *L. monocytogenes* were not always correlated [49]. Based on the cadmium or BC resistance results, this result could be due to the genetic diversity of the *L. monocytogenes* strains associated with the genetic determinants of cadmium resistance, such as *cadA*1 (plasmid-transposon Tn5422), *cadA*2 (plasmid pLM80), *cadA3* (at the chromosome level of *L. monocytogenes*), and *cadC* [48,62]; or BC resistance, such as qac*A/B*, *qacC/D*, *qacE*, *qacE1Δ-sul*, *qacF*, *qacG*, *bcrABC*, transposon Tn6188 (containing the *qacH* gene), or *mdrL* (chromosome- and plasmid-borne; encodes an efflux pump) [50,60,63,64]. In addition to the various breakpoints specific to determining resistance to disinfectants (MIC = 4–32 μg/mL), this multitude of factors may interfere with the prevalence of resistance phenomena regarding

BC in *L. monocytogenes*, as they are established according to the number of *L. monocytogenes* isolates, the origins of strains, the susceptibility testing medium, etc. [65].

Additionally, the decrease in QAC efficiency is related to (*i*) environmental niches, with sites that are difficult to clean and disinfect, along with the inability to remove cells; (*ii*) the presence of organic matter on food contact surfaces; (*iii*) exposure to sublethal concentrations of QACs on food contact surfaces that, in turn, confer BC tolerance to *L. monocytogenes*. Moreover, this phenomenon is associated with the persistence of *L. monocytogenes* in the food industry, along with the subsequent adaptation and formation of biofilms [18,66–68]. Our results indicate that all *L. monocytogenes* isolates can form biofilms, as they harbor the genes *inlA*, *prfA, plcA*, *hly*, *plcB*, and *actA*, which are associated with biofilm formation. Previous research indicated that *inlA*, *inlL*, *prfA*, *plcA, actA, Imo0673*, *bapL*, *recO, Imo2504,* and *luxS* play roles in the different stages of *L. monocytogenes* biofilm formation [35,46,69], though Price et al. [70] argued that the presence of LIPI-1 genes *hly* and *prfA* are required. However, biofilm formation is a complex and dynamic process that is contingent upon a number of factors, including the availability of nutrients in the environment, the origin and biodiversity of the strain, and quorum sensing (QS), which activate and regulate biofilm-associated genes and virulence factors [19]. Several studies have demonstrated that *L. monocytogenes* forms biofilms that exhibit significantly greater resistance to sanitizing and antibiotic compounds than free-floating cells [44,71]; therefore, it could represent a source of concurrent food contamination, thus increasing the risk to the consumer and impacting public health, in addition to the economic losses associated with voluntary recalls or damage to equipment within the food industry.

Moreover, in this study, *L. monocytogenes* strains isolated from fresh fruits and vegetables could have caused severe human infection; however, the severity of the clinical manifestation of *L. monocytogenes* is related to genetic diversity, immune system status, and host comorbidities. Indeed, in Mexico, listeriosis is not notified within the National Epidemiological Surveillance System, which has limited the characterization of the danger and risk it may pose for its population. Castañedas-Ruelas et al. [72] argued that the dearth of data concerning the significance of *L. monocytogenes* in Mexico underscores the necessity of sensitizing authorities to the risks associated with food and human exposure to *L. monocytogenes*, thereby facilitating an understanding of the clinical and epidemiological impacts of listeriosis in Mexico. Notably, it is essential to incorporate techniques with whole-genome sequencing (WGS) and multilocus sequence typing (MLST) that allow us to determine the biodiversity of *L. monocytogenes*, enabling the identification of clonal complexes (CCs), sublineages (SLs), and analysis of virulence genes of *L. monocytogenes* that contribute to the microbiological surveillance of Listeriosis, severity of the disease, sources, or the continuous improvement in control measures based on hazard characterization [34,46,73].

In addition to the continuous improvements in good agricultural practices, including the incorporation of agricultural or sanitary inputs such as phytogenic products, plant antimicrobial products can provide growth-promoting and pathogen-controlling effects, or substances generally recognized as safe (GRAS), such as citric acid, gallic acid, and lactic acid, can be incorporated, which show an antiplanktonic cell effect on *L. monocytogenes* or control of its biofilm, and can therefore be used among the disinfection methods implemented within the industry, with the intent of reducing antimicrobial resistance and its impacts on the environment and the consumer [46,74].

4. Materials and Methods

4.1. Sample Collection and Isolation of Listeria

Overall, 132 samples were collected, comprising 88 vegetables and 44 fruits, purchased from a local supermarket in Ocotlán, Jalisco, from May to August 2023. The raw vegetables and fresh fruits included Hass avocados, lettuce, parsley, cilantro, broccoli, and cucumber (twenty-two samples each). *L. monocytogenes* was isolated from foods according to the methods described in the Bacteriological Analytical Manual (individual subsample analysis, enrichment procedure, isolation procedure with Oxford agar (OXA; Becton Dickinson

Bioxon, Le Pont de Claix, France) after 24–48 h incubation at 35 °C, with selection of up to 5 typical colonies for identification). The colonies were transferred into a tryptic soy broth (TSB; Becton Dickinson Bioxon, Le Pont de Claix, France) with 0.6% yeast extract (TSBYE), the incubated at 30 °C for 24–48 h and examined for morphological and biochemical characteristics using Gram staining, with hemolysis determined with 5% sheep blood agar, CAMP test, motility, catalase, and carbohydrate fermentation (mannitol, rhamnose, and xylose). Finally, the strains were confirmed via PCR using *hly* (listeriolysin O) and *prs* (putative phosphoribosyl pyrophosphate synthetase) [24,25,75]. Stocks were stored in TSB containing 30% glycerol at −80 °C.

4.2. Genomic Characterization: Genes Involved in Pathogenicity Islands, Biofilm Formation, and Antibiotic Resistance

L. monocytogenes strains were reactivated in TSBYE (Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 30 °C. According to the manufacturer's instructions, genomic DNA was extracted from *L. monocytogenes* using a Bacteria DNA Preparation Kit (Jena Bioscience, Jena, Germany). All *L. monocytogenes* strains were investigated for the detection of genes (*prfA*, *hly*, *plcA*, *plcB*, *mpl*, *actA*, *inlA*, *inlB*, *inlC*, *inlJ*, *llsA*, *llsG*, *llsH*, *llsX*, *llsB*, *llsY*, *llsD*, *llsP*, *licC*, *licB*, *licA*, and *glvA*) that harbored *L. monocytogenes* pathogenicity islands (LIPIs) via PCR using the protocol of Zhang et al. [21] (Table 4). Subsequently, *L. monocytogenes* phylogenetic groups (I.1 (172a-3a), I.2 (1/2c-3c), II.1 (4b-4d-4e), II.2 (1/2b-3b-7), and III (4a-4c)), and the genes associated with their antibiotic resistance (efflux pump Ide (*Ide*), chloramphenicol acetyltransferase (*cat*), macrolide-lincosamide-streptogramin B efflux pump (*msrA*), rRNA adenine-N-6-methyltransferase (*ermA*), and ribosomal protection protein tetM (*tetM*)) were determined using the protocols of Doumith et al. [24] and Boháčová et al. [39]. After amplification, the products were electrophoresed on 1% (*w*/*v*) agarose gel (UltraPure agarose, Invitrogen, Carlsbad, CA, USA) using SYBR Green (Sigma-Aldrich, St. Louis, MO, USA) and visualized using transillumination under UV light (UVP, DigiDoc-It Darkroom, Upland, CA, USA).

Table 4. Primers for the amplification of *L. monocytogenes* virulence-associated genes.

Gene	Primer Sequences (5′-3′)	Protein Coded by Target Gene (Gene)	Biological Function	References
LIPI-1				
prfA	F: 5′-AACGGGATAAAACCAAAACCA-3′ R: 5′-TGCGATGCCACTTGAATATC-3′	Transcriptional regulator A (prfA)	Controls and regulates the expression levels of *L. monocytogenes* virulence factors.	[21,29]
hly	F: 5′-GTTAATGAACCTACAAGACCTTCC-3′ R: 5′-ACCGTTCTCCACCATTCCCA-3′	Listeriolysin O (hly)	Phagosome lysis.	[21,26–28]
plcA	F: 5′-TCCCATTAGGTGGAAAAGCA-3′ R: 5′-CGGGGAAGTCCATGATTAGA-3′	Phosphatidyl inositol phospholipase C (plcA)	Phagosome lysis.	[21,26–28]
plcB	F: 5′-CAGCTCCGCATGATATTGAC-3′ R: 5′-CTGCCAAAGTTTGCTGTGAA-3′	Phosphatidyl choline phospholipase C (plcB)	Phagosome lysis.	[21,26–28]
mpl	F: 5′-AAAGGTGGAGAAATTGATTCG-3′ R: 5′-AGTGATCGTATTGTAGGCTGCTT-3′	Metalloprotease (mpl)	Processes the PC-PLC precursor to its mature form.	[21,26–28]
actA	F: 5′-AAACAGAAGAGCAGCCAAGC-3′ R: 5′-TTCACTTCGGGATTTTCGTC-3′	Protein for actin nucleation (actA)	Facilitates the motility of the bacterial cell to the host cell's cytoplasm.	[21,26–28]

Table 4. *Cont.*

Gene	Primer Sequences (5′-3′)	Protein Coded by Target Gene (Gene)	Biological Function	References
LIPI-2				
inlA	F: 5′-ACGAGTAACGGGACAAATGC-3′ R: 5′-CCCGACAGTGGTGCTAGATT-3′	Internalin A (inlA)	Adhesion and invasion of *L. monocytogenes* cells to the host cell and dissemination.	[5,7,21,22,29–31]
inlB	F: 5′-CATGGGAGAGTAACCCAACC-3′ R: 5′-GCGGTAACCCCTTTGTCATA-3′	Internalin B (inlB)	Adhesion and invasion of *L. monocytogenes cells* to the host cell, and dissemination.	[5,7,21,22,29–31]
inlC	F: 5′-AATTCCCACAGGACACAACC-3′ R: 5′-CGGGAATGCAATTTTTCACTA-3′	Internalin C (inlC)	Contribute postintestinal stages of infection.	[6,21,22,32]
inlJ	F: 5′-TGTAACCCCGCTTACACACAGTT-3′ R: 5′-AGCGGCTTGGCAGTCTAATA-3′	Internalin J (inlJ)	Involved in passage through the intestinal barrier, as well as in subsequent stages of infection.	[6,21,22,32]
LIPI-3				
llsA	F: 5′-ATGAATATTAAATCACAATCATCA-3′ R: 5′-TTACATTTTGGTTGCAGCAG-3′	Operon coding LLS (contributes to the expression of listeriolysin S (LLS))	Bacteriocin, with hemolytic and cytotoxic factors that alter the host intestinal microbiota and promote the survival of *L. monocytogenes* in polymorphonucleocytes.	[21,22,30,33]
llsG	F: 5′-GAGACTGGGCTTACTTGC-3′ R: 5′-TACCTCCTGTTCACTGCTTG-3′			
llsH	F: 5′-ATGATGTTCGCTATGGTT-3′ R: 5′-ACATTCCTACTGGCATCA-3′			
llsX	F: 5′-TTATTGCATCAATTGTTCTAGGG-3′ R: 5′-CCCCTATAAACATCATGCTAGTG-3′			
IIsB	F: 5′-TTACAATCAACCACCAGG-3′ R: 5′-AGTGAACCGAATGACAGA-3′			
IIsY	F: 5′-ATTAGAATAGGAACGCAGAC-3′ R: 5′-TCATAGCACCCAGTTTCG-3′			
IIsD	F: 5′-TATGGTGGTATGGAGGGT-3′ R: 5′-ATCACCCTGCTTATTTCA-3′			
IIsP	F: 5′-TTTCCAGGTATGCTTCTT-3′ R: 5′-CAATTACGGTGGTTCTCA-3′			
LIPI-4				
licC	F: 5′-GGGATTCCGAAACTACCT-3′ R: 5′-CGAGTGCTCCTGTAACCC-3′	Cellobiose family phosphotransferase system (PTS)	Involved in infection of the host's neuronal and placental tissues.	[21,34,35]
licB	F: 5′-ATTGCGGCATCTGAGAAA-3′ R: 5′-CAGCGATTAGAATTGGTACTGC-3′			
licA	F: 5′-GCCTCTTCCTCGTTTCTA-3′ R: 5′-GACTTAACTAAATCGCAGTA-3′			
glvA	F: 5′-TTACTATTGCTGGCGGAGGA-3′ R: 5′-TGCTCACGACCATCCATT-3′			

4.3. Phenotypic Characterization for the Persistence of L. monocytogenes

4.3.1. Disinfectant and Heavy Metal Sensitivity

Benzalkonium chloride (BC) (Sigma-Aldrich, St. Louis, MO, USA) was used to determine the sensitivity of *L. monocytogenes* strains to a quaternary ammonium compound

(QAC) using the protocol of Gray et al. [30] with modifications. *L. monocytogenes* strains were grown overnight in Mueller–Hinton broth (MHB; Becton Dickinson Bioxon, Le Pont de Claix, France) at 30 °C and subsequently diluted to ~10^8 CFU/mL. A BC stock concentration of 100 µL was added to the microtiter plates (Corning® 96-Well Assay Microplate, Lowell, MA, USA) with concentrations of 100, 50, 25, 12.5, 6.2, 3.1, 1.5, and 0.7 µg/mL. The microtiter plates were then incubated at 30 °C/24 h, and growth was monitored by measuring the OD_{560} using a Multiskan FC (Thermo Fisher Scientific, Inc., Madison, WI, USA) in order to determine the minimum inhibitory concentration (MIC). Each strain was tested in triplicate, with the positive (100 µL of MHB + 100 µL of *L. monocytogenes* ATCC 19111 (~10^8 CFU/mL)) and negative control wells containing only 200 µL of MHB. Cadmium chloride ($CdCl_2$; Sigma-Aldrich, St. Louis, MO, USA) was used to determine the resistance of *L. monocytogenes* to the heavy metal cadmium. Mueller–Hinton agar (MHA; Becton Dickinson Bioxon, Le Pont de Claix, France) was supplemented with different concentrations of $CdCl_2$ (400, 200, 100, 70, 50, 25, and 12.5 µg/mL); each *L. monocytogenes* isolate was adjusted to ~10^8 CFU/mL and inoculated onto the $CdCl_2$ plates, which were then incubated at 37 °C/24 h in triplicate. Resistance to cadmium was interpreted as $\geq$70 µg/mL [49,50].

4.3.2. Phenotypic Antibiotic Sensitivity and Resistance Analysis

The antibiotic resistance and susceptibility of the *L. monocytogenes* strains were determined using the agar diffusion method, following guidelines from the Clinical and Laboratory Standards Institute (CLSI) [76]. Bacterial suspensions, adjusted to 0.5 McFarland, were inoculated onto MHA, where antibiotics were incorporated, and the specimens were incubated at 35 °C/24 h. Thereafter, among the eleven classes of antimicrobials, the following thirteen antibiotics were selected for testing: phenicols (chloramphenicol (CL, 30 µg)); cephalosporines (1st generation) (cephalothin (CF, 30 µg)); lincosamides (clindamycin (CLM, 30 µg)); sulfonamides (trimethoprim–sulfamethoxazole (SXT, 2.5/23.75 µg)); cyclic peptides (tetracycline (TE, 30 µg)); macrolides (erythromycin (E, 15 µg)); aminoglycosides (gentamicin (GE, 10 µg)); fluoroquinolones (ciprofloxacin (CPF, 5 µg)); cephalosporines (3rd generation) (cefotaxime (CFX, 30 µg)); glycopeptides (vancomycin (VA, 30 µg)); and β-lactams (penicillin (P, 10 U), ampicillin (AM, 10 µg), and dicloxacillin (DC, 1 µg)) (BBL^{TM} Sensi-$Disc^{TM}$). The inhibition zones were interpreted as resistance (R), intermediate resistance (I), and susceptible (S), according to CLSI [76]. *L. monocytogenes* ATCC 19111 was used as the positive control. The multiple antibiotic resistance (MAR) index of each *L. monocytogenes* isolate was determined using the methods described by Krumperman [41] and Blasco et al. [77]; the MAR index is defined as a/b, where a is the number of antibiotics the isolate was resistant to, and b represents the total number of antibiotics to which the isolate was exposed.

4.3.3. Biofilm Formation Assay

Each strain's ability to form a biofilm was evaluated in a polystyrene microtiter plate (Corning® 96-Well Assay Microplate, Lowell, MA, USA) using crystal violet (CV) staining, following the protocol described by Avila-Novoa et al. [78]. For each strain, 230 µL of TSB and 20 µL bacterial suspension (~10^8 CFU/mL) were added to a polystyrene microtiter plate and incubated at 30 °C for 240 h. The planktonic bacteria were removed using 200 µL of phosphate-buffered saline (PBS; 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, and 130 mM NaCl, pH 7.4). The biofilm was fixed with 200 µL of methanol for 10 min, dried at 55 °C for 15 min, and stained with 200 µL of 0.1% crystal violet for 45 min. Excess stain was rinsed off with PBS and resolubilized with 200 µL of 95% ethanol. Absorbance was measured at 570 nm (OD_{570}) using the Multiskan FC. The assay was performed in triplicate, including positive (230 µL of TSB and 20 µL of *L. monocytogenes* ATCC 19111 (~10^8 CFU/mL)) and negative control wells, which contained TSB only. The cut-off O.D. (O.D.c) value was determined using the protocol described by Stepanović et al. [79], defined as three standard deviations above the mean O.D. of the negative control. Based on the O.D. values of the bacterial films, strains were classified into the following categories: nonbiofilm producers (O.D. < O.D.c), weak biofilm producers ((O.D. c < O.D.c < (2 × O.D.c)), moderate biofilm producers ((2 × O.D.c) < O.D. < (4 × O.D.c)), and strong biofilm producers ((4 × O.D.c) < O.D.).

5. Conclusions

In the present study, we obtained data on virulence-associated genes (LIPI-1, LIPI-2, LIPI-3, and LIPI-4) and resistance mechanisms to antibiotics used in clinical or veterinary medicine, as well as the environmental impacts of fertilizer residues on the antimicrobial resistance of *L. monocytogenes* isolated from fruits and vegetables. With this study, we aimed to raise awareness of the continuous improvement needed regarding treatments and sanitary prerequisites in areas such as agricultural practices, food farming practices, and standard operating procedures for sanitation. Future research should consider vali-dating and rotating disinfectants to reduce the risk of *L. monocytogenes* niche establishments in the environment and disinfectant tolerance that promotes the survival of *L. monocytogenes* biofilms. Additionally, a genomic analysis should be conducted of *L. monocytogenes* considering various sources of contamination (humans, animals, food, and the environment) within the traceability (production, transformation, and distribution of food) of fruits and vegetables to characterize the biological hazard, associate the source of contamination, and establish an efficient control measure to reduce the risk to the consumer.

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Article

Identification of Antimicrobial-Resistant Zoonotic Bacteria in Swine Production: Implications from the One Health Perspective

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Abstract: Antimicrobial resistance poses a major threat to global health and food security and is primarily driven by antimicrobial use in human and veterinary medicine. Understanding its epidemiology at farm level is crucial for effective control measures. Despite the significant reduction in antibiotic use in conventional livestock production, the swine sector traditionally has a higher level of antibiotic use in veterinary medicine. Consequently, multidrug resistance (MDR) among microbial isolates of swine origin has been relatively frequent. The aim of this study was to assess the presence of multidrug-resistant (MDR) bacteria, enteric pathogens and resistance genes to the main antibiotics used in clinical practice, both within the environment and in animals across pig farms characterized by varying degrees of sanitary status. A total of 274 samples were collected. Of these, 34 samples were collected from the environment (wall swabs, slat swabs and slurry pit), and 240 samples were collected from animals (sows' and piglets' rectal faeces). All samples were analysed for MDR bacteria and enteric pathogens. The study revealed a high frequency of extended-spectrum beta-lactamases (ESBL)-producing *Enterobacterales* and *Campylobacter* spp., with ESBL-producing *Enterobacterales* predominating in high health status farms (environment and animals) and *Campylobacter* spp. in both high health status and low health status environments. Additionally, a high percentage of methicillin-resistant *Staphylococcus aureus* (MRSA) was found, mainly in environmental samples from high health status farms, and *Clostridioides difficile* was distributed ubiquitously among farms and samples. Furthermore, though less frequently, vancomycin-resistant *Enterococcus faecium* (VRE) was isolated only in high health status farms, and Gram-negative bacilli resistant to carbapenems were isolated only in environmental samples of high health status and low health status farms. This study underscores the importance of surveillance for MDR bacteria in farm animals and their environment, including their waste. Such ecosystems serve as crucial reservoirs of bacteria, requiring national-level surveillance to promote responsible antibiotic use and pandemic control.

Keywords: global health; pig farms; multidrug resistant microorganism

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1. Introduction

Antimicrobial resistance (AMR) is one of the largest threats to global health and food security [1]. The Organisation for Economic Cooperation and Development suggests that without the adoption of new policies, approximately 2.4 million individuals across North America, Australia and Europe could succumb to untreatable infections in the next three decades. Yet, the repercussions extend beyond human loss, encompassing an economic impact projected as exceeding USD 3.5 billion annually [1,2].

The primary contributor to AMR is antimicrobial use (AMU) [3], with its utilisation in human and veterinary medicine as the predominant factor [4]. Furthermore, AMU in veterinary medicine also plays an important role in the prevalence of AMR in human health [3]. Long-term AMU in food-producing animals fosters the emergence and dissemination of AMR through animals and environmental sources, since, for instance, a slurry is usually applied as a fertiliser for crops in agriculture. Notably, similarities between AMR bacteria found in humans and animals have been found in foodborne pathogens and commensal bacteria, including *Escherichia coli*, *Enterococcus* spp., or *Salmonella* [5].

The multidrug resistance (MDR) phenomenon diminishes treatment options in veterinary medicine as well as in human medicine, contributing to the spread of resistant bacteria through the food chain or by direct contact [6]. Traditionally, the swine sector has had a high level of antibiotic use in veterinary medicine [7,8]. As a consequence, MDR has relatively frequently been observed among microbial isolates from swine farms [9,10]. In addition, the post-weaning period is critical for piglets (21–28 days old), as they face stressors such as transport, diet changes, litter mixing and reduced maternal immunity, which can lead to susceptibility to Enterotoxigenic Escherichia coli (ETEC) and post-weaning diarrhea (PWD). Although antimicrobial treatments and management practices, such as biosecurity and diet adjustments, help control infectious diseases [11], some Spanish farms, despite standardising practices, experience frequent outbreaks and are classified as low health status (LHS). In contrast, others remain unaffected and are considered high health status (HHS). A farm with HHS must excel in production metrics and health records, with mortality rates under 4%, substandard pig percentages between 2–3% and treatment costs of EUR 1–1.5 per pig. HHS farms focus on selection and breeding and show superior biosecurity and resilience to major swine pathogens. In contrast, LHS farms experience recurrent post-weaning diarrhea and morbidity rates of 10–15%.

The three primary zoonotic bacteria in swine production are *Campylobacter*, *Salmonella* and livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) [12]. *Campylobacter* and *Salmonella* are also the most frequently reported foodborne zoonotic pathogens in human medicine in the European Union (EU), responsible for 61.3% and 29.2% of the foodborne infections reported in 2022, respectively [13]. In addition, zoonotic LA-MRSA can infect workers who have occupational contact with pigs [14]. Coagulase-negative *staphylococci*, such as *S. epidermidis*, *S. haemolyticus* and *S. saprophyticus*, are also being considered a major nosocomial burden, playing an important role as a reservoirs of AMR genes, which can be then transferred to *S. aureus* [6].

As antimicrobials are essential for the treatment of bacterial diseases [15], current policies focus on reducing AMU in livestock [16,17], particularly in the swine industry, which is the most extensive agricultural user of antimicrobials in the EU [18]. In this sense, the AMR surveillance of targeted zoonotic or bioindicator bacteria through European programmes [19] constitutes a fundamental pillar in the evaluation of the trends in AMR due to antimicrobial selection pressure [5,10].

Current policies have established strong control measures at farm level to minimise the administration of antibiotics, highlighting European [17] and national regulations, such as the National Action Plan against the Emergence of Antibiotic Resistance (PRAN) in Spain [20]. These plans are based on reducing the misuse of antibiotics in various sectors, including livestock farming. They focus on promoting responsible prescription and use of antibiotics, gathering data on AMU and collaborating across sectors to implement preventive measures to preserve the effectiveness of these drugs in human and animal health.

However, to combat AMR, it is not only necessary to consider reducing AMU in animals but also to address the surrounding environment, which plays a key role as a potential reservoir for these MDR microorganisms [6]. The environment represents a stable microbial population among different animal batches and can sometimes host significant reservoirs of AMR [21]. Additionally, the prohibition of a large number of disinfectants by European authorities makes it increasingly difficult to combat these bacteria, especially those that are resistant and stable in the environment [6]. These stable communities exhibit characteristics specific to each type of production and, in some cases, characteristics unique to a particular company, production type or on-farm sanitary status [22].

Considering the aforementioned factors, understanding the epidemiology of AMR at the farm level represents a challenge crucial for the establishment of a robust field-level control programme. Consequently, the aim of this study was to assess the presence of multidrug-resistant (MDR) bacteria, enteric pathogens and resistance genes to the main antibiotics used in clinical practice both within the environment and in animals across pig farms characterized by varying degrees of sanitary status.

2. Results

Detection of Multidrug Resistant Bacteria and Enteric Pathogens

After collecting 274 samples from 6 different pig farms (3 HHS and 3 LHS) and 2 different origins (environment and animals), a total of 9 groups of microorganisms were isolated. From farm environments, 36 samples were collected. On each farm, 10 samples from walls (2 pooled samples/farm), 10 from slats (2 pooled samples/farm) and 2 from slurry pit were collected. The groups most frequently detected were the ESBL-producing *Enterobacterales* (n = 210, 76.6 %), followed by *Campylobacter* spp. (n = 77, 28.1 %), MRSA (n = 32, 11.7 %), *C. difficile* (n = 22, 8 %), VRE (n = 5, 1.8 %), Gram-negative bacilli producing carbapenemases (n = 5, 1.8 %) and colistin-resistant *Enterobacterales* (n = 1, 0.4 %) (p-value < 0.05). Neither *Salmonella* nor *Yersinia* spp. were isolated in any sample.

Regarding the species of *Enterobacterales* studied, *E. coli* was identified in 98.6% of the positive samples and *Klebsiella pneumoniae* in 1.4%. ESBL-producing *Enterobacterales* significantly predominated in HHS farms compared to LHS farms (p-value < 0.001) (Figure 1). In addition, ESBLs were isolated from environmental and animal samples. However, no statistically significant differences were found between the presence of ESBL-producing *Enterobacterales* and the sample origin (environment vs. animals) (Figure 2). The data for each sample type related to the antimicrobial resistance (AMR) obtained, the sample origin and the status of the farm can be found in the Supplementary Materials.

All *Campylobacter* isolates were identified as *C. coli*. The percentage of *C. coli* isolated did not vary significantly between farm status (Figure 1). However, statistically significant differences were found between the percentage of the bacteria isolated and the origin of the sample (p-value < 0.001) (Figure 2).

MRSA was isolated at a higher percentage in HHS farms than in LHS farms (p-value < 0.001) (Figure 1), and it was also significantly detected colonising environmental samples compared to samples from animals (p-value < 0.001) (Figure 2). It should be noted that none of the MRSA strains isolated expressed the PBP2a protein.

The presence of *C. difficile* was evenly distributed both according to the farm status and the sample origin, with no significant differences found between any of the studied variables (Figures 1 and 2). Only one strain of *C. difficile* tested positive for the presence of B toxin and negative for binary toxin.

In the case of VRE, all of them belonged to the species *Enterococcus faecium*, and they were only isolated in HHS farms (p-value = 0.032, Figure 1). Regardless of the presence of the bacteria and the origin of the sample, no statistically significant differences were found (Figure 2). None of the isolates expressed the vanA/B protein.

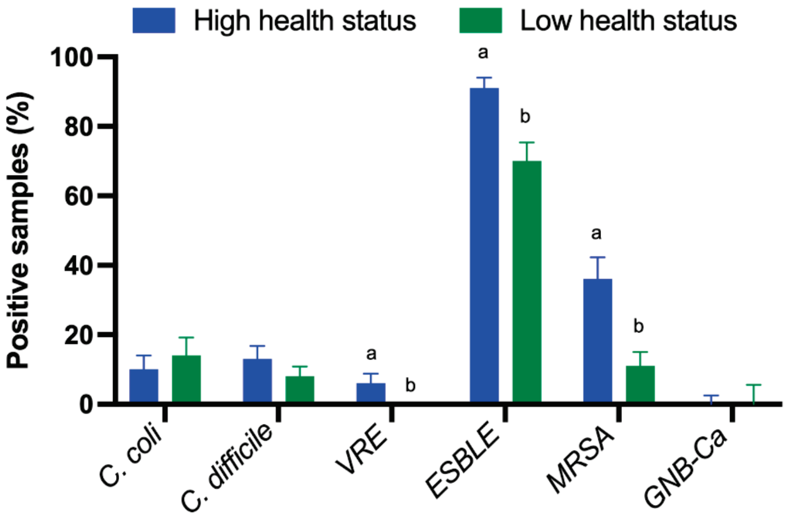

Figure 1. Percentage of each of the studied bacterial groups according to the farm status (High Health Status vs. Low Health Status). *C. coli—Campylobacter coli*. *C. difficile—Clostridioides difficile.* VRE—Vancomycin-resistant *Enterococcus*. ESBLE—ESBL-producing *Enterobacterales,* including *E. coli* and *K. pneumoniae*. MRSA—Methicillin-resistant *Staphylococcus aureus*. GNB-Ca—Gram-negative bacilli resistant to carbapenems. Different letters (a and b) represent significant differences within each bacterial group (*p*-value < 0.05). Data are presented as least squares means ± SE of the least squares means.

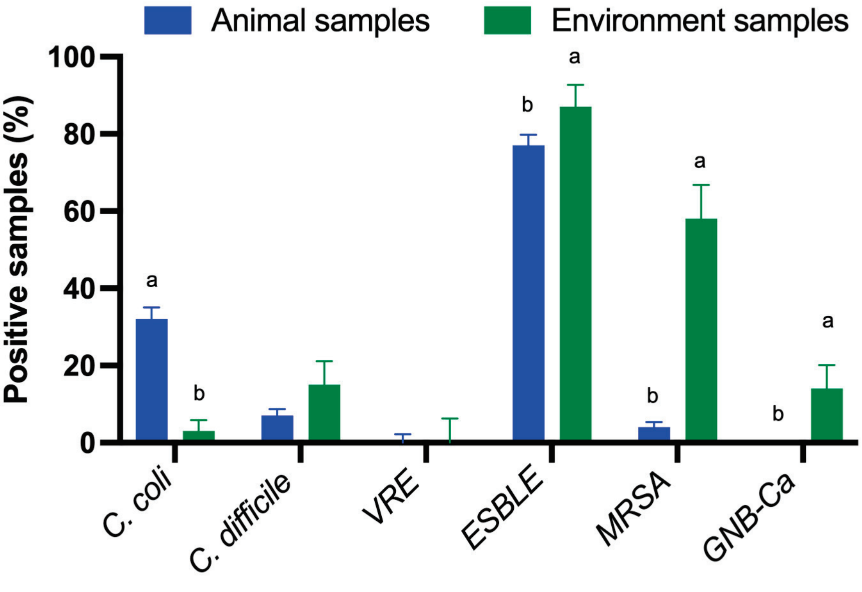

Figure 2. Percentage of each of the studied bacterial group according to the sample origin (environment vs. animals). *C. coli—Campylobacter coli*. *C. difficile—Clostridioides difficile*. VRE—Vancomycin-resistant *Enterococcus*. ESBLE—ESBL-producing *Enterobacterales,* including *E. coli* and *K. pneumoniae*. MRSA—Methicillin-resistant *Staphylococcus aureus*. GNB-Ca—Gram-negative bacilli resistant to carbapenems. Different letters (a and b) represent significant differences within each bacterial group (*p*-value < 0.05). Data are presented as least squares means ± SE of the least squares means.

In the case of Gram-negative bacilli resistant to carbapenemases, they were isolated at similar rates in both HHS and LHS farms. However, the bacteria were present only in environmental samples (*p*-value < 0.0001, Figure 2). The isolated strains belonged to the genus *Aeromonas* in 60% of cases and to the genus *Pseudomonas* in 40% of cases, with one of them expressing the carbapenemase OXA-48.

Among all the samples studied, only one isolate of colistin-resistant *Enterobacterales* was detected, which was identified as *E. coli*, and the presence of the MCR-1 enzyme was not detected. This strain was isolated from an environmental sample obtained at one of the HHS farms.

3. Discussion

The results of this field study demonstrate that resistant zoonotic bacteria, such as ESBL-producing *Enterobacterales*, *C. coli*, MRSA, *C. difficile*, VRE and Gram-negative bacilli resistant to colistin or producing carbapenemases, are important microorganisms to control in pig production. The results obtained reveal that the presence of resistant zoonotic bacteria is not confined only to livestock, which are the primary focus of the main European programmes for controlling AMR. Instead, it highlights the critical importance of the farm environment in perpetuating these bacteria among consecutive batches of animals, posing a danger to both public and animal health.

E. coli is part of the intestinal microbiota in humans and animals, making it one of the most likely vectors for the dissemination of ESBLs [23]. Moreover, *E. coli* is the most frequent cause of human urinary tract infections and bloodstream infections, and it is one of the leading causative agents of foodborne infections worldwide [24]. Therefore, several institutions, such as the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), include ESBL-producing *E. coli* in their surveillance programmes [25,26]. Similarly, it is considered a candidate microorganism to assess the impact on policies for responsible antimicrobial use in the swine sector [6,25]. The data obtained in this study demonstrate the high prevalence of ESBL-producing *E. coli* in the pig farms studied, although none of them have used cephalosporins to treat the animals. Perhaps they are being co-selected by the use of other antimicrobials. These results support the importance of including ESBL *E. coli* screening in surveillance programmes. Additionally, it has been shown that strains isolated in animals can be transmitted to humans through direct contact or via the food chain, such as the *E. coli* lineage ST131, which is an extra-intestinal pathogen that can colonise the gastrointestinal tract of food-producing animals and humans [23]. In this line, the USA Center for Disease Control and Prevention reports showed a continuous increase in community-associated human infections caused by ESBL-producing *Enterobacterales*, and the high prevalence of infections by this bacterial group in humans has been attributed to the frequent use of third-generation cephalosporins in dairy farms [27].

Campylobacter and *Salmonella* are the two main enteric pathogens causing zoonoses in humans [12]. Pork is considered a major source of *Salmonella typhimurium* infection in humans in the EU, including monophasic strains (mST) and *Campylobacter* spp. [10,28,29]. Although our study did not identify any *Salmonella*, a widespread distribution of virulent serotypes, such as *S. typhimurium* and its monophasic variant, has emerged as a public health threat [10,28–30]. Despite the current situation, within the EU, there is no mandatory programme for the control of *Salmonella* at pork production level. On the other hand, our study shows that the swine sector constitutes an important reservoir of *C. coli*, but its clinical significance is unknown since most human infections are associated with the presence of *C. jejuni* [31,32], mainly related with chicken consumption [13]. It should be noted that the majority of *Campylobacter* spp. strains have been isolated from animal samples, but this does not indicate that they are not present in the environment, since *Campylobacter* spp., due to its physiological requirements, remains in the environment as a viable non-culturable form [33], which justifies its low recovery from environmental samples.

Regarding infection with *C. difficile*, an enteric pathogen also found on the studied farms, it is the leading cause of human nosocomial diarrhea, mainly associated with antibiotic use. Its occurrence in the community setting is becoming increasingly common [34]. This microorganism is also becoming a pathogen to watch out for in animal species, including pigs, horses and dairy calves [35–37]. It has been postulated that livestock could be one of the main reservoirs of *C. difficile* [34]. In line with this, studies have shown that both animals and farm workers can be colonised by identical clones of *C. difficile* [36]. This emphasises the need for additional research into the connection between this microorganism and the food chain. This study provides data demonstrating the significant prevalence of this pathogen on pig farms. In fact, there are studies reporting even higher rates of this pathogen in swine, with up to 87% of positive samples from pig farms [38–41]. Our work supports the importance of *C. difficile* as a pathogen to be considered within the One Health setting, as it has been isolated in all types of farms and samples.

Among Gram-positive cocci, the two main pathogens isolated therein were MRSA and VRE. Both are human pathogens, and studies have reported that patients infected with these resistant microorganisms present higher risk of mortality compared to those infected with non-resistant strains [42]. MRSA, frequently isolated in environmental samples from the studied farms, is an important pathogen causing infectious diseases in both humans and animals, leading to high economic costs in both public health and livestock. However, there is little information regarding the risk of transmission of these strains to humans, although the strains colonising animals are rarely isolated in humans [43]. Since the early 2000s, numerous studies have pointed to an increased risk of LA-MRSA colonisation among individuals who have prolonged, repeated contact with livestock, especially pigs [14]. The presence of this microorganism has been associated with factors including the administration of tetracycline and zinc oxide, the size and type of the pig herd and the management practices in place. Measures to control the spread of LA-MRSA on pig farms include conducting periodic tests for the detection of LA-MRSA in pigs and avoiding certain antibiotics [44,45].

Regarding human infections caused by VRE, they are highly prevalent in the USA and are gradually increasing in Europe. VRE poses a major healthcare problem due to the difficulty of treating serious conditions associated with this microorganism, given the significant therapeutic limitations available [46,47]. Traditionally, this difference is explained by the varying use of drugs from this family in different geographical areas. In addition to this phenomenon, one must consider the potential implication of using this microorganism as a probiotic, which may promote the emergence of these strains in the intestinal microbiome of animals [48]. These strains, after acquiring virulence factors, can be transmitted through direct contact between farmers, veterinarians and workers [49,50]. This risk becomes more serious considering that the dominant species of Enterococcus in pigs and humans are the same [51].

Finally, we have detected five isolates resistant to carbapenems, although only one of them was a carbapenemase producer, which is of major clinical interest. Thus, carbapenems have never been used in animal husbandry, and these isolates were detected in the environment of the farms, not in faecal samples. In particular, the OXA-48 isolate belonging to the genus *Aeromonas* is typically environmental. This finding is of concern, as horizontal transmission of resistance from environmental bacteria to commensal bacteria inhabiting the animal gut could result in pigs colonised with carbapenemase producing bacteria and posing a risk to consumers. Little is known about the influence of the food chain on the spread of carbapenem-resistant strains, although they have been isolated in pigs, cattle, poultry, seafood, pets and wildlife [52–54]. Due to the significant clinical importance of their spread, and although the frequency is very low, control measures should be strictly enforced to minimise their spread, especially in the environment through cleaning and disinfections procedures [55–57].

Regarding colistin resistance, it is important to note that it is one of the antibiotics used in the clinical management of so-called difficult-to-treat bacteria due to the scarcity of therapeutic alternatives. Therefore, the dissemination of strains resistant to this drug constitutes a significant healthcare problem. In fact, there are reports of emerging *E. coli* and *Klebsiella pneumoniae* strains resistant to this compound in human healthcare [58,59]. In our study, we detected the presence of a colistin-resistant *E. coli* that did not carry the MCR-1 plasmid. The presence of these types of proteins would add an additional component of severity due to the ease of transmission among other bacteria [60]. However, the high fitness cost associated with the acquisition and expression of this plasmid in *Enterobacterales* hinders the expansion of these strains [61]. The results obtained in this study demonstrate the excellent outcomes achieved in recent years in Spain following implementation of the "REDUCE-COLISTIN" programme led by the Spanish Agency of Medicines and Medical Products within the PRAN, which all Spanish swine companies have voluntarily joined [16].

The prevention and control of infections associated with MDR bacteria is a highly complex and challenging phenomenon. It is a multifactorial process involving diverse agents, such as the microbiome of all involved parties (patients, medical staff, healthcare workers, environment, etc.), ecosystem characteristics, dissemination pathways, clinical approaches and microbial resistome. The importance of the food chain is still poorly understood, especially in community-associated infections involving these types of bacteria [62]. To minimise this problem, the implementation of surveillance programmes that integrate data from humans (both nosocomial and community) and animals, especially those circulating in the food chain, are key. Currently, these surveillance programmes are multiple and scattered and should evolve with a One Health perspective [63]. Another aspect to consider is the improvement of antibiotic usage control in humans and animals. In fact, various, agencies such as the World Health Organisation (WHO) and the European Medicines Agency (EMA), have drawn up documents on the prudent use of antibiotics, both in humans and animals, as reported above [64].

Our study supports the need for surveillance for the presence of resistant zoonotic bacteria in both farm animals and their environment, including the waste generated. These results demonstrated that MDR bacteria were mainly found in environmental samples, suggesting that screening the environment is more sensitive than screening the animals. MDR bacteria from the environment provide a representation of the entire pool of animals coexisting in that space. In fact, different studies conducted solely with animal samples showed lower percentages of this type of microorganisms [65,66]. Furthermore, species typically exhibiting MDR profiles, such as clinically important ESBL-producing *Enterobacterales* or MRSA, are often physiologically adapted to harsh environmental conditions, being more difficult to eliminate from environmental reservoirs. Hence, it is necessary to introduce some of these microorganisms and resistance traits in national surveillance programmes through collaboration between governments and private companies in order to progress towards more sustainable farming with more responsible use of antibiotics, which will contribute to controlling this significant pandemic [32,57,67].

4. Materials and Methods

4.1. Study Sample

Over a four-month period, six houses from six pig farms were environmentally sampled. Farms were affiliated with one of the production companies that handle the majority of pigs slaughtered in Spain. The present study was approved by the Ethics Committee for Animal Experimentation from the Universitat Autònoma of Barcelona and the Animal Experimentation Commission from the local government (Departament d'Acció Climàtica, Alimentació i Agenda Rural from the Generalitat de Catalunya; Reference 12234).

4.2. Selection of High and Low Sanitary Status Farms to Define "Healthy" and "Unhealthy" Microbiomes

A farm attaining high health status (HHS) must meet stringent criteria. Firstly, it must demonstrate exceptional production performance, with metrics like average daily gain and feed conversion rate ranking in the top 25% of all pig farms. Secondly, it should maintain outstanding health records, placing among the top 10% for parameters such as mortality rate, substandard pig percentage during rearing and antimicrobial treatment costs. For these farms, expected mortality rates from weaning to slaughter should not exceed 4%, with substandard pig percentages between 2–3% and treatment costs ranging from EUR 1–1.5 per pig. Typically, such farms focus on selection and breeding. Conversely, low health status (LHS) farms are characterized by recurrent post-weaning diarrhea and morbidity rates of at least 10–15%. While standardized management practices are implemented across all farms, those with HHS exhibit superior performance in various aspects of biosecurity, including the acquisition of breeding pigs, visitor management, compartmental measures, equipment maintenance and sanitation practices. Additionally, HHS farms demonstrate resilience against major swine pathogens such as PRRSV, swine dysentery and Mycoplasma hyopneumoniae, with mortality rates below 4%.

4.3. Sampling

During January and June 2023, six pig farms were enrolled for in study (three HHS and three LHS). One house from each farm was selected according to the described criteria. At each farm, one house from the nursery facility containing sows with 3 week-old piglets was selected.

4.3.1. Environmental Sampling

Thereafter, 10 pens within each house were sampled (walls and slats), i.e., 4 and 6 pens from the corners and the middle of the house, respectively (Figure 3). In addition, two slurry pit samples were collected from different sampling points in sterile pots (500 mL each). Wall and slat samples were collected by wiping 1 m^2 of surfaces using both sides of a sterile wipe (Whirl-Pak®, Scharlab, Barcelona, Spain). First, wall samples (10 per farm, 1 per pen) were taken at an approximate height of 70 cm above the pen floor. Then, the slat samples (10 per farm, 1 per pen) were collected between and under the grates whenever possible. Once collected, all the samples were placed individually in a sterile bag with sterile diluent and transported to the laboratory in refrigerated conditions and processed within 12 h.

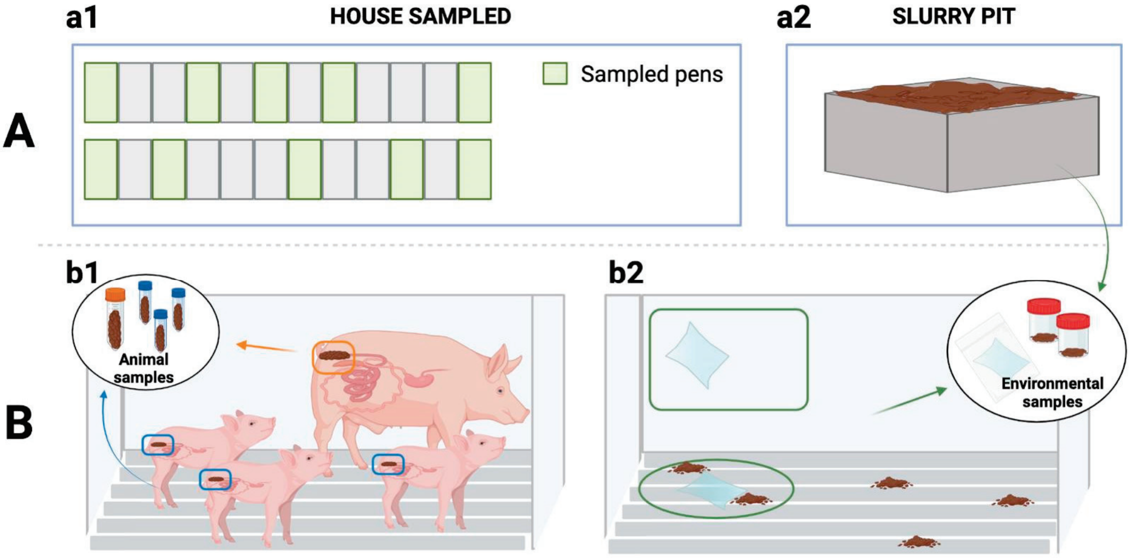

Figure 3. Sampling procedure. (**A**) Sampling points: (**a1**) Sampling within the house, (**a2**) Sampling outside the building (slurry pit). (**B**) Samples collected: (**b1**) from the animals (sows' and piglets' rectal faeces) and (**b2**) from the farm environment with swabs (wall and slat samples) and from slurry.

Once in the laboratory, the wall and slat samples were processed identically. Two pools of five wipes each (one quarter of a wipe per pool) were generated for each type of sample. The remaining wipes were stored in the freezer for further studies. Then, each pool and each slurry sample were placed in a stomacher (BagFilter® 400 mL, Scharlab, Barcelona, Spain) and homogenised with 15 mL of PBS (5 min, 260 rpm).

4.3.2. Animal Sampling

In each environmentally sampled pen, the sows and piglets were also sampled (Figure 3). Thus, faecal samples were collected directly from the rectum of sows (n = 10) and their piglets (n = 30, three piglets/sow). Each sample collected was homogenised, and a swab from each homogenised sample (Cary Blair sterile transport swabs, Deltalab®, Barcelona, Spain) was transported to the laboratory under refrigerated conditions and analysed within 24 h of collection.

4.4. Multidrug Resistant Bacteria and Enteric Pathogens Screening

When screening for MDR bacteria and enteric pathogens, a total of 274 samples were analysed (34 samples from the environment and 240 samples from the animals). The screening for MDR bacteria was carried out using selective plates for each microorganism. After this screening, the presence of the main antibiotic resistance mechanisms was analyzed in the phenotypically resistant strains through PCR and/or immunochromatography [68–70]

All the samples collected were cultured and analysed following the scientific protocols of the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC) (https://seimc.org (accessed on 10 June 2024)) for detection of the bacterial groups related to antibiotic resistance: *Enterobacterales* resistant to third-generation cephalosporins (producing extended-spectrum beta-lactamases (ESBL)) and plasmid-mediated AmpC, Gram-negative bacilli resistant to carbapenems and/or colistin, MRSA, vancomycin-resistant *Enterococcus* (VRE) and enteric pathogens: *Clostridioides difficile*, *Salmonella*, *Yersinia* spp. and *Campylobacter* spp.

Isolation of ESBL-producing *Enterobacterales* was performed by seeding on a specific chromogenic medium, CHROMID ESBL (Biomerieux, Craponne, France), and selection of colistin-resistant strains was carried out by seeding on MacConkey Agar (Biomerieux, Craponne, France) combined with a colistin disc. For the detection of Gram-negative bacilli resistant to carbapenems, the chromogenic medium CROMID CARBA SMART (Biomerieux, Craponne, France) was used. Isolation of MRSA and VRE was performed using the CROMID MRSA SMART (Biomerieux, Crapone, France) and CHROMID VRE (Biomerieux, Crapone, France), respectively.

Regarding the detection of enteric pathogens, selective media were used for the isolation of *Campylobacter* spp. (Campylogel agar, Biomerieux, Crapone, France), *Yersinia* spp. (*Yersinia* CIN agar, Biomerieux, Crapone, France) and *C. difficile* (CHROMID *C. difficile* agar, Biomerieux, Crapone, France). In the case of *Salmonella*, prior to seeding on the chromogenic plate (BD Chromagar *Salmonella*, Becton, Dickinson, Franklin Lakes, NJ, USA), the sample underwent enrichment by broth (Selenite F broth, Biomerieux, Crapone, France) and subsequent incubation at 37 °C for 24 h.

All plates were incubated at 37 °C for 24 h, except for the selective plates for *Campylobacter*, which were maintained at 42 °C for 48 h under microaerophilic conditions, and the selective plates for *C. difficile*, which were maintained at 37 °C for 48 h under anaerobic conditions. From plates showing growth, the isolation of a colony was performed and the microorganism was subsequently identified using mass spectrometry (MALDI-TOF, Bruker, Karlsruhe, Germany).

Finally, rapid tests were conducted for confirmation and determination of resistance mechanisms in all the isolated phenotypically resistant microorganisms. Specifically, in MRSA, an immunochromatography test was performed to detect the PBP2a protein (Clearview PBP2a, Abbott, Lake County, IL, USA). Vancomycin resistance was confirmed by e-test (Biomerieux, Crapone, France) and detection of vanA/B genes was carried out using multiplex PCR (FilmArray multiplex PCR, Biomerieux, Crapone, France). ESBL-producing *Enterobacterales* were confirmed using a microdilution of cefotaxime and ceftazidime with and without clavulanic acid (MicroScan, Beckman Coulter, Indianapolis, IN, USA), and the presence of major carbapenemases (VIM, NDM, KPC, IMP and OXA-48) was confirmed using immunochromatography (NG-Test® CARBA-5, NG-BIOTECH, Guipry-Messac, France). Colistin resistance was confirmed using microdilution with the UMIC colistin kit (Bruker, Karlsruhe, Germany), and the presence of the MCR 1 enzyme was determined using immunochromatography (NG-Test® MCR-1, NG-BIOTECH, Guipry-Messac, France). Detection of genes encoding toxin B and binary toxin in *C. difficile* strains was performed using the Xpert C. difficile BT kit based on real-time PCR (GeneXpert, Cepheid, Sunnyvale, CA, USA).

4.5. Statistical Analysis

A generalised linear model (GLM), which assumed a binomial distribution for each AMR zoonotic bacterium presence, was fitted to the data to determine whether there was an association among the status of the farm (HHS and LHS), the sample origin (environment and animals) and the AMR zoonotic bacteria status of the batch. For this analysis, the error was designated as having a binomial distribution, and the probit link function was used. Binomial data for each sample were assigned: one if they had AMR zoonotic bacterium or zero if they did not. A *p*-value of less than 0.05 was considered statistically significant. Data are presented as least squares means ± standard error of the least squares means. All statistical analyses were carried out using a commercially available software program (SPSS 27.0.1.0; SPSS Inc., Chicago, IL, USA).

5. Conclusions

This study has identified *Campylobacter* and *C. difficile* among the enteric pathogens studied, while *Salmonella* or *Yersinia* were not isolated. Additionally, antimicrobial-resistant zoonotic bacteria of great importance in public and animal health were isolated, especially in the farm environment, suggesting the persistence of these microorganisms after cleaning and disinfection. Moreover, the presence of these microorganisms was particularly prevalent on those farms where the selective pressure of cleaning and disinfection was higher. Therefore, our results highlight the need for a holistic approach within the One Health paradigm, which acknowledges the interconnectedness between human, animal and environmental health in addressing zoonotic diseases and AMR.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13090883/s1, Figure S1: Percentage of each of the studied bacterial groups according to the farm status (High Health Status vs. Low Health Status) and the animal sampled (sows or piglets). Figure S2: Percentage of each of the studied bacterial groups according to the farm status (High Health Status vs. Low Health Status) and the environmental sample collected (walls, slats or slurry pit).

Author Contributions: Conceptualization: C.M., L.M.-G., S.V., M.T.P.-G. and J.C.R.; methodology: M.P.V., I.G., I.E., L.M.-D. and L.L.-R.; software: M.P.V., C.M. and N.G.; validation: L.M.-G., C.T.-M. and N.G.; formal analysis: M.P.V., C.M., J.C.R. and L.M.-G.; investigation: M.P.V., C.T.-M., I.G., I.E., A.M.-F., L.M.-D. and N.G.; resources: A.M.-F., L.M.-D., L.L.-R. and C.M.; data curation: J.C.R., C.M., M.P.V. and C.T.-M.; writing—M.P.V., C.M. and J.C.R.; writing—review and editing: all authors; supervision: C.M., L.M.-G., S.V. and M.T.P.-G.; project administration: C.M. and L.M.-G.; funding acquisition, C.M., L.M.-G. and M.P.V. 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.

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Article

Antibiotic Residues and Resistance in Three Wastewater Treatment Plants in Romania

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Abstract: This study evaluates antibiotic residues and bacterial loads in influent and effluent samples from three wastewater treatment plants (WWTPs) in Romania, across four seasons from 2021 to 2022. Analytical methods included solid-phase extraction and high-performance liquid chromatography (HPLC) to quantify antibiotic concentrations, while microbiological assays estimated bacterial loads and assessed antibiotic resistance patterns. Statistical analyses explored the impact of environmental factors such as temperature and rainfall on antibiotic levels. The results showed significant seasonal variations, with higher antibiotic concentrations in warmer seasons. Antibiotic removal efficiency varied among WWTPs, with some antibiotics being effectively removed and others persisting in the effluent, posing high environmental risks and potential for antibiotic resistance development. Bacterial loads were higher in spring and summer, correlating with increased temperatures. Eight bacterial strains were isolated, with higher resistance during warmer seasons, particularly to amoxicillin and clarithromycin.

Keywords: antibiotics; antibiotic resistance; risk quotient; environment; wastewaters

1. Introduction

Antibiotics are essential tools in modern medicine, significantly decreasing mortality and morbidity rates from infectious diseases [1]. But, antibiotic pollution in aquatic environments has emerged as a global environmental challenge, with implications for human and ecological well-being.

The rise of antibiotic usage has led to the exposure of bacterial communities and ecosystems to a significant amount of antibiotic residues. Also, the widespread use of antibiotics has led to the emergence and spread of antibiotic-resistant bacteria (ARB), posing a severe threat to human health. Wastewater treatment plants (WWTPs) are essential in eliminating antibiotics and various contaminants from wastewater prior to its release into natural ecosystems. However, there is a substantial possibility that WWTPs could themselves become major contributors to the dispersion of antibiotic residues across various environmental compartments when the treatment process is inadequate. Even if contaminated wastewater is treated in WWTPs, a complete removal of antibiotics, ARB, and ARGs (antibiotic resistance genes) is impossible in conventional WWTPs [2,3]. WWTPs are considered major reservoirs of ARB and ARGs due to the discharge of untreated or partially treated wastewater containing antibiotic residues [4]. It is crucial to monitor antibiotic concentrations in both influents and effluents and to assess the effectiveness of the treatment processes in reducing the antibiotic contamination of wastewater. The incomplete removal of antibiotics from wastewater is a major concern because antibiotics can re-enter the environment through wastewater treatment plants and thus contaminate drinking water, soil, and waterways [5]. Antibiotic environmental contamination could increase the resistant bacterial population or maintain the selective pressure on it [6].

The analysis of influents and effluents from WWTPs could provide useful information about medication use and misuse [7]. Antibiotics are not fully metabolized in animals or humans, resulting in the excretion of 30–90% of the administered antibiotics as parent compounds through urine and feces. This introduces antibiotics into the environment, where they can persist due to their varying half-lives. For instance, the half-lives of azithromycin, amoxicillin, and ciprofloxacin are less than 5 h, less than a day, and less than 46 h, respectively, while norfloxacin persists for up to 77 days [5]. These half-life variations underscore antibiotics' diverse temporal behavior in aquatic systems. The degradation of antibiotics in WWTPs and the environment is influenced by several interconnected processes, including seasonal variations, which can affect the fate and transport of antibiotics within WWTPs. Therefore, a thorough investigation into the impact of the changing seasons on antibiotic removal and environmental risk is essential for optimizing treatment processes. [5]. The release of antibiotics into the environment, coupled with their persistence and potential to generate environmentally concerning metabolites, necessitates concerted efforts to regulate and control these contaminants.

This study focuses on evaluating the presence of the following antibiotics, amoxicillin, piperacillin, ciprofloxacin, norfloxacin, azithromycin, clarithromycin, and doxycycline, in three Romanian WWTPs, over the four seasons in the years of 2021–2022. These antibiotics were chosen due to their widespread use in human medicine, veterinary medicine, and animal farms, leading to their frequent detection in wastewater treatment plants and the environment. These antibiotics were selected through inquiries made to hospital pharmacies with high antibiotic consumption, such as those in infectious disease and pneumology hospitals, as well as community pharmacies. These inquiries focused on the most frequently dispensed antibiotics during 2020 and 2021, covering the COVID-19 pandemic period, which saw an increase in antibiotic usage in Romania compared to average European consumption. National reports from the ECDC further confirm this increased usage [8]. Also, some of these antibiotics, including amoxicillin, ciprofloxacin, azithromycin, and clarithromycin, are listed on the environmental watch list due to their potential as aquatic pollutants, further justifying their inclusion in this study [9].

For the first time, the central-western region of Romania is considered for analyzing antibiotics within this study in the influents and effluents of three WWTPs which are discharging their effluents into the same river. Our objectives were to quantify the selected antibiotics in influent and effluent samples, assess the removal efficiency of the WWTPs, and evaluate the risk posed by the identified residues to aquatic ecosystems. Additionally, we aimed to understand the seasonal variation in antibiotic concentrations and their correlation with the development of antibiotic resistance in bacterial populations. A preliminary microbiological assay was conducted as an initial step in this study to lay the groundwork for the planned advanced molecular analyses. The comprehensive approach of the present study combines chemical analysis, microbiological assays, and environmental risk assessment to provide an understanding of the challenges posed by antibiotic residues in wastewater.

2. Results

2.1. Occurrence and Seasonal Variation of Antibiotics in Wastewaters

The present study measured the residual antibiotic concentrations that occurred in the influents and effluents of three WWTPs across four seasons. Table 1 shows that only one antibiotic, amoxicillin, displayed a statistically significant concentration difference between the influent and effluent samples. The mean difference in concentration for amoxicillin was 7.11 μg/mL, with a 95% confidence interval ranging from 2.84 to 19.98 μg/mL. Therefore, despite the confidence interval suggesting a potential difference in concentration, the *p*-value of 0.093 indicates that this result is not statistically significant. Also, the remaining six antibiotics (AZT, CIP, CLT, DOX, PIP, and NOR) did not exhibit statistically significant differences between influent and effluent concentrations.

Table 1. Statistical analysis of antibiotic concentrations (μg/mL) in WWTP influents and effluents.

	Median		**Difference (95% CI)**	***p*-Value**
	Influent	**Effluent**		
AMX	7.11 (<LOQ–16.61)	<LOQ (<LOQ–2.1)	7.11 (2.84–19.98)	0.093
AZT	0.56 (<LOQ–1.12)	<LOQ (<LOQ–1.12)	0.56 (NaN–NaN)	1
CIP	<LOQ (<LOQ–0.26)	0.41 (<LOQ–1.05)	−0.41 (−1.05–3.55)	0.854
CLT	1.27 (<LOQ–1.27)	1.27 (<LOQ–1.27)	<LOQ (−1.27–1.28)	0.584
DOX	2.18 (<LOQ–2.47)	<LOQ (<LOQ–2.51)	2.18 (−1.47–2.32)	0.933
NOR	<LOQ (<LOQ–0.79)	0.89 (0.43–1)	0.89 (−1–6.02)	0.824
PIP	0.87 (0.2–1.87)	0.24 (<LOQ–0.9)	0.64 (−15.73–1.79)	0.286
	Mean		**Difference (95% CI)**	***p*-Value**
	Influent	**Effluent**		
AMX	9.5 (10.47)	3.88 (9.12)	5.62 (−0.28–11.53)	0.06
AZT	0.56 (0.58)	0.47 (0.57)	0.09 (−0.11–0.3)	0.339
CIP	0.94 (2.64)	0.51 (0.53)	0.44 (−1.13–2.01)	0.553
CLT	0.89 (0.74)	0.84 (0.69)	0.05 (−0.31–0.41)	0.764
DOX	1.5 (1.36)	1.32 (1.73)	0.18 (−0.61–0.97)	0.622
NOR	2.23 (5.09)	1.05 (1.13)	1.19 (−2.11–4.48)	0.445
PIP	1.21 (1.18)	3.26 (10.02)	−2.05 (−8.53–4.43)	0.501

AMX—amoxicillin, AZT—azithromycin, CIP—ciprofloxacin, CLT—clarithromycin, DOX—doxycycline, NOR—norfloxacin, PIP—piperacillin.

Regarding the seasonal variations in antibiotic concentrations, most of the *p*-values (Table 2) are less than 0.05, indicating statistically significant results. Differences in seasonal antibiotic concentrations were observed for both influent and effluent samples. In the spring and summer, high concentrations of amoxicillin were detected in the influents of all WWTPs, with particularly high effluent levels observed in WWTP C (31.177 μg/mL) and WWTP B (31.163 μg/mL). Piperacillin showed extremely high concentrations in the effluent of WWTP B during autumn (35.056 μg/mL), while ciprofloxacin and norfloxacin were generally low or undetectable, except for notable influent levels in WWTP B during spring (9.213 μg/mL and 16.578 μg/mL, respectively). Azithromycin and clarithromycin were largely undetected, except for trace levels in specific influents, and doxycycline exhibited higher effluent levels in autumn and winter in WWTP C and WWTP B.

Figure 1 includes error bars representing the median variability in antibiotic concentrations across different seasons and sampling points. The lower error bars indicate the first quartile (Q1), and the upper error bars indicate the median value's third quartile (Q3). Significant variability was observed for amoxicillin (Q3 median values reaching up to 22.16 μg/mL) in both influents and effluents (Q3 median values reaching up to 20.82 μg/mL), particularly during the summer of 2022 and the autumn of 2021, highlighting fluctuating concentrations. Azithromycin and ciprofloxacin showed consistent low concentrations and high variability in specific seasons. Clarithromycin and doxycycline exhibited moderate to high variability, indicating inconsistent detection and removal efficiency across seasons. Norfloxacin and piperacillin also displayed significant variability, with influent concentrations peaking in the spring of 2022 and the autumn of 2021.

Table 2. Antibiotic concentrations in WWTP influents (I) and effluents (E): a statistical descriptive analysis by season (µg/mL).

	Median				***p*-Value**
	Q4 (n = 3)	**Q1 (n = 3)**	**Q2 (n = 3)**	**Q3 (n = 3)**	***p* {(1, 2)/(1, 3)/(1, 4)/(2, 3)/(2, 4)/(3, 4)}**
AMX I	n.d.	n.d.	14.65 (14.43–19.99)	19.98 (17.74–22.16)	0.005 {1/<0.001/<0.001/<0.001/<0.001/0.998}
AMX E	n.d.	n.d.	10.47 (6.79–20.82)	<LOQ (<LOQ–0.88)	0.018 {1/<0.001/0.889/<0.001/0.889/<0.001}
AZT I	1.12 (1.12–1.12)	n.d.	1.12 (1.12–1.12)	n.d.	0.005 {<0.001/1/<0.001/<0.001/1/<0.001}
AZT E	1.12 (1.12–1.12)	n.d.	1.12 (0.56–1.12)	n.d.	0.044 {<0.001/0.827/<0.001/0.548/1/0.548}
CIP I	n.d.	n.d.	1.05 (1.05–5.13)	n.d.	0.018 {1/<0.001/1/<0.001/1/<0.001}
CIP E	1.05 (0.93–1.05)	n.d.	1.05 (1.05–1.05)	n.d.	0.007 {<0.001/0.928/<0.001/<0.001/1/<0.001}
CLT I	1.27 (1.27–1.27)	1.27 (0.9–1.54)	1.27 (0.64–1.63)	n.d.	0.108 {1/1/<0.001/1/<0.001/0.594}
CLT E	1.27 (1.27–1.27)	0.52 (0.26–0.89)	1.27 (1.27–1.6)	n.d.	0.017 {0.629/0.886/<0.001/0.408/0.629/<0.001}
DOX I	2.2 (2.18–2.72)	2.74 (2.56–2.89)	n.d.	<LOQ (<LOQ–1.13)	0.025 {0.995/<0.001/0.916/<0.001/<0.001/0.895}
DOX E	3.9 (3.38–4.17)	2.23 (1.11–2.31)	n.d.	n.d.	0.004 {<0.001/<0.001/<0.001/0.629/0.629/1}
NOR I	n.d.	n.d.	8.19 (4.1–12.38)	0.55 (0.28–1.02)	0.107 {1/0.459/0.459/0.459/0.459/0.878}
NOR E	1 (0.89–1)	1 (0.93–2.48)	0.92 (0.75–1.7)	n.d.	0.049 {0.984/1/<0.001/0.988/<0.001/<0.001}
PIP I	1.74 (1.29–2.62)	1.69 (0.85–1.98)	0.9 (0.58–1.82)	<LOQ (<LOQ–0.28)	0.228 {0.983/0.983/<0.001/1/0.865/0.638}
PIP E	0.9 (0.9–17.98)	n.d.	<LOQ (<LOQ–0.45)	0.45 (0.24–0.68)	0.024 {<0.001/0.37/0.37/0.853/<0.001/0.951}

n.d. = not detected, AMX—amoxicillin, AZT—azithromycin, CIP—ciprofloxacin, CLT—clarithromycin, DOX—doxycycline, NOR—norfloxacin, PIP—piperacillin, I—influent, E—effluent.

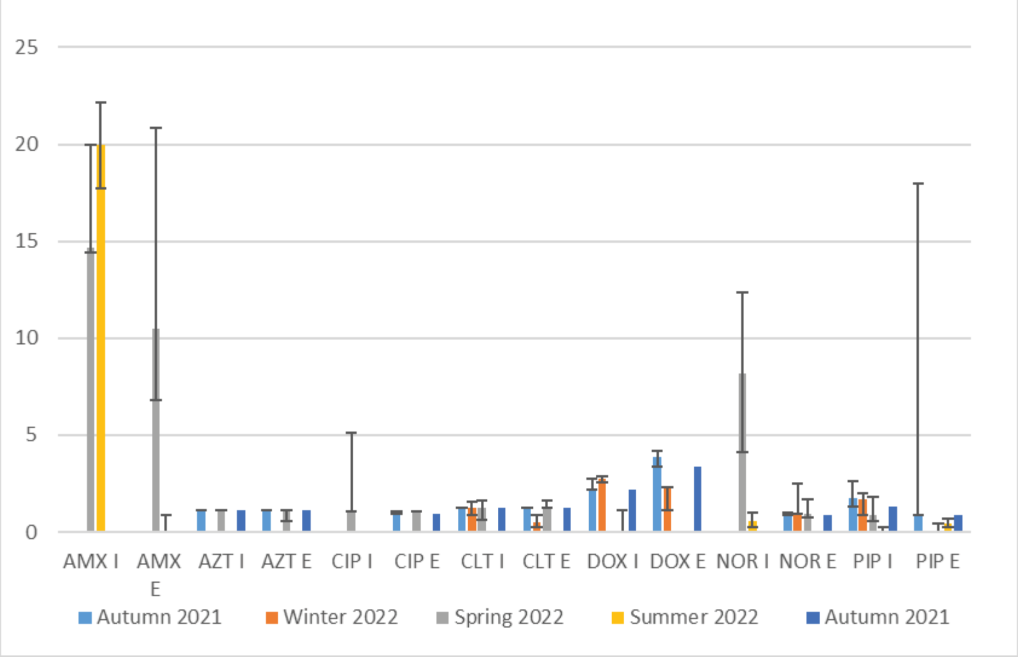

Figure 1. The median variability in antibiotic concentrations across different seasons and sampling points.

2.2. Antibiotic Removal Efficiency from Wastewater Treatment Plants

Antibiotic removal efficiency (RE%) was calculated based on the equation described by Douziech et al. [10],

$$\text{RE} = (1 - \exp\,(\text{LN}(\text{CEWW}/\text{CIWW})) \times 100\%)\ \text{CEWW}$$

CIWW—Influent antibiotic concentration
CEWW—Effluent antibiotic concentration

Table 3 shows the variations of the antibiotic removal rates during four seasons for the three WWTPs, which varied between 3% and −315%. Attempts to establish statistical significance in the correlation between wastewater treatment efficacy and antibiotic levels yielded no significant results (Table 4). Piperacillin had very high removal rates in all seasons except for the autumn season in WWTP C, where a low negative removal rate was obtained. Norfloxacin had removal rates that varied between 70% and 100%. Clarithromycin (3% and 100%) and doxycycline (19% and −105%) had a large range of removal rates, which could be explained by the fact that they are persistent antibiotics and have the capacity of being adsorbed by solid matrices such as sludge. We noticed negative antibiotic removal efficiency for amoxicillin (−23% in WWTP B in Q2), for doxycycline (−30% in WWTP A in Q4, −105% in WWTP B in Q4, and −20% in WWTP C in Q4) and piperacillin (−315% in WWTP C in Q4).

Table 3. Antibiotic removal efficiency (RE %) from three WWTPs during four seasons.

Location	AMX	PIP	CIP	NOR	AZT	CLT	DOX
WWTP A Q4	n/a	74%	n/a	n/a	n/a	n/a	−30%
WWTP B Q4	n/a	−315%	n/a	n/a	n/a	n/a	−20%
WWTP C Q4	n/a	48%	n/a	n/a	n/a	n/a	−105%
WWTP A Q1	n/a	100%	n/a	n/a	n/a	100%	21%
WWTP B Q1	n/a	100%	n/a	n/a	n/a	71%	100%
WWTP C Q1	n/a	n/a	n/a	n/a	n/a	n/a	19%
WWTP A Q2	26%	−237%	n/a	n/a	n/a	3%	n/a
WWTP B Q2	78%	100%	89%	97%	n/a	n/a	n/a
WWTP C Q2	−23%	n/a	n/a	70%	n/a	n/a	n/a
WWTP A Q3	100%	n/a	n/a	n/a	n/a	n/a	100%
WWTP B Q3	100%	96%	n/a	100%	n/a	n/a	n/a
WWTP C Q3	93%	n/a	n/a	100%	n/a	n/a	n/a

AMX—amoxicillin, AZT—azithromycin, CIP—ciprofloxacin, CLT—clarithromycin, DOX—doxycycline, NOR—norfloxacin, PIP—piperacillin, WWTP: Wastewater treatment plant, Q1—Winter, Q2—Spring, Q3—Summer, Q4—Autumn, n/a—not applicable.

Table 4. Statistical data for the antibiotic removal efficiency.

Antibiotics	Mean (SD)/Median (IQR)
AMX	85.74 (39.45–98.19)
AZT	0 (0–0)
CLT	0 (0–20.34)
DOX	18.69 (−25.38–60.72)
NOR	98.27 (89.87–100)
PIP	95.54 (48.11–100)

AMX—amoxicillin, AZT—azithromycin, CLT—clarithromycin, DOX—doxycycline, NOR—norfloxacin, PIP—piperacillin.

2.3. Antibiotic Resistance Profiles of Isolated Bacteria

For samples where CFU were observed (Table 5), the isolation of at least one bacterial strain was conducted. There were a total of 24 isolates, out of which 8 could be further investigated by Gram coloration, antibiograms, and SEM.

Table 5. Logarithm of CFU/mL across the four seasons during which the samples were collected.

	WWTP A		WWTP B		WWTP C	
	Influent	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**
Autumn Q4	6.903	6.322	6.114	6.301	6.041	6.041
Winter Q1	-	6	6.477	-	7.491	-
Spring Q2	8.857	6.699	9.230	-	8.146	7.681
Summer Q3	7.633	-	8.447	-	6	-

2.4. Gram Staining

The eight isolates (six isolates from the spring season and two from the summer season) were tested to determine their Gram group (Table 6). Out of these, five strains were Gram-negative (most probably Gram-negative coliforms), one strain was Gram-positive, and two were inconclusive.

Table 6. Gram staining and distribution of the eight bacterial isolates.

	WWTP A		WWTP B		WWTP C	
	Influent	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**
Autumn Q4	-	-	-	-	-	-
Winter Q1	-	-	-	-	-	-
Spring Q2	G+	G−	G−	-	G−	G−
Summer Q3	-	-	G−	-	-	-

G+ = Gram-positive, G− = Gram-negative, "-" inconclusive isolates.

3. Discussions

3.1. Comparison of Antibiotic Concentrations with Other Studies

The data from our study reflect the trends observed in other studies, confirming the presence of these antibiotics in urban wastewater systems [11–15]. In the study by Mirzaei et al. [16], antibiotic concentrations in WWTPs were found to vary seasonally, with our findings indicating higher concentrations detected in the warmer months compared to the winter season. A study by Chukwu et al. [17] reported mean concentrations of antibiotics such as sulfamethoxazole reaching up to 0.28618 μg/mL in WWTP effluent, indicating substantial pollution levels. Also, seasonal variations in antibiotic concentrations were observed, with influent levels reaching several μg/mL. A study by Faleye et al. [18] reported influent antibiotic concentrations ranging from 1.3 ng/L of azithromycin to 81,748 ng/L of ciprofloxacin. A review by Madikizela et al. [19] reported pharmaceutical concentrations in the range of ng/L to μg/L in African water bodies. A comprehensive study conducted across Europe has reported various antibiotics such as ciprofloxacin, azithromycin, and clarithromycin in WWTP effluents with concentrations reaching up to several μg/mL, although these were lower than the levels observed in our study [20]. However, it is important to note that most European studies reported antibiotic levels in WWTP effluents that were lower than those observed in our study. This discrepancy could be attributed to regional differences in antibiotic usage and disposal practices, as well as variations in the efficiency of wastewater treatment processes. Our results align with other studies, which indicate significant pollution levels in pharmaceutical effluents [21].

3.2. Variability of Antibiotic Removal Efficiencies

The statistical mean and median data (Table 4) for antibiotic removal efficiency for three WWTPs during four seasons confirm that the WWTPs' methods were not highly effective in removing most antibiotics from the wastewater. Also, the antibiotic removal efficiencies of the WWTPs were highly variable, depending on the specific antibiotic. The removal efficiency was very high for some antibiotics, such as amoxicillin, norfloxacin, and piperacillin, with mean and median values above 95%. Although good removal efficiency by treatment processes was observed for several antibiotics, most antibiotics were still present in WWTP effluents. The removal levels, such as azithromycin, ciprofloxacin, doxycycline, and clarithromycin, were much lower, with mean removal efficiencies of 20% or less.

The negative antibiotic removal for doxycycline and piperacillin during the autumn season (Q4) can be attributed to higher organic loads and runoff, which overload microbial communities and reduce their efficiency. Increased amounts of suspended solids lead to the adsorption of antibiotics onto particles, making them less available for degradation. Lower temperatures in autumn further slow microbial activity. These factors collectively reduce antibiotic removal efficiency, whereas other seasons show removal rates between 19% and 100%. Amoxicillin showed lower or negative removal rates for the spring season, but the removal rates were higher for the summer season, from 93% to 100%.

Similar results to those obtained by the present study were reported by other research groups [3,22–25]. Zheng et al. noticed the highest antibiotic removal rate during the summer season [26]. Also, Mozaz et al. [20] mentioned that better antibiotic removal rates were obtained for temperatures of 15–20 °C than for those below 10 °C. Higher antibiotic removal rates during the summer season can be attributed to elevated temperatures, which enhance the activity of microorganisms responsible for degrading antibiotics in wastewater treatment processes. Warmer temperatures improve the metabolic rates of these microorganisms, facilitating a more efficient breakdown of antibiotic compounds.

Several factors contribute to the overall low effectiveness of antibiotic removal in WWTPs. Firstly, processes like dilution, degradation, and various treatment methods (physical, chemical, and biological) affect antibiotic removal efficiency. Higher antibiotic levels in effluents than in influents may result from the cleavage of phase II metabolites (glucuronides and sulfates) during treatment, releasing parent compounds. Secondly, certain antibiotics can be metabolized or transformed by microorganisms, reducing their concentration or removing them completely. Antibiotics can also be trapped within biofilms on WWTP surfaces, protecting them from degradation and allowing accumulation. Thirdly, adsorption–desorption processes can cause antibiotics to adhere to solid particles like sludge, extracting them from the liquid phase. Lastly, many WWTPs are not specifically designed for antibiotic removal, making this a particularly complex challenge [5,12,16,27,28]. Although they have the potential to degrade, antibiotics are frequently detected in the environment, placing them in the category of pseudo-persistent pollutants. A study of the effluents from 90 WWTPs in the European Union revealed a high detection frequency for antibiotics such as trimethoprim, ciprofloxacin, and sulfamethoxazole, with rates of 93%, 90%, and 83%, respectively [5].

3.3. Factors Influencing the Variation of Antibiotic Concentrations

The fluctuation in antibiotic concentrations across different seasons can be attributed to various factors, including antibiotic usage patterns, seasonal consumption spikes, pandemic-related increases, the population served, anthropogenic activities, agricultural discharges, inadequate environmental regulations, poor pharmaceutical waste management, increased amounts of travel, and higher illness susceptibility [29]. In 2021–2022, Romania had the highest antibiotic consumption in Europe at 25.9 DDD per 1000 inhabitants per day, surpassing the EU average of 19.4 DDD. Contributing factors include over-the-counter availability and socio-economic barriers. Inappropriate disposal of antibiotics further exacerbates environmental contamination. Romania's pharmaceutical waste management needs urgent reform to meet European standards [30,31].

It is important to note that the differences in concentrations of antibiotics in wastewater can vary widely, even within the same season, and can be caused by fluctuations in antibiotic usage levels [32]. The error bars (Figure 1) explain the seasonal variability of antibiotic concentrations in the influents and effluents of WWTPs. High variability suggests fluctuating usage patterns or inconsistent removal efficiencies, while low variability indicates more stable conditions. Therefore, these observed patterns are essential for assessing the impact of wastewater treatment on antibiotic levels. Also, the analysis emphasizes the need for continuous monitoring and adaptive management in WWTP operations to address the challenges posed by antibiotic residues.

A limitation of the study is that single seasonal sampling may not capture the full range of antibiotic concentrations due to potential fluctuations in consumption. Increased sampling frequency or real-time monitoring could provide a more accurate understanding of the relationship between antibiotic consumption and wastewater concentrations [33]. Similar trends have been observed regarding the correlation between the fluctuation in antibiotic concentrations and the prevalence of bacterial infections, as noted in various studies [20,34]. This correlation suggests that as antibiotic levels vary, there is a corresponding change in the incidence of bacterial infections. Such findings underscore the interconnected relationship between antibiotic use and the dynamics of bacterial infection rates [1,35].

3.3.1. The Influence of Air Temperature and Rainfall on Antibiotic Concentration Variability

Statistical analysis was performed to explore the possible associations between antibiotic concentrations and the mean monthly temperature or the mean monthly rainfall. Statistically significant associations were found between the mean monthly temperature or the mean monthly rainfall and amoxicillin and doxycycline concentrations, both in univariate and multivariate models (Tables 7–10). For both independent variables, higher values are associated with increased concentrations of amoxicillin but with decreased concentrations of doxycycline. The models, including mean monthly temperatures, had the highest determination coefficients, indicating an important effect of this variable on antibiotic concentrations. The determination coefficients were higher in the models predicting doxycycline concentrations than in those predicting amoxicillin. The treatment process of wastewater was not associated in a statistically significant manner with the antibiotic concentrations. No associations with the temperature and rainfall were noticed for the other antibiotics.

Table 7. Simple and multiple linear regression models predicting amoxicillin levels based on treatment and mean monthly temperature.

Variables	B Unadjusted	(95% CI)	*p*	R^2	B Adjusted	(95% CI)	*p*
Intercept					1.66	(–7.65–10.96)	0.707
Treated	–5.62	(−13.94–2.69)	0.175	0.08	−4.5	(–14.27–5.28)	0.338
Mean monthly temperature (°C)	0.79	(0.2–1.37)	0.012	0.37	0.79	(0.19–1.38)	0.003

CI, confidence interval; R^2, determination coefficient. The multivariate model included both variables. The adjusted determination coefficient was 0.32.

Table 8. Simple and multiple linear regression models predicting amoxicillin levels based on treatment and mean monthly rainfall.

Variables	B Unadjusted	(95% CI)	*p*	R^2	B Adjusted	(95% CI)	*p*
Intercept					2.11	−5.07	0.574
Treated	−5.62	(−3.48–2.23)	0.175	0.08	−4.5	(−4.58–5.59)	0.398
Mean monthly rainfall (L/m^2)	0.15	(0.06–0.24)	0.006	0.2	0.15	(0.07–0.23)	0.003

CI, confidence interval; R^2, determination coefficient. The multivariate model included both variables. The adjusted determination coefficient was 0.13.

Table 9. Simple and multiple linear regression models predicting doxycycline levels based on treatment and mean monthly temperature.

Variables	B Unadjusted	(95% CI)	*p*-Value	R^2	B Adjusted	(95% CI)	*p*
Intercept					2.99	(1.98–4)	<0.001
Treated	−0.18	(−1.5–1.14)	0.777	0.004	−0.06	(−1.12–1.01)	0.907
Mean monthly temperature (°C)	−0.14	(−0.2–−0.08)	<0.001	0.62	−0.14	(−0.2–−0.07)	<0.001

CI, confidence interval; R^2, determination coefficient. The multivariate model included both variables. The adjusted determination coefficient was 0.56.

Table 10. Simple and multiple linear regression models predicting doxycycline levels based on treatment and mean monthly rainfall.

Variables	B Unadjusted	(95% CI)	*p*	R^2	B Adjusted	(95% CI)	*p*
Intercept					3.11	(1.7–4.51)	<0.001
Treated	−0.18	(−1.5–1.14)	0.777	0.004	−0.06	(−1.35–1.24)	0.924
Mean monthly rainfall (L/m^2)	−0.03	(−0.05–−0.01)	0.006	0.43	−0.03	(−0.05–−0.01)	0.008

CI, confidence interval; R^2, determination coefficient. The multivariate model included both variables. The adjusted determination coefficient was 0.35.

The model with mean monthly temperature as an independent variable shows a positive correlation with amoxicillin levels and has a higher determination coefficient, suggesting a predictive power of temperature on amoxicillin concentration. This might indicate that temperature increases lead to conditions that either promote persistence or reduce the degradation of amoxicillin in the environment. Rainfall also shows a significant positive effect on amoxicillin concentrations but with a lower determination coefficient than temperature. This might suggest that while rainfall impacts amoxicillin levels, it does so less consistently or predictably than temperature.

For doxycycline, the negative coefficient with temperature indicates an inverse relationship, where higher temperatures might enhance the degradation or reduce the persistence of doxycycline in wastewater. The high determination coefficient in these models highlights temperature as a critical factor affecting doxycycline levels. Therefore, temperature has a more consistent and predictable influence on the degradation and persistence of antibiotics in wastewater compared to rainfall, which can vary in its effects.

These results indicate that these environmental factors could play a role in the variability of antibiotic concentrations in wastewater, specifically for amoxicillin and doxycycline. Both antibiotics exhibit different stability profiles in wastewater. This difference could be due to the chemical properties of the antibiotics, such as their solubility and stability under varying environmental conditions. For instance, a study on the ecological degradation of doxycycline demonstrated that temperature and H_2O_2 concentration significantly affect its residual concentration [36]. Similar to temperature, higher rainfall correlates with lower doxycycline concentrations. This could be due to dilution effects or enhanced microbial degradation stimulated by increased water content. Enhanced degradation of some antibiotics, leading to lower concentrations in the final effluent because of higher temperatures and increased rainfall, was reported in the literature [11,37].

Also, in the present study, higher concentrations of norfloxacin during the spring season (Q2) in WWTP C were observed, coinciding with high rainfall compared to other seasons and WWTPs, but without statistical significance. This suggests a potential influence of seasonal rainfall on the mobility and persistence of this antibiotic in aquatic environments. Effluents could show higher antibiotic concentrations due to inefficient wastewater treatment, while influents have reduced concentrations due to dilution. Additionally, seasonal variations in wastewater flow can influence the removal efficiency of ciprofloxacin and norfloxacin in WWTPs, with higher concentrations observed during periods of high rainfall [11].

Understanding these dynamics is crucial for optimizing wastewater treatment processes. For instance, treatments can be designed to take advantage of higher temperatures for more efficient degradation during warmer seasons and to manage the influx of antibiotics due to rainfall. Temperature plays a critical role in chemical reactions and biological treatment processes in wastewater plants, and there is a growing scientific recommendation for proactive wastewater temperature management, which would be essential for efficient treatment [38–41].

3.3.2. Influence of pH and Physicochemical Characteristics on Antibiotic Removal

During autumn, the high organic load in WWTP B correlates with poor antibiotic removal efficiency due to the potential overloading of microbial communities, competition for degradation pathways, and adsorption onto particulate matter. WWTP B shows COD, chemical oxygen demand, to be at 228.16 mg/L, BOD, biochemical oxygen demand, to be at 122 mg/L, suspended solids at 170 mg/L, fixed residues at 792 mg/L, ammonia at 181 mg/L, nitrites at 31.7 mg/L, phosphorus at 84.81 mg/L, and sulfates at 64.04 mg/L, indicating significant organic and nutrient loads that lead to low or negative antibiotic removal rates. In contrast, WWTPs A and C have lower CODs (181.9 mg/L and 259 mg/L, respectively) and BODs (95.35 mg/L and 85 mg/L, respectively), suggesting better performance but still showing variable antibiotic removal rates. Additionally, WWTP A's fixed residue is 417 mg/L, and WWTP C's is 740 mg/L, with lower ammonia (40.88 mg/L and 45.32 mg/L, respectively) and nitrite levels (0.208 mg/L in WWTP A). Despite this, high levels of fixed residues and moderate levels of suspended solids contribute to the observed variability in antibiotic removal rates.

WWTP A, with the largest capacity of 115,000 cbm/24 h serving 300,000 inhabitants, generally performs well but requires optimization in solid matter handling and adaptation to seasonal variations. It faces challenges with high levels of fixed residues (417 mg/L) and moderate levels of suspended solids. WWTP C, with a capacity of 4772 cbm/24 h serving 31,000 inhabitants, shows reasonable performance but struggles with high solid matter and requires improved sludge management. It also deals with high levels of fixed residues (740 mg/L) and moderate levels of suspended solids. Both plants exhibit variable antibiotic removal efficiency, typically higher in warmer months. WWTP B, with a capacity of 5673 cbm/24 h serving 19,000 inhabitants, faces significant challenges due to high organic and nutrient loads. It has poor antibiotic removal efficiency, especially in autumn (Q4), with negative rates for doxycycline (−20% in Q4) and piperacillin (−315% in Q4). All three plants share issues with high levels of fixed residues and moderate levels of suspended solids, and WWTP C requires advanced treatment technologies and seasonal adjustments to improve its performance. In our case, the high amounts of turbidity and sediment in the water samples further challenge the extraction. This interaction between the antibiotics' inherent properties and the treatment environment elucidates why ciprofloxacin and norfloxacin might persist through or even appear in higher concentrations after wastewater treatment [42].

The seasonal pH values and antibiotic removal efficiencies in three WWTPs (Figures 2 and 3) show the essential role of maintaining stable pH levels for effective treatment. WWTP A, with relatively stable and neutral pH values (influent: 6.76–7.34, effluent: 7.05–7.18), shows high removal efficiencies for amoxicillin and clarithromycin, particularly in Q3, while experiencing lower efficiencies and negative removal for doxycycline in Q2 and Q4. WWTP B maintains slightly consistent pH levels (influent: 6.20–7.11, effluent: 6.36–6.93), correlating with a high removal of norfloxacin in Q2 and Q3, but a negative removal for doxycycline in Q4. WWTP C, which exhibits pH fluctuations (influent: 6.45–7.47, effluent: 5.86–7.59), achieves a high removal of several antibiotics in Q2 and Q3, but poor removal efficiency for piperacillin and doxycycline in Q4 due to the low effluent pH of 5.86. These findings highlight the importance of stable, neutral pH levels in optimizing the microbial degradation of antibiotics, as significant pH fluctuations and consistently low pH conditions can reduce treatment efficiency [43,44].

For example, at a low pH, doxycycline becomes more stable and less bioavailable for microbial degradation. This could lead to its persistence in the effluent, as observed in Q4 for WWTP B. This can explain why the removal efficiency for antibiotics such as doxycycline is poor under acidic conditions in WWTP C. Regarding the effectiveness of the WWTPs, WWTP A appears to be the most effective in maintaining conditions conducive to antibiotic removal, except for DOX. WWTP B shows potential for good removal efficiency but with a slight decline in pH values across the seasons. In WWTP C, during Q4, a high organic load and a low pH correlates with poor antibiotic removal and underscores the need for better pH and physicochemical property management.

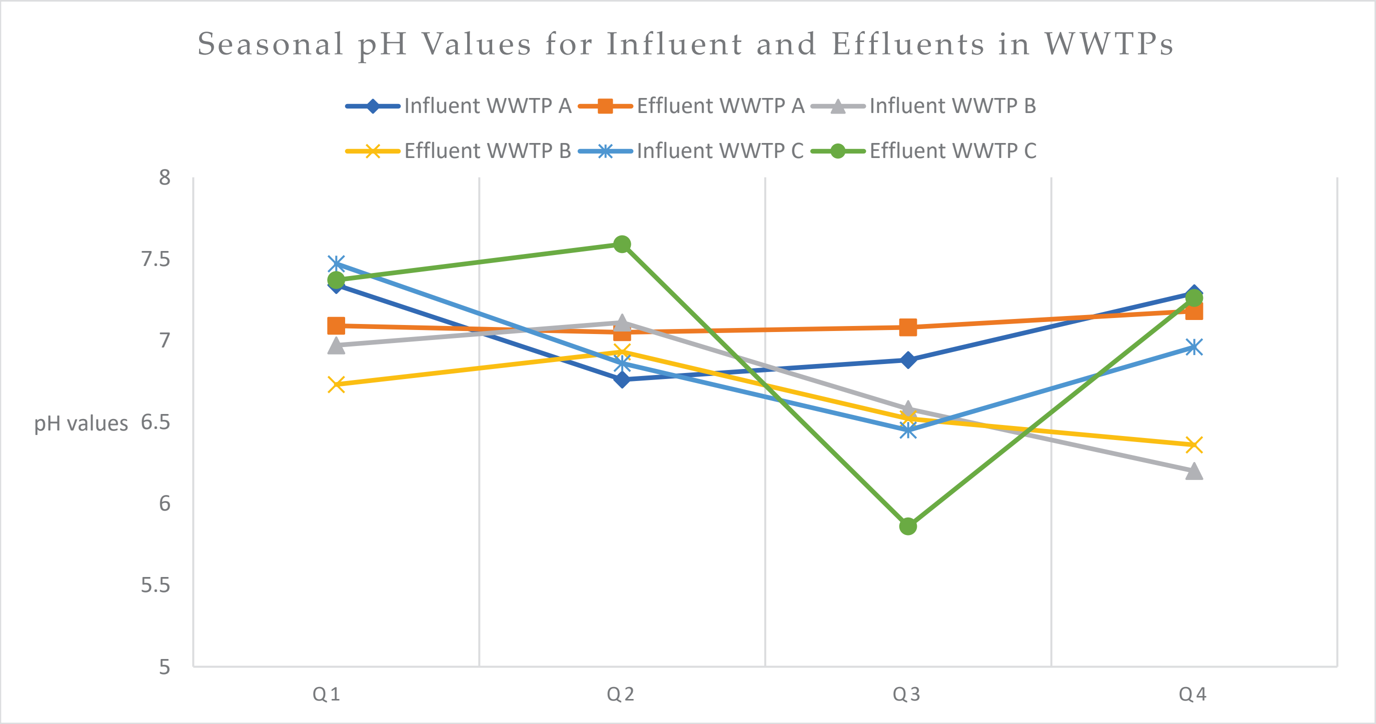

Figure 2. Seasonal variations in pH values in WWTPs. WWTP: Wastewater treatment plant, Q1—Winter, Q2—Spring, Q3—Summer, Q4—Autumn.

Different wastewater treatment techniques vary in their ability to remove antibiotics, with some being more effective than others. Methods such as biological processes, membrane technologies, and advanced treatments like ozonation or activated carbon adsorption can notably decrease antibiotic concentrations. However, conventional treatment methods, like those used in the WWTPs examined in this study, might not achieve the same level of effectiveness [24,45–47]. Also, there are several ways to improve the removal of antibiotics from wastewater. One way is to upgrade WWTPs with new technologies with advanced treatments that are designed to remove antibiotics. Also, recent studies confirm that high-rate algae ponds (HRAPs) are effective in removing antibiotics from wastewater and have potential for integration into real wastewater treatment plants [48]. Another way is to reduce the amount of antibiotics that are discharged into WWTPs. This can be achieved by educating the public about the proper use of antibiotics and by developing alternatives to antibiotics, such as bacteriophages [24,45,49,50].

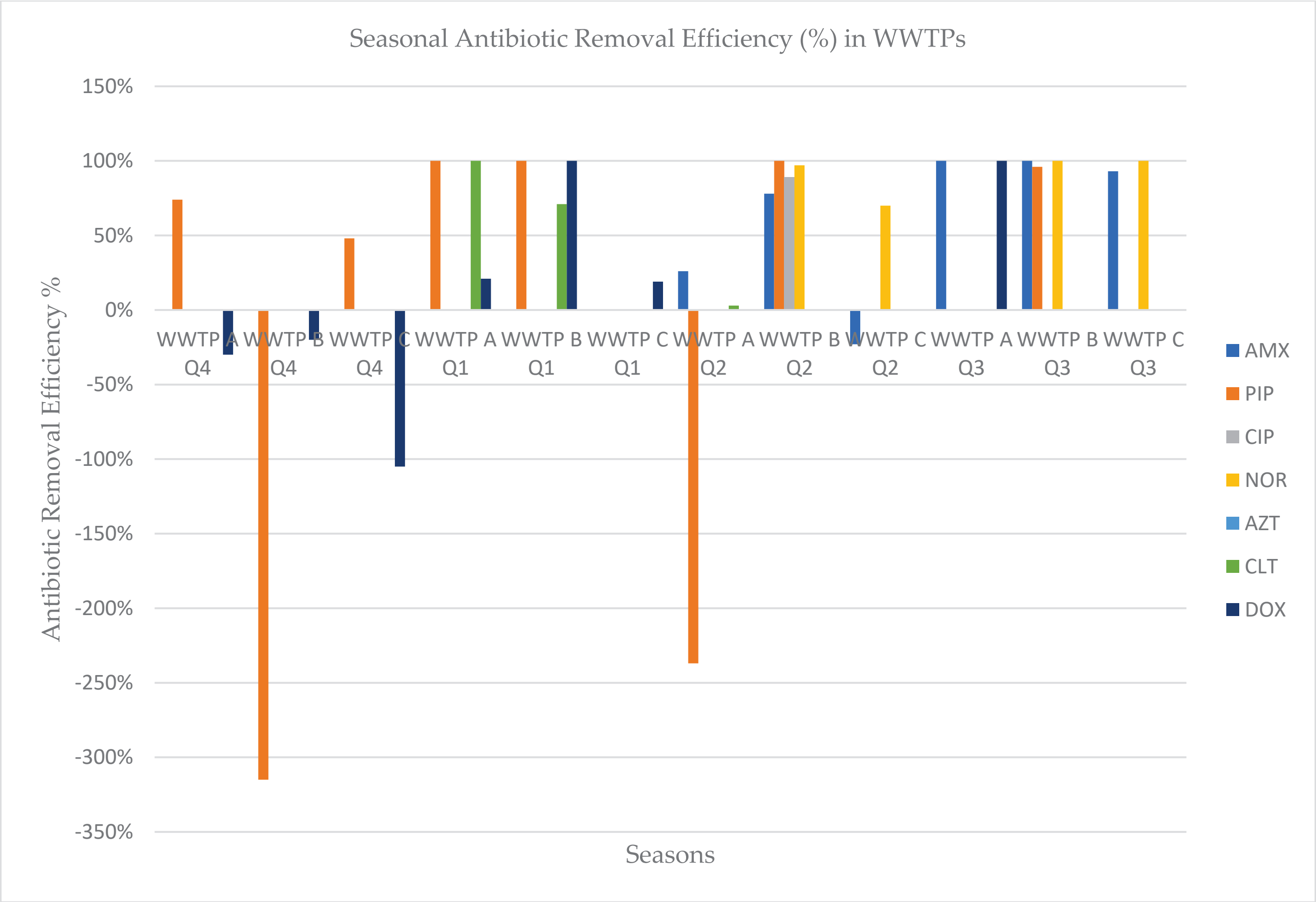

Figure 3. Seasonal variations in antibiotic removal efficiency in WWTPs. AMX—amoxicillin, AZT—azithromycin, CIP—ciprofloxacin, CLT—clarithromycin, DOX—doxycycline, NOR—norfloxacin, PIP—piperacillin, WWTP: Wastewater treatment plant, Q1—Winter, Q2—Spring, Q3—Summer, Q4—Autumn.

3.4. Impact of Antibiotic Residues in Effluents of WWTPs on the Aquatic Environment

The antibiotic concentrations in the effluents were used for the assessment of their impact on the aquatic environment as it was assumed that effluents are discharged into river waters. The environmental risk associated with the occurrence of antibiotic residues in wastewaters was assessed using the risk quotient (RQ).

All the targeted antibiotics at some point showed a high level of risk based on the ecological risk assessment (RQ > 1), as can be observed in Table 11. In the Q4 season, AMX showed high risk in WWTP C, but medium risk in WWTPs A and B, even though AMX showed high concentrations in the effluents. This could be explained by the high value of the PNEC for amoxicillin (Table 11). The PNEC represents the concentration below which no adverse effects are expected; a high PNEC is indicating that the organism or endpoint has a relatively high tolerance to the antibiotic [51]. We observed the opposite effect for antibiotics like NOR, CIP, and CLT, which were detected at lower concentrations than AMX, but showed low PNEC values, which led to remarkably high RQs. As can be observed, most of the selected antibiotics showed high RQ levels; therefore, antibiotic release into the environment poses both ecological and human health risks and requires more constant biological monitoring through ecological and antibiotic resistance risk assessments.

For the protection of environmental and public health, it is crucial to evaluate the selection pressure caused by antibiotic pollution in various scenarios. The extent and magnitude of the impact at the discharge point are influenced by factors such as water depth, currents, and sediment characteristics [52]. Adopting a precautionary approach that considers dilution in the aquatic environment, this study emphasizes the need for efforts to eliminate antibiotic residues during the wastewater treatment process.

Table 11. Risk quotients calculated for the antibiotics in the effluents of three WWTPs.

Effluent		AMX	PIP	CIP	NOR	AZT	CLT	DOX
WWTP A	Q1	0	0	0	111.1	0	0	16.74
	Q2	9.17	0	0	216.03	0	337.14	0
	Q3	0	12.68	0	0	0	0	0
	Q4	0	0	0	0	0	0	20.1
WWTP B	Q1	0	0	0	0	0	91.08	0
	Q2	2.73	0	0	16.07	0	0	0
	Q3	0	0.7	0	0	0	0	0
	Q4	0	98.32	0	0	0	0	27.35
WWTP C	Q1	0	0	0	23.97	0	0	0.02
	Q2	27.33	0	0	69.29	0	0	0
	Q3	1.54	0	0	0	0	0	0
	Q4	0	0	190.2	22.13	0	0	27.35

AMX—amoxicillin, AZT—azithromycin, CIP—ciprofloxacin, CLT—clarithromycin, DOX—doxycycline, NOR—norfloxacin, PIP—piperacillin, WWTP: Wastewater treatment plant, Q1—Winter, Q2—Spring, Q3—Summer, Q4—Autumn.

3.5. Analytical Limitations and Implications

In this study, a significant portion of measurable concentrations of samples fell below the LOQ. Differences below the quantification limit could be partially attributed to analytical error, hindering the accurate quantification of the actual removal efficiency. Therefore, it is difficult to definitively conclude whether observed increases in antibiotic concentration in the effluent solely reflect inefficient treatment or involve contributions from other factors like accumulation in sludge. Also, the inability of the current method to monitor the complete range relevant for environmental risk assessments—specifically, levels above PNEC-ENVs and PNEC-MICs—means that potentially harmful concentrations are often not detected and falsely reported as absent. This limitation impacts the accuracy of assessing environmental risks associated with these antibiotics.

3.6. Seasonal Variations in Estimated Bacterial Loads

Table 5 presents the logarithm of CFU/mL values across the four seasons for the influents and effluents of WWTPs A, B, and C. Different WWTPs show significant variations in bacterial loads both in influents and effluents.

Higher bacterial counts were observed in warmer months (spring and summer), with the highest values recorded in spring (Q2) for both the WWTP B influent (9.230 log CFU/mL) and the WWTP A influent (8.857 log CFU/mL). This increase is likely due to higher temperatures (15.5–15.4 °C in Q2 and 21.3–21.6 °C in Q3) which enhance bacterial growth, consistent with the findings of López et al. [53], who reported similar seasonal variations with bacterial concentrations reaching up to 10^8 CFU/100 mL during warmer periods. In contrast, winter and autumn showed lower bacterial loads (3.6–4.2 °C in Q4 and 0.8–1.8 °C in Q1), likely due to inhibited bacterial activity at lower temperatures [54]. Effluent bacterial loads were generally lower, demonstrating the partial effectiveness of wastewater treatment processes. However, exceptions were noted in Q1 for WWTP A, and Q4 for WWTPs B and C, where effluent levels were similar to or slightly higher than influent levels, indicating variable treatment efficacy.

3.6.1. SEM Analysis

The SEM micrographs (Figure 4) show the bacterial morphologies and densities from various WWTPs across different seasons. Some effluent sample micrographs (Figure 4d,e) display clusters of cells, suggesting biofilm formation, which can protect bacteria from treatment processes and promote the spread of antibiotic resistance. Biofilm formation is a well-known survival strategy that protects from physical stresses and antimicrobial agents, both of which are common in WWTPs and can also facilitate horizontal gene transfer among bacteria, potentially spreading resistance traits [55,56].

Overall, the SEM analysis highlights the need to consider both seasonal variations and biofilm formation when assessing the efficiency of wastewater treatment processes. These detailed morphological observations complement the quantitative CFU data, providing a potential understanding of microbial dynamics in WWTPs.

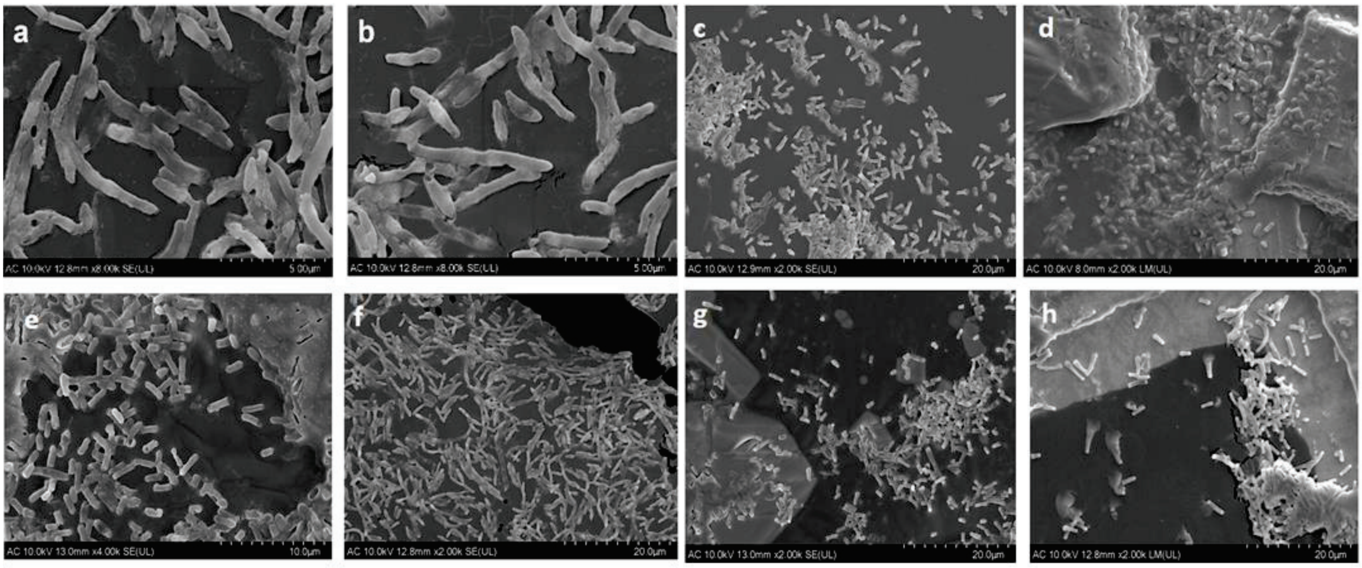

Figure 4. SEM micrographs of the eight isolated samples: (**a**) Q2 IWWTP A (**b**) Q2 EWWTP A (**c**) Q4 EWWTP A (**d**) Q2 EWWTP C (**e**) Q4 EWWTP C (**f**) Q2 IWWTP C, (**g**) Q3 IWWTP B, (**h**) Q2 IWWTP B. I WWTP- influent wastewater treatment plant, EWWTP—effluent wastewater treatment plant.

3.6.2. Analysis of Antibiotic Sensitivity Patterns

After characterization, bacterial isolates were assessed for sensitivity to seven antibiotics (Table 12). The most bacterial isolates during warmer months (Q2 and Q3) showed higher resistance to at least one antibiotic compared to those from colder months. Most isolates were generally sensitive to the antibiotics tested, but notable resistance was observed for amoxicillin and clarithromycin. Resistance to norfloxacin and ciprofloxacin varied, with some samples showing resistance or intermediate resistance. This is significant, as fluoroquinolone resistance is a critical public health issue [57]. These findings suggest that warmer conditions may facilitate the proliferation of antibiotic-resistant bacteria, potentially due to the presence of these antibiotics in the influent.

The data indicates increased resistance during the warmer seasons (Q2, Q3), likely due to factors such as higher bacterial growth rates at elevated temperatures, which can accelerate mutation rates and the spread of resistance genes [58].

The presence of antibiotic-resistant bacteria in treated effluents suggests the need to review and potentially enhance the treatment processes to better address bacterial resistance [59]. This initial microbiological assay was carried out with a narrow focus, laying the groundwork for more extensive future research. Furthermore, the SEM analysis and antibiotic sensitivity patterns provided additional support for microbiological research, particularly in the evaluation of CFU counts. These findings have also identified crucial areas for our future research on antibiotic-resistant bacteria and resistance genes.

Regular monitoring of antibiotic resistance in WWTPs is crucial for the early detection and management of resistant bacterial populations. Implementing robust antimicrobial stewardship and surveillance programs in WWTPs could help manage the risk of spreading antibiotic resistance [20,60]. The effluents from WWTPs often discharge into natural water bodies; thus, the presence of antibiotic-resistant bacteria could pose risks to ecosystems and human health. Strategies to mitigate these risks include improving disinfection processes and public health policies focusing on reducing antibiotic usage [53].

Table 12. Antibiograms of the eight isolated bacteria incubated with the seven most abundant antibiotics.

Location	AMX	PIP	CIP	NOR	CLT	AZT	DOX
Q4 EWWTP C	*IR*	S	S	S	S	S	S
Q4 EWWTP A	S	S	S	S	S	S	S
Q2 EWWTP A	R	S	S	S	R	R	S
Q2 IWWTP A	S	S	R	R	S	S	*IR*
Q2 IWWTP C	R	R	S	S	S	S	S
Q2 EWWTP C	R	*IR*	*IR*	*IR*	R	*IR*	*IR*
Q2 IWWTP B	S	S	S	S	R	S	S
Q3 IWWTP B	R	S	S	S	R	S	S

R = resistant, *IR* = intermediate resistance, S = sensitive, AMX—amoxicillin, AZT—azithromycin, CIP—ciprofloxacin, CLT—clarithromycin, DOX—doxycycline, NOR—norfloxacin, PIP—piperacillin, WWTP: Wastewater treatment plant, Q1—Winter, Q2—Spring, Q3—Summer, Q4—Autumn, I—influent, E—effluent.

In the same sampling season, high RQs of antibiotics were observed, and resistant or intermediate-resistant strains were identified. We identified resistant or intermediate-resistant strains to all monitored antibiotics in WWTP C effluents from the Q2 spring season and high RQs for amoxicillin and norfloxacin. Also, for the Q2 season, strains resistant to amoxicillin, clarithromycin, and azithromycin were isolated in WWTP A effluents and high RQs were determined for amoxicillin, norfloxacin, and clarithromycin. There are several studies which analyzed the seasonal variation of the interrelation of antibiotics and bacteria in WWTPs [61]. For example, Shen et al. found a positive correlation between bacteria, antibiotics, and antibiotic resistance in summer due to higher microbial activity and a negative correlation in winter due to reduced microbial activity and lower temperatures [62]. Another study by Rizzo et al. found that the levels of antibiotic resistance genes in wastewater effluent were highest during the summer months. The authors suggested that this was caused by the higher concentrations of antibiotics in wastewater effluent during the summer months, which can promote the development and spread of antibiotic resistance [12].

However, it is important to note that long-term exposure, even to low concentrations of antibiotics, can also facilitate the development and dissemination of antibiotic-resistant bacteria and antibiotic resistance genes. During prolonged exposure, antibiotics exert selective pressure, thereby stimulating bacterial metabolism and the proliferation of bacteria, which can adapt to antibiotic pressure through gene mutations or horizontal gene transfer. The continuous exposure to low concentrations of antibiotics, known as sub-minimal inhibitory concentration (sub-MIC), is believed to drive the development of antimicrobial resistance in environmental microbiota. However, the relationship between antibiotic exposure and resistance selection in environmental bacterial communities is still not well understood and requires further investigation [63–68].

4. Materials and Methods

4.1. Location and Collection of Samples

A total of twenty-four influent and effluent wastewater composite samples were seasonally collected from autumn (Q4) of 2021 to summer (Q3) of 2022 at three different WWTPs (A, B, C) located in Central-western region of Romania (Figure 5). The selection of these WWTPs was strategic, as all discharge their effluents into the same river.

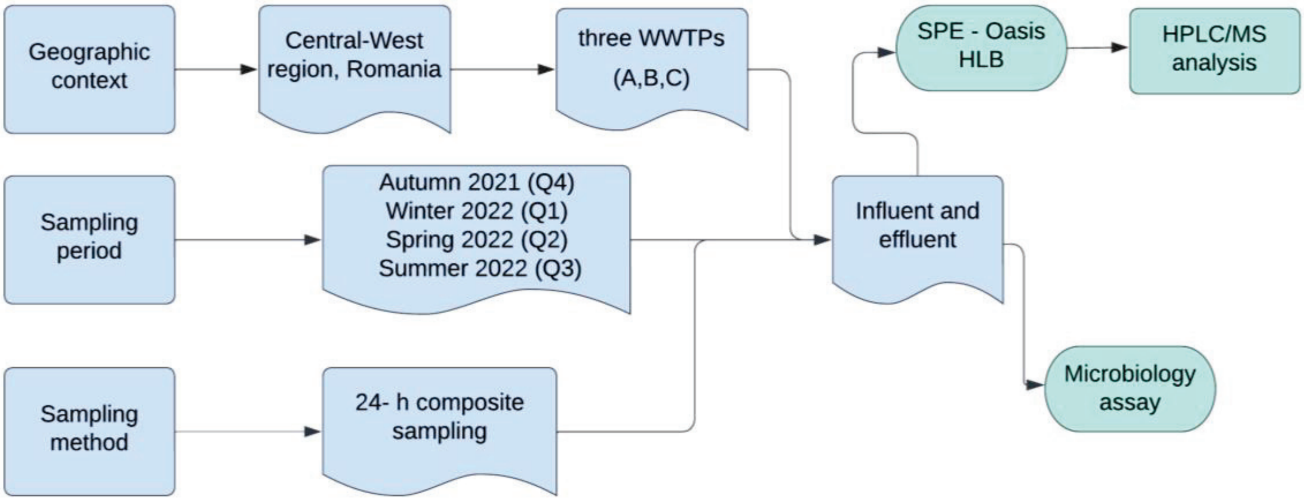

Figure 5. Workflow for sampling and analysis in WWTPs.

Samples were collected using continuous 24 h composite sampling methods to ensure the representativeness of daily variations and were stored in sterile polyethylene bottles. Upon collection, samples were immediately chilled to 4 °C to prevent microbial activity and chemical degradation and transported promptly to the laboratory, where they were processed within 24–48 h to minimize changes in antibiotic concentrations. This required the processing of both water and sediment components for each sample, which could have contributed to the observed variability in antibiotic concentrations, as sediment fractions likely contained different levels of antibiotics. Thus, throughout this study, these samples will be referred to as 'water samples'. It is important to note their composite nature and the potential influence of sediment on antibiotic concentrations.

4.2. Description of WWTP and Physicochemical Measurements

The selected WWTPs are used to treat wastewater originating from households, hospital areas, agricultural runoff, and rural areas connected to a public sewerage network. WWTP A is designed to process around 115,000 cubic meters (cbm) of wastewater/24 h, from an average of 300,000 inhabitants. WWTP B is currently treating around 4772 cbm/24 h, from around 19,000 inhabitants, while WWTP C processes around 5673 cbm/24 h, from an average 31,000 inhabitants. Specifically, WWTP A collects wastewater from many hospitals, including general, specialized, and referral hospitals. The key difference among the three WWTPs is that WWTP A manages a substantially larger volume of wastewater than the other two plants.

Table 13 presents a detailed comparative description of the treatment stages employed in three different WWTPs: WWTP A, WWTP B, and WWTP C. In the selected WWTPs, sewage treatment is performed using conventional procedures, including mechanical pre-treatment, biological, sludge, and tertiary treatment stages.

pH measurement: The samples' pHs (Table 14) were measured using a calibrated digital pH meter: Sension+ PH3 Basic laboratory pH and ORP Meter. Prior to measurement, the pH meter was calibrated with standard buffer solutions at pHs of 4.0, 7.0, and 10.0. All measurements were conducted at room temperature. The measurements for total nitrogen, nitrites, nitrates, ammonium, biochemical oxygen demand (BOD), chemical oxygen demand (COD), and suspended solids were provided just for the autumn season by the laboratories of the respective WWTPs. These labs followed standard operating procedures to ensure accuracy and reliability.

Table 13. Comparative description of treatment stages in selected wastewater treatment plants.

WWTP	Mechanical Pre-Treatment	Biological Treatment	Sludge Treatment	Tertiary Treatment
WWTP A	Screening, sand and grease removal, primary sedimentation	Activated sludge process, biological reactors	Anaerobic digesters, sludge thickening, dewatering	Tertiary filtration, disinfection
WWTP B	Coarse screening, fine screening, grit chamber, primary sedimentation	Aeration tanks	Sludge thickening, dewatering, stabilization	Chemical phosphorus removal, disinfection
WWTP C	Mechanical bar screen, grit chamber, primary sedimentation	Aeration tanks	Sludge thickening, anaerobic digestion, dewatering	Tertiary filtration, UV disinfection

WWTP: Wastewater treatment plant.

Table 14. Seasonal pH values of influents and effluents in three WWTPs.

Season	WWTP A		WWTP B		WWTP C	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
Q1	7.34	7.09	6.97	6.73	7.47	7.37
Q2	6.76	7.05	7.11	6.93	6.86	7.59
Q3	6.88	7.08	6.58	6.52	6.45	5.86
Q4	7.29	7.18	6.2	6.36	6.96	7.26

WWTP: Wastewater treatment plant, Q1—Winter, Q2—Spring, Q3—Summer, Q4—Autumn.

4.3. Air Temperature and Rainfall Data

This study analyses the seasonal variations in rainfall and air temperature recorded at two WWTPs, WWTP A and WWTP C, during the period of 2021–2022. The data (Table 15) include the average rainfall (mm) and air temperature (°C) for each quarter (Q4, Q1, Q2, and Q3). The monthly average temperature and precipitation data utilized in this study were provided by the National Meteorological Administration of Romania. The data were available for WWTP A and C.

Table 15. Seasonal rainfall (mm) and air temperature (°C) at WWTPs A and C.

	Season	Recording Month	Rainfall (R24) *	Air Temperature (°C) *
WWTP A	Q4	November	23	4.2
	Q1	February	11.4	1.8
	Q2	May	97.4	15.4
	Q3	August	87.8	21.3
WWTP C	Q4	November	51.4	3.6
	Q1	February	13.8	0.8
	Q2	May	52.6	15.5
	Q3	August	77.6	21.6

WWTP: Wastewater treatment plant, Q1—Winter, Q2—Spring, Q3—Summer, Q4—Autumn, * Monthly average.

4.4. Chemicals and Reagents

Acetonitrile of an HPLC grade was purchased from Sigma-Aldrich (Darmstadt, Germany), and formic acid was purchased from Cristal R Chim (Bucharest, Romania). For the SPE method, methanol (MeOH) was purchased from Merck (Darmstadt, Germany), the ammonium hydroxide was purchased from Primexchim (Bucharest, Romania), and the hydrochloric acid (HCl) was purchased from Poch (Gliwice, Poland). Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) was purchased from Fluka (Buchs, Switzerland). All experiments' high-purity water was prepared using a Mili-Q Ultrapure water purification system (Millipore, Billerica, MA, USA). Six antibiotic standards, amoxicillin (AMX), ciprofloxacin (CIP), norfloxacin (NOR), azithromycin (AZT), clarithromycin (CLT), doxycycline (DOX), were purchased from Sigma-Aldrich (Darmstadt, Germany) and piperacillin was purchased from Alpha Aesar (Kandel, Germany) (Table 16).

The stock solutions of antibiotics were obtained by dissolving 1 mg of the powder form of each substance in 1 mL of the appropriate solvent. Amoxicillin and piperacillin were dissolved in acetonitrile: aqueous formic acid 0.1% (50:50, *v*/*v*), ciprofloxacin and norfloxacin were dissolved just in aqueous formic acid 0.1%, azithromycin and clarithromycin were dissolved in acetonitrile, and doxycycline was dissolved in ultrapure water. The final stock standard solutions were stored at −20 °C. For the HPLC analysis, we used the following working standard solutions of each antibiotic: 100 µg/mL, 16 µg/mL, 14 µg/mL, 12 µg/mL, 10 µg/mL, 9 µg/mL, 8 µg/mL, 7 µg/mL, 6 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.8 µg/mL, and 0.6 µg/mL. Working standard solutions were obtained by diluting the stock solutions and then stored in dark containers in a refrigerator at 2–8 °C to prevent degradation.

Table 16. Characteristics of the antibiotics studied.

Chemical Group	Antibiotic	Use	Chemical Formula	Molecular Weight (g mol^{-1})
β-lactams	Amoxicillin	Human and Veterinary	$C_{16}H_{19}N_3O_5S$	365.4
	Piperacillin	Human	$C_{23}H_{27}N_5O_7S$	516.54
Fluoroquinolones	Ciprofloxacin	Human and Veterinary	$C_{17}H_{18}FN_3O_3$	331.34
	Norfloxacin	Human	$C_{16}H_{18}FN_3O_3$	319.33
Macrolides	Azithromycin	Human	$C_{38}H_{72}N_2O_{12}$	748.99
	Clarithromycin	Human	$C_{38}H_{69}NO_{13}$	747.95
Tetracyclines	Doxycycline	Human and Veterinary	$C_{22}H_{24}N_2O_8 \cdot H_2O$	444.4

4.5. Analytical Procedures

Solid-phase extraction of antibiotics from water samples was performed following a previously described method [69]. Briefly, the preconcentration of antibiotics from the water samples was carried out using Oasis HLB SPE cartridges (500 mg, 6 mL; Waters, Milford, MA, USA) on a SupelcoVisiprep SPE vacuum manifold from Sigma-Aldrich (Darmstadt, Germany). Before sample application, cartridges were conditioned with 25 mL of methanol and 25 mL of ultrapure water and then loaded with 250 mL of sample (Table 17). Prior to analysis, 0.2 g of EDTA was added to 250 mL of sample, and the pH was adjusted to 5.5 with 0.5 N of HCl or 5% NH_4OH. The samples were passed through the cartridges at a flow rate of 2 mL/min. The antibiotics retained on the cartridges were eluted with 25 mL of methanol, the obtained methanolic solutions were evaporated to dryness using a rotary evaporator (Laborota 4011-digital; Heidolph, Schwabach, Germany) at 40 °C, and the residues were dissolved in 2 mL of ultrapure water: acetonitrile (1.25:0.75, *v*/*v*). Before analysis, the extracts were passed through nylon syringe filters (13 mm, 45 mm; Phenomenex, Torrance, CA, USA). Extractions for each sample were performed in triplicate. Antibiotics were assayed using a high-performance liquid chromatography (HPLC) system, Shimadzu 2010 (Shimadzu, Kyoto, Japan), equipped with a diode array and mass spectrometry single quadrupole (MS) detectors (Shimadzu, Kyoto, Japan). The antibiotics were separated on a Zorbax SB C18 column (100 × 3 mm, 3.5 µm) thermostated at 40 °C. The mobile phase consisted of acetonitrile: ultrapure water (90:10, *v*/*v*) (A) and 0.1% aqueous formic acid (B). The gradient program started with 5% A for 1 min, increased up to 50% A in 8 min, and then at 6 min, it reached 85% A, which was maintained for 5 min. The flow rate of the mobile phase was 0.3 mL/min and the injected sample volume was 10 µL. The mass spectrometric detection parameters were a capillary voltage 1.5 kW, a dissolution temperature of 250 °C and an interface temperature 200 °C, and positive electrospray ionization (ESI^+). The concentrations of antibiotics in influent and effluent samples from WWTPs were determined by the standard addition method as described by Soran et al. [69,70] alongside a validation of the analytical parameters (Table 17).

Table 17. Analytical method validation parameters.

Compound	Linearity (R^2)	LOD µg/mL	LOQ µg/mL	Retention Time (min)	Molecular Ion (*m/z*)
Amoxicillin	0.9989	3.139	4.240	5.5	366 $[M + H]^+$
Piperacillin	0.9993	0.908	1.804	13.75	518 $[M + H]^+$
Ciprofloxacin	0.999	1.060	2.104	10.8	332 $[M + H]^+$
Norfloxacin	0.9991	1.008	2.001	11.3	320 $[M + H]^+$
Azithromycin	0.9989	1.125	2.232	12.5	749 $[M + H]^+$ 375 $[M + 2H]^+$
Clarithromycin	0.9986	1.282	2.540	15.5	748 $[M + H]^+$
Doxycycline	0.9987	4.230	5.445	13	445 $[M + H]^+$

LOD—Limit of detection, LOQ—Limit of quantification.

4.6. Microbiology Assay

The bacterial load in each collected water sample was estimated using the colony forming units (CFU) technique. Nutrient agar (NA) was used as growth media for all experiments because all aerobic heterotrophs grow on this type of media. The samples were diluted ten-fold in physiological serum and 1 mL samples of the 5th to 10th dilution were transferred on plates with NA. The plates were left to incubate for 24 to 72 h at 35 °C, after which the CFU were counted via a manual method using the following formula:

$$\text{colony forming units (CFU/mL)} = \frac{\sum(n \times d)}{N \times V}$$

where

n—Number of colonies in a Petri plate,
d—The inverse of the dilution of the inoculated sample,
N—The number of Petri plates considered,
V—The volume of the sample used, in mL [71].

Bacterial strains were isolated from the plates that showed CFU, performing multiple transfers until only one type of colony was developed. For the characterization of the isolated strains, the following methods were used, as outlined by Carpa et al. [71]:

1. Gram staining is a double staining that helps distinguish Gram-positive and Gram-negative bacteria from the samples. This method was used to distinguish coliforms, knowing that most of them are Gram-negative bacteria.
2. Antibiogram assay—The diffusimetric method was used, on Mueller–Hinton agar medium [72] with antibiotic disks, which is considered a method with large applicability in practice to test the efficacy of antimicrobial substances. The bacterial strain suspensions were adjusted to 0.5 McFarland turbidity, and their susceptibility for the seven antibiotics (detected through the HPLC method) was tested. Incubation was performed at 37 °C for 18–24 h; thereafter, the diameter of the inhibition zone was measured. The interpretation of the results was carried out in accordance with the EUCAST guidelines [73]. The diameter of the inhibition area is correlated with the sensitivity of the bacterium to the tested antibiotics.
3. Scanning electron microscopy (SEM) technique. The microscopic examination was performed to support the observations made by the microbiological procedures. The bacteria were taken from the plates with NA where CFU calculations were made. Then, the samples were fixed with 2.7% glutaraldehyde, washed with phosphate-buffered saline (PBS), dehydrated with 30 to 100% ethanol, and examined using a SEM Hitachi SU8230 (Hitachi, Tokyo, Japan) operated at 30 kV.

4.7. Calculations and Statistical Analyses

Antibiotic concentrations detected from different environments and at different dates were exported to Microsoft Excel and then analyzed in the R environment for statistical computing and graphics (R Foundation for Statistical Computing, Vienna, Austria), version 4.2.1 [74] software for statistical analysis. Continuous data were presented as median, interquartile ranges, means, and standard deviations. Comparisons between dependent observations (influent vs. effluent) were performed with the Wilcoxon rank-sum test and t-tests for dependent samples. Comparisons between seasons were performed with the Kruskal–Wallis test, followed by post hoc nonparametric tests. A p-value below 0.05 was considered to be statistically significant. For all tests, two-tailed p-values were computed.

To explore the associations between antibiotic concentrations and the mean monthly temperature or the mean monthly rainfall, we built simple and multiple models with antibiotic concentrations as dependent variables, and the treatment of wastewaters and the mean monthly temperature or the mean monthly rainfall as independent variables. We verified the models' assumptions: the normality of the residuals, the presence of heteroskedasticity, the multicollinearity, and the linearity of continuous predictors with the dependent variable. The coefficients, their confidence intervals, and p-values were presented for all models. The determination coefficients were reported for univariate models, while the adjusted ones were reported for multivariate models.

4.8. Environmental Risk Assessment (ERA)

To examine the potential impact of the antibiotics detected in the effluents from WWTPs on the aquatic ecosystem, ecological and antibiotic resistance risk assessments were performed, using the following equation [20]:

$$RQ = PEC/PNEC$$

where PEC is the "Predicted Environmental Concentration" for each antibiotic, and PNEC is the "Predicted No-Effect Concentration" (Table 18). Regarding the PNEC value used for each substance, it was either the environmental predicted no-effect concentration (PNEC-ENVs) used for the evaluation of the impact on microbial communities in aquatic systems or the PNECs based on the minimal inhibitory concentrations (PNECs-MICs) used in the assessment of the selective pressure for antibiotic resistance in microbial populations, whichever had the lower value.

The PEC was calculated using the following equation:

$$PEC = MC/DF$$

where MC is the "Measured Concentration" in the wastewater effluents for each antibiotic, and DF is the "National annual median dilution factor" calculated for each country by Keller et al. [20,75]. For Romania, the dilution factor is 71.31. Risk categorization for selected antibiotics was divided into three categories based on the RQ (risk quotient) values: low or insignificant risk to organisms ($RQ \leq 0.1$), moderate risk to organisms ($0.1 \leq RQ \leq 1$), and high risk to organisms ($RQ > 1$).

Table 18. Environmental predicted no-effect concentration (PNEC-ENV) and predicted no-effect concentration based on minimum inhibitory concentration (PNEC-MIC) for the seven antibiotics selected in this study (extracted from [76]).

Antibiotic	PNEC-ENV (μg/mL)	PNEC-MIC (μg/mL)	Lowest PNEC Value (μg/mL)
Amoxicillin	N/A	0.016	0.016
Piperacillin	N/A	0.0005	0.0005
Ciprofloxacin	0.00045	0.00006	0.00006
Norfloxacin	0.0012	0.0005	0.0005
Azithromycin	0.00002	0.00025	0.00002
Clarithromycin	0.00008	0.00025	0.00008
Doxycycline	N/A	0.002	0.002

5. Conclusions

This study examined the occurrence of antibiotic residues and bacterial loads in influent and effluent samples from three WWTPs in the central-western region of Romania across four seasons. Our analysis has encompassed antibiotic removal efficiency, seasonal variations of antibiotic residues, environmental risk assessment, and the isolation and characterization of some bacterial strains potentially involved in antibiotic resistance.

The findings revealed seasonal variations in antibiotic concentrations and bacterial loads, with higher levels detected during warmer seasons. Statistically significant differences in antibiotic concentrations were observed, particularly for amoxicillin, which showed a mean difference of 7.11 μg/mL between influents and effluents (p-value = 0.093). The antibiotic removal efficiency varied among the WWTPs, with some antibiotics like amoxicillin being partially removed, while others like doxycycline and piperacillin persisted, especially in the autumn season, showing negative removal rates. Statistical analysis demonstrated correlations between antibiotic concentrations and environmental factors. Higher temperatures and rainfall were associated with increased concentrations of amoxicillin and decreased concentrations of doxycycline, indicating that these factors play a role in the variability of antibiotic levels in wastewater. Notably, the observed variations in pH across different seasons and treatment plants highlight the need for the careful management of pH and other physicochemical properties to enhance the overall effectiveness of antibiotic removal in wastewater treatment processes. High antibiotic concentrations in effluents pose environmental risks and potentially contribute to the development of antibiotic-resistant bacteria, with notable resistance observed for amoxicillin and clarithromycin, particularly during warmer seasons. The presence of bacterial strains was more pronounced during these warmer periods, highlighting the need for optimized treatment processes and continuous monitoring. Overall, the study underscores the importance of improving wastewater treatment methods to mitigate the environmental and public health risks associated with antibiotic residues and resistant bacteria.

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antibiotics

Article

Influence of CO_2 and Dust on the Survival of Non-Resistant and Multi-Resistant Airborne *E. coli* Strains

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Abstract: The airborne transmission of bacterial pathogens poses a significant challenge to public health, especially with the emergence of antibiotic-resistant strains. This study investigated environmental factors influencing the survival of airborne bacteria, focusing on the effects of different carbon dioxide (CO_2) and dust concentrations. The experiments were conducted in an atmospheric simulation chamber using the non-resistant wild-type *E. coli* K12 (JM109) and a multi-resistant variant (JM109-pEC958). Different CO_2 (100 ppm, 800 ppm, 3000 ppm) and dust concentrations (250 $\mu g\ m^{-3}$, 500 $\mu g\ m^{-3}$, 2000 $\mu g\ m^{-3}$) were tested to encompass a wide range of CO_2 and dust levels. The results revealed that JM109-pEC958 exhibited greater resilience to high CO_2 and dust concentrations compared to its non-resistant counterpart. At 3000 ppm CO_2, the survival rate of JM109 was significantly reduced, while the survival rate of JM109-pEC958 remained unaffected. At the dust concentration of 250 $\mu g\ m^{-3}$, JM109 exhibited significantly reduced survival, whereas JM109-pEC958 did not. When the dust concentration was increased to 500 and 2000 $\mu g\ m^{-3}$, even the JM109-pEC958 experienced substantially reduced survival rates, which were still significantly higher than those of its non-resistant counterpart at these concentrations. These findings suggest that multi-resistant *E. coli* strains possess mechanisms enabling them to endure extreme environmental conditions better than non-resistant strains, potentially involving regulatory genes or efflux pumps. The study underscores the importance of understanding bacterial adaptation strategies to develop effective mitigation approaches against antibiotic-resistant bacteria in atmospheric environments. Overall, this study provides valuable insights into the interplay between environmental stressors and bacterial survival, serving as a foundational step towards elucidating the adaptation mechanisms of multi-resistant bacteria and informing strategies for combating antibiotic resistance in the atmosphere.

Keywords: antimicrobial resistance; bio-aerosols; survival rate; environmental factors; atmospheric simulation chamber

1. Introduction

The spread of bacterial pathogens through the air presents a significant challenge to public health, particularly with the emergence and proliferation of antibiotic-resistant strains [1]. Understanding the environmental factors that influence the survival of these bacteria in aerosolized form is crucial for devising effective strategies to mitigate their transmission and impact on human health.

Airborne bacterial survival is influenced by a myriad of environmental factors, both abiotic and biotic [2–5]. Among these, temperature, relative humidity (RH), and exposure to UV radiation have been extensively studied [4,6–8]. Temperature and RH play pivotal roles in bacterial viability, with warm temperatures and moderate RH levels generally favoring bacterial survival and growth. Conversely, extreme temperatures or RH levels outside the optimal range can lead to decreased viability and increased susceptibility to environmental stressors [8–10]. UV radiation, particularly in the UV-C spectrum, is known for its germicidal effects, effectively reducing bacterial viability upon exposure [11,12].

While much research has focused on understanding bacterial survival on solid surfaces or in liquid media, fewer studies have explored the dynamics of bacterial aerosols. Analyzing influencing factors on bacterial survival to airborne scenarios requires careful consideration of additional variables unique to aerosolized environments, including particle size distribution, air flow dynamics, and the presence of atmospheric gases [4,13–15].

In this context, the influence of carbon dioxide (CO_2) concentrations and dust levels on bacterial survival in the air represents a relatively understudied area even though it might play a significant role in climate change [16]. CO_2, a natural constituent of the atmosphere, can accumulate to elevated levels in indoor environments, particularly in poorly ventilated spaces or areas with high occupant density [17]. It serves not only as a vital component of cellular respiration for many organisms but also plays a role in modulating environmental conditions. In laboratory settings, CO_2 is often regulated to maintain physiological conditions conducive to bacterial growth and viability in culture media [18,19]. It is routinely supplied to incubators and growth chambers to maintain optimal pH conditions for cell culture and bacterial growth and to enhance microbial proliferation [20]. However, the implications of fluctuating CO_2 concentrations on airborne bacterial survival remain poorly understood. Investigating the influence of CO_2 on airborne bacteria is essential not only for understanding their survival dynamics in indoor environments but also for optimizing laboratory conditions to accurately simulate real-world scenarios.

Similarly, dust particles, comprising a complex mixture of organic and inorganic materials, ubiquitous in indoor and outdoor air, have been implicated as carriers for microbial contaminants, including bacteria and viruses [21]. Dust particles can originate from various sources, including skin flakes, textile fibers, pollen, and soil particles, and can harbor a diverse array of microbial species [22,23]. Dust can provide a substrate for microbial attachment and growth, providing nutrients, and protection from environmental stressors, and potentially prolonging the survival of airborne pathogens [21]. Furthermore, the physicochemical properties of dust particles, including size, composition, and surface characteristics, may influence microbial adhesion and survival dynamics [24]. Moreover, dust particles can facilitate the dispersal of bacteria over long distances, contributing to the transmission of infectious agents in both indoor and outdoor environments [25,26]. Despite the recognized role of dust in microbial dissemination, our understanding of its interactions with airborne bacteria and its influence on their survival remains limited.

This work aims to bridge this knowledge gap by investigating the influence of varying concentrations of CO_2 and dust levels on the survival of two distinct bacterial strains: non-resistant and multi-resistant *Escherichia coli* (*E. coli*). *E. coli* was used for this study as it is a very common laboratory bacterial strain frequently employed in experiments, including experiments on airborne bacteria due to the extensive knowledge available about this species [8–12,27,28]. By systematically altering CO_2 concentrations and dust levels within an atmospheric simulation chamber with controlled settings, this study aims to provide insights into the complex interplay between environmental factors and bacterial survival in aerosolized form.

2. Results and Discussion

2.1. Effect of CO_2 on the Survival Rate

The effect of three different concentrations of CO_2 (100, 800, 3000 ppm) on the survival rates of both strains (JM109 and JM109-pEC958) was assessed by comparing them to the survival rates of the baseline experiments [29]. These specific concentrations were chosen to encompass a wide range of CO_2 levels in comparison to the global mean CO_2 concentration of about 400 ppm, from very low to very high, to ensure that any potential effects on survival rates could be distinctly observed. The results from experiments with CO_2 set to 100 ppm revealed a reduction in survival rates for both strains, approximately 58% and 46%, respectively. (Figure 1), suggesting that very low CO_2 concentrations might notably decrease survival rates. CO_2 plays a crucial role in bacterial metabolism, carbon assimilation, pH regulation, and ecological interactions, with most bacteria requiring a certain level of CO_2 for survival [30–33]. The two primary biological processes reliant on CO_2 for bacteria are the biosynthesis of biomolecules and carbon fixation [34,35]. Although *E. coli* is primarily heterotrophic and typically does not fix carbon from CO_2, it requires CO_2 as a carbon source for the biosynthesis of essential biomolecules such as amino acids and nucleotides [36–38]. Insufficient CO_2 concentration (100 ppm) inside the chamber may thus hinder growth and metabolism, explaining the observed reduction in survival rates for both strains.

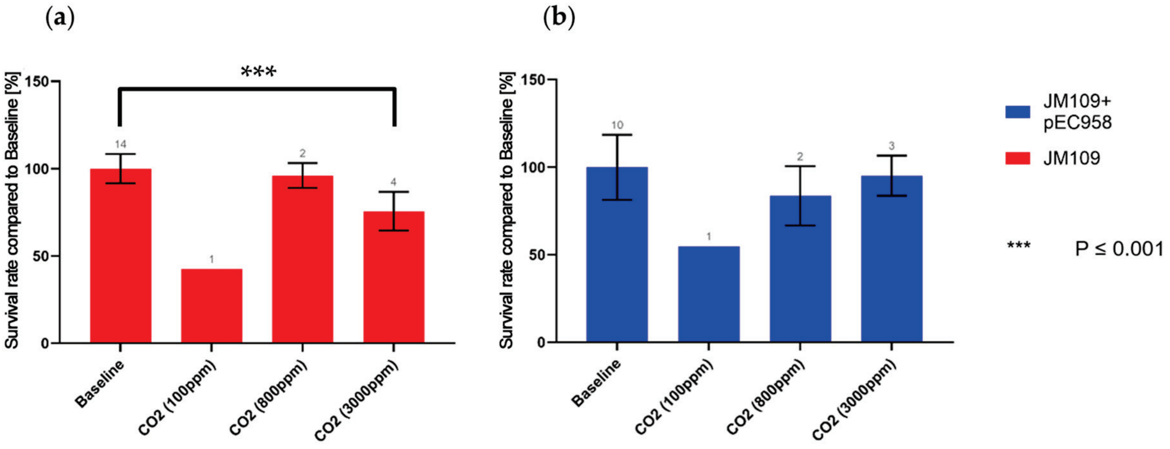

Figure 1. Comparison of the survival rate at different CO_2 concentrations of (**a**) the wild-type *E. coli* K12 (JM109), (**b**) the modified *E. coli* K12 (JM109-pEC958); the numbers above the histograms are the numbers of experiments.

While CO_2 is typically essential for bacteria survival at appropriate levels, excessive concentrations can be detrimental due to environmental acidification, disrupting bacterial cellular processes, pH homeostasis, respiration, and membrane integrity [39].

Increasing CO_2 to 800 ppm had no significant effect on either strain, while increasing it to 3000 ppm significantly reduced the survival rate of JM109 by approximately 24% (Figure 1). Conversely, the JM109-pEC958 was not significantly affected by this CO_2 concentration, suggesting that JM109-pEC958 can withstand exceptionally high levels of CO_2 better than its non-resistant counterpart.

The *E. coli* K12 genome contains several CO_2 utilization-related genes such as those encoding carbonic anhydrases [38]. Carbonic anhydrases are enzymes that catalyze the interconversion between CO_2 and HCO_3^-, crucial for efficient utilization of CO_2 as a carbon source and intracellular pH regulation [40]. Although pEC958 does not provide additional CO_2 utilization-related genes, it might contain genes that could potentially regulate the expression of the CO_2 utilization-related genes on the chromosome, potentially increasing carbonic anhydrases inside the cell and subsequently enhancing resilience to high CO_2 levels. Additionally, other genes on the plasmid, while not directly related to CO_2 utilization, might indirectly impact the utilization of CO_2. Further research is warranted to fully elucidate the mechanism enabling better survival of resistant *E. coli* at exceptionally high CO_2 levels and how antibiotic resistance genes (ARGs) relate to it.

This finding is significant because it suggests that resistant strains of *E. coli* could outcompete non-resistant ones in high CO_2 environments, such as poorly ventilated indoor areas, thereby posing a significant health risk.

2.2. Effect of Dust on the Survival Rate

Arizona Road Dust comprises relatively large irregular-shaped particles, simulating real-world environmental dust and air pollution scenarios [41]. The experiments were conducted with three different dust concentrations: 250, 500 and 2000 µg m^{-3}.

The survival rate of JM109 was significantly reduced, by approximately 26%, at a dust concentration of 250 µg m^{-3} (Figure 2). At this dust concentration, the survival rate of JM109-pEC958 was not yet significantly reduced. At a dust concentration of 500 µg m^{-3} both strains displayed a significant reduction in their survival rates with JM109-pEC958 exhibiting a remaining survival rate of approximately 53%, while that of JM109 was further significantly reduced to 27% compared to the baseline survival rates. The difference in survival rates between both strains was significant at this dust concentration (Figure 3), indicating that the multi-resistant *E. coli* strain can maintain a significantly higher survival rate at high dust concentrations, despite also experiencing a reduction in its own survival rate. This phenomenon was even more pronounced in the experiments with 2000 µg m^{-3} dust, where JM109 exhibited only 13% survival, whereas JM109-pEC958 maintained an approximately 60% survival rate.

These results suggest that while the survival rates of both strains decrease with increasing dust concentrations, the effect starts at a lower dust concentration for the non-resistant strain. Additionally, the degree of survival rate decrease is more severe for the non-resistant strain compared to its multi-resistant counterpart at 500 and 3000 µg m^{-3}.

The presence of dust in ambient air can impose various stressors on bacterial cells, challenging their survival and metabolic activity, thereby explaining the observed reduction in survival rates during our experiments [42]. Stressors may include oxidative stress due to reactive oxygen species (ROS) contained in dust particles, chemical contaminants accumulated in the dust, and physical damage to the bacteria cells through abrasion from the particles' abrasive surfaces [43–45]. Arizona Road Dust used in this study, derived from road surfaces, may contain mineral dust, organic matter, and pollutants that might have undergone photochemical reactions in sunlight, leading to the production of ROS such as superoxide radicals (O_2^-), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2) [43,46,47]. These ROS can induce oxidative stress and damage cellular components in the bacterial cells [48]. Furthermore, the dust may contain various chemical contaminants from vehicle emissions, industrial activities, and atmospheric deposition, such as heavy metals and polycyclic aromatic hydrocarbons (PAHs) [44,49]. Exposure to such chemical contaminants can exert toxic effects on cells and disrupt cellular functions. A combination of these stressors in the dust particles might be responsible for survival reduction in the strains during our study.

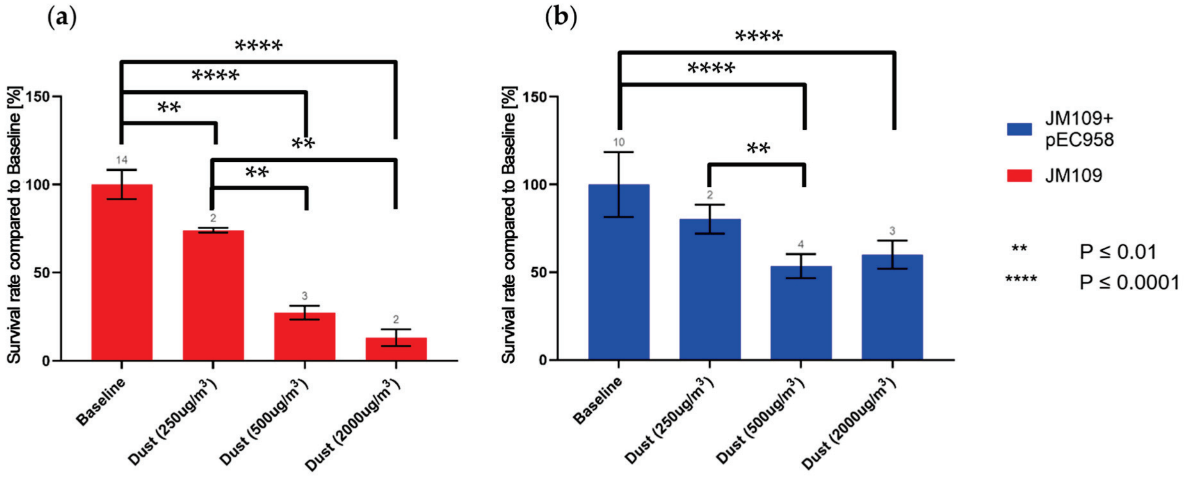

Figure 2. Comparison of the survival rate at different dust concentrations of (**a**) the wild-type *E. coli* K12 (JM109), (**b**) the modified *E. coli* K12 (JM109-pEC958); the numbers above the histograms are the numbers of experiments.

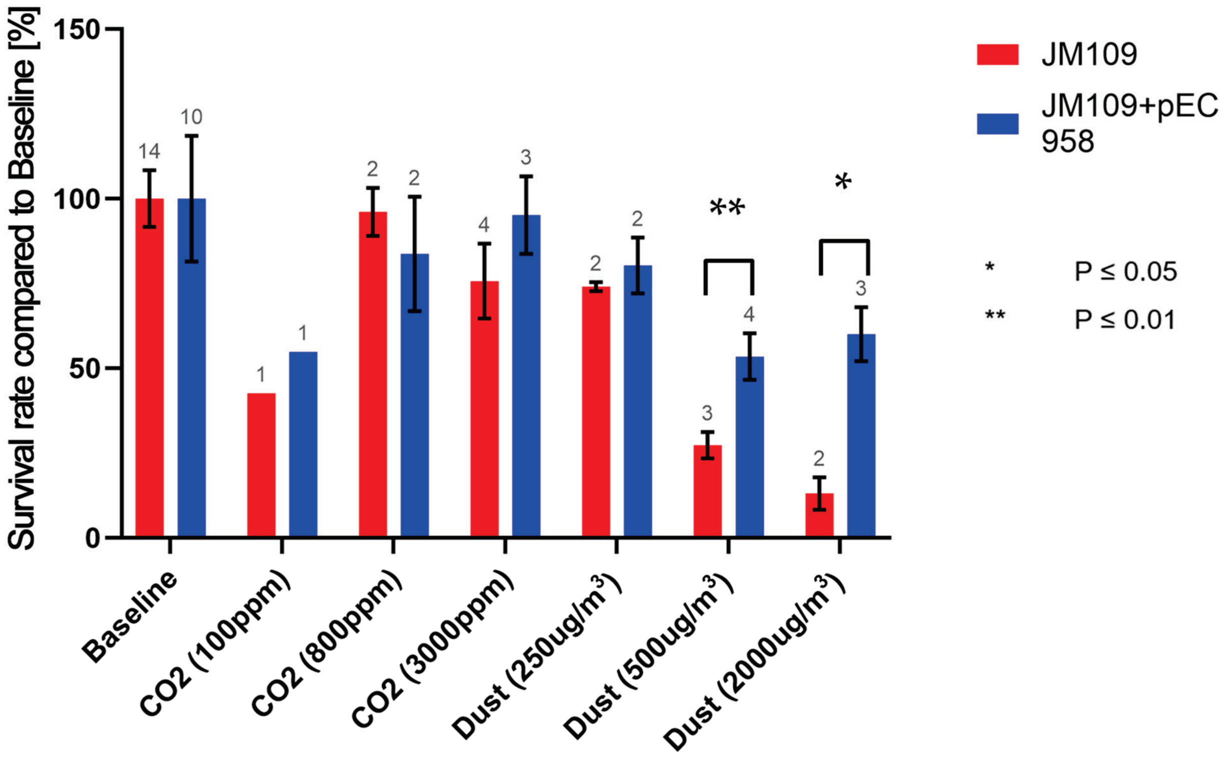

Figure 3. Comparison of the survival rates of both strains (JM109 and JM109-pEC958) at different CO_2 and dust concentrations; The numbers above the histograms are the number of experiments.

The fact that JM109-pEC958 was less affected and maintained a higher survival rate than its non-resistant counterpart may be attributed to efflux pumps encoded on the plasmid. Efflux pumps can extrude chemical contaminants from cells, reducing intracellular concentrations of harmful substances [50]. JM109 lacks the genes encoding efflux pumps, whereas pEC958 contains *tetA* and its regulatory gene *tetR*, potentially increasing its ability to remove contaminants from the cells and thus contributing to better survival than JM109 without pEC958 [51,52]. Further research is needed to identify the exact mechanisms provided by pEC958 and potentially other plasmids and resistance genes for better survival at high dust concentrations.

3. Material and Methods

3.1. Preparation of Bacterial Strains

To assess the disparity in survival between multi-resistant bacteria and their non-resistant counterparts, we utilized the reference strain *E. coli* K12. *E. coli* K12 is a well-studied non-pathogenic *E. coli* strain commonly employed in laboratory settings [53]. For this study, two distinct variants of the strain were utilized: the wild-type *E. coli* K12 and a genetically modified variant containing the plasmid pEC958, which encodes multi-resistance genes. The pEC958 plasmid is derived from the highly resistant *E. coli* variant ST131, which is responsible for numerous infection outbreaks in hospitals [51].

The JM109 High Efficiency Competent Cells (Promega, Madison, WI, USA) served as the non-resistant wild-type strain. To generate the multi-resistant strain, the JM109 strain underwent a transformation by integrating the pEC958 plasmid, following the standard transformation protocol for single-use cells from the competent cells' manufacturer. The pEC958 plasmid was previously extracted from a clinical sample of *E. coli* ST131 using Qiaprep spin miniprep kit (Qiagen, Hilden, Germany).

3.2. Preparation of Bacterial Suspension

To prepare the bacterial suspension for the injection into the chamber, the bacterial strain was cultured overnight on a petri dish containing appropriate media: LB (Merck KgaA, Darmstadt, Germany) media plates for JM109 and LB media plates spiked with 1 mL ampicillin (100 mg mL^{-1}, Sigma-Aldrich, Burlington, MA, USA) per liter of agar for JM109-pEC958. Subsequently, the bacterial cells were suspended in 25 mL of LB broth and incubated at 37 °C with continuous shaking until they reached the logarithmic (log) phase of growth. The log phase was determined by achieving an OD_{600nm} (Shimadzu 1900, Columbia, MD, USA) reading of 0.6 [54,55].

Subsequently, 20 mL of the bacterial suspension was centrifuged at 4000 rpm for 10 min, and the resulting pellet was resuspended in 20 mL 0.9% NaCl solution.

3.3. Chamber Operations

The experiments were conducted in the Chamber for Aerosol Modelling and Bio-aerosol Research (ChAMBRe) [56–58], an atmospheric simulation chamber installed at the National Institute of Nuclear Physics in Genoa, Italy, in collaboration with the Environmental Physics Laboratory of the Physics Department of the University of Genoa. The chamber has a total volume of 2.2 m^3 and is equipped with several in- and outlets that facilitate aerobiological simulations under controlled conditions. Photos of the setup and a scheme of the experimental procedure can be found in Figures 4 and S1, while a recent detailed description of the facility can be found in [59].

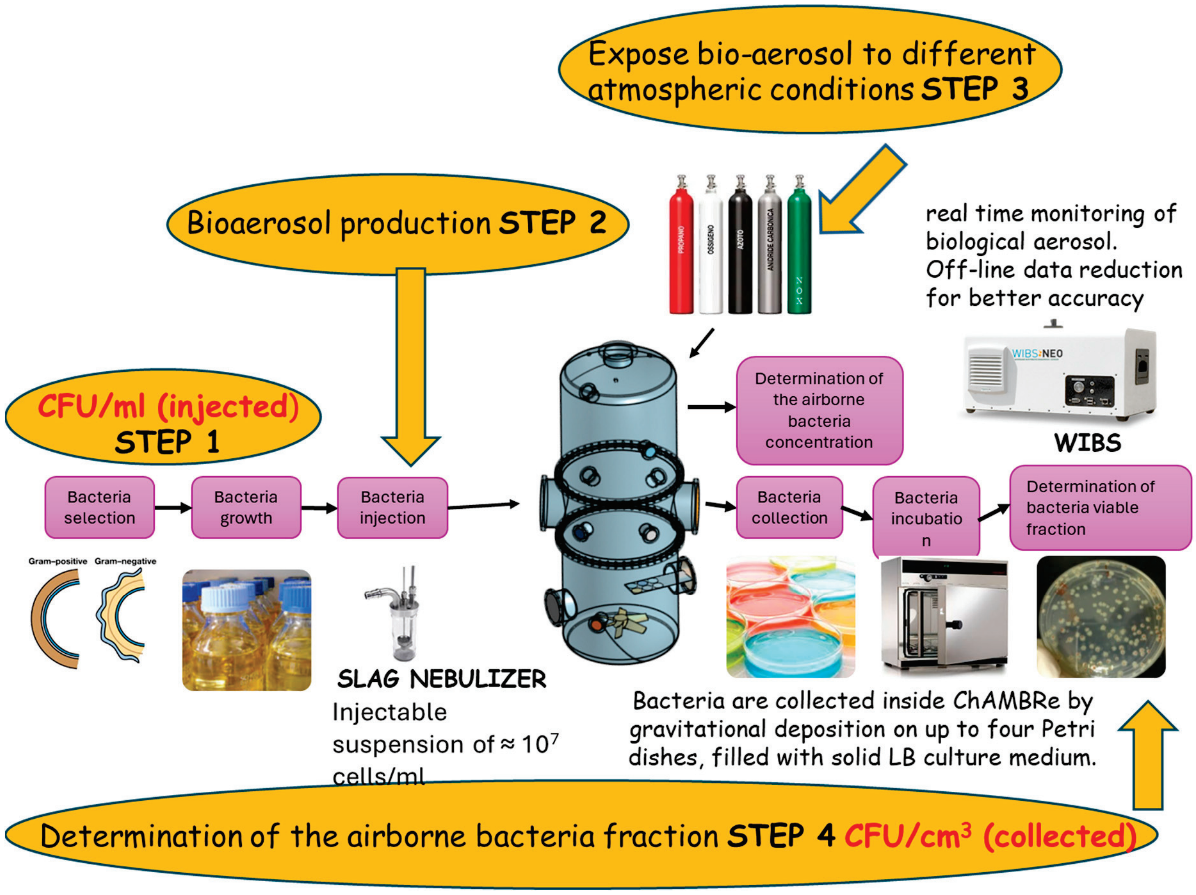

Figure 4. Scheme of experimental procedure.

The bacterial cells suspended in 0.9% NaCl were injected into the chamber using the SLAG nebulizer (Sparging Liquid Aerosol Generator, CH Technologies, Westwood, NJ, USA) and an automatic syringe pump (NE-300 Just Infusion™ Syringe Pump, New Era, Farmingdale, NY, USA). The injection process typically lasted 5 min, with a working airflow of 3.5 Lpm. These parameters were automatically controlled by a mass flow controller (MFC, Bronkhorst, Ruurlo, The Netherlands; model F201C-FA) managed via NI LabView^{TM} SCADA (Supervisory Control And Data Acquisition). The syringe rate was set at 0.4 mLpm, resulting in the nebulization of 2 mL of the bacterial suspension. Thereby, a bacterial cell concentration of approximately 10^6 colony-forming unit (CFU) mL^{-1} inside the ChAMBRe was achieved.

The concentration of the total bacteria inside the ChAMBRe was monitored using a waveband-integrated bio-aerosol sensor (WIBS-NEO; Droplet Measurement Technologies, Longmont, CO, USA), which utilizes fluorescence signals to identify and differentiate bio-aerosol components. A custom procedure for WIBS data reduction, written in Igor PRO 8.0 and previously published by Vernocchi et al. (2023) [59], optimized for the JM109 strain, was employed to retrieve the time series of bacteria concentration throughout the experiment.

A series of "baseline" experiments (i.e., in clean air) was carried out to assess the stress induced by the experimental procedure on the bacteria and to determine, through comparison, the effects of atmospheric components or pollutants on the bacteria viability. "Clean" air was introduced into the chamber through the following procedure [56]: first the chamber was evacuated at least down to 10^{-2} mbar, then, pure N_2 from a compressed gas cylinder was flushed in, until a pressure of 5 mbar was reached, and then the ambient air re-entered the chamber through an absolute HEPA filter (Kurt J. Lesker, Dresden, Germany, model: PFIHE842, NW25/40 Inlet/Outlet—25/55 SCFM, 99.97% efficient at 0.3 μm) and a zeolite trap (right before the HEPA filter). The ambient conditions during each baseline experiment were set at atmospheric pressure, with CO_2 concentrations at 400 ppm, temperatures around 20 °C and relative humidity ranging from 60 to 70%. Other gases (O_3, NO_x and SO_x) and the dust concentration were maintained below the minimum detectable level (MDL) of the respective detector/monitor [56].

Subsequently, the effects of different CO_2 and dust concentrations were evaluated by maintaining the chamber conditions identical to those during the baseline experiments, except for varying CO_2 or dust concentrations.

During CO_2 experiments, the gas concentration was kept constant thanks to an automatic feedback control system [59], and monitored by a non-dispersive carbon monoxide and dioxide analyzer (CO12e, ENVEA, Poissy, France). CO_2 levels that were tested were 100, 800 and 3000 ppm.

For experiments involving exposure to dust, an OPS (Optical Particle Sizer, model 3330, TSI Inc., Shoreview, MI, USA) was utilized to obtain the dust mass concentration inside the chamber. The dust was generated using a solid particle disperser (Palas, model RBG 1000, Karlsruhe, Germany), which injected Arizona Road Dust (ISO 12103-1, A1) [60] a typical laboratory dust, into the chamber [41,61]. Three different dust concentrations were tested, 250, 500 and 2000 μg m^{-3}.

A fan installed at the bottom of the chamber, operating constantly at 5 Hz, ensured homogenization of the concentration and proper mixing within the chamber.

Bacteria were collected via gravitational settling on four petri dishes filled with the appropriate culture medium (LB or LB+ampicillin), positioned at the bottom of the chamber via an automated shelf. The typical duration of an experiment was approximately 5 h. The extracted petri dishes were then incubated overnight at 37 °C, and CFUs were counted the following day.

3.4. Determination of Survival Rates

The amount of the bacteria injected into the chamber varied from experiment to experiment. Therefore, a proper correlation procedure was needed to determine the survival rates. First, the concentration of bacterial cells inside the chamber was determined through the following steps:

(1) Measurement of OD_{600nm} of the suspended cells in 0.9% NaCl solution to assess the total bacterial concentration injected into the chamber. OD_{600nm} is proportional to the bacteria concentration, thus the equation below was employed to determine the total bacterial concentration:

$$\left(\frac{cells}{ml}\right)_{estimated} = OD_{measured} \times 8{\cdot}10^8 \frac{cells}{ml}, \tag{1}$$

based on the empirical relation that 1 $OD_{600nm} = 8 \times 10^8$ cells mL^{-1} for *E. coli* [62]. This method determines the total number of cells, irrespective of viability, providing information solely about the total amount of cells injected into the chamber.

(2) Preparation of appropriate serial dilutions of the bacterial suspension in 0.9% NaCl, followed by plating on LB culture medium-filled petri dishes (LB media plates for JM109 and LB media plates spiked with ampicillin for JM109-pEC958). After overnight incubation at 37 °C, CFUs were counted the next morning to estimate the viable fraction of the bacterial suspension in terms of CFU mL^{-1}.

(3) Calculation of the dead bacteria concentration by subtracting the viable bacterial concentration from step 2 from the total bacterial concentration obtained in step 1.

(4) Determination of a correlation factor, Cf, using the following equation:

$$Cf = \frac{\beta}{1+\beta} \tag{2}$$

where:

$$\beta = \frac{viable\ bacteria\ concentration}{dead\ bacteria\ concentration} \tag{3}$$

(5) Determination of the total airborne bacteria concentration inside the ChAMBRe by the WIBS data analysis as # cm^{-3}, considering data at 3 min after the injection's conclusion to ensure proper mixing within the chamber volume.

(6) Calculation of the airborne viable bacteria concentration (# cm^{-3}) inside the chamber using the measured airborne bacterial concentrations from step 5 and the correlation factor from step 4:

$$\left(\frac{cells}{cm^3}\right)_{viable\ airborne\ bacteria} = Cf \times \left(\frac{cells}{cm^3}\right)_{total\ airborne\ bacteria} \tag{4}$$

This represents the final calculated airborne concentration of viable bacterial cells inside the chamber exposed to different ambient conditions tested during the experiments (see Section 3.3).

Next, the fraction of bacterial cells that survived the 5 h period of each experiment was determined through the following steps:

(1) Using the bacteria culturable fraction collected on the four petri dishes inside the chamber (see Section 3.3), determined by CFU visual counting and inserted into the following equation to determine the ratio of the fraction of surviving bacteria:

$$Ratio = \frac{CFUs_{petri\ dishes\ inside\ the\ chamber}}{\left(\frac{cells}{cm^3}\right)_{viable\ airborne\ bacteria}} \tag{5}$$

(2) Calculation of the survival rate (%) of the bacterial cells under specific environmental conditions by comparing the ratios during those experiments to the ratios obtained during baseline experiments:

$$Survival\ rate\ (\%) = \frac{ratio_{experiment}}{ratio_{baseline}} \times 100 \tag{6}$$

The results were presented as percentages. The baseline survival rate is considered 100%, and the survival rates obtained during experiments with varying concentrations of CO_2 and dust were compared to assess their effect on the survival of the bacterial cells. Several repetitions of each experiment were conducted to increase statistical significance. The variation in the number of repetitions between experiments arose because some experiments reached statistical significance with fewer repetitions, while others required more repetitions to achieve the same level of significance. More details about the baseline determination as well as limitations and challenges of this study can be found in S2.

3.5. Statistical Analysis

All statistical analyses and graphical visualizations were conducted using Graphpad Prism (v10.0.2, available at https://www.graphpad.com/, accessed on 2 April 2024). The results were deemed statistically significant when $p \leq 0.05$.

4. Conclusions

To our knowledge, this study represents the first investigation into the impact of varying CO_2 and dust concentrations on the survival of airborne bacteria, with a comparative analysis between non-resistant and multi-resistant bacterial strains.

The results suggest that multi-resistant *E. coli* strains exhibit greater resilience to high CO_2 and dust levels compared to their non-resistant counterparts. Notably, at CO_2 concentrations of 3000 ppm, the survival rate of JM109 was significantly reduced by approximately 24%, whereas the survival rate of JM109-pEC958 remained unaffected. This disparity was even more pronounced during the dust experiments. JM109 experienced a 26% reduction in survival rate already at 250 $\mu g\ m^{-3}$, whereas the multi-resistant counterpart showed a significant reduction with the dust concentration at or higher than 500 $\mu g\ m^{-3}$. At this concentration, the non-resistant strain exhibited a survival rate of 27%, which further decreased to 20% at 2000 $\mu g\ m^{-3}$. In contrast, the multi-resistant strain maintained significantly higher survival rates, approximately 55% at high dust concentrations.

These findings suggest that the JM109-pEC958 strain possesses mechanisms enabling it to endure extreme ambient conditions such as high CO_2 and dust concentrations better than its non-resistant counterpart. These mechanisms may include regulatory genes or genes encoding efflux pumps. Further research is warranted to elucidate the precise mechanisms underlying this enhanced resilience.

Understanding their mechanisms is crucial for developing effective mitigation strategies to prevent the enrichment and dissemination of antibiotic-resistant bacteria in the air. Additionally, future studies could explore the impact of other environmental factors, such as additional gases and UV light, to determine if similar effects are observed when comparing non-resistant and multi-resistant bacterial strains. Such investigations would provide valuable insights into the adaptation strategies employed by multi-resistant bacteria in response to various environmental stressors, and this study represents the initial step in that direction.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics13060558/s1.

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

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antibiotics

Article

Inactivation of Bacteria and Residual Antimicrobials in Hospital Wastewater by Ozone Treatment

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Abstract: The emergence and spread of antimicrobial resistance (AMR) has become a persistent problem globally. In this study, an ozone treatment facility was established for an advanced hospital wastewater treatment in a core hospital facility in an urban area in Japan to evaluate the inactivation of antimicrobial-resistant bacteria and antimicrobials. Metagenomic DNA-seq analysis and the isolation of potential extended-spectrum *β*-lactamase (ESBL)-producing bacteria suggested that ozone exposure for at least 20 min is required for the adequate inactivation of DNA and ESBL-producing bacteria. *Escherichia coli* and *Klebsiella* species were markedly susceptible to 20-min ozone exposure, whereas *Raoultella ornithinolytica* and *Pseudomonas putida* were isolated even after an 80-min exposure. These ozone-resistant bacteria might play a pivotal role as AMR reservoirs in the environment. Nine antimicrobials (ampicillin, cefdinir, cefpodoxime, ciprofloxacin, levofloxacin, clarithromycin, chlortetracycline, minocycline, and vancomycin) were detected at 373 ng/L to 27 μg/L in the hospital wastewater, and these were removed (96–100% removal) after a 40-min treatment. These results facilitate a comprehensive understanding of the AMR risk posed by hospital wastewater and provides insights for devising strategies to eliminate or mitigate the burden of antimicrobial-resistant bacteria and the flow of antimicrobials into the environment. To the best of our knowledge, this is the first report on the implementation of a batch-type, plant-scale ozone treatment system in a hospital facility to execute and evaluate the inactivation of drug-resistant bacteria and antimicrobials.

Keywords: hospital wastewater; ozone treatment; metagenomics; *enterobacteriaceae*; extended-spectrum *β*-lactamase (ESBL); carbapenemase; residual antimicrobials

1. Introduction

Research on health and environmental risk assessment and countermeasures against antimicrobial resistance (AMR) is ongoing on a global scale. The spread of AMR is a cause of increasing concern for the future use of antimicrobials and other measures to control infectious diseases. AMR is an important issue requiring immediate and effective action by the World Health Organization (WHO) and the G7 group of industrialized nations. In recent years, it has been observed that as soon as a new antimicrobial is used in clinical practice, resistant bacterial strains emerge, which has resulted in the spread of multiple antimicrobial-resistant strains. In addition to the clinical use of antimicrobials, community-acquired infections caused by healthy carriers and outbreaks of AMR, originating from livestock, fisheries, and other industries, are also becoming problematic, making it essential to comprehensively understand the trend of AMR on a global scale and a “One Health” approach is desired to combat these problems [1–3].

Antimicrobial-resistant bacteria and antimicrobial residues have been reported in medical effluents from hospitals and other medical facilities [4,5]. Conventional wastewater treatment, which aims to remove organic matter derived from human waste, as reflected by biochemical oxygen demand, could not completely remove environmental pollutants. The discharge of treated water into aquatic environments, such as rivers, lakes, and oceans, creates new environmental pollution problems [5,6]. The water pollution control law in Japan for discharges to general wastewater by organizations, such as hospitals, regulates pH, heavy metals, the coliform group, etc., but does not set regulatory values for pathogenic bacteria or antimicrobial-resistant bacteria, as in other countries [7,8]. At municipal wastewater treatment plants located in Japan, treated wastewater is discharged in compliance with the regulations on the coliform group (<3000 viable bacteria count/mL). However, in areas where a confluence system is adopted, rainwater flows into the same waterway as sewage and the wastewater is discharged into rivers and the ocean without sufficient treatment, during rainy weather. It is, therefore, important to understand the actual situation of AMR originating from hospital wastewater, evaluate its risk, and consider effective countermeasures for assessing and solving the AMR problem, not only at the environmental site but also at the clinical site [7–11].

In the abovementioned context, the treatment of AMR-related factors in hospital wastewater before discharging it into the sewage system could be one of the measures for the effective reduction in AMR. In addition, it is expected that information on AMR inherent in hospital wastewater, as environmental AMR in the medical field, will not only support nosocomial infection control measures for the early detection and prevention of nosocomial infection strains that may occur, but will also enable discussions on the fundamental measures to be taken against AMR. However, owing to the associated difficulties, limited studies have been conducted on hospital wastewaters worldwide [7,8,12].

Various wastewater treatment systems that could be effective in treating hospital wastewater have been developed and studied; these include systems involving the use of the Fenton process [13,14], electrolysis [15], TiO_2 [16], persulfate [17], UV/chlorine [18], and ozone [14,19]. Ozone treatment has been the focus of much research in recent years because it does not require the addition of any chemicals, the wastewater is free from residues after treatment, and the treatment has strong sterilizing and deodorizing effects [20]. However, the efficacy of ozone treatment has been primarily evaluated in small-scale test systems in laboratories and has yet to be studied on the actual hospital wastewater scale. As such, there is a lack of knowledge on the practical application of ozone treatment [21–23]. If the ozone treatment of hospital wastewater on a practical scale is demonstrated to be a reasonable solution for the environmental AMR concern, it will be possible to solve the problem of AMR and contribute to the One Health approach [24]. Furthermore, the results can potentially contribute to the public interest in regional security and would guarantee the safety of the local population [25,26]. Toward this end, in the present study, a plant-scale ozone treatment system was implemented in a hospital facility using a batch-type process to execute and evaluate the inactivation of antimicrobial-resistant bacteria and antimicrobial residues and mitigate the environmental impact of AMR.

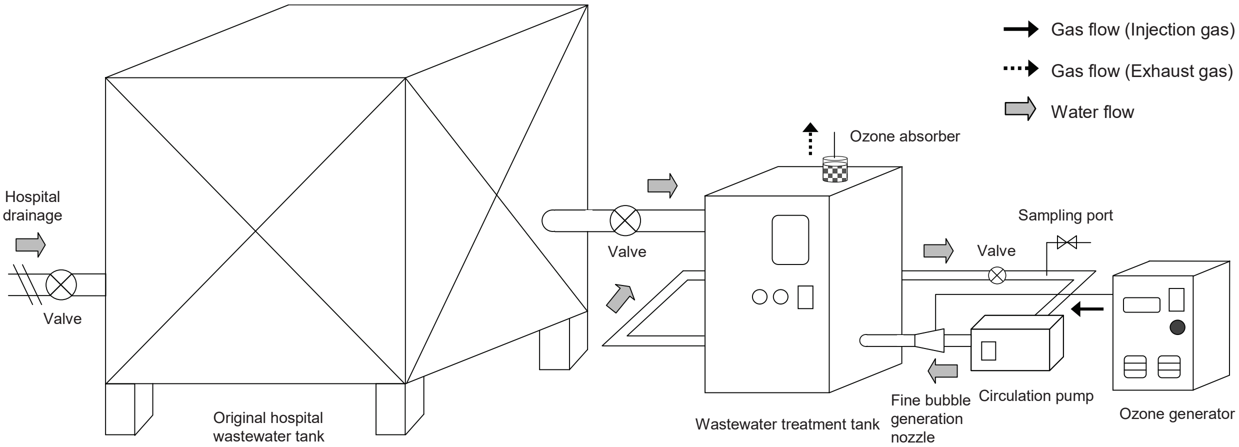
2. Materials and Methods

2.1. Sample Collection

Hospital wastewater samples were collected at the Ohashi Medical Center (BN; 35.652578°N, 139.683959°E), with a capacity of 319 beds, in Toho University, located in Jonan area, Tokyo, Japan. In the hospital, various wastewater types (stool and urine), generated as a result of hospital activities, are stored in two underground wastewater tanks, with an effective volume of 22.5 m^3, without mixing with other drainage. The supernatant is pumped directly into the public sewage system several times a day, and the settled sediments are collected and incinerated by a specialized waste management company. It was impossible to quantify the daily inflow and outflow of wastewater tanks because of the lack of any system for regular measurements.

2.2. Ozone Treatment

Inactivation of bacteria and antimicrobials present in the hospital wastewater by ozonation was performed using an ozone treatment system installed in the hospital facility. Wastewater from one of the two storage tanks was introduced into a wastewater treatment tank, with an effective volume of 1 m^3, for semi-batch ozone treatment. The appearance and configuration of the ozone treatment system used in this study are shown in Figure 1.



Equipment	Technical specification
Ozone generator Ozonia® TOGC45X (Suez Environment, France)	Ozone concentration range: 3-150 mg/L Ozone production range: 1-45 g/h Gas flow: 5 L/min
Fine bubble generation nozzl EE091 (For EARTH Co., Ltd., Japan)	Spitting flow rate: 80 L/min Suction gas flow rate: 28 L/min
Circulation pump 32LPS5.75E (Ebara Corporation, Japan)	Flow rate: 80 L/min
Original hospital wastewater tank	Volume: 45 m^3 (22.5 m^3 × 2)
Wastewater treatment tank	Volume: 1 m^3

Figure 1. Schematic representation of the batch ozone treatment system implemented in a hospital facility. The picture shows the appearance of the advanced hospital wastewater treatment system equipped with the ozone treatment system that was tested in this study. The technical specifications of the equipment used in the system are shown in detail.

Ozone was generated using an ozone generator (Ozonia® TOGC45X, Suez Environment, Paris, France). The hospital wastewater in the wastewater treatment tank was circulated using a circulation pump (32LPS5.75E, Ebara Corporation, Tokyo, Japan) at a flow rate of 80 L/min, and ozone gas was fine-bubbled through fine-bubble generating nozzles (EE091, For EARTH Co., Ltd., Tokyo, Japan) and introduced into the wastewater treatment tank. The ozone treatment was performed at an ozone generation rate of 34 g/h and an effective ozone gas concentration of 111 mg/L. The experiments were initiated by sparging ozone gas continuously into the filled wastewater treatment tank. A portion (100 mL) of the solution in this tank was sampled at 0, 10, 20, 40, and 80 min after the start of the experiment. These durations were determined based on the average contact times in Japanese wastewater treatment plants that implement ozonation before discharging their effluent into rivers [27] and by considering previously reported values [11,28]. Dissolved ozone and gaseous ozone concentrations and water quality parameters (COD, BOD, SS, and the coliform group) measured during ozonation prior to conducting this study is shown in Table S1. Sodium thiosulfate or tryptic soy broth was immediately added to mitigate the effects of any residual ozone in the samples [29,30]. The samples were then stored at 4 °C in the dark and further processed within 12 h.

2.3. Metagenomic DNA-Seq Analysis of Wastewater Samples

To collect organisms larger than bacteria, ozone-treated wastewater samples were passed through TPP Rapid Filtermax Vacuum Filtration systems (TPP, Trasadingen, Switzerland) in 500 mL bottles fitted with 49 cm^2, 0.2 µm polyethersulfone membranes. The membranes were removed from the bottles and stored at −30 °C until DNA extraction. One-fourth of the collected membrane was cut into small pieces and placed in ZR-96 BashingBead Lysis Tubes (0.1 and 0.5 mm; Zymo Inc., Irvine, CA, USA). Bacterial lysis buffer (800 µL; Roche, Basel, Switzerland) was added to the bead tube, which was frozen at −30 °C and thawed at 23 °C. The tube was subjected to bead-beating (1500 rpm for 10 min) using a GenoGrinder 2010 homogenizer. After brief centrifugation (8000× *g* for 3 min), 400 µL of the supernatant was collected. The DNA in the supernatant was purified using a Roche MagNa Pure Compact instrument (DNA_Bacteria_v3 protocol; Elution: 50 µL). DNA concentrations and purity were measured using the Qubit DNA HS kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Metagenomic DNA-Seq libraries were prepared using the QIAseq FX DNA library kit (Qiagen, Hilden, Germany), followed by short-read sequencing using the iSeq platform (2 × 150-mer paired-end) (Illumina, San Diego, CA, USA). Adapter and low-quality sequences were trimmed using Sickle version 1.33 (https://github.com/najoshi/sickle), considering the following parameters: average quality threshold "-q 20" and minimum length threshold "-l 40" Metagenomic DNA-Seq analysis was performed using cleaned reads for homology search without de novo assembly in all subsequent analyses. Detailed scripts and databases are described below.

Taxonomic classification of every single read from metagenomic analysis was performed using mega-BLAST (e-value threshold, 1E^{-20}; identity threshold, 95%) against the NCBI nt database using MePIC2 [31], and subsequently analyzed using MEGAN 6 [32].

2.4. Resistome Analysis

Metagenomic DNA-seq analysis was performed using cleaned reads for homology searches without de novo assembly. Before resistome analysis, an ARG database was constructed using the bacterial antimicrobial resistance reference gene (National Center for Biotechnology Information (NCBI) BioProject ID, PRJNA313047) and ResFinder (https://bitbucket.org/genomicepidemiology/resfinder_db/src/master/). The study database was constructed using Makeblastdb in the basic local alignment search tool (BLAST+). The operational taxonomic units (OTUs) in the ARG database (AMROTU ver. 2022-04-11) were created by clustering at ≥90% sequence identity and ≥80% coverage using vsearch version 2.10.4. The metagenomic DNA-seq reads were searched using mega-BLAST (e-value threshold, 1E^{-20}; identity threshold, 95%) against the customized ARG database. The detected genes were summarized for each OTU of the ARGs. Reads per kilobase of gene per million (RPKM) counts were calculated using the following formula for normalization:

RPKM = number of detected reads against OTUs/[average gene length of detected OTUs (bp) × total number of trimmed reads] × 10^9.

2.5. Whole-Genome Analysis of Bacterial Isolates

Whole-genome sequencing of bacterial isolates was performed using the NextSeq 1000 platform (Illumina). The draft genome sequence was assembled using A5-miseq with Illumina short-read data. Gene annotation was performed using DFAST version 1.2.3 [33] using the following databases: DFAST default database, ResFinder database [34], Bacterial Antimicrobial Resistance Reference Gene (BARRG) database (PRJNA313047), and Virulence Factors Database [35]. Multilocus sequence typing (MLST) was performed using "mlst" program version 2.16.2 (Seemann T, mlst Github https://github.com/tseemann/mlst) with PubMLST database (https://pubmlst.org/).

2.6. Analytical Procedures for Antimicrobials

A total of 15 antimicrobials were investigated on the basis of a previous report on their concentrations and detection frequencies in hospital effluent, wastewater, and river water, both in Japan and around the world, as well as on the basis of antimicrobial use in Japan [6,36–38]. β-lactams (ampicillin, cefdinir, cefpodoxime, cefpodoxime proxetil, and ceftiofur), new quinolones (ciprofloxacin and levofloxacin), macrolides (azithromycin and clarithromycin), tetracyclines (chlortetracycline, doxycycline, minocycline, oxytetracycline, and tetracycline), and glycopeptide (vancomycin) (> 8%) were targeted in the present study.

The concentrations of target antimicrobials in the wastewater were determined using a combination of solid phase extraction (SPE) and ultra-performance liquid chromatography–tandem mass spectrometry, as described previously [36]. Briefly, 10 mL of wastewater was filtered through a glass-fiber filter (GF/B, 1 μm pore size, Whatman, Maidstone, UK). The solutions were then passed through SPE cartridges (OASIS HLB, 200 mg; Waters Corp., Milford, MA, USA) at a flow rate of 1 mL/min. The cartridges were washed with 6 mL of Milli-Q water, preadjusted to pH 3, and then dried using a vacuum pump. Finally, the adsorbed antimicrobials were eluted with 3 mL acetone and 3 mL methanol or with 2 mL of 10% (*v*/*v*) formic acid in acetone, 2 mL of 10% (*v*/*v*) formic acid in methanol, and 2 mL of 5% ammonia–methanol (*v*/*v*). Each combined eluted solution was evaporated mildly to dryness under a gentle stream of N_2 gas at 37 °C. The residue was solubilized in 200 μL of a 90:10 (*v*/*v*) mixture of 0.1% formic acid solution in methanol, and 10 μL of this solution was subjected to analysis using a UPLC system coupled to a tandem quadrupole mass spectrometer (TQD, Waters Corp.), equipped with an electrospray ionization source operated in positive ion mode.

Quantification was performed by subtracting the blank data from the corresponding data yielded by the spiked sample solutions to account for matrix effects and losses during sample extraction [39,40]. The recovery rates of antimicrobials in the wastewater influent ranged from 48% to 98% (Table S2) The limits of detection (LODs) and limits of quantification (LOQs) were calculated as the concentrations at signal-to-noise ratios of 3 and 10, respectively [41,42]. These values are also summarized in Table S2.

3. Results

3.1. Proportion of Bacteria in Hospital Wastewater after Ozone Treatment

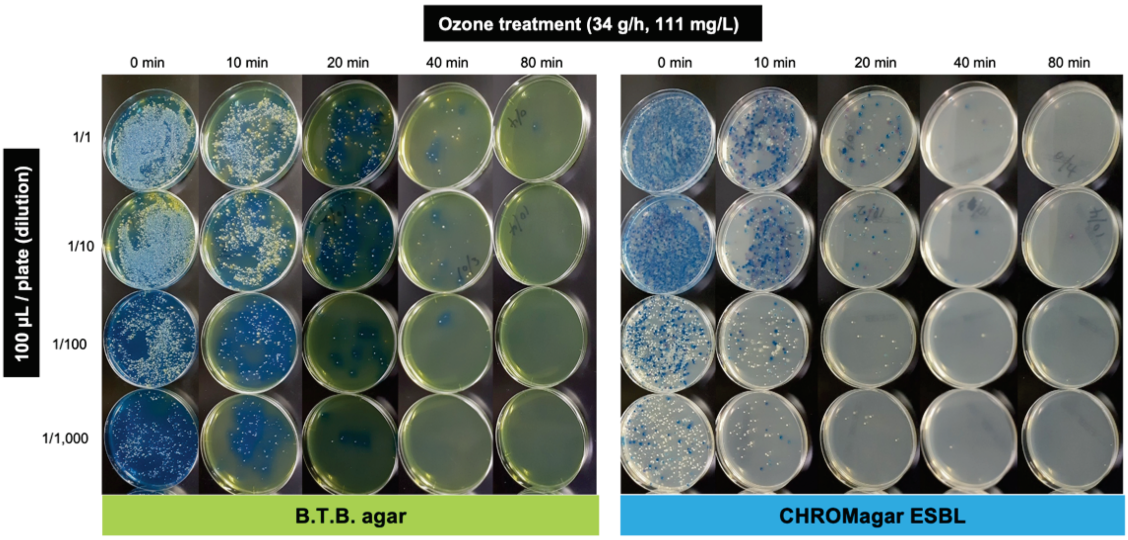
Hospital wastewater (1 m^3) in a batch reactor was treated with ozone fine bubbles (Figure 1), and the treated sample was subjected to metagenomic DNA-seq analysis (Table 1). Exposure to ozone for 20 min was sufficient to inactivate bacteria to <0.01% of the original number (for instance, those of the genus *Bacteroides*). Other bacteria genera were also reduced up to 0.02% of the original number upon 20-min exposure, indicating that most organisms were effectively inactivated in a short duration of exposure (Table 1). Besides bacterial genera, we also investigated the value of RPKM for each operational taxonomic unit (OTU) of an AMR gene (ARG) after ozone treatment (Table S3). In original raw wastewater before ozone treatment, the *tet*(Q) gene that is related to *Bacteroides* species in human feces was predominantly detected and the $bla_{\text{GES-1}}$ variants that are related to environmental bacteria such as *Aeromonas* and *Pseudomonas* species were secondly predominant. After 20 min of exposure to ozone, all ARGs were not detected in metagenomic DNA-Seq analysis (Table S3).

Gram-negative *Enterobacteriaceae* from urine and feces were cultured on B.T.B. agar (Bromothymol Blue, lactose agar; Drigalski Agar, Modified) and colony forming units (CFU) were determined. The percentage of viable bacteria was reduced to 76.2% (179,000/235,000 CFU) and 11.9% (27,900/235,000 CFU) upon a 10- and 20-min exposure to ozone, respectively (Figure 2).

Table 1. Metagenomic DNA-seq analysis of bacteria trapped on a 0.2 µm filter after ozone treatment.

Ozone Treatment (Min)	0	10	20	40	80
DNA Concentration (ng/µL)	0.5	1	<0.1	<0.1	<0.1
Metagenome DNA-Seq (Total Reads)	1,544,832	2,845,016	79,798	12,568	3060
Megablast Search of Bacteria (Genus) *					
Bacteroides	141,342	366,044	19	42	18
Parabacteroides	25,428	60,011	1	4	4
Acidovorax	14,454	25,271	1	195	28
Aeromonas	4125	18,455	2	41	6
Citrobacter	3939	7889	0	18	2
Escherichia	13,791	27,109	6	9	0
Klebsiella	13,049	24,336	8	23	5
Raoultella	12,630	14,533	0	37	12
Acinetobacter	19,510	30,185	4	73	29
Pseudomonas	10,162	14,073	0	528	213
Bifidobacterium	21,541	41,180	8	5	0
Enterococcus	3322	5245	0	0	2
Ruminococcus	17,829	33,639	2	8	2

* Next-generation sequencing-read counts for the detected notable bacterial genera are shown.



Viable colony forming units (CFU/mL) on the selected media after ozone treatment

	Ozone treatment				
Media selection	0 min	10 min	20 min	40 min	80 min
BTB agar	235000 (100%)	179000 (76.2%)	27900 (11.9%)	11600 (4.9%)	6500 (2.8%)
CHROMagar ESBL					
Total	90000 (100%)	51000 (56.7%)	18200 (20.2%)	7000 (7.8%)	4900 (5.4%)
● Dark Blue	55000 (100%)	31000 (56.4%)	3100 (5.6%)	700 (1.3%)	100 (0.2%)
● Pink	12000 (100%)	4000 (33.3%)	100 (0.8%)	0 (0%)	0 (0%)
● White	23000 (100%)	16000 (69.6%)	15000 (65.2%)	6300 (27.4%)	4800 (20.9%)

Figure 2. Isolation of bacteria from ozone-treated wastewater samples on B.T.B. agar and CHROMagar ESBL. An aliquot (100 µL) of ozone-treated wastewater sample was spread on the agar plate at the indicated dilution. Colony forming units (CFU/mL) were determined at the appropriate dilution for each time-point of ozone treatment. A colony on CHROMagar ESBL plate exhibited variable pigmentation, namely dark blue, pink, or white.

To determine the efficacy of the ozone microbubble treatment in inactivating potential β-lactam-resistant bacteria, the treated sample was spread on a CHROMagar ESBL plate, and the number of pigmented colonies was counted (Figure 2). Based on the CFU values, the percentage of all bacteria was reduced to 56.7% (51,000/90,000 CFU) and 20.2% (18,200/90,000 CFU) upon a 10- and 20-min exposure, respectively (Figure 2). The percentage of colonies showing dark blue or pink pigmentation was reduced to 5.6% (3100/55,000 CFU) and 0.8% (100/12,000 CFU), respectively, upon a 20-min exposure, whereas that of white colonies (no pigmentation) was not significantly reduced (65.2% upon a 20-min exposure) compared with that of the abovementioned pigmented colony types.

3.2. Susceptibility of Bacterial Species in Hospital Wastewater to Ozone Treatment

To elucidate the susceptibility of different bacterial species to ozone treatment, a markedly pigmented colony from each time-point was selected for whole genome sequence analysis (Table S4). Based on the results described in Section 2.1, the 20-min treatment was considered a reasonable time-point to investigate the susceptibility of notable bacterial species to ozone. CTX-M-producing *E. coli* isolates (pink pigmentation on CHROMagar ESBL plate) were found to be highly susceptible to ozone treatment compared with other isolates (Figure 2 and Table S4). Although variable ESBL/carbapenemase-producing *Enterobacteriaceae* isolates were identified up to 20 min after treatment, *Raoultella ornithinolytica* (dark blue pigmentation) and *Pseudomonas putida* (white, no pigmentation) were remarkably isolated even at the 40- and 80-min time points of ozone treatment (Table S4).

3.3. Removal of Antimicrobials by Ozone Treatment

Nine antimicrobials (ampicillin, cefdinir, cefpodoxime, ciprofloxacin, levofloxacin, clarithromycin, chlortetracycline, minocycline, and vancomycin) were detected at a wide range of concentrations (from ng/L to µg/L levels; 373 ng/L to 27 µg/L) in the hospital wastewater (Table 2).

Table 2. Concentrations of targeted antimicrobials in hospital wastewater during ozone treatment (N.D.: Not detected).

Classification	**Antimicrobials**	**Treatment Time (Min)**				
		0	**10**	**20**	**40**	**80**
β-lactams	Ampicillin	27,106	11,366	5522	148	N.D.
	Cefdinir	443	59	N.D.	N.D.	N.D.
	Cefpodoxime	6603	2040	20	N.D.	N.D.
	Cefpodoxime proxetil	N.D.	N.D.	N.D.	N.D.	N.D.
	Ceftiofur	N.D.	N.D.	N.D.	N.D.	N.D.
New quinolones	Ciprofloxacin	505	134	N.D.	N.D.	N.D.
	Levofloxacin	16,818	1676	92	N.D.	N.D.
Macrolides	Azithromycin	N.D.	N.D.	N.D.	N.D.	N.D.
	Clarithromycin	2933	1724	832	114	N.D.
Tetracyclines	Chlortetracycline	373	4	N.D.	N.D.	N.D.
	Doxycycline	N.D.	N.D.	N.D.	N.D.	N.D.
	Minocycline	2577	1185	35	N.D.	N.D.
	Oxytetracycline	N.D.	N.D.	N.D.	N.D.	N.D.
	Tetracycline	N.D.	N.D.	N.D.	N.D.	N.D.
Glycopeptides	Vancomycin	541	50	N.D.	N.D.	N.D.

The higher concentrations of antimicrobials were consistent with those previously reported from other countries [10,43,44]. The removal of antimicrobials detected in hospital wastewater by ozone treatment is summarized in Figure 3. The results show that ozone treatment reduced the residual antimicrobials in the wastewater over time, with a 96–100% removal of all targeted antimicrobials at 40 min after the treatment. The removal rate of cefdinir, levofloxacin, chlortetracycline, and vancomycin reached 90% within 10 min after the treatment, suggesting that these components are rapidly removed by ozone treatment.

The removal rates of cefpodoxime, ciprofloxacin, and minocycline were 69% (cefpodoxime), 73% (ciprofloxacin), and 54% (minocycline) at 10 min after the start of treatment, and the removal rate of all components reached over 99% at 20 min after the treatment. On the contrary, ampicillin and clarithromycin were detected (20–22%) at 20 min after the start of the treatment, but the removal rate reached 96–99% after 40 min. These results suggest that ozone treatment can effectively remove antimicrobials in hospital wastewater in a short time.

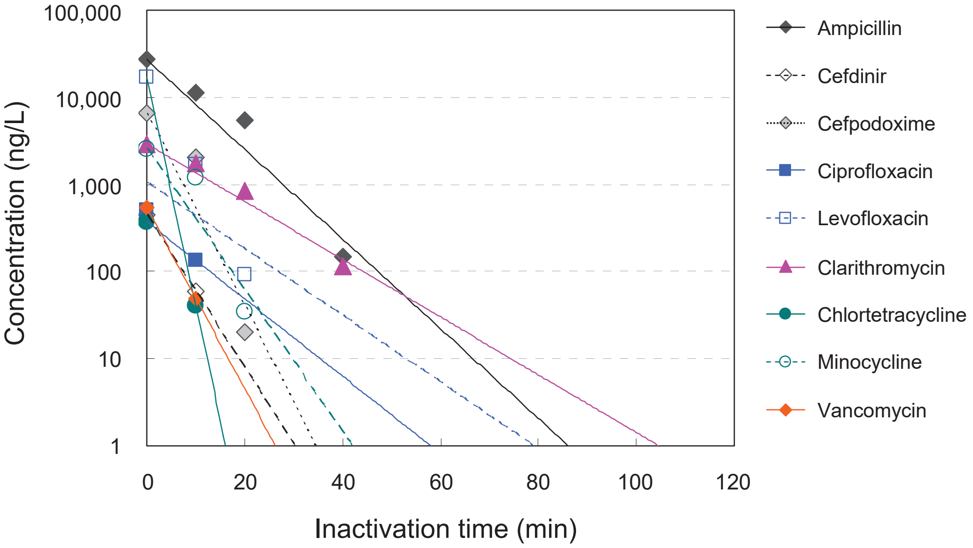


Figure 3. Time course of the concentration of antimicrobials in hospital wastewater during ozone treatment. Removal of antimicrobials over time during ozone treatment of hospital wastewater. The vertical axis shows the logarithmic value of the detected concentration, and the horizontal axis shows the time after the start of treatment.

4. Discussion

In this study, we investigated the efficacy of ozone fine bubble treatment (Figure 1) of hospital wastewater in mitigating the environmental AMR issue and evaluated the treatment by performing metagenome DNA-seq analysis (Table 1), culture of AMR isolates (Figure 2), and quantitative measurement of residual antimicrobial agents (Table 2 and Figure 3). The results of metagenome DNA-seq analysis suggested that ozone exposure for at least 20 min is required for adequate inactivation (less than 0.02% of the original concentration) of the DNA molecule (Table 1). Complete inactivation of the DNA molecule is ideal because the residual AMR genes could be utilized by bacteria to acquire AMR through horizontal gene transfer, although these are partial DNA fragments.

In addition, viable AMR bacteria in wastewater effluents are the most important and constitute a primary cause of environmental AMR burden because these bacteria can potentially grow in the environment. Therefore, these bacteria should be controlled to limit the spread of AMR bacteria in the environment. ESBL-producing bacteria were apparently reduced by 10-min of ozone exposure in a time-dependent manner (Figure 2). Intriguingly, CTX-M-producing *E. coli* isolates (pink pigment on CHROMagar ESBL plate) were found to be significantly susceptible to ozone treatment compared to the other isolates (Figure 2 and Table S4). Notably, *R. ornithinolytica* (dark blue pigment) and *P. putida* (white, no pigment) were isolated even at 40- and 80-min of ozone treatment (Table S4). Both these species (or specific features of these isolates) may be intrinsically resistant to ozone or perhaps a microbial biofilm or aggregation phenotype could confer them with resistance to ozone.

R. ornithinolytica isolates carry $bla_{\text{CTX-M-62}}$ (Table S4), alluding to the risk of horizontal gene transfer via plasmid transconjugation. *P. putida* has a low virulence, and its isolates are negative for potential β-lactamases and other marked ARGs, suggesting that it is an environmental bacterium with a very low risk for pathogenesis and AMR.

These results indicate that the susceptibility to ozone varies among bacterial species. The finding that ESBL-producing pathogenic bacteria, such as *E. coli* (pink pigment) and *Klebsiella* (dark blue pigment) exhibit a marked susceptibility to ozone and is consistent with previous reports [28,45,46] supports the proposal for the installation of an ozone treatment system in hospital wastewater tanks.

The identification of ozone-resistant bacteria, such as *Raoultella* and *Pseudomonas*, in this study, implies that these bacteria might play a pivotal role as an AMR reservoir in the environment and should be extensively monitored.

Residual antimicrobials detected in hospital wastewater are thought to originate from the antimicrobials used to treat diseases in clinical settings [47]. We did not detect cefpodoxime proxetil, ceftiofur, azithromycin, oxytetracycline, and tetracycline in the wastewater samples in the present investigation probably because of the non-usage of these antimicrobials in the hospital at the time the present study was conducted or because of the fact that the concentrations of antimicrobials present in wastewater represent the concentration at a particular time of the day due to grab sampling of wastewater [48,49]. In addition, it should also be noted that some antimicrobials, such as β-lactam antimicrobials, are attenuated in the water environment within a few hours [50,51]. Previous studies have reported that ozone treatment generally reduces the ecotoxicity compared to that of untreated compounds [52–54], but toxicity may increase in some cases [54]. On the other hand, the strong oxidizing action of ozone can decrease the formation of residual intermediate products by providing a sufficient processing time and by acting in combination with catalysts such as UV and hydrogen peroxide [20,55,56]. Our results support the need for further, conclusive research performed by taking experimental, technical, regional customs, bias, and unknown factors into consideration.

Studies evaluating the removal of antimicrobial-resistant bacteria and antimicrobials from wastewater using ozone treatment have primarily focused on wastewater treatment in wastewater treatment plants [53,57,58]. In these reports, the wastewater that has undergone processes such as the removal of solids by primary treatments such as sand filtration, the removal of organic matter by secondary treatments such as biological treatments, and disinfection after discharge into the water environment has been considered [58–60]. Moreover, as it is generally difficult to perform studies on hospital wastewater, the diversity and prevalence of antimicrobial-resistant bacteria and antimicrobials in hospital wastewaters have been evaluated only in a few studies worldwide [7,8,12]. The present study clarifies the effect of direct ozonation on hospital wastewater without pretreatment, and the treatment included fine ozone bubbles to achieve high removal efficiency; this has not been reported previously.

The problem of environmental pollution caused by the flow and persistence of antimicrobial-resistant bacteria and antimicrobials in rivers, lakes, and sea areas via wastewater systems has been reported worldwide [4,61–63], with reports pointing to the possibility of toxic effects on ecosystems [47,64,65]. Recent reports have suggested the possibility of the promotion of an unexpected emergence of antimicrobial-resistant bacteria from the environment [66–68]. Therefore, it is important to conduct a detailed assessment of the environmental risk posed by both antimicrobial-resistant bacteria and antimicrobials and to evaluate advanced wastewater treatment techniques that are effective in reducing or eliminating these risks.

5. Conclusions

A plant-scale ozone wastewater treatment facility based on a batch-type process was implemented in a core hospital located in the heart of Japan, and the inactivation effect on antimicrobial-resistant bacteria and antimicrobial residues was evaluated. The ozone treatment was effective in inactivating both antimicrobial-resistant bacteria and antimicrobials in hospital wastewater. Even when direct treatment, without filtration or biological pretreatment, was employed, most of the clinically problematic antimicrobial-resistant bacteria and residual antimicrobials were inactivated within 20–40 min of direct ozone treatment. The fact that a variety of antimicrobial-resistant bacteria and antimicrobials were detected at high concentrations in the hospital wastewater is a key point related to the development and spread of AMR. The fact that these organisms and antimicrobials can be inactivated by advanced wastewater treatment is significant in terms of taking feasible and effective countermeasures for addressing AMR in the environment.

The overall results signify a novel approach for preventing the environmental risks associated with the spread of AMR and could facilitate the early detection of nosocomial infection risk and the reduction of the environmental impact of AMR. Our findings could help enhance the effectiveness of introducing advanced wastewater treatment systems, not only at wastewater treatment plants but also at medical facilities, to reduce the discharge of pollutants into rivers, thereby contributing to the safety of environmental and human health.

Supplementary Materials: The following supporting information can be downloaded from https://www.mdpi.com/article/10.3390/antibiotics11070862/s1, Table S1. Summary of water quality parameters during hospital wastewater treatment with ozone (N.D.: Not detected); Table S2. Validation of the method characteristics for analysis of antimicrobials in hospital wastewater; Table S3. Comparison of RPKM value after ozone treatment; Table S4. Whole genome sequence of bacteria isolated using CHROMagar ESBL after ozone treatment.

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Data Availability Statement: All raw read sequence files are available from the DRA/SRA database (accession numbers DRR376744–DRR376803 (see Table S4)).

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antibiotics

Brief Report

A Universal LC-MS/MS Method for Simultaneous Detection of Antibiotic Residues in Animal and Environmental Samples

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Abstract: Detecting and monitoring the usage of antibiotics is a critical aspect of efforts to combat antimicrobial resistance. Antibiotic residue testing with existing LC-MS/MS methods is limited in detection range. Current methods also lack the capacity to detect multiple antibiotic residues in different samples simultaneously. In this study, we demonstrate a methodology that permits simultaneous extraction and detection of antibiotic residues in animal and environmental samples. A total of 30 different antibiotics from 13 classes could be qualitatively detected with our methodology. Further study to reduce analytes' matrix effect would allow for quantification of antibiotic residues.

Keywords: antibiotic residue; solid-phase extraction; LC-MS/MS

1. Introduction

Antibiotics have been in use for nearly a century and have been an important means to treat and prevent bacterial infection in both humans and animals [1]. However, the misuse and overuse of antibiotics have driven the rapid development of drug-resistant bacteria [2]. This has contributed to the bigger problem of antimicrobial resistance (AMR), which is currently a severe global public health issue. In order to tackle the issue of AMR, a One Health holistic approach, which covers human, animal, and environmental sectors, is necessary, due to inter-sectoral transmission [3].

Under the One Health framework, governments and organizations have taken a primary approach to mitigating AMR by reducing antimicrobial use (AMU) in human and animal sectors [4,5]. However, AMU and antimicrobial consumption have been difficult to measure. AMU survey or antimicrobial procurement data serve as a proxy to measure AMU but are limited by data availability and reliability. Thus, testing of antibiotic residues would be a complementary or alternative option.

In antibiotic residue testing, most of the fundamental laboratory detection methods involve an initial extraction followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Figure 1). Although the fine details of the methodology differ between different studies, this fundamental approach has been used to detect the chemical composition of and antibiotic residues in different samples, including porcine muscle [6], duck meat [7], aquaculture products [8], bovine milk [9], milk [10], honey [11], natural water [12,13], swine manure [14], and distiller grains [15].

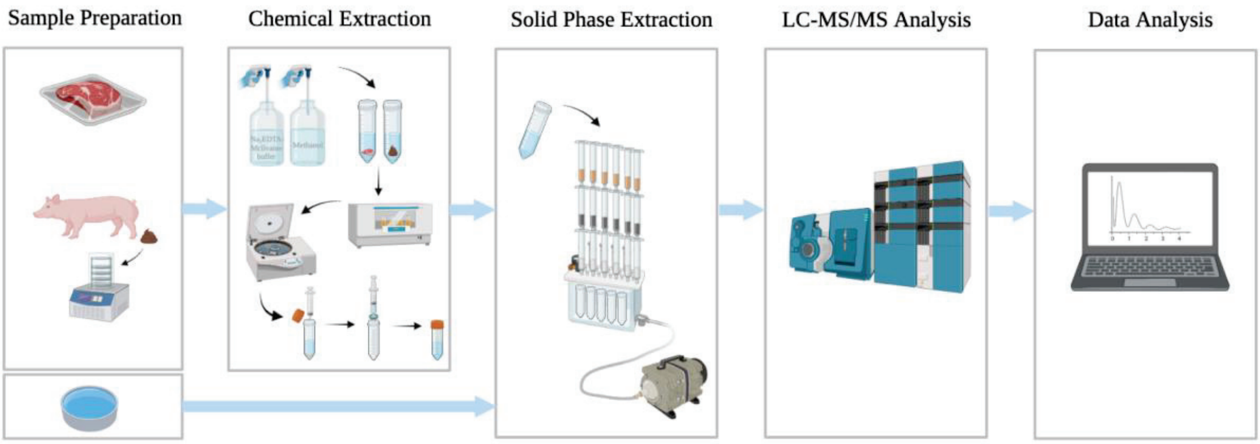

Figure 1. Flowchart showing the key steps in antibiotic residue testing. The figure was created with BioRender.com.

However, the common limitations of antibiotic residue testing in these studies are the lack of proven applicability of the testing protocol to detect different sample types and the wide number of antibiotic classes used in human and animals. For example, effectively extracting target chemicals from solid samples is different from that of water samples, and the classes of antibiotic that can be detected vary. Moreover, antibiotics and their residues can be unstable. For example, these substances may be unstable in water [16] and could be unstable based on their storage conditions [17,18]. To address these shortcomings, we demonstrate how a single extraction and detection method can be used in AMU surveillance to simultaneously detect multiple antibiotics in both animal and environmental samples.

2. Materials and Methods

2.1. Chemicals and Reagents

Acetonitrile, ammonia solution, citric acid monohydrate, dibasic sodium hydrogen phosphate, and LC-MS grade methanol were purchased from VWR (Radnor, PA, USA) for chemical extraction. The methanol that was used as the solvent in LC-MS was procured from Wako Chemicals (Osaka, Japan). Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) was purchased from BDH (Radnor, PA, USA), while formic acid was purchased from Fisher Scientific (Waltham, MA, USA). A total of 43 antibiotics belonging to 16 different antibiotic classes were used for this study (Table 1). Amoxicillin, ceftazidime, cefuroxime sodium salt, chloramphenicol, ciprofloxacin hydrochloride, colistin sulfate, doxycycline monohydrate, levofloxacin, florfenicol, metronidazole, oxytetracycline, spectinomycin hydrochloride pentahydrate, and sulfadiazine were purchased from Abcam (Cambridge, UK). Mequindox was ordered from Huawen Chemical (Henan, China), while tylvalosin was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Erythromycin, gentamicin sulfate, tylosin tartrate salt, and trimethoprim were purchased from MP Biomedicals (Irvine, CA, USA). Ampicillin, caffeine-(trimethyl-$^{13}C_3$) solution (used as internal standard), cefalexin, cefquinome sulfate, ceftiofur sodium, chlortetracycline hydrochloride, clindamycin phosphate, enrofloxacin, kanamycin sulfate, lincomycin hydrochloride, meropenem, neomycin trisulfate salt hydrate, norfloxacin, ofloxacin, penicillin G sodium salt, streptomycin sulfate salt, sulfachloropyridazine, sulfadimidine, sulfamethoxazole, sulfamonomethoxine, tetracycline, tiamulin, tilmicosin, and vancomycin were purchased from Sigma-Aldrich (Darmstadt, Germany).

Table 1. List of antibiotics tested.

Classes	Antibiotics
Aminoglycosides	Gentamicin
	Kanamycin sulfate
	Neomycin trisulfate salt hydrate
	Spectinomycin hydrochloride pentahydrate
	Streptomycin sulfate salt
Amphenicols	Chloramphenicol
	Florfenicol
Antifolate	Trimethoprim
Carbapenems	Meropenem
Cephalosporins	Cefalexin
	Cefquinome sulfate
	Ceftazidime
	Ceftiofur sodium
	Cefuroxime
Fluoroquinolones	Ciprofloxacin
	Enrofloxacin
	Levofloxacin
	Norfloxacin
	Ofloxacin
Glycopeptides	Vancomycin
Lincosamides	Clindamycin phosphate
	Lincomycin hydrochloride
Macrolides	Erythromycin
	Tilmicosin
	Tylosin tartrate salt
	Tylvalosin
Nitroimidazole	Metronidazole
Penicillins	Amoxicillin
	Ampicillin
	Penicillin G sodium salt
Pleuromutilins	Tiamulin
Polymyxins	Colistin A
	Colistin B
Quinoxaline 1,4-di-N-oxides (QdNOs)	Mequindox
Sulfonamides	Sulfachloropyridazine
	Sulfadiazine
	Sulfadimidine
	Sulfamethoxazole
	Sulfamonomethoxine
Tetracyclines	Chlortetracycline hydrochloride
	Doxycycline
	Oxytetracycline
	Tetracycline

2.2. Sample Preparation

Swine feces were collected from local farms and freeze-dried before processing. Pork was purchased from local markets. River water, animal drinking water, and Milli-Q water were used as water samples. An antibiotic mixture (~0.01 mg/mL concentration) containing 43 different antibiotics was used for the spike-in. For solid samples, the antibiotic mixture was directly spiked into 1 g of solid sample (i.e., fecal sample and meat sample) by adding the aqueous antibiotic mixture into a Falcon tube containing the solid sample and then mixing by vortex. The sample was allowed to stand for 1 h to allow for the antibiotic mixture to be absorbed into the solid sample. For liquid samples, the antibiotic mixture was spiked into 100 mL of liquid sample at three volumes: 1 mL of 0.01 mg/mL (i.e., 10 μg), 0.5 mL of 0.01 mg/mL (i.e., 5 μg), and 0.1 mL of 0.01 mg/mL (i.e., 1 μg). All samples were stored at −20 °C and extracted within 1 week.

2.3. Chemical Extraction

Chemical extraction was carried out according to the literature [14] with modifications. The chemical extraction buffer that was used was a mixture of Na_2EDTA-McIlvaine buffer solution (10 mL), which contains 10.93 mg/mL anhydrous dibasic sodium phosphate, 12.93 mg/mL citric acid monohydrate, and 37.22 mg/mL Na_2EDTA, and 100% methanol (10 mL). For extraction, 20 mL of this chemical extraction buffer was added into 1 g of sample (fecal sample or meat sample). Extraction was carried out in a shaking incubator at 300 rpm for 30 min at room temperature. Supernatants were collected after centrifugation at 3200 g for 5 min at 4 °C. Residues were further extracted two more times with the same protocol. Supernatants of the same sample were then combined and stored at −20 °C until solid-phase extraction (SPE) was performed. For liquid samples, chemical extraction was not performed. All samples were filtered through a 0.2 μm PES syringe filter, purchased from Sartorius (Göttingen, Germany), prior to solid-phase extraction.

2.4. Solid-Phase Extraction (SPE)

An Oasis MAX 6cc (150 mg) cartridge (cat. no.: 186000369), Oasis PRiME HLB 6cc (200 mg) cartridge (cat. no.: 186008057), and Oasis PRiME MCX 6cc (150 mg) cartridge (cat. no.: 186008919) were purchased from Waters (Milford, MA, USA) and used to form a MAX-HLB-MCX combined cartridge for solid-phase extraction. All cartridges were preconditioned with acetonitrile and Milli-Q water separately. To investigate the retention of antibiotics in different SPE cartridges, 10 mL of the antibiotic mixture containing 43 targeted antibiotics was loaded to the MAX-HLB-MCX combined cartridge. For solid samples, supernatants collected from chemical extractions were diluted with Milli-Q water to reduce the methanol content to less than 5% (*v*/*v*). All of the diluted supernatant flowed through the MAX-HLB-MCX combined cartridge at a flow rate of 3 mL/min. For liquid samples, all of the samples were passed through the MAX-HLB-MCX combined cartridge directly at a flow rate of 3 mL/min. Analytes were eluted from the MAX cartridge, HLB cartridge, and MCX cartridge separately. To elute the analytes, 4 mL of 2% formic acid in methanol, acetonitrile/methanol (60%/40%; *v*/*v*), and 5% ammonia solution in methanol were used, respectively. The elution process was repeated three times. The ratio in which elutes were mixed from the MAX, HLB, and MCX cartridges was 1:1:1. Elutes from the same sample were combined for LC-MS/MS analysis.

2.5. LC-MS/MS Analysis

The Acquity I-Class ultra-high-performance liquid chromatographic system by Waters (Milford, MA, USA), coupled with the QTRAP® 6500+ MS system from AB Sciex (Framingham, MA, USA), was used for LC-MS/MS analysis. A Phenomenex Synergi 4 μm Fusion-RP 80 Å (2 mm × 50 mm) column was used for separation, and the column oven temperature was set at 40 °C. The elution gradient (solvent A: 0.1% aqueous formic acid, solvent B: acetonitrile) was set up as follows: 0 min, 0% B; 0.1 min, 0% B; 1 min, 10% B; 6.5 min, 50% B; 7 min, 100% B; 8 min, 100% B. This gradient was re-equilibrated to 0% B for

2 min after each run. The flow rate was 0.5 mL/min, and the injection volume was 5 µL. In terms of electrospray ionization, the parameters were the following: (CUR), nitrogen, 12; collision gas (CAD), high; electrospray voltage, +5500 V; ion source temperature, 550 °C; curtain gas of 25, CAD gas medium, and gas 1 and 2 of 45 and 50 psi, respectively. Retention time and transitions are shown in Table 2.

Table 2. Liquid chromatography and mass spectrometry of 43 antibiotics.

Antibiotics	Retention Time (min)	Transition 1 (m/z)	Transition 2 (m/z)	Limit of Detection (ppb)
Amoxicillin	0.83	366.1 > 348.9	366.1 > 208	8.51
Ampicillin	1.78	350 > 191.9	350 > 160	0.49
Cefalexin	1.79	348 > 158	348 > 174	0.9
Cefquinome sulfate	2.33	529 > 396	529 > 134	1.11
Ceftazidime	2.14	547.1 > 467.8	547.1 > 396	3.44
Ceftiofur sodium	3.53	524 > 241	524 > 285	0.31
Cefuroxime	2.57	447 > 385.7	447 > 342	4.1
Chloramphenicol	2.56	323.1 > 274.9	323.1 > 304.8	5.41
Chlortetracycline hydrochloride	2.66	479 > 444	479 > 462	4.06
Ciprofloxacin	2.35	332.1 > 313.9	332.1 > 231.1	0.4
Clindamycin phosphate	3.16	505.1 > 457	505.1 > 487.1	0.55
Colistin A	1.95	585.6 > 535.5	585.6 > 576.4	862.53
Colistin B	1.77	578.5 > 528.4	578.5 > 569.5	793.3
Doxycycline	2.94	445.1 > 428	445.1 > 267	0.49
Enrofloxacin	2.58	360 > 316	360 > 245	0.4
Erythromycin	3.83	734.3 > 576.3	734.3 > 157.9	14.52
Florfenicol	2.26	358 > 340	358 > 241	13.21
Gentamicin	2.79	500.1 > 456	500.1 > 227.1	320
Kanamycin sulfate	0.27	485 > 324	485 > 163	6.54
Levofloxacin	2.36	362.1 > 318.2	362.1 > 261.1	0.47
Lincomycin hydrochloride	1.43	407 > 126	407 > 359	0.45
Mequindox	2.18	219 > 143	219 > 185	1.7
Meropenem	1.79	384.1 > 340.1	384.1 > 297.7	1.76
Metronidazole	1.06	172 > 128.2	172 > 82.1	0.46
Neomycin trisulfate salt hydrate	0.26	615 > 293	615 > 161	345.18
Norfloxacin	2.3	320 > 302	320 > 231.2	0.54
Ofloxacin	2.35	362 > 318	362 > 261	0.43
Oxytetracycline	2.02	461 > 426	461 > 444	1.98
Penicillin G sodium salt	2.36	335 > 160	335 > 176	10.79
Spectinomycin hydrochloride pentahydrate	0.3	333 > 189	333 > 140	2.52
Streptomycin sulfate salt	4.13	582 > 174	582 > 156	425.98
Sulfachloropyridazine	2.3	285 > 156	285 > 108	0.6
Sulfadiazine	1.45	251 > 156	251 > 92	0.45
Sulfadimidine	2.08	279 > 186	279 > 156	0.36
Sulfamethoxazole	2.4	254.1 > 155.8	254.1 > 108.2	0.43
Sulfamonomethoxine	2.38	281 > 156	281 > 126	0.62
Tetracycline	2.14	445 > 410	445 > 269	0.49

Table 2. *Cont.*

Antibiotics	Retention Time (min)	Transition 1 (m/z)	Transition 2 (m/z)	Limit of Detection (ppb)
Tiamulin	4.12	494 > 192	494 > 119	0.59
Tilmicosin	3.38	869.4 > 696	869.4 > 174	5.73
Trimethoprim	1.92	291.1 > 230	291.1 > 260.9	0.41
Tylosin tartrate salt	4.13	916.3 > 772	916.3 > 174	11.63
Tylvalosin	5.2	1042.3 > 814	1042.3 > 174	64.27
Vancomycin	1.95	726 > 144	725 > 144	26.63

For transition ranges, each pure antibiotic compound purchased commercially was first injected into the LC-MS/MS instrument for preliminary testing. From this preliminary test, information about transition ranges was obtained. Two transitions of each antibiotic with sharp peaks shown were chosen as references to identify the antibiotic.

2.6. Data Analysis

Data analysis for LC-MS/MS was performed using the SciEX OS-Q Analysis Software (Framingham, MA, USA). Analytes were confirmed by comparing the retention time and the ratio of characteristic transitions between the sample and the standard.

3. Results

3.1. Limit of Detection

This study began with the determination of the detection limit (i.e., limit of detection, LOD) of the MS system. The LOD was determined by injecting a low concentration of antibiotic standard into the mass spectrometer directly and then reviewing the peak generated. If the signal was three times higher than the background base noise level, then we accepted the peak as an actual peak. Infusion was only used for optimizing the MRM parameter before LC-MS/MS. The results of LOD are shown in Table 2. Generally, the detection limit varied. Most of the 43 antibiotics were detectable at levels lower than 70 ppb, with only six antibiotics having a level of detection higher than 70 ppb. These six antibiotics were colistin A, colistin B, neomycin trisulfate, gentamicin, and streptomycin sulfate. Nevertheless, all 43 targeted antibiotics were detectable.

3.2. Solid-Phase Extraction

The percentage of recovery was calculated by comparing the concentration of antibiotic recovered after passing through SPE and without passing through SPE. In other words, the percentage of recovery = total concentration of antibiotic in elutes from SPE/concentration of antibiotic in antibiotic mixture before passing through SPE. The recoveries of amoxicillin, ampicillin, cefquinome sulfate, meropenem, and tiamulin from SPE were poor, with loss being >75% (Table 3). Apart from those which had a poor recovery, 11 antibiotics were retained in the MAX cartridge; five antibiotics were retained in the HLB cartridge; and 17 antibiotics were retained in the MCX cartridge. For chlortetracycline hydrochloride, doxycycline, and mequindox, the MAX cartridge could not completely retain all of the residues, and a significant portion flowed through the MAX cartridge and were retained in the HLB cartridge. For sulfadiazine and sulfadimidine, they could be detected in the elutes of all three cartridges in the MAX-HLB-MCX tandem. Overall, 38 out of the 43 antibiotics had a recovery that was satisfactory or good after SPE.

Table 3. Solid-phase extraction of 43 antibiotics mixture.

Antibiotics	SPE Recovery	Mainly Retained
Amoxicillin	Poor	MAX
Ampicillin	Poor	MAX
Cefalexin	Good	MCX
Cefquinome sulfate	Poor	MAX/HLB
Ceftazidime	Good	MAX
Ceftiofur sodium	Good	MAX
Cefuroxime	Good	MAX
Chloramphenicol	Good	MAX
Chlortetracycline hydrochloride	Satisfactory	MAX/HLB
Ciprofloxacin	Good	MCX
Clindamycin phosphate	Good	MAX
Colistin A	Satisfactory	MCX
Colistin B	Satisfactory	MCX
Doxycycline	Satisfactory	MAX/HLB
Enrofloxacin	Satisfactory	MCX
Erythromycin	Good	HLB
Florfenicol	Good	MAX
Gentamicin	Satisfactory	MAX
Kanamycin sulfate	Good	MCX
Levofloxacin	Good	MCX
Lincomycin hydrochloride	Good	MCX
Mequindox	Satisfactory	MAX/HLB
Meropenem	Poor	MAX/HLB
Metronidazole	Good	MCX
Neomycin trisulfate salt hydrate	Good	MCX
Norfloxacin	Good	MCX
Ofloxacin	Good	MCX
Oxytetracycline	Satisfactory	MCX
Penicillin G sodium salt	Good	MAX
Spectinomycin hydrochloride pentahydrate	Satisfactory	MCX
Streptomycin sulfate salt	Good	HLB
Sulfachloropyridazine	Good	MAX
Sulfadiazine	Good	MAX/HLB/MCX
Sulfadimidine	Good	MAX/HLB/MCX
Sulfamethoxazole	Good	MAX
Sulfamonomethoxine	Good	MAX
Tetracycline	Good	MCX
Tiamulin	Poor	MCX
Tilmicosin	Satisfactory	HLB
Trimethoprim	Good	MCX
Tylosin tartrate salt	Good	HLB
Tylvalosin	Good	HLB
Vancomycin	Good	MCX

Concentration of antibiotics mixture: 10 mL, 0.01 mg/mL. Recovery < 25% is considered to be “Poor”; recovery $\geq$25% and $\leq$60% is considered to be “Satisfactory”; and recovery >60% is considered to be “Good”.

3.3. Chemical Extraction

The integrated recovery of antibiotic residues (i.e., including the limitation of chemical extraction, SPE, and LOD) is shown in Table 4. Using our methodology, 30 out of 43 targeted antibiotics could be detected (Table 4). The 13 antibiotics that could not be detected were: amoxicillin, cefquinome sulfate, ceftazidime, cefuroxime, ciprofloxacin, colistin (A and B), gentamicin, kanamycin sulfate, meropenem, neomycin trisulfate, norfloxacin, and vancomycin. Among these 13 undetectable antibiotics, amoxicillin, cefquinome sulfate, and meropenem were found to have a poor recovery from SPE.

Table 4. Detection of antibiotic mixture for spike-in of water, fecal, and meat sample.

Antibiotics	Water Sample	Fecal Sample	Meat Sample
Amoxicillin	N.D.	N.D.	N.D.
Ampicillin	Detected	Detected	Detected
Cefalexin	Detected	Detected	Detected
Cefquinome sulfate	N.D.	N.D.	N.D.
Ceftazidime	N.D.	N.D.	N.D.
Ceftiofur sodium	Detected	Detected	Detected
Cefuroxime	N.D.	N.D.	N.D.
Chloramphenicol	Detected	Detected	Detected
Chlortetracycline hydrochloride	Detected	Detected	Detected
Ciprofloxacin	N.D.	N.D.	N.D.
Clindamycin phosphate	Detected	Detected	Detected
Colistin A	N.D.	N.D.	N.D.
Colistin B	N.D.	N.D.	N.D.
Doxycycline	Detected	Detected	Detected
Enrofloxacin	Detected	Detected	Detected
Erythromycin	Detected	Detected	Detected
Florfenicol	Detected	Detected	Detected
Gentamicin	N.D.	N.D.	N.D.
Kanamycin sulfate mixture of kanamycin A (main component) and kanamycin B and C	N.D.	N.D.	N.D.
Levofloxacin	Detected	Detected	Detected
Lincomycin hydrochloride	Detected	Detected	Detected
Mequindox	Detected	Detected	Detected
Meropenem	N.D.	N.D.	N.D.
Metronidazole	Detected	Detected	Detected
Neomycin trisulfate salt hydrate	N.D.	N.D.	N.D.
Norfloxacin	N.D.	N.D.	N.D.
Ofloxacin	Detected	Detected	Detected

Table 4. *Cont.*

Antibiotics	Water Sample	Fecal Sample	Meat Sample
Oxytetracycline	Detected	Detected	Detected
Penicillin G sodium salt	Detected	Detected	Detected
Spectinomycin hydrochloride pentahydrate	Detected	Detected	Detected
Streptomycin sulfate salt	Detected	Detected	Detected
Sulfachloropyridazine	Detected	Detected	Detected
Sulfadiazine	Detected	Detected	Detected
Sulfadimidine	Detected	Detected	Detected
Sulfamethoxazole	Detected	Detected	Detected
Sulfamonomethoxine	Detected	Detected	Detected
Tetracycline	Detected	Detected	Detected
Tiamulin	Detected	Detected	Detected
Tilmicosin	Detected	Detected	Detected
Trimethoprim	Detected	Detected	Detected
Tylosin tartrate salt	Detected	Detected	Detected
Tylvalosin	Detected	Detected	Detected
Vancomycin	N.D.	N.D.	N.D.

1 mL of antibiotic mixture (0.01 mg/mL) was spiked into 1 g of solid sample or 100 mL of liquid sample. N.D. represents "not detected".

3.4. Sensitivity

The sensitivities of antibiotic classes were calculated by taking the raw data and then summing the data for all of the tests performed for all antibiotics in a class. Overall, the sensitivity of the protocol in this study was high (i.e., >60%) for 30 out of the 43 antibiotic residues (Table 5). When the spike-in concentration was high, i.e., 10 μg, our approach showed a sensitivity of 100% in most of the antibiotics in all three types of sample. For ampicillin, ceftiofur, chloramphenicol, chlortetracycline, doxycycline, erythromycin, metronidazole, penicillin, spectinomycin, streptomycin, tetracycline, tilmicosin, tylosin tartrate, and tylvalosin, the sensitivities of the protocol were only reduced when the spiked-in antibiotic content was reduced to 1 μg (Table S1).

Out of 16 classes of antibiotic tested, antibiotics from 13 classes could be detected (Table 6). The sensitivity of detecting antibiotics from the antifolate, lincosamide, pleuromutilin, quinoxaline 1,4-di-N-oxide (QdNO), and sulfonamide classes was relatively high in all three types of sample at three different concentrations. The sensitivity of detection of antibiotics from the amphenicol, macrolide, nitroimidazole, and tetracycline classes was high (around 100%) when the spiked-in content was 10 μg and 5 μg, but it was less sensitive when the spiked-in content was reduced to 1 μg. The sensitivity of our detection method was relatively lower for aminoglycosides, cephalosporins, fluoroquinolones, and penicillins, while carbapenems, glycopeptides, and polymyxins could not be detected.

Table 5. Sensitivity of overall protocol for 43 antibiotics at spike-in concentrations of 10 µg, 5 µg, and 1 µg.

Antibiotics	Sensitivity								
	10 µg Spiked-in			**5 µg Spiked-in**			**1 µg Spiked-in**		
	Water Sample	**Fecal Sample**	**Meat Sample**	**Water Sample**	**Fecal Sample**	**Meat Sample**	**Water Sample**	**Fecal Sample**	**Meat Sample**
Amoxicillin	0	0	0	0	0	0	0	0	0
Ampicillin	100	100	100	100	100	100	66.7	0	33.3
Cefalexin	100	100	100	100	100	100	100	100	100
Cefquinome sulfate	0	0	0	0	0	0	0	0	0
Ceftazidime	0	0	0	0	0	0	0	0	0
Ceftiofur sodium	100	100	100	100	100	100	66.7	66.7	0
Cefuroxime	0	0	0	0	0	0	0	0	0
Chloramphenicol	100	100	100	100	100	100	0	0	66.7
Chlortetracycline hydrochloride	100	100	100	100	100	100	100	100	66.7
Ciprofloxacin	0	0	0	0	0	0	0	0	0
Clindamycin phosphate	100	100	100	100	100	100	100	100	100
Colistin A	0	0	0	0	0	0	0	0	0
Colistin B	0	0	0	0	0	0	0	0	0
Doxycycline	100	100	100	100	100	100	0	33.3	0
Enrofloxacin	100	100	100	100	100	100	100	100	100
Erythromycin	100	100	100	100	33.3	100	33.3	0	33.3
Florfenicol	100	100	100	100	100	100	100	100	100
Gentamicin	0	0	0	0	0	0	0	0	0
Kanamycin sulfate	0	0	0	0	0	0	0	0	0

Table 5. *Cont.*

Antibiotics	Sensitivity								
	10 µg Spiked-in			5 µg Spiked-in			1 µg Spiked-in		
	Water Sample	Fecal Sample	Meat Sample	Water Sample	Fecal Sample	Meat Sample	Water Sample	Fecal Sample	Meat Sample
Levofloxacin	100	100	100	100	100	100	100	100	100
Lincomycin hydrochloride	100	100	100	100	100	100	100	100	100
Mequindox	100	100	100	100	100	100	100	100	100
Meropenem	0	0	0	0	0	0	0	0	0
Metronidazole	100	100	100	100	100	100	100	33.3	66.7
Neomycin trisulfate salt hydrate	0	0	0	0	0	0	0	0	0
Norfloxacin	0	0	0	0	0	0	0	0	0
Ofloxacin	100	100	100	100	100	100	100	100	100
Oxytetracycline	100	100	100	100	100	100	100	100	100
Penicillin G sodium salt	100	100	100	100	33.3	100	0	0	0
Spectinomycin hydrochloride pentahydrate	100	66.7	100	66.7	66.7	100	0	0	0
Streptomycin sulfate salt	66.7	100	100	33.3	0	66.7	0	0	0
Sulfachloropyridazine	100	100	100	100	100	100	100	100	100
Sulfadiazine	100	100	100	100	100	100	100	100	100
Sulfadimidine	100	100	100	100	100	100	100	100	100
Sulfamethoxazole	100	100	100	100	100	100	100	100	100
Sulfamonomethoxine	100	100	66.7	100	100	100	100	100	100
Tetracycline	100	100	100	100	100	100	100	100	66.7

Table 5. *Cont.*

Antibiotics	Sensitivity								
	10 µg Spiked-in			5 µg Spiked-in			1 µg Spiked-in		
	Water Sample	Fecal Sample	Meat Sample	Water Sample	Fecal Sample	Meat Sample	Water Sample	Fecal Sample	Meat Sample
Tiamulin	100	100	100	100	100	100	100	100	100
Tilmicosin	66.7	100	100	66.7	100	100	0	33.3	33.3
Trimethoprim	100	100	100	100	100	100	100	100	100
Tylosin tartrate salt	100	100	100	100	100	100	100	100	66.7
Tylvalosin	100	100	100	100	100	100	33.3	100	100
Vancomycin	0	0	0	0	0	0	0	0	0

Sensitivity = [number of true positives/(number of true positives + number of false negatives)] × 100%. Note: the number of tests performed to calculate detection sensitivity was 3.

Table 6. Sensitivity of overall protocol for 16 groups of antibiotics at spike-in concentrations of 10 µg, 5 µg, and 1 µg.

Antibiotics Group	**Sensitivity**								
	10 µg Spiked-in			**5 µg Spiked-in**			**1 µg Spiked-in**		
	Water Sample	**Fecal Sample**	**Meat Sample**	**Water Sample**	**Fecal Sample**	**Meat Sample**	**Water Sample**	**Fecal Sample**	**Meat Sample**
Aminoglycosides	33.3	33.3	40	20	13.3	33.3	0	0	0
Amphenicols	100	100	100	100	100	100	50	50	83.3
Antifolate	100	100	100	100	100	100	100	100	100
Carbapenems	0	0	0	0	0	0	0	0	0
Cephalosporins	40	40	40	40	40	40	33.3	33.3	20
Fluoroquinolones	60	60	60	60	60	60	60	60	60
Glycopeptides	0	0	0	0	0	0	0	0	0
Lincosamides	100	100	100	100	100	100	100	100	100
Macrolides	91.7	100	100	91.7	83.3	100	41.7	58.3	58.3
Nitroimidazole	100	100	100	100	100	100	100	33.3	66.7
Penicillins	66.7	66.7	66.7	66.7	44.4	66.7	22.2	0	11.1
Pleuromutilins	100	100	100	100	100	100	100	100	100
Polymyxins	0	0	0	0	0	0	0	0	0
Quinoxaline 1,4-di-N-oxides (QdNOs)	100	100	100	100	100	100	100	100	100
Sulfonamides	100	100	93.3	100	100	100	100	100	100
Tetracyclines	100	100	100	100	100	100	75	83.3	58.3

Sensitivity = [number of true positives/(number of true positives + number of false negatives)] × 100%.

4. Discussion

In this study, we have developed a LC-MS/MS-based working protocol that could detect residues of 30 antibiotics from 13 classes in animal meat and environmental samples. Although using LC-MS/MS to detect antibiotic residues in food and water samples is not a novel technique, our protocol has two significant improvements. The first improvement is that we have developed a methodology that can cover different sample types. Previous developed methods can detect 34 veterinary drugs from six distinct groups in porcine muscle [6]; 75 antibiotics from six groups in meat and aquaculture products [8]; 63 pharmaceuticals in natural water [12]; 58 antibiotics from eight groups in milk [10]; and 20 antibiotics from three different groups in honey [11]. Most of these methods targeted food samples and are not sufficient in monitoring antibiotic contaminations, particularly in various types of environmental sample.

The second improvement is that we have developed a methodology that can cover a wider range of different antibiotics. Different antibiotics have different chemical properties, and they may require different extraction and detection methods [19,20]. In our study, we used a single extraction and detection protocol to cover 30 antibiotics from 13 families, which makes it an improvement over existing methodologies in terms of efficiency and convenience [7,9,13–15]. To provide an insight into why we could make the aforementioned two improvements in detecting antibiotic residues, we provide an interpretation of the results after SPE and chemical extraction were conducted in our protocol.

In theory, SPE is a common procedure performed to clean up the samples before LC-MS/MS analysis [21,22]. However, the major purpose of using SPE in this study was to concentrate the analytes from large-volume but low-concentration samples, such as environmental water and wastewater with diluted concentrations of antibiotic residues. Thus, in order to increase the sensitivity of detection, SPE was an essential step of our developed method. The chemical nature of the antibiotics and the type of SPE cartridge could influence the final recovery of antibiotic residues for detection. Considering that 43 antibiotics with varying properties were targeted in this study, we used a MAX-HLB-MCX tandem consisting of the MAX cartridge, the MCX cartridge, and the HLB cartridge to retain as much of the antibiotic residues as possible. The rationale behind this tandem formation was that the different cartridges would target compounds with different pH properties: the MAX cartridge targets acidic compounds; the MCX cartridge targets basic compounds; and the HLB cartridge targets relatively neutral compounds (i.e., those that are neither basic nor acidic).

Our SPE method was able to recover a majority of the antibiotics (38 out of 43 antibiotics) with satisfactory or good performance, indicating its ability to successfully detect antibiotics of different pH properties. For those five antibiotics that had a poor recovery after SPE (i.e., >75% loss), we hypothesized that the chemical structure of these antibiotics may not be compatible with the tandem formation that we designed. Most of these belonged to the β-lactam class of antibiotics, of which amoxicillin, ampicillin, cefquinome sulfate, and meropenem possess a chemically unstable β-lactam ring that spontaneously undergoes hydrolysis [23]. Thus, this is a plausible reason explaining why it was very difficult to recover these antibiotics in water samples.

Generally, the extraction method of using Na_2EDTA-McIlvaine buffer solution/methanol (1:1; *v*/*v*) provided a detection of 30 different antibiotics. There were three antibiotics that were found to have a poor SPE recovery and could not be detected in spiked-in samples: amoxicillin, cefquinome sulfate, and meropenem. It would not be possible to determine the reason of zero sensitivity for these three antibiotics, since it could be related to the chemical extraction method, poor SPE recovery, or both. Thus, it is difficult to determine whether the three antibiotics could be extracted using our chemical extraction protocol. For the rest of the nine antibiotics that could not be detected, it seems that the extraction method was insufficient in recovering these antibiotics. We arrived at this conclusion because we observed a satisfactory recovery rate from SPE for these antibiotics, and the spike-in concentrations were higher than the LOD. Based on these results, the extraction method may not be suitable for extracting the residues of ceftazidime, cefuroxime, ciprofloxacin, colistin, gentamicin, kanamycin sulfate, neomycin trisulfate, norfloxacin, and vancomycin from samples. Further study for modifying the chemical extraction method to further increase the range of recovery may be necessary.

The proposed method has notable limitations, such as the inability to conduct precise quantitative analysis and a decline in sensitivity with lowering the spiked-in concentration. Although SPE is commonly performed to remove impurities from analytes in order to reduce a potential matrix effect, the use of the MAX-HLB-MCX tandem resulted in strong matrix effects. Moreover, it is difficult to obtain corresponding isotopes for all tested antibiotics to correct the matrix effect of each antibiotic during quantification. However, an estimation of the antibiotic concentrations may still be made by performing calculations. From LC-MS/MS results, one will have the information of the total amount of antibiotics in the elute of each sample. The concentration of an antibiotic can be estimated by the following equation: (concentration of antibiotic in sample = amount of antibiotic/sample weight or sample volume). Thus, the difference in volume between solid and liquid samples during the whole process does not affect the estimation of the antibiotic concentration in samples after calculation.

Only 15 out of the 30 detectable antibiotics could maintain a sensitivity of 100% when the spiked-in content was reduced from 10 μg to 5 μg and 1 μg for all three types of sample. It seems that, when the spiked-in concentrations were gradually lowered, the sensitivity of the protocol decreased. From the results in Tables 3 and 4, the loss of antibiotic residues during the extraction process and SPE was expected. The explanation is that the likelihood of a false negative result increased when the residue content decreased. Having stated these limitations, our method could be useful in AMU surveillance in livestock farms as a first-line qualitative assessment tool—especially for detecting residues in farm waste.

5. Conclusions

In conclusion, 30 different antibiotics from 13 classes could be detected with high sensitivity with our sample processing method when the residue content was 10 ppm or above. When the residue content was reduced to 1 ppm, 27 different antibiotics could still be detected, and 21 of them had a sensitivity higher than 50%. The developed chemical extraction method, together with SPE, allowed us to detect at least 30 antibiotic residues from 13 families qualitatively in foods and environmental samples at the same time. Nevertheless, further study to reduce the matrix effect of analytes is necessary so that quantification of antibiotic residues could be possible.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11070845/s1, Table S1. Reduction in sensitivity of overall protocol for 43 antibiotics at spike-in concentrations of 10 μg, 5 μg, and 1 μg.

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