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Special Issue Reprint

Antibiotic Resistance in a One Health Context

Bridging Environmental, Agricultural, Nutritional,
Veterinary, and Clinical Perspectives

Edited by
Pedro A. Jiménez Gómez and Marina Robas Mora

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Antibiotic Resistance in a One Health Context: Bridging Environmental, Agricultural, Nutritional, Veterinary, and Clinical Perspectives

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Guest Editors

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About the Editors

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Preface

The growing threat of antibiotic resistance continues to challenge human, animal, and environmental health worldwide. Despite significant advances in surveillance, diagnostics, and stewardship programs, antimicrobial resistance remains a dynamic and multifactorial problem that transcends traditional disciplinary boundaries. The complexity of its emergence and dissemination reinforces the need for integrated approaches capable of addressing the interconnected nature of humans, animals, food systems, and the environment.

This Reprint builds upon the success of the first edition, which brought together valuable contributions from researchers working across diverse fields related to antimicrobial resistance. The positive response and scientific impact of the previous collection highlighted the importance of maintaining an interdisciplinary forum where environmental microbiologists, clinicians, veterinarians, food scientists, ecologists, and public health professionals can exchange knowledge and perspectives.

This second edition will provide an updated overview of current research on antibiotic resistance within the One Health framework, including emerging drivers, novel methodological approaches, surveillance strategies, microbial ecology, resistome dynamics, and innovative mitigation measures. Particular emphasis is placed on understanding both the environmental dimensions of resistance and the mechanisms linking anthropogenic activities with the evolution and spread of resistant microorganisms and resistance genes.

This Reprint is targeted at researchers, healthcare professionals, environmental scientists, policymakers, and other stakeholders interested in advancing scientific knowledge and developing effective solutions to one of the most pressing global challenges of our time.

Pedro A. Jiménez Gómez and Marina Robas Mora

Guest Editors

Article

Effective Control of *Salmonella* Enteritidis in Poultry by Dietary Supplementation with Microencapsulated Essential Oils

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Abstract: Background/Objectives: *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S. Enteritidis*) is a major pathogen associated with poultry products, and the rise of antimicrobial-resistant strains has intensified the need for effective natural control strategies. Essential oils (EOs) are recognized for their antimicrobial potential, but their volatility, instability, and risk of toxicity at high concentrations limit their practical application. Therefore, the objective of this study was to evaluate the antimicrobial efficacy of EOs in broilers infected with *S. Enteritidis* and to characterize potential synergistic or antagonistic interactions between the oils. **Methods:** To achieve this, the oils were first assessed through Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), and Fractional Inhibitory Concentration (FIC) assays, and the most effective ones against *S. Enteritidis* were selected. These selected oils were then microencapsulated and incorporated into the broiler feed for the in vivo assay. **Results:** The encapsulated formulation retained key bioactive compounds and significantly reduced bacterial shedding and intestinal colonization when administered to broilers experimentally infected with *S. Enteritidis*. Broilers receiving the optimized half-dose supplementation exhibited a 36% reduction in fecal shedding and a 4 log₁₀ decrease in cecal bacterial counts compared to untreated controls. A transient reduction in liver colonization was also observed, while feed intake remained unaffected. **Conclusions:** These findings demonstrate that microencapsulated EOs can serve as an effective natural strategy to control *S. Enteritidis* in poultry. The results support the broader application of lipid-based encapsulation technologies for improving the functional performance of phytobiotics in animal production.

Keywords: cinnamon; clove; oregano; phytobiotics; poultry farm; synergistic effect

1. Introduction

Salmonella enterica subsp. *enterica* serovar Enteritidis (*S. Enteritidis*) is among the most frequently isolated paratyphoid serovars in poultry products and human foodborne infections [1]. Although infection in poultry is often asymptomatic, the bacterium is still excreted and disseminated within and beyond the poultry environment [2]. In addition to its rapid spread, antimicrobial-resistant or multidrug-resistant *Salmonella enterica* strains

are frequently isolated from poultry production, likely due to the indiscriminate use of antimicrobials, posing a challenge to One Health [3–5].

Given the need to control the spread of *Salmonella* strains and the restrictions on conventional antimicrobials in poultry farming, novel antimicrobial products have been investigated worldwide. Among the natural alternatives, essential oils (EOs) have been proposed due to their reported antimicrobial activity against various avian paratyphoid *Salmonella* strains [6,7]. Different applications of these phytobiotics have been suggested in poultry farming, including carcass decontamination [8], performance enhancement in broilers [9], litter sanitization [10], egg disinfection [11], and antimicrobial agents to control *Salmonella* in poultry [12]. Although their precise mechanisms of action have not been fully elucidated, the antimicrobial activity of EOs is hypothesized to be associated with their interaction with the bacterial cell wall [13,14]. This effect may be even more potent when EOs are synergistically combined with conventional antimicrobials or other EOs, enhancing the efficacy of one or both agents, such as tetracycline and thyme [15].

Some limitations associated with the use of EOs as antimicrobials in poultry include high volatility, sensitivity to oxygen and/or luminosity, low water solubility, and rapid degradation in the upper gastrointestinal tract [16]. To mitigate these challenges, EO encapsulation technologies have been proposed as a promising approach to reduce volatilization, enhance bioavailability, and improve the delivery of these compounds within the host organism [17]. Microencapsulation allows the active compounds to be embedded within a protective matrix, reducing evaporation and degradation while facilitating controlled release at target sites, such as the intestinal tract [18].

Lipid-based matrices have shown favorable physicochemical compatibility with EO components, making them suitable carriers for feed applications [19–22]. Additionally, encapsulation can enhance storage stability, mask strong odors, and minimize adverse effects related to EO overdosing [23–25]. In this context, the present study aimed to evaluate the antimicrobial efficacy of microencapsulated EOs against *S. Enteritidis* in experimentally infected poultry and characterize the occurrence of synergism or antagonism among phytobiotics in vitro.

2. Results

2.1. In Vitro Results

Among the EOs evaluated, cinnamon EO exhibited the strongest antimicrobial activity against *S. Enteritidis*, with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 0.22 and 0.44 mg/mL, respectively. Oregano (MIC: 0.49 mg/mL), white thyme (0.57 mg/mL), clove (0.69 mg/mL), and mentha (0.69 mg/mL) also demonstrated significant inhibitory effects. In contrast, no inhibitory activity was detected for black pepper, ginger, copaiba, or sweet pear orange EOs, even at a maximum tested concentration of 10% (*v/v*) (Table 1).

Bactericidal activity, as indicated by MBC values, varied among the EOs. While cinnamon, oregano, and tea tree oils eliminated the pathogen at relatively low concentrations, other EOs—including basil, mentha, clove, white thyme, and eucalyptus—required concentrations four times higher than their respective MICs to achieve the same effect. These findings underscore a diverse range of bactericidal potential among the tested oils. All MIC and MBC values are summarized in Table 1.

In addition to individual efficacy, selected EOs were evaluated in combination to determine potential interactive effects against *S. Enteritidis*. Among the top-performing EOs—cinnamon, clove, oregano, white thyme, and mentha—synergistic, commutative, indifferent, and antagonistic interactions were observed, as detailed in Table 2. A synergistic interaction was identified between mentha and oregano, where the MIC values of

mentha and oregano were reduced by four- and two-fold, respectively, yielding a Fractional Inhibitory Concentration Index (FICI) of 0.75. In contrast, combinations of mentha with white thyme or cinnamon resulted in antagonistic effects, with FICI values of 8 and 6, respectively, and a four-fold increase in their MICs related to the individual oils. Several pairings—including oregano/cinnamon, oregano/white thyme, cinnamon/clove, white thyme/clove, and mentha/clove—exhibited commutative effects (FICI = 2), indicating additive but non-synergistic interactions. The combination of oregano and clove was classified as indifferent (FICI = 1), suggesting that the combined effect was neither enhanced nor diminished relative to the individual activities.

Table 1. MIC and MBC values of tested EOs against *S. Enteritidis*.

EOs	MIC (mg/mL)	MBC (mg/mL)
Oregano	0.49	0.99
Cinnamon	0.22	0.44
Clove	0.69	2.76
Bay laurel	4.56	4.56
Citronella	4.48	4.48
White thyme	0.57	2.28
Lemongrass	2.25	2.25
Rosemary	11.52	23.05
Tea tree	1.37	1.37
Black pepper *	-	-
Eucalyptus	5.83	23.32
Ginger *	-	-
Basil	5.79	23.17
Copaiba *	-	-
Sweet pear orange *	-	-
Mentha	0.69	2.77

*: no effect against *S. Enteritidis* was observed at a 10% (*v/v*) concentration of EOs.

Table 2. Fractional Inhibitory Concentration Index (FICI) and interaction classification of selected essential oil combinations against *S. Enteritidis*.

EOs Combinations	FICI	Classification
Oregano/Cinnamon	2	Commutative
Oregano/White thyme	2	Commutative
Cinnamon/White thyme	4	Antagonistic
Cinnamon/Clove	2	Commutative
Oregano/Clove	1	Indifferent
White thyme/Clove	2	Commutative
Mentha/White thyme	8	Antagonistic
Mentha/Clove	2	Commutative
Mentha/Cinnamon	6	Antagonistic
Mentha/Oregano	0.75	Synergistic

2.2. Encapsulation and Quantification of Active Compounds

Following the identification of synergistic effects between mentha and oregano EOs, a lipid-based encapsulation system was developed to enhance the stability of the bioactive compounds. For this, a hydrophobic matrix was employed to encapsulate a blend composed of cassia cinnamon, oregano, and clove EOs. The selected mass proportions were 92.26 g (cassia cinnamon), 104.84 g (oregano), and 146.77 g (clove), totaling 343.9 g of EOs. The encapsulation matrix consisted predominantly of fat (86%), with the EO blend comprising 11% of the total mass and silica acting as an adsorbent at 3%. The final powdered product reached a total mass of 3194.88 g. This approach aimed to protect the active compounds from volatilization and degradation.

The selection of cassia cinnamon, oregano, and clove EOs for encapsulation was based on their individual antimicrobial activity against *S. Enteritidis*, the chemical stability of their major constituents, and their compatibility with lipid-based matrices. Although the combination of mentha and oregano demonstrated *in vitro* synergism, mentha EO was excluded from the encapsulated formulation due to its antagonistic interaction with cassia cinnamon and white thyme EOs.

The average particle diameter of the microcapsules was $28.34 \pm 2.65 \mu\text{m}$ ($n = 20$), based on the mean of two perpendicular measurements per capsule. The observed values ranged from 25.45 to 33.81 μm . These results indicate a relatively uniform size distribution of the lipid-encapsulated EO particles (Figure S1).

High-Performance Liquid Chromatography (HPLC) analysis was performed to assess the retention of active constituents' post-encapsulation (Figure 1). The quantified markers included carvacrol and thymol (from oregano oil), eugenol (from clove oil), and cinnamaldehyde (from cinnamon oil). In the unencapsulated blend, the concentrations of carvacrol, thymol, eugenol, and cinnamaldehyde were 20.7%, 3.98%, 35.3%, and 19.9%, respectively (Table 3). Following encapsulation, the corresponding values decreased to 2.02%, 0.39%, 3.49%, and 1.99%, reflecting an approximately 10-fold dilution. It is important to clarify that the observed decrease in the concentrations of carvacrol, thymol, eugenol, and cinnamaldehyde post-encapsulation (approximately 10% of their initial values) reflects the dilution effect resulting from the incorporation of the essential oil blend into a much larger lipid matrix. This reduction does not represent compound degradation or poor recovery during encapsulation. Due to confidentiality agreements with the encapsulating company, quantitative recovery data (e.g., encapsulation efficiency) could not be disclosed. However, the consistent biological activity observed *in vivo* indicates that a functionally relevant proportion of the active compounds was retained and effectively delivered. This is consistent with previous reports describing encapsulation efficiencies ranging from 20% to 85% for lipid-based systems, depending on formulation strategies and analytical conditions.

Table 3. Percentage of active compounds in the EO blend (cinnamon + clove + oregano EO) before and after encapsulation.

Compounds	Before Encapsulation (%)	Post-Encapsulation (%)
Carvacrol	20.7	2.02
Thymol	3.98	0.39
Eugenol	35.3	3.49
Cinnamaldehyde	19.9	1.99

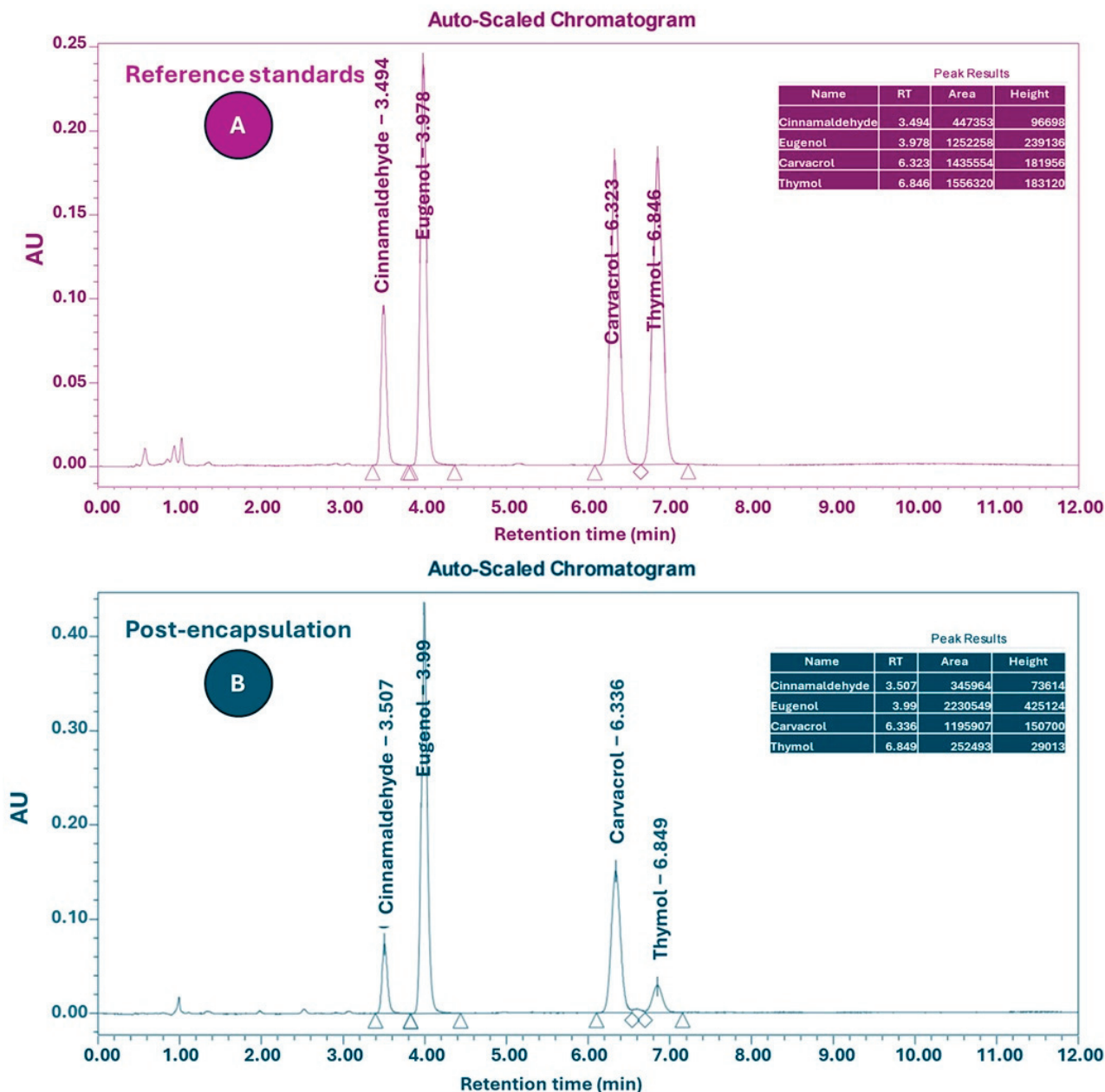


Figure 1. (A) HPLC retention profiles of analytical markers: cinnamaldehyde, eugenol, carvacrol, and thymol. (B) Chromatographic profile of the encapsulated EO blend analyzed using HPLC. Detection performed at 203 nm; major peaks correspond to retained active compounds within the lipid matrix.

Due to the different dilution factors applied to the sample and standards, the peak areas were not directly proportional. Therefore, the final percentages shown in Table 3 reflect concentration values corrected by both the dilution factors and standard curves, rather than raw or normalized peak area ratios.

Based on the concentration of active compounds in the encapsulated product, the recommended inclusion level in animal feed was calculated as 41 g/kg. This corresponds to 1.1 g of cinnamon oil, 1.25 g of oregano oil, and 1.75 g of clove oil per kg of feed, ensuring delivery of 4.1 g of the EO blend per kg. The encapsulated product was protected from light, heat, and moisture to maintain stability until use.

2.3. Chick In Vivo Results

To investigate the in vivo antimicrobial efficacy of the microencapsulated EO blend, a broiler infection model was used, involving oral challenge with *S. Enteritidis*. The EO blend—comprising cassia cinnamon (1.1 g/kg), clove (1.75 g/kg), and oregano (1.25 g/kg)—was administered in feed at two concentrations. HPLC analysis previously confirmed that encapsulation retained approximately 10% of the initial concentration of active compounds (see Section 2.2).

Fecal Shedding—Cloacal swab analysis revealed significant differences in *S. Enteritidis* fecal excretion across groups (Figure 2). Group A exhibited a 54.44% positivity rate (49/90), substantially lower than the 77.77% in group B (70/90) and 90% in group C (81/90). The 36% fewer positive swabs from fecal excretion in group A relative to the control was statistically significant ($p < 0.05$), indicating that the lower EO dose was more effective at limiting pathogen shedding.

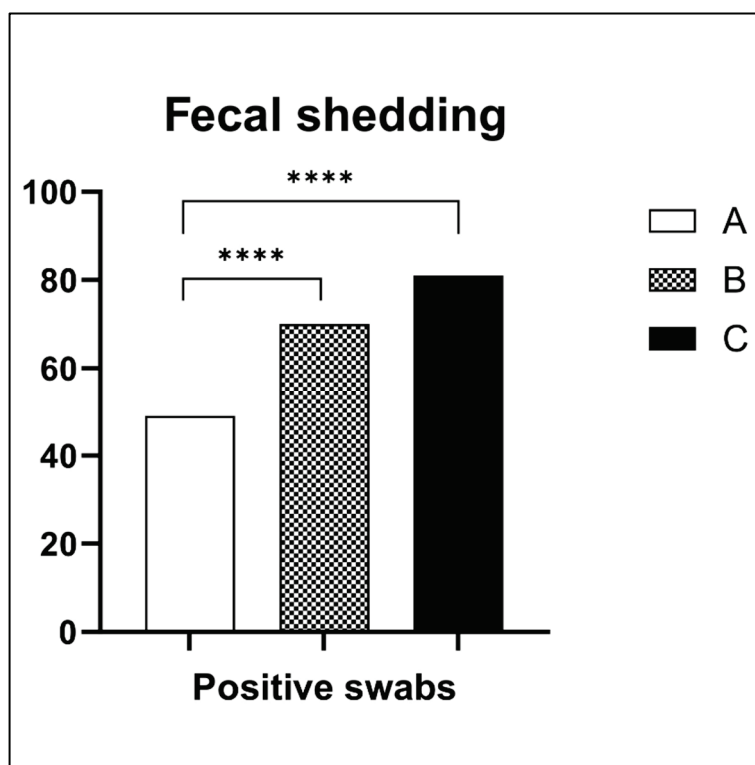


Figure 2. Fecal shedding of *S. Enteritidis* during 21 days of the experiment. Groups A (half dose of EO blend), B (full dose of EO blend), and C (non-treatment). A = 54.44% positive swabs for *S. Enteritidis*; B = 77.77%; C = 90%. **** High statistical difference between groups A/B and A/C.

Cecal Colonization—Bacterial enumeration in cecal contents (Figure 3I) confirmed the superior effect of the lower EO dose. At 7 dpi, group A chicks presented a significant 4 log₁₀ reduction in *S. Enteritidis* counts compared to group C ($p < 0.01$). This effect remained statistically significant at 14 dpi ($p < 0.01$) and persisted through 21 dpi ($p < 0.05$), demonstrating a sustained inhibitory effect of the microencapsulated EOs on intestinal colonization. In contrast, group B showed no significant improvement compared to the control.

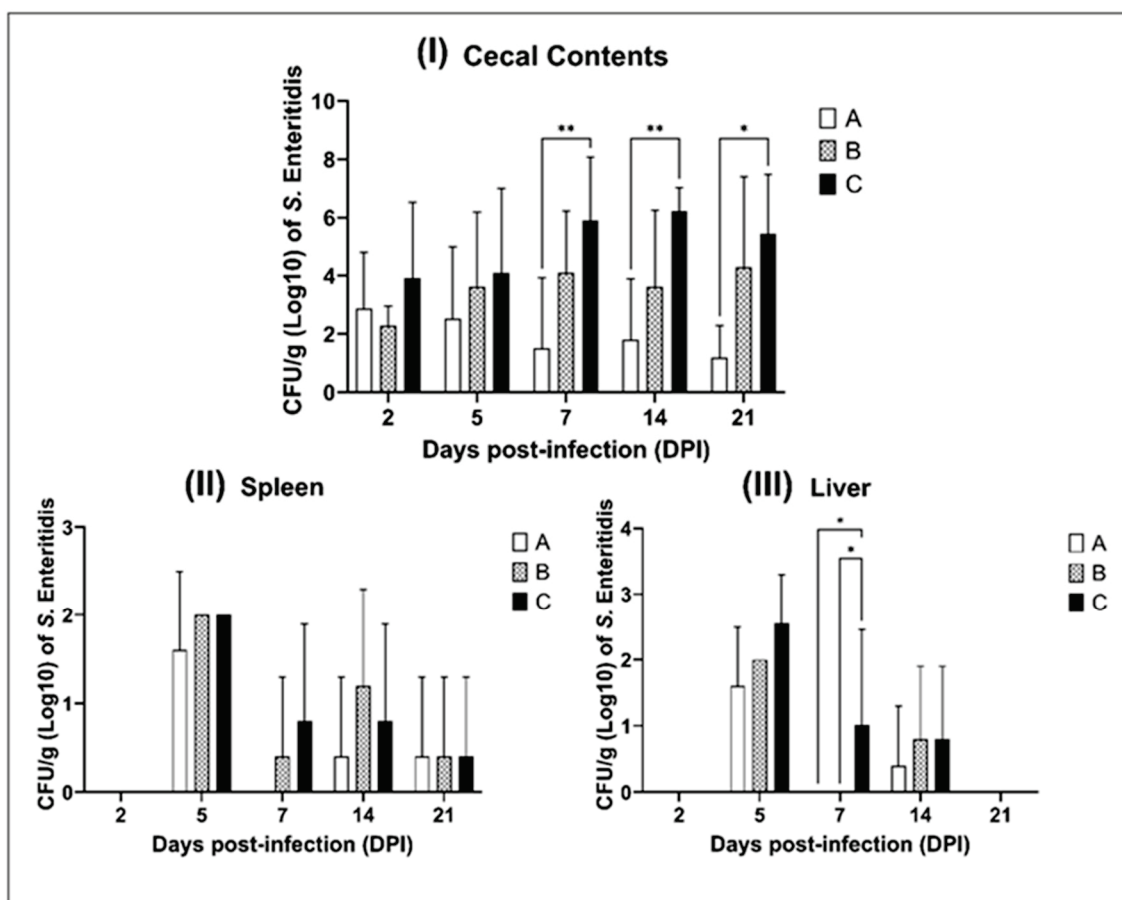


Figure 3. The viable CFU/g of cecal contents from chickens inoculated with *S. Enteritidis* (SE P125 109): (I); spleen (II); and liver (III). Group A (half dose of EO blend), group B (full dose of EO blend), and group C (non-treatment). * ($p < 0.05$); ** ($p < 0.01$); Statistical difference in comparison between groups A, B, and C using two-way ANOVA with Bonferroni's Multiple Comparisons test at 5% probability.

Systemic Dissemination: Liver and Spleen—Bacterial translocation to systemic organs was evaluated by enumerating CFUs in liver and spleen samples (Figure 3II,III). In the spleen, no significant differences in bacterial load were observed among groups at any time point. In the liver, however, a notable transient effect was detected at 7 dpi. Both groups A and B demonstrated significantly lower *S. Enteritidis* counts compared to group C ($p < 0.05$). While the reduction was more pronounced in group A, no significant differences were observed among groups at 14 and 21 dpi.

Daily feed Intake—Daily feed intake measurements (Figure 4) revealed no statistically significant differences between treated and untreated groups throughout the 23-day experiment. All groups followed a similar feed intake trajectory, indicating that the inclusion of the EO blend, regardless of concentration, did not impair feed palatability or broiler appetite. This supports the potential feasibility of EO inclusion in poultry diets from a production standpoint.

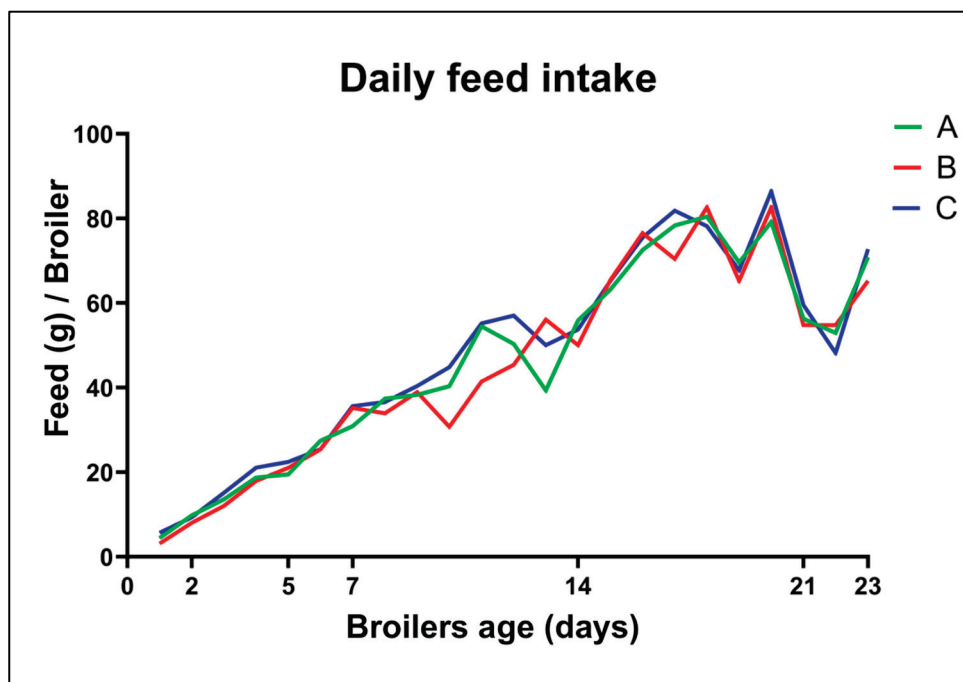


Figure 4. Average daily feed intake of broilers of groups A (half dose of EO blend), B (full dose of EO blend) and C (non-treatment).

Although no histological evaluation was performed, necropsy findings provided valuable insights into tissue-level responses. Macroscopic examination of all broilers from group B (7–14 dpi) revealed signs of intestinal mucosal congestion, hepatic discoloration, and yolk sac retention in several animals. These lesions were not observed in group A and were minimal in group C.

3. Discussion

EOs derived from culinary herbs—such as cinnamon, clove and oregano — have long been utilized for their antimicrobial properties, particularly in food preservation and safety applications [26]. Their increasing use as phytobiotics in poultry production aims to control enteric pathogens, such as *Salmonella enterica*, while minimizing antibiotic reliance [16]. However, their practical use is often limited by volatility, poor solubility, and challenges in maintaining effective concentrations at target sites. Microencapsulation technologies have emerged as a strategic solution, allowing gradual release along the gastrointestinal tract and improved systemic bioavailability [27]. This is especially relevant considering that non-encapsulated EO treatments often show lower reductions in cecal and cloacal colonization by *Salmonella* [12,28].

Our results confirm the *in vitro* activity of certain EOs against *S. Enteritidis*, with cinnamon EO demonstrating the strongest inhibitory and bactericidal activity (MIC = 0.22 mg/mL). This result aligns with previous findings attributing cinnamon's efficacy to cinnamaldehyde, a major active compound known to compromise bacterial membrane integrity and modulate gene expression [7,16,29]. Oregano, white thyme, mentha, and clove also displayed relevant antimicrobial effects, in line with reports documenting their phenolic components—such as thymol, carvacrol, menthol, and eugenol—as responsible for disrupting bacterial homeostasis [30]. In contrast, black pepper, ginger, copaiba, and sweet pear orange EOs exhibited no inhibitory activity against *S. Enteritidis* under the tested conditions, despite previous reports of their antimicrobial efficacy against other pathogens [8,31]. These discrepancies highlight the strain-specific nature of EO

activity, which can be influenced by factors such as bacterial membrane composition, pH, emulsification capacity, and oil solubility [32].

The bactericidal activity profile revealed that all inhibitory EOs could eliminate *S. Enteritidis* at concentrations ranging from $1 \times$ to $4 \times$ MIC, consistent with earlier studies showing similar MIC–MBC relationships for *Salmonella* spp. [6,33,34]. This suggests that although some EOs act as bacteriostatic agents at low concentrations, increasing the dose can shift their activity toward bactericidal effects, an important consideration for dosage optimization in feed applications. Moreover, we highlight that the study’s strength lies in the combinatorial evaluation of EOs interactions through FICI analysis — an approach seldom addressed in poultry or veterinary microbiology trials despite the common use of EO blends — and, notably, a synergistic interaction was observed between mentha and oregano EOs, resulting in substantial MIC reduction.

On the other hand, antagonistic effects were observed in combinations such as mentha with white thyme or cinnamon, where the MICs of both components increased significantly. This highlights a critical but often overlooked issue: the empirical mixing of EOs without mechanistic compatibility testing may lead to reduced effectiveness, or even counterproductive interactions. This reinforces prior warnings against arbitrary EO combinations [16] and emphasizes the need for rational formulation design; also, the indifferent interaction observed between oregano and clove ($FICI = 1$) and the predominance of commutative (additive) effects further underscore the complexity of EO interactions. Interestingly, while clove and cinnamon exhibited additive behavior in this study, previous research has shown them to be synergistic or antagonistic depending on the target microorganism [35,36]. For instance, synergy was reported against *S. Typhimurium* and *Aspergillus niger*, whereas antagonism was noted against *Escherichia coli* but not *Listeria monocytogenes* or *Yersinia enterocolitica*. These observations support the notion that EO interactions are highly context-dependent, influenced by microbial physiology, EO composition, and testing conditions [37], which can pose difficulty in the use of EOs. Overall, the initial findings of this study provide mechanistic and practical insights into the antimicrobial potential of individual and combined EOs. While some combinations can significantly enhance activity through synergism, others may compromise efficacy due to antagonistic interactions.

Although mentha EO demonstrated in vitro synergism with oregano, it was excluded from the final EO blend due to antagonistic interaction with cinnamon. This decision was based on preliminary FICI results that indicated a reduced antimicrobial effect when mentha and cinnamon were combined. Nevertheless, this exclusion may have overlooked potentially effective combinations, such as mentha + oregano without cinnamon. Future studies should consider testing alternative binary or ternary EO blends to further optimize antimicrobial efficacy while avoiding antagonistic interactions.

The encapsulation approach applied in this study represents a critical technological intervention to enhance the usability of bioactive compounds in animal nutrition and food science [38]. By embedding the bioactive compound blend into a lipid or polymeric matrix, the strategy aimed not only to reduce volatility and degradation but also to enable site-specific release in the intestinal tract—a key requirement for targeting enteric pathogens such as *Salmonella* sp. [18,39,40]. Rather than focusing on the quantitative loss during encapsulation—an inherent characteristic of the dilution process—this study demonstrates that biological efficacy can still be preserved even when active compound concentrations are significantly reduced. Although we did not assess the controlled release of the EO blend in this study, previous research using similar capsules has reported that their degradation occurs gradually along the gastrointestinal tract [23–25]. The observed antimicrobial performance in vivo, particularly in group A, supports the hypothesis that controlled

release and local delivery may compensate for lower nominal concentrations by improving compound stability and bioaccessibility at the site of infection [41].

The formulation, based on hydrogenated vegetable fat and silica, enables cold dispersion in feed mixtures and offers protection during storage and thermal processing, and these attributes are particularly relevant for industrial-scale feed production, where compound volatility and oxidative instability typically limit the phytogenic use [42]. Although the process preserved four key analytical markers (carvacrol, thymol, cinnamaldehyde, and eugenol), it is plausible that minor volatile or synergistic compounds were lost or chemically altered during encapsulation, potentially modifying the overall spectrum of activity [43]. In the present study, the microcapsules produced had an average diameter of 28 μm , which is considerably smaller than those reported in previous research [38,44], where capsule sizes reached approximately 32–2000 μm for poultry administration. Smaller particle sizes, such as those obtained in our formulation, may offer advantages in terms of more uniform blending into feed and easier ingestion by broilers, potentially improving the distribution and bioavailability of the active compounds [45,46]. Although exact encapsulation efficiency values could not be disclosed due to confidentiality agreements with the partnering company, the observed antimicrobial efficacy *in vivo* supports the hypothesis that the major bioactive compounds were adequately preserved and delivered. The apparent reduction in compound concentration to approximately 10% of the original values was due to intentional dilution in the lipid matrix, not compound loss. This aligns with previous reports showing encapsulation recoveries ranging from 20% to 85% in lipid- and polymer-based systems [23–25,38,44], indicating that the formulation applied here falls within the expected range for effective encapsulation in animal feed applications.

For the *in vivo* trial, a full dose of EOs was selected compared to those typically reported in poultry studies [9,12,47,48] to evaluate anti-*Salmonella* efficacy at elevated doses. While most studies focusing on growth performance use EO blends at 150–800 mg/kg [9,12], antimicrobial effects have been observed with much lower concentrations, such as 50–100 mg/kg of cinnamaldehyde or thymol, which reduced *S. Enteritidis* prevalence by up to 25% [49]. Another study combining 45 mg/kg of carvacrol, 100 mg/kg of cinnamaldehyde, 200 mg/kg of cineole, and 2 g/kg of capsaicin achieved a 3 log reduction in *S. Enteritidis* [28]. These findings suggest that concentrations ranging from 100 to 600 mg/kg per EO may be sufficient for pathogen control in poultry, although outcomes may vary depending on environmental challenges and the bioavailability of the active compounds in the host organism [50].

In our study, supplementation with the EO blend significantly reduced fecal shedding of *S. Enteritidis* by approximately 36% compared to the untreated control. This result is consistent with previous reports showing reductions in the shedding of *S. Heidelberg*, *S. Minnesota*, and *S. Typhimurium* following treatment with EOs and organic acids [51]. These findings underscore the potential of EO-based strategies to decrease horizontal transmission of paratyphoid *Salmonella* serovars in poultry production systems. A notable finding was the significant 4 log₁₀ reduction in cecal *S. Enteritidis* counts observed in broilers treated with half the recommended EO dose. This reduction was evident from 7 dpi and persisted through 21 dpi. Similar time-dependent antimicrobial effects have been reported in other studies, including reductions observed at 7 and 14 dpi [51] and even earlier, as reported by Hu et al. [12], where declines began at 3 dpi and extended to 10 dpi. These results reinforce the importance of defining an optimal dose–response curve to guide the future standardization of EO-based antimicrobials for poultry applications. Although the EO blend significantly reduced cecal bacterial load, no significant changes were observed in spleen colonization. However, a transient decrease in liver colonization was noted at 7 dpi in the treated groups. This may reflect a reduction in the translocation

of bacteria from the intestine to systemic organs, possibly due to improvements in gut integrity and microbial balance promoted by EOs [12].

Interestingly, the most pronounced antimicrobial effect was observed at the lower dose of the EO blend. This paradox may be explained by the non-selective antimicrobial properties of EOs, which, at higher concentrations, could disrupt the intestinal microbiota and damage the mucosal barrier. Supporting this hypothesis, Laptev et al. [52] reported marked shifts in the gut microbiota of EO-treated chickens infected with *S. Enteritidis*, including reductions in beneficial genera such as *Bifidobacterium* and *Lactobacillus*. These findings suggest that high EO doses may inadvertently compromise gut health and indicate potential dose-dependent toxicity associated with higher EO concentrations, aligning with previous reports of mucosal irritation or microbiota disruption linked to phenolic EO components [53–55]. The lower effectiveness and apparent adverse effects in group B may reflect the non-selective antimicrobial nature of the blend at excessive doses, potentially leading to gut dysbiosis or epithelial damage [56]. Daily feed intake remained unaffected by EO supplementation, indicating good palatability and compatibility of the blend at both tested concentrations. Similar results have been reported in the literature [9,57]. Although this study did not include zootechnical performance assessments due to the infection-focused design, prior research has shown that EO supplementation can improve the feed conversion ratio, enhance gut morphology, and reduce carcass fat content in broilers [9,58].

A key limitation of this study is the extrapolation of in vitro findings to in vivo conditions, which involve complex physiological variables such as digestive enzymatic activity, pH fluctuations, and interactions with the gut microbiota—factors that can significantly influence the efficacy of EOs. Moreover, the observed signs of mucosal and hepatic damage in broilers administered the higher EO dose highlight the importance of comprehensive toxicity assessments. Additional studies are needed to clarify the mechanisms of action of the encapsulated compounds; verify their systemic bioavailability, degree of homogenization of EO blends in poultry feed, and encapsulation efficiency; and evaluate their safety across a range of physiological conditions. Future research should also address the stability of the microcapsules during feed processing and storage, as well as their scalability and cost-effectiveness for commercial applications. Despite these limitations, the present findings remain robust and support the conclusion that lipid-encapsulated EO blends—when administered at appropriate doses—represent a promising, natural, and effective alternative to conventional antimicrobials for *Salmonella* control in poultry production systems.

4. Materials and Methods

4.1. Chemical Reagents

Mueller–Hinton Broth (MHB) was purchased from Oxoid (Hampshire, UK), and Brilliant Green Agar (BGA) from Kasvi (Spain). Selenite broth was obtained from Hi-Media (Mumbai, India), and Luria–Bertani (LB) broth from Sigma-Aldrich (St. Louis, MO, USA). Analytical standards, including carvacrol (W224502), thymol (T0501), cinnamaldehyde (C80687), and eugenol (E51791), were also obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS, pH 7.2) was prepared using analytical-grade reagents.

4.2. Essential Oils

Sixteen EOs were purchased from Destilaria Bauru[®] (São Paulo, Brazil), and detailed specifications from each oil, such as origin and plant species, are arranged in Table 4 (extracted by steam distillation). For characterization, 1 mL aliquots of each EO were placed into individual Eppendorf tubes and weighed to determine their densities (mg/mL). EO

aliquots (1 mL each) were individually placed into Eppendorf tubes, weighed to determine their densities (mg/mL), and stored under refrigeration (<5 °C) until the preparation of stock solutions. Stock solutions were initially prepared at a 1% EO (*v/v*) concentration, containing 1% Tween 80 as an emulsifier, with PBS (pH 7.2) completing the final volume; for EOs lacking antimicrobial activity at 1%, the concentrations were increased up to 10% EO. Finally, the stock solution was homogenized using a vortex mixer at 3800 rpm for five minutes to promote oil emulsification.

Table 4. Characteristics of each essential oil tested in vitro for antimicrobial activity against *Salmonella* Enteritidis.

Common Name	Scientific Name	Botanical Source	Geographical Origin
Oregano	<i>Origanum vulgare</i>	Leaf	Spain
Cinnamon	<i>Cinnamomum zeylanicum</i>	Bark	Brazil
Clove	<i>Eugenia caryophyllus</i>	Flower	Brazil
Mentha	<i>Mentha arvensis</i>	Leaf	Brazil
White thyme	<i>Thymus vulgaris</i>	Leaf	India
Citronella	<i>Cymbopogon nardus</i>	Leaf	Brazil
Lemongrass	<i>Cymbopogon flexuosos</i>	Leaf	Brazil
Rosemary	<i>Rosmarinus officinalis</i>	Leaf	Spain
Tea tree	<i>Melaleuca alternifolia</i>	Leaf	India
Black pepper	<i>Piper nigrum</i> L.	Fruit	India
Eucalyptus	<i>Eucalyptus globulus</i>	Leaf	Brazil
Ginger	<i>Zingiber officinale</i>	Root	China
Basil	<i>Ocimum basilicum</i> L.	Leaf	India
Copaiba	<i>Copaifera officinalis</i>	Leaf	Brazil
Sweet pear orange	<i>Citrus sinensis</i>	Fruit bark	Brazil
Bay laurel	<i>Laurus nobilis</i>	Leaf	Brazil

4.3. In Vitro Studies

4.3.1. Bacterial Strain

For this study, we selected an *S. Enteritidis* strain (SE P125 109) originally isolated from contaminated eggs and associated with human salmonellosis outbreaks in England. An overnight culture of *S. Enteritidis* was prepared by incubating 10 mL of MHB at 37 °C with orbital agitation at 150 rpm. After incubation, the bacterial suspension was diluted in phosphate-buffered saline (PBS, pH 7.2) and adjusted to approximately 10^{33} colony-forming units (CFU/mL) for use in vitro assays. All experimental procedures were carried out at the Department of Pathology, Reproduction and One Health (DPRSU), School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV/Unesp).

4.3.2. Antimicrobial Activity

MIC was performed in sterile 96-well microplates following the Clinical and Laboratory Standards Institute guidelines [59], with minor adaptations based on Munive Nuñez et al. [60]. Briefly, 95 µL of MHB was added to each well, then 100 µL of the EO stock solution was added to the first well of each row, except for the positive control, which contained MHB and bacterial suspension only. Using a multichannel micropipette, we slowly homogenized the stock solution into the MHB. After homogenization, a two-fold

serial dilution was performed by transferring 100 µL from the first to the subsequent wells. Next, 5 µL of the standardized bacterial suspension was added to all wells, except for the negative control (MHB + EO only), resulting in a final volume of 100 µL per well. Plates were incubated at 37 °C for 24 h. After incubation, 30 µL of 0.01% resazurin solution was added to each well, and results were obtained after 30 min, based on the visual reading of the colorimetric reaction occurrence, where blue indicated bacterial inhibition and pink indicated the absence of inhibition. The MIC was defined as the lowest EO concentration at which the well maintained a blue color.

For MBC, 10 µL measures from the last four wells showing no visible growth in the MIC assay were spot inoculated onto BGA plates, as described by Munive Nuñez et al. [60]. Plates were incubated at 37 °C for 24 h, and the MBC was defined as the lowest concentration at which no bacterial colonies were observed.

4.3.3. Synergism Assessment of Essential Oils

To evaluate the synergistic interactions between EOs, a Checkerboard assay was conducted using a 6 × 6 microplate format. Five EOs exhibiting the lowest MIC values against *S. Enteritidis* were selected, resulting in ten possible combinations. Stock solutions were individually prepared for each oil at sixteen times their respective MIC values (16 × MIC), mixed with 1% Tween 80, and adjusted to final volume with PBS.

For each EO combination, three 96-well microplates were prepared by initially dispensing 100 µL of MHB per well. On the first plate, 100 µL of EO from those exhibiting the lowest MIC values (in stock solution) was added into the first six wells of the initial column. After thorough homogenization, serial dilutions were performed horizontally by transferring 100 µL to subsequent columns. Similarly, on the second plate, 100 µL of another one from the lowest-MIC-value EO stock solution was added to the first six wells of the initial row, followed by vertical serial dilutions transferring 100 µL downward into subsequent rows. Afterwards, 50 µL from each of the first six columns of the first plate and 50 µL from each of the first six rows of the second plate were combined into corresponding wells on a third plate. This ensured that the highest concentrations of both oils were in the first well (top-left), progressively decreasing toward the lowest concentrations in the last well (bottom-right), as depicted in Figure 5. After combining these aliquots, 5 µL of bacterial suspension was added into each well, excluding the negative controls, maintaining identical conditions as previously described for MIC determination.

To validate the EO stock solutions, MIC tests were repeated on separate plates using the same five oil solutions. Checkerboard and MIC assay plates were incubated at 37 °C for 24 h. Following incubation, 30 µL of 0.01% resazurin solution was added to each well to interpret visually bacterial growth inhibition, employing the same criteria as the standard MIC assay. The FICI Index was calculated using Equation (1), where “first EO” and “second EO” denote the MIC values for each EO in the mixture and individually. The calculated FICI values were interpreted as synergistic ($FICI < 1$), indifferent ($FICI = 1$), commutative ($1 < FICI \leq 2$), or antagonistic ($FICI > 2$), according to Maggio et al. [15].

$$FICI = \frac{MIC^A_{mixture}}{MIC^A_{alone}} + \frac{MIC^B_{mixture}}{MIC^B_{alone}} \quad (1)$$

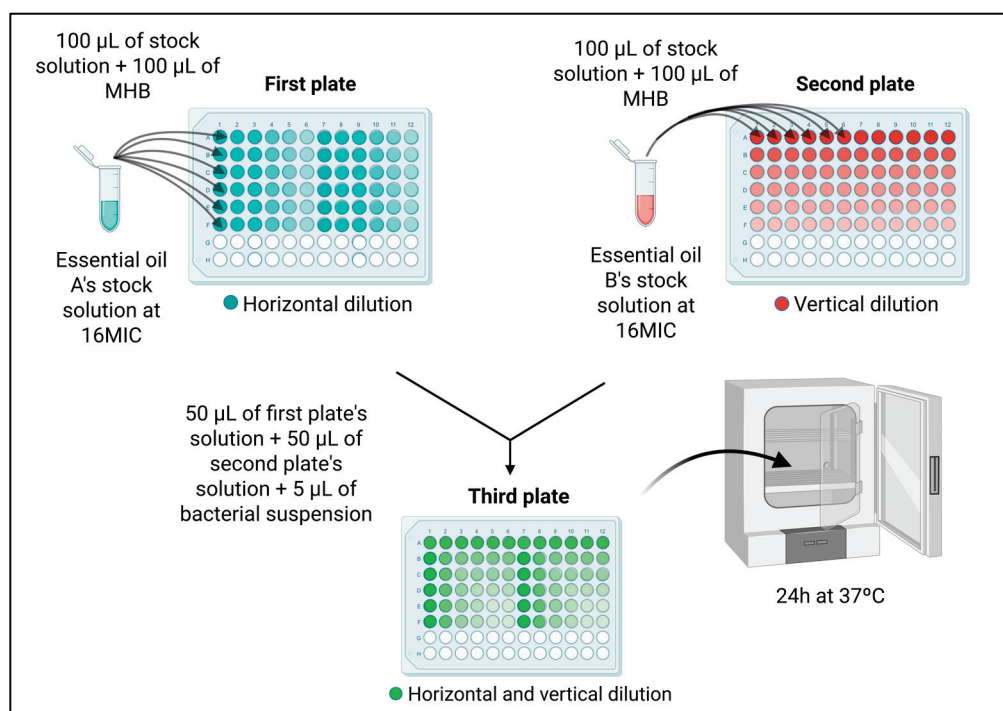


Figure 5. Method of the Checkboard assay. MHB = Mueller–Hinton Broth; MIC = minimum inhibitory concentration. This figure was created using Biorender.com.

4.4. Encapsulation Strategy and Analytical Assessment

Of five EOs exhibiting the lowest MIC values, three of them were selected based on their favorable interaction profiles, with no antagonism observed in previous in vitro assessments. These oils were encapsulated within a hydrophobic matrix designed to prevent volatilization and promote the bioavailability of EOs. The formulation strategy considered physicochemical compatibility and additive behavior among components, guided by principles derived from the FICI.

EOs were encapsulated in collaboration with Agrocere's Multimix Nutrição Animal Ltda., using hydrogenated vegetable fat as the encapsulation matrix. The process consisted of melting the fat, followed by the incorporation of EOs under continuous stirring until complete homogenization. The mixture was then cooled under controlled agitation, leading to the solidification of the lipid matrix. Agitation continued until a powdered product was obtained. Due to industrial confidentiality agreements, specific formulation details and processing conditions cannot be disclosed.

The particle size of the microcapsules was evaluated using optical microscopy (Olympus, DP73—Tokyo, Japan) with CellSens Standard[®] software, version 4.2 CS-ST-V4.2 (Olympus LS). Approximately 0.5 g of the encapsulated EO blend was deposited onto clean glass slides and imaged under a 20× objective lens. Two orthogonal diameters (length and width) were measured per capsule to account for possible asymmetry. Measurements were taken directly using the software and expressed in micrometers (µm). A total of 20 individual microcapsules were randomly selected and analyzed.

4.5. Analytical Performance

To evaluate the retention and stability of key active compounds, the encapsulated and non-encapsulated oils were analyzed in triplicate using chromatography (HPLC; Waters 2695 Separations Module), coupled with a photodiode array UV–Vis detector (Waters 2998, Photodiode Array Detector). Chromatographic separation was achieved using a C18 column (Agilent Technologies, 150 × 4.6 mm, 5 µm SB-C18) under isocratic conditions,

with detection at 203 nm. The column oven was maintained at 45 °C, with a flow rate of 1.0 mL/min and an injection volume of 10 µL. The concentrations of active compounds in the EO blend were determined by integration of the chromatographic peak areas obtained via HPLC, similar to the method described by Wang et al. [61]. Quantification was carried out using external standards for carvacrol, thymol, eugenol, and cinnamaldehyde, with calibration curves prepared from standard solutions at known concentrations. For sample preparation, 103.5 mg of the EO blend was extracted in 25 mL of methanol in an ultrasonic bath for 30 min. A second dilution was performed by adding 0.10 mL of this extract to 10 mL of mobile phase (composed of 50% acetonitrile and 50% Milli-Q water), resulting in a final concentration of 41.4 µg/mL. The standards were prepared independently, with final concentrations of 9.9 µg/mL for carvacrol and thymol, 3.2 µg/mL for eugenol, and 10.5 µg/mL for cinnamaldehyde. Quantification was performed both before and after encapsulation.

4.6. Ethical Approval

The in vivo study was reviewed and approved by the Ethics Committee on Animal Experimentation (CEUA) of the School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV/Unesp), under protocol number 2931/2024, on June 16, 2024.

4.6.1. Bacterial Inoculum

To prepare the inoculum, we used the same strain of *S. Enteritidis* (SE P125 109) as in the in vitro assay. This strain is resistant to nalidixic acid and spectinomycin (SE^{NalSpcR}). The strain was inoculated in LB broth and incubated at 37 °C for 24 h under constant agitation at 150 rpm. After incubation, the inoculum was diluted 10³.

4.6.2. Experimental Design

For this study, 135 one-day-old broiler chicks were obtained from a commercial hatchery. These were randomly distributed into three groups (A, B, and C) of 45 animals each. Poultry in group A received a half dose of the EO blend (205 g/10 kg of feed), poultry in group B received the full dose of the EO blend (410 g/10 kg of feed), and poultry in group C received no treatment (the control group).

Upon arrival, sterile swabs were used to collect samples from the bottom of the transport boxes to confirm that the newly hatched chicks were free of *Salmonella* spp. The poultry chicks were housed in acclimatized rooms, separately in metal cages and provided with water and feed *ad libitum*, following species and lineage-specific recommendations. Poultry treated with the EO blend received the product mixed with feed throughout the experiment. To this end, the EO blend was incorporated into the feed and manually homogenized for approximately ten minutes.

On the second day of age, chicks were inoculated into the crop using oral gavage needles with 0.2 mL *S. Enteritidis*^{NalSpcR} culture containing approximately 1×10^6 CFU. Twice weekly after the challenge, cloacal swabs were collected from 15 broilers per group to inspect the excretion of *S. Enteritidis*^{NalSpcR}. Then, the swabs were placed in tubes containing 3 mL of selenite broth plus 0.4% novobiocin. Samples were then streaked onto BGA plates supplemented with nalidixic acid and spectinomycin (BGA^{Nal/Spc}). The plates and swab-containing tubes were incubated at 37 °C for 24 h.

To assess cecal colonization and systemic infection, five broilers per group were euthanized at 2-, 5-, 7-, 14-, and 21-day post-infection (dpi) for *S. Enteritidis*^{NalSpcR} enumeration in the liver, spleen, and ceca. Bacterial enumeration was carried out according to the methodology adopted by Berchieri Junior et al. [62]. The obtained CFU/mL values were converted to Log₁₀ for statistical analysis and result interpretation.

To measure daily feed intake, the residual feed volume in the feeders was weighed daily and subtracted from the amount added the previous day at standardized times, ensuring a 24 h evaluation period. Data from all three groups were recorded daily in spreadsheets from the beginning to the end of the experiment.

4.6.3. Statistical Analysis

Statistical analysis was conducted using GraphPad Prism© software, version 10.2.3 for Windows©. Fecal shedding data from the three groups were plotted as positive and negative swabs and compared using the Chi-square test. Log-transformed bacterial counts in the liver, spleen, and ceca were analyzed using a two-way ANOVA, followed by Bonferroni's multiple comparison test. Data on daily feed intake were analyzed using one-way ANOVA and compared using the Chi-square test. Statistical significance was admitted when the p -value was lower than 5% ($p < 0.05$).

5. Conclusions

This study provides important insights into the *in vitro* and *in vivo* antimicrobial activity of various EOs against *S. Enteritidis*. Our results indicate that microencapsulated cinnamon, clove, and oregano EOs are an effective natural alternative for reducing the fecal excretion of *S. Enteritidis* and its systemic infection in broilers, without affecting the daily feed intake of treated broilers. These findings strongly contribute to the maintenance of the One Health approach and minimizing the emergence of resistant or multidrug-resistant bacteria originating from animal production. We emphasize the need for further research to determine optimal and potentially toxic concentrations for poultry, including the assessment of the effects of these phytobiotics on the gut microbiota and tissue integrity in broiler chickens. Therefore, further research exploring lower concentrations and their effects on infection and poultry's health is needed to establish optimal therapeutic dosing standards in broiler chickens.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/antibiotics14060552/s1>: Figure S1: Optical microscopy of the microcapsules present in the EO blend under a 20× objective lens. (A) Microcapsules; (B) Measurement of orthogonal diameters (length and width) using CellSens Standard© software, version 4.2 (CS-ST-V4.2).

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Institutional Review Board Statement: This study was conducted in accordance with current national and international guidelines for the use of animals in research, and with full respect for the welfare of the species involved. Moreover, the study was reviewed and approved by the Ethics Committee on Animal Experimentation (CEUA) of the School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV/Unesp), under protocol number 2931/2024, on 16 June 2024.

Informed Consent Statement: Not applicable.

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Conflicts of Interest: Nayla Pádua Del Bianco Gontijo Souki and Tarley Araújo Barros are affiliated with Agrocere Multimix Animal Nutrition, Rio Claro, Brazil, and declare a potential conflict of interest due to their employment. All other authors declare no conflicts of interest.

Abbreviations

The following abbreviations have been used in this article:

SE	<i>Salmonella enterica</i> subsp. <i>enterica</i> serotype Enteritidis
EOs	Essential oils
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
FICI	Fractional Inhibitory Concentration Index
CEUA	Ethics Committee on Animal Experimentation
MHB	Mueller–Hinton Broth
LBB	Luria–Bertani Broth
BGA	Brilliant Green Agar
Nal/Spc	Nalidixic acid/Spectinomycin
CFU	Colony-Forming Unit
v/v	Volume by volume
HPLC	High-Performance Liquid Chromatography

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Article

High Prevalence of Antimicrobial Resistance Genes in Rabbit Farms from Sumy Region, Ukraine

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Abstract: Background/Objectives: Antimicrobial resistance (AMR) poses a significant threat to public health, food security, and sustainable livestock production. Despite increasing concern, AMR remains poorly studied in cuniculture, particularly in regions where rabbit farming is predominantly small-scale and household-based. This study aimed to assess the prevalence and diversity of antimicrobial resistance genes (ARGs) in rabbit farms in northeastern Ukraine. **Methods:** A total of 100 fecal samples were collected from clinically healthy rabbits housed in two farms and one vivarium. DNA was extracted and analyzed using real-time PCR targeting 21 ARGs associated with resistance to major antibiotic classes, including tetracyclines, sulfonamides, β -lactams, macrolides, quinolones, carbapenems, and multidrug efflux systems. **Results:** A diverse and widespread resistome was identified. The most prevalent genes included *sul1* (96%), *blaTEM* (95%), *tetM* (94%), and *ermB* (93%). *AcrB* (78%) and *qnrS/oqxS* (approximately 67%) were also frequently detected. Carbapenemase genes (*blaKPC*, *blaNDM*, *blaVIM*) were identified in 6% of samples, despite limited veterinary use of carbapenems. Notably, 96% of samples harbored ARGs from three or more antibiotic classes, indicating a high potential for multidrug resistance. **Conclusions:** The findings demonstrate a high prevalence and diversity of ARGs in rabbit farming systems in northeastern Ukraine. The presence of clinically significant resistance genes, including those conferring carbapenem resistance, underscores the urgent need for targeted AMR surveillance and improved antibiotic stewardship in cuniculture, particularly in regions with minimal regulatory oversight of antimicrobial use.

Keywords: antimicrobial resistance genes; cuniculture; rabbits; Ukraine

1. Introduction

Antimicrobial resistance (AMR) is one of the most critical threats to global health, food safety, and sustainable animal production. It arises when microorganisms evolve mechanisms that render antimicrobials ineffective. This phenomenon severely compromises the ability to treat infections, leading to prolonged illness, increased mortality, and higher healthcare costs [1]. In addition to its impact on human health, AMR has major implications in the veterinary and agricultural sectors, particularly through the transmission of resistant pathogens from animals to humans via direct contact, food products, and environmental

routes [2]. Recognizing its complex and interconnected nature, the issue of AMR has been placed at the center of the One Health approach, which highlights the need for integrated efforts across human, animal, and environmental health disciplines [3].

The use of antibiotics in veterinary medicine plays a crucial role in ensuring animal welfare and productivity. Antimicrobials are employed not only for therapeutic purposes but also as metaphylactic and prophylactic agents to prevent disease spread and onset, particularly in high-density and intensive farming systems [4]. On livestock farms, antibiotics have also been employed for non-therapeutic purposes such as enhancing growth and feed efficiency, leading to prolonged exposure of animals to low, sub-lethal concentrations, particularly in the gastrointestinal tract [5–7]. These conditions suppress susceptible bacteria while favoring the survival of resistant strains, accelerating the development and spread of AMR. The risk is further amplified by the chemical similarity between many veterinary antibiotics and those used in human medicine, increasing the potential for cross-resistance and posing significant public health concern [8].

Rabbit farming in Ukraine is mainly carried out by private households and backyards, which account for about 97% of production, mostly for family consumption, while only a small fraction (3%) is produced by agricultural enterprises [9,10]. In 2018, the country produced around 12 thousand tonnes of rabbit meat (slaughter mass), accounting for 0.5% of the total domestic meat production and placing Ukraine 8th in global rabbit meat production, contributing 1.1% of the total [6,9]. The development of organic rabbit meat farming presents a significant economic opportunity for the Ukrainian industry compared to other animal sectors [7,11]. Nevertheless, the rabbit farming sector faces critical challenges related to antimicrobial usage. Rabbits raised for meat in industrial systems exhibit the highest levels of antimicrobial consumption among food-producing animals, contributing to the emergence of AMR within the industry [12–14]. This is particularly concerning given rabbits' high susceptibility to bacteria, which necessitates frequent antibiotic use, especially during stressful phases. The limited availability of veterinary medicines authorized specifically for rabbits often results in off-label and empirically guided antimicrobial use, further aggravating the risk of selecting multidrug-resistant bacteria [15]. These practices also raise environmental concerns, as resistance genes may be disseminated through fecal excretion and manure application. Despite the scale and intensity of rabbit farming, this sector remains underrepresented in AMR research and surveillance, and there is a notable lack of systematic data on antimicrobial use and resistance profiles, making it difficult to develop targeted mitigation strategies [9,16–18].

Understanding the use and impact of antibiotics in rabbit production is essential for developing evidence-based guidelines and interventions. This study aims to address this gap by investigating the administration, use practices, and resistance patterns associated with antimicrobial agents in rabbit farming. This work contributes to a broader understanding of AMR within animal production and highlights the need for species-specific surveillance and policy approaches.

2. Material and Methods

2.1. Sampling

In April 2024, 100 fresh fecal samples were collected from clinically healthy Californian domestic rabbits (*Oryctolagus cuniculus*), born and raised on two private farms (Farm 1 $n = 9$; Farm 2 $n = 80$) and in a vivarium ($n = 11$) inside a laboratory animal facility with uninfected animals in the Sumy region of northeastern Ukraine. Samples were collected directly from rabbit cages during routine cleaning, without handling or disturbing the animals, and transferred into individual sterile tubes to minimize the risk of contamination.

They were immediately stored at 4 °C and transported to the laboratory within 12 h of collection. Upon arrival, samples were preserved at −20 °C until DNA extraction.

The vivarium housed clinically healthy rabbits kept in cages in separate rooms and fed with specialized commercial feed and purchased vegetables; these animals were not intended for meat production. Farm 1 was a small family-type with rabbits kept in individual cages, fed with commercial feed, farm-grown vegetables, and hay from local fields, with slaughtering carried out on-site for household use only. Farm 2 was a larger family-type holding, with rabbits kept in a separate building in three-tiered cages, fed with alfalfa, hay, wheat, and barley mixture purchased from local farmers, and farm-grown vegetables; slaughtering was also limited to household consumption.

Although the rabbits themselves were confined to cages, environmental exposure may occur indirectly through feed sourced from local fields that could be fertilized with animal manure. This sample set provides an illustrative example of how the degree of animal contact with the external environment may influence resistance.

2.2. DNA Extraction

Fecal samples (0.5 g) were suspended in 560 µL of Buffer AVL (QIAGEN, Venlo, The Netherlands) containing carrier RNA, thoroughly mixed, and incubated at room temperature for 10 min to ensure complete lysis and pathogen inactivation. The suspensions were subsequently centrifuged at 8000 × *g* for 5 min, and 200 µL of the clarified supernatant was used for nucleic acid extraction. Total nucleic acids were isolated with the QIAamp Cador Pathogen Mini Kit (QIAGEN) on the QIAcube automated platform, following the manufacturer's instructions. The purified DNA was eluted in RNase-free water and stored at −80 °C until further analysis.

2.3. Molecular Detection of AMR Genes

The molecular detection of antimicrobial resistance (AMR) genes targeted several antibiotic classes: Tetracycline resistance genes (*tetA*, *tetC*, *tetM*, and *tetW*), Carbapenemases resistance genes (*blaNDM*, *blaVIM*, *blaKPC*), β-lactamase genes (*blaCTX-M-9-like*, *blaCTX-M-15-like*, and *blaTEM*), Macrolide resistance genes (*ermB* and *ermC*), Quinolones resistance genes (*qnrD*, *qnrS* and *oqxB*), Sulfonamide resistance genes (*sul1*, *sul2* and *sul3*), the Aminoglycosides resistance gene *aadD* and Multidrug resistance genes (*acrA* and *acrB*). For the amplification process, 0.5 µL of purified DNA was used in a real-time PCR (qPCR) reaction containing 5 µL of iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) and 0.4 µL of each primer (10 µM), making up a final volume of 10 µL. For each gene analyzed, positive controls consisted of environmental samples previously validated by Sanger sequencing as containing the respective resistance genes [19]. A negative template control, composed of the PCR mixture and RNase-free water in place of DNA, was included to check for potential contamination. The melting temperatures of ARGs were carefully analyzed, and amplification products outside the expected range were considered negative to minimize the risk of false positives. In addition, PCR products obtained in this study were confirmed by Sanger sequencing to ensure the reliability of the results by BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi> accessed 27th July 2025). The thermal cycling conditions were as follows: an initial denaturation step at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 10 s, annealing at a gene-specific temperature for 30 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 10 min and a hold at 4 °C. Primer sequences, annealing temperatures and expected product sizes for each gene are provided in Table 1. PCR products were separated on a 2% agarose gel prepared in 1 × TAE buffer (0.04 mol/L Tris-acetate, 0.001 mol/L EDTA, pH 8.0), stained with GreenSafe (Nzytech, Lisbon, Portugal), and visualized under UV light.

Table 1. Sequences of primers, annealing temperatures, and expected sizes of ARGs associated with various classes of antibiotics.

Class	Gene	Sequence (5' > 3')	Annealing Temperature (°C)	Product Size (bp)	Reference
Tetracyclines	<i>tetA</i>	F:GCTACATCCTGCTTGCCCTTC R:CATAGATCGCCGTGAAGAGG	60	210	[6]
	<i>tetC</i>	F:TGCAACTCGTAGGACAGGTG R:ACCAGTGACGAAGGCTTGAG	60	139	
	<i>tetM</i>	F:ACAGAAAGCTTATTATATAAC R:GGCGTGTCTATGATGTTTAC	51	171	
	<i>tetW</i>	F:GAGAGCCTGCTATATGCCAGC R:GGGCGTATCCACAATGTTAAC	60	168	
Carbapenemases	<i>blaNDM</i>	F:CAGTCGCTTCCAACGGTTTG R: AACGCATTGGCATAAGTCGC	60	217	[20]
	<i>blaVIM</i>	F:AAAACACAGCGGCACCTTCTC R: AATCTCGTTCCCTCTACCTC	60	182	
	<i>blaKPC</i>	F:CATTTCGCTAAACTCGAACAGG R: TTTTGCCGTAACGGATGG	60	201	
Extended-spectrum β-lactamases (ESBL)	<i>blaCTX-M-15like</i>	F:GCTGGTGACATGGATGAAAG R: TAGGTTGAGGCTGGGTGAAG	60	87	[20]
	<i>blaCTX-M-9like</i>	F:GTTGGTGACGTGGCTCAAAG R: GTTGCGGCTGGGTAAAATAG	60	89	
	<i>blaTEM</i>	F:TCTGACAACGATCGGAGGAC R: TGCCGGGAAGCTAGAGTAAG	60	86	
Macrolides	<i>ermB</i>	F:AGGGTTGCTCTTGACACTC R: CTGTGGTATGGCGGGTAAGT	58	119	
	<i>ermC</i>	F:GAAATCGGCTCAGGAAAAGG R: TAGCAAACCCGTATTCCACG	56	292	
Quinolones	<i>qnrD</i>	F:GCTGGTGACATGGATGAAAG R: TAGGTTGAGGCTGGGTGAAG	57	373	
	<i>qnrS</i>	F:GTTGGTGACGTGGCTCAAAG R: GTTGCGGCTGGGTAAAATAG	55.3	134	
	<i>oqxB</i>	F:TCTGACAACGATCGGAGGAC R: TGCCGGGAAGCTAGAGTAAG	60	131	
Sulfonamides	<i>sul1</i>	F:TGTGCAACCTTCAAAGCTG R: TGGACCCAGATCCTTTACAG	60	113	[6]
	<i>sul2</i>	F:ATCTGCCAAACTCGTCGTTA R: CAATGTGATCCATGATGTCG	60	89	
	<i>sul3</i>	F:AGGCTTGGCAAAGTCAGATT R: CACCAGCCTCAACTAAAGCA	57	152	
Aminoglycosides	<i>aadD</i>	F:ATGGGGATGATGTTAAGGCT R: TCACTTCCACCTTCCACTCA	55	153	
Multi-drug	<i>acrA</i>	F:CTCTCAGGCAGCTTAGCCCTAA R: TGCAGAGGTTTCACTTTGACTGTT	60	107	
	<i>acrB</i>	F:GGTCGATTCCGTTCTCCGTTA R:CTACCTGGAAGTAAACGTCATTGGT	60	105	

2.4. Statistical Analysis

Each sample was examined for the presence or absence of ARGs, and the results were reported as the proportion of positive samples out of the total analyzed, along with the corresponding 95% confidence interval (95% CI). Data processing and analysis were carried out using Microsoft Excel[®] for Microsoft 365 MSO (Redmond, WA, USA), and Fisher's exact test was used to assess differences in the presence of antimicrobial resistance genes and classes between farms using RStudio version 4.4.2 (Boston, MA, USA).

3. Results

A comprehensive analysis of rabbit stool samples by PCR revealed a widespread and diverse set of ARGs encompassing multiple antibiotic resistance classes (Figure 1A). The

most abundant resistance genes were detected within the tetracycline, sulfonamide and β -lactamase classes. Among tetracycline resistance genes, *tetA*, *tetM* and *tetW* were highly prevalent, being present in 94, 87 and 76 samples, respectively. Sulfonamide resistance genes *sul1* ($n = 96$), *sul2* ($n = 88$), and *sul3* ($n = 49$) were also frequently detected.

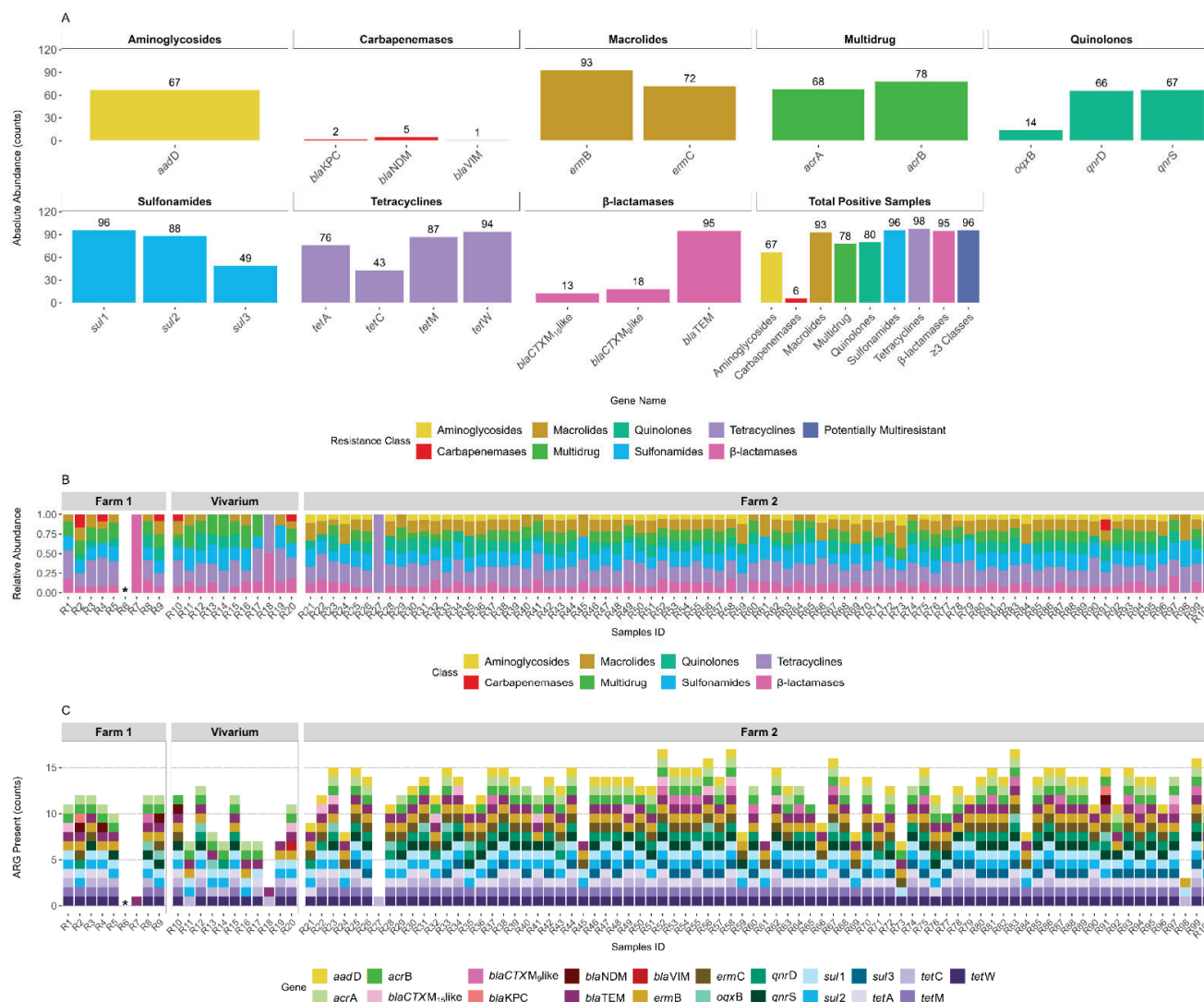


Figure 1. Abundance and distribution of antibiotic resistance genes (ARGs) across sampling sites and environments. **(A)** Absolute abundance of detected ARGs grouped by resistance class: aminoglycosides, carbapenemases, macrolides, multidrug resistance, quinolones, sulfonamides, tetracyclines and β -lactamases. For each gene within a class, bar height represents the total number of detections across all samples, with the numeric value indicating detection count. The rightmost panel summarizes the number of positive samples per resistance class and samples carrying ARGs from three or more classes. **(B)** Relative abundance of ARG classes in each sample across Farm 1, Vivarium and Farm 2 environments. Each bar represents the proportional composition of ARG classes within a single sample. Samples are sorted by source and sample ID. **(C)** ARG richness per sample, shown as the total count of distinct ARGs detected per sample. Colored segments within each bar represent specific ARGs, with colors corresponding to resistance classes as shown in the legend. * Represents the absence of ARGs.

Within the β -lactamase class, *blaTEM* was the most abundant gene ($n = 95$), followed by *blaCTX-M-15like* ($n = 18$) and *blaCTX-M-9like* ($n = 13$). Macrolide resistance was primarily driven by *ermB* ($n = 93$) and *ermC* ($n = 72$), while multidrug efflux pump genes *acrA* ($n = 68$) and *acrB* ($n = 78$), were detected at similar frequencies.

Aminoglycoside resistance was represented by the gene *aadD* ($n = 67$), whereas quinolone resistance genes *qnrB*, *oqxB*, and *qnrS* were identified at similar levels ($n = 66$ – 67). In contrast, carbapenemase genes were scarce, with *blaKPC* ($n = 5$), *blaNDM* ($n = 2$), and *blaVIM* ($n = 1$) detected at low frequencies, indicating limited but non-negligible dissemination of resistance to last-resort antibiotics.

The total number of positive samples per ARG class showed high levels of resistance gene diversity in nearly all samples, with the majority (93–98%) testing positive for genes in the tetracycline, sulfonamide and β -lactamase classes. Multidrug and macrolide resistance genes were also widespread, detected in 78% and 80% of samples, respectively. Only six percent of samples harbored carbapenemase genes, underscoring their relative rarity. Overall, 96% of the samples carried more ARG from three or more antibiotic classes, falling in the scope of multiresistant potential.

The relative abundance of ARGs across samples, normalized to total ARG content (Figure 1B), demonstrated consistent compositional patterns within and between sampling sources. Samples from both Farm 1 and Farm 2 exhibited relatively even distributions across resistance classes, although tetracycline, sulfonamide, and β -lactamase genes typically accounted for the largest proportional shares. In contrast, samples from vivarium animals displayed a slightly lower representation of multidrug and macrolide resistance genes.

Despite variations in individual gene presence, the class-level profiles remained relatively stable across management conditions, suggesting a core resistome structure shared across different environments.

Antibiotic resistance genes (ARG) richness, defined as the number of distinct ARGs detected per sample (Figure 1C), ranged from zero to 17 genes. Samples from Farm 2 exhibited the highest overall ARG diversity, with many samples presenting 12 or more distinct ARGs. In contrast, vivarium-derived samples displayed a lower richness (typically under 10 ARGs per sample). Farm 1 showed intermediate values but with considerable inter-sample variability, exception for sample R6 that did not harbor any ARG. In total, the number of ARG detections across all samples was $n = 81$ for Farm 1, $n = 93$ for the vivarium and $n = 1018$ for Farm 2.

Analysis of antimicrobial resistance across the three farms revealed significant variation at both the class and gene levels. Among resistance classes, aminoglycosides, carbapenemases, macrolides, quinolones, sulfonamides and tetracyclines showed adjusted p values < 0.05 indicating uneven distribution across farms while multidrug resistance genes and β -lactamases showed adjusted p values > 0.05 suggesting similar prevalence between farms. At the gene level several determinants including *aadD*, *blaNDM*, *ermB*, *ermC*, *qnrD*, *qnrS*, *sul1*, *sul3*, *tetA*, *tetC*, *tetM* and *tetW* had adjusted p values < 0.05 indicating significant differences between farms with *aadD*, *ermC*, *sul3*, *qnrD* and *ermB* exhibiting particularly strong associations. In contrast *acrA*, *acrB*, *blaCTX M 15 like*, *blaCTX M 9 like*, *blaKPC*, *blaTEM*, *blaVIM*, *oqxB* and *sul2* had adjusted p values > 0.05 consistent with a more stable distribution across the three farming environments.

4. Discussion

The present study characterizes ARGs in rabbit stool samples collected from both farming environments and laboratory facilities in Sumy region, Ukraine. The findings reveal a rich and diverse profile of ARGs, dominated by those conferring resistance to tetracyclines, sulfonamides, β -lactams, and macrolides. These results confirm a widespread presence of resistance determinants in rabbit stool, mirroring patterns observed in other livestock, and likely shaped by ongoing antimicrobial usage in animal production systems [21–24].

In commercial rabbit farming, three bacterial pathogens stand out due to their prevalence and clinical impact namely the most common, *Pasteurella multocida*, *Escherichia coli*,

and *Staphylococcus aureus* [1,25,26]. These bacteria are responsible for respiratory, enteric and cutaneous infections, respectively [27–29], and are commonly treated with antibiotics from the same classes in which we detected a high prevalence of ARGs (tetracyclines, β -lactams, sulfonamides, and macrolides). Providing biological context to our findings, as the frequent therapeutic use of these antimicrobials against such pathogens in rabbits may have contributed to the selective pressure shaping the resistome profile observed in this study. Although information on antibiotic use in Ukraine cuniculture is scarce, previous studies reported the widespread use of tetracyclines, β -lactamase, sulfonamides, macrolides, aminoglycosides and quinolones [29,30].

The highest prevalence of ARGs corresponds to the tetracycline class, detected in almost every sample. These findings suggest selective pressure and align with the literature, which identifies tetracyclines as among the most frequently used antibiotics against diverse pathogens in this animal species. This prevalence likely stems from the extensive use of these antibiotic classes in Ukrainian agriculture. Tetracyclines represent one of the most extensively used classes of antimicrobials in veterinary medicine worldwide and hold a particularly dominant position in Ukrainian agriculture, where they account for the largest proportion of veterinary antimicrobial sales [31]. Although antimicrobial use has been regulated since 2006, some authors suggest that reductions have been modest [18,32,33]. Furthermore, recent conflict in Ukraine may have facilitated increased antibiotic acquisition and use, potentially weakening regulatory oversight. In some cases, therapeutic use has been replaced by metaphylactic or prophylaxis administration.

The high detection rate of *blaTEM*, a gene encoding resistance to commonly used β -lactam antibiotics and the presence of extended-spectrum β -lactamase (ESBL) genes like *blaCTX-M-15like* and *blaCTX-M-9like*, raise particular concern [34,35]. Although these ESBLs were found with less prevalence ($n = 13$ and $n = 18$, respectively), their detection in a non-priority species such as rabbits highlights a potentially overlooked reservoir for resistance genes of high clinical importance. The co-occurrence of macrolide resistance genes (*ermB*, *ermC*) and multidrug efflux pumps (*acrA*, *acrB*) in a substantial proportion of samples further indicates that multidrug resistance profiles may be maintained within this animal population. Indeed, 96% of samples harbored ARGs from three or more resistance classes, suggesting that many of the microbial communities associated with these animals may already possess broad resistance phenotypes.

Although carbapenemase genes were detected at low frequencies, their presence should not be overlooked. The identification of *blaKPC*, *blaNDM*, and *blaVIM*, albeit in a handful of samples, is troubling, given that carbapenems are not licensed for veterinary medicine due to risks to public health [36]. Their presence may be attributed to co-selection via mobile genetic elements or environmental contamination linked to human or industrial sources [37,38]. Given the mobility of these genes and their association with hospital-acquired infections, even low-level detection in animal reservoirs demands attention [39].

The relative abundance data confirmed that tetracycline, sulfonamide, and β -lactam ARGs consistently dominated the resistome across all sources, including both farms and laboratory animals. This distribution likely reflects differences in the frequency of use of these antibiotic classes in rabbit husbandry, the long-term persistence of their resistance determinants in bacterial populations, and the frequent association of tetracycline and sulfonamide resistance genes with mobile genetic elements that facilitate their dissemination [40]. Laboratory animals showed a slightly lower representation of macrolide and multidrug resistance genes, likely reflecting minimal or no direct antibiotic exposure in controlled settings. While class-level resistome profiles were broadly consistent between farms, supporting the existence of a core resistome maintained across environments, some variation emerged. For instance, aminoglycoside resistance genes were detected only in

Farm 2, possibly indicating differences in antibiotic administration. However, due to the lack of antibiotic usage data, this remains speculative.

The number of ARGs per sample ranged from 0 to 17. Farm 2 exhibited the highest richness, likely owing to greater environmental microbial diversity, the number of samples being superior or more frequent antimicrobial exposure. Animals at this farm cohabit with other livestock, share manure, and receive the same feed, which may contribute to prominent diversity. In contrast, Farm 1 showed more variable ARG profiles, while laboratory animals presented the lowest diversity, consistent with their limited environmental exposure. These findings hold several important implications for public health, particularly considering the role of rabbits in the Ukrainian food system [9]. In 2023 rabbit meat production reached an estimated 8.400 tones according to FAOSTAT [41]. Because rabbit meat is often processed outside industrial slaughter systems usually in private households, increasing the risk of fecal contamination and subsequent human exposure to resistant bacteria or resistance genes [42]. Inadequate hygiene during slaughter or insufficient cooking could lead to the ingestion of ARG-carrying bacteria, with potential colonization of the human gut microbiome [43]. Farm workers and individuals in close contact with rabbits are particularly at risk of direct transmission through handling of animals, bedding, or feed [44].

In addition to direct contact with rabbits or their environment, humans may be exposed to these ARGs through multiple indirect pathways. Consumption of meat or other products, especially if processed under suboptimal hygiene conditions or insufficiently cooked, can facilitate the ingestion of ARG-carrying bacteria [45]. Environmental dissemination is also plausible, as manure used as fertilizer or runoff from farms may contaminate soil and water sources, creating opportunities for ARGs to enter the broader human microbiome [46,47]. Occupational exposure remains a critical route, particularly for farm workers and laboratory personnel handling animals, bedding, or feed, where repeated contact could increase the likelihood of colonization or transfer of resistant bacteria [44,48,49]. Comparing our findings to existing studies reveals a consistent pattern of dominant resistance classes across different regions. In Italy [50], the most prevalent antibiotic resistance classes in *S. aureus* isolates from rabbits were tetracyclines (95.8%), macrolides (93.8%), and quinolones (63.5%). These data closely mirror our own results, where tetracycline and macrolide resistance genes such as *tetM*, *tetW*, *ermB*, and *ermC* were among the most frequently detected, suggesting the presence of a conserved resistome structure within rabbit production systems.

Similarly, studies in Portugal have reported widespread *E. coli* resistance to multiple key antibiotics, including tetracycline (91.6%), ampicillin (100%), aztreonam (97.8%), streptomycin (93.7%), tobramycin (64.58%), trimethoprim/sulfamethoxazole (75%), amoxicillin-clavulanic acid (54.16%), and chloramphenicol (72.9%) [24]. These phenotypic resistance patterns correspond closely with our detection of sulfonamide genes (*sul1*, *sul2*, *sul3*), β -lactamase genes (*blaTEM*, *blaCTX-M-15like*, *blaCTX-M-9like*), and aminoglycoside resistance genes such as *aadD*. The identification of extended-spectrum β -lactamase (ESBL) genes like *blaCTX-M* variants in both our study and the Portuguese literature further underscores the presence and circulation of clinically significant resistance determinants in rabbit-associated microbial communities.

The identification of multiple clinically significant ARGs, including ESBLs and even carbapenemases, supports the potential for these resistance determinants to spread to human pathogens through direct contact with rabbits or their feces during husbandry and handling, or via the food chain through the consumption of contaminated meat products [51]. The possibility of horizontal gene transfer, particularly through plasmids or integrative elements not characterized in this study, remains high and warrants further investigation. While phenotypic resistance, gene expression, and genomic context were

beyond the scope of this study, the co-detection of multiple ARGs within individual samples suggests the involvement of mobile genetic elements that facilitate gene clustering and horizontal transfer. This underscores the need to address these gaps in future research.

Despite the interesting findings of this study and the notable prevalence of ARGs, several limitations must be addressed in future research. Namely, the aim of this study was limited to the assessment of presence or absence of ARGs but not quantification of their abundance. While this approach is suitable for assessing the diversity and distribution of resistance genes across farms, it does not capture differences in gene load or relative prevalence, which may have important implications for antimicrobial resistance risk. Future work should therefore incorporate abundance-based analyses to complement presence/absence data and provide a more quantitative understanding of the resistome. The stronger focus on Farm 2 reflects the greater availability of animals and practical constraints at the time of sampling; however, this imbalance may limit the representativeness of the findings. To estimate ARG distribution patterns across the Ukrainian cuniculture industry, extensive geographical sampling is needed, increasing both the number of samples and sampling locations. Finally, to gain a more comprehensive understanding of the gut resistome, metagenomic approaches using next-generation sequencing should be employed, as they could provide a broader and more detailed characterization of ARGs.

In the context of Ukraine's evolving veterinary regulatory framework, this study provides molecular evidence that rabbits carry a diverse array of antimicrobial resistance genes (ARGs), including several with known clinical relevance. Although the study is limited by the absence of phenotypic resistance profiles and records of antimicrobial usage, the breadth of ARGs detected highlights the need to include non-traditional livestock species such as rabbits in national AMR surveillance systems. Current monitoring efforts primarily target major food-producing animals, while rabbits are often raised in small-scale or informal production systems that fall outside the scope of regulatory oversight. This gap presents a risk, as these animals may contribute to the dissemination of resistance. Expanding surveillance to include rabbit farming would enable earlier identification of high-risk resistance patterns and support more effective mitigation strategies. It is also essential to improve traceability of antimicrobial use in rabbit production and to encourage practices that align with international standards for responsible antimicrobial stewardship. These steps are necessary to better assess AMR risks from small livestock and reduce the potential for transmission to human and environmental systems.

5. Conclusions

This study reveals a high prevalence and diversity of ARGs in rabbit farms in north-eastern Ukraine, highlighting notable risks to both animal and public health. Nearly every fecal sample harbored ARGs from multiple antibiotic classes, including clinically significant genes such as *bla*CTX-M, *erm*B, and efflux pumps like *acr*A and *acr*B. Although genes conferring resistance to carbapenems were seldom detected, their presence is especially concerning, given their restricted use in veterinary medicine and their use as a last resort for a variety of different human bacterial infections. The consistency of ARG profiles across farms suggests a stable resistome shaped by ongoing antimicrobial pressure. Current veterinary oversight may be insufficient, particularly in small-scale or informal production systems.

These findings underscore the need for stronger antibiotic stewardship, targeted surveillance in non-traditional livestock, and One Health-aligned strategies to curb the spread of resistance and safeguard both human and animal health.

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Article

Gut Resistome and Hearing Loss in Young Adults: A Preliminary Study on the Interplay Between Microbial Resistance and Auditory Health

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Abstract: Background: Hearing loss (HL) affects more than 1.5 billion people worldwide and represents a major global health concern. Recent evidence suggests that alterations in gut microbial composition and antimicrobial resistance (AMR) may be linked to inflammatory and metabolic pathways that could influence auditory physiology. **Objectives:** This study aimed to explore the relationship between auditory function and the antimicrobial resistance in the gut microbiome of young adults. **Methods:** Fecal and auditory data were collected from young adults. Auditory function was assessed through pure-tone audiometry, and participants were classified according to the presence or absence of HL based on the American Speech-Language-Hearing Association (ASHA) criteria. Bacterial resistance was analyzed under aerobic and anaerobic conditions using disk diffusion and E-test methods to determine minimum inhibitory concentrations (MICs) for a panel of antibiotics. Gut microbiota composition was further characterized using quantitative polymerase chain reaction (qPCR) to quantify 15 key microbial taxa. **Results:** Overall, 40.9% of participants presented some degree of HL, with mild or slight HL being more frequent in women (53.3%) than in men (14.3%). Participants with HL exhibited significantly higher MICs for nalidixic acid, amoxicillin, and ciprofloxacin, as well as trends toward increased MIC variability for several other agents. Principal component analysis demonstrated distinct clustering of individuals without HL and greater dispersion among those with HL, suggesting higher interindividual variability in resistance profiles. These findings suggest potential associations between antimicrobial resistance and auditory function, possibly mediated through gut microbiome alterations. qPCR analyses demonstrated that *Faecalibacterium prausnitzii* abundance was significantly higher in individuals with HL and in those exhibiting greater resistance to amoxicillin. **Conclusions:** These findings provide preliminary evidence connecting the gut resistome with auditory function, supporting the emerging concept of a gut–ear–brain axis and underscoring the need for further research into microbiome-related mechanisms underlying HL.

Keywords: hearing loss; microbiome; resistome; microbial diversity

1. Introduction

Hearing loss (HL) affects more than 1.5 billion people worldwide and represents a major global health problem due to its profound social, economic, and healthcare implications [1]. Its prevalence is rising globally, particularly among women and individuals aged 15 to 49 years, reflecting a concerning trend in younger populations [2]. This increase is largely attributed to recreational and occupational noise exposure, including attendance at loud music events and the use of personal listening devices (PLDs) or other unsafe listening practices [3–5]. The World Health Organization (WHO) recognizes recreational noise exposure as one of the leading preventable causes of sensorineural HL in young people, contributing to noise-induced HL. It is estimated that more than one billion individuals aged 12–35 years are exposed to potentially harmful sound levels through PLD use, posing serious risks to both physical and mental health [6].

The etiology of sensorineural HL is multifactorial, involving factors such as age, genetic predisposition, acute or chronic noise exposure, use of ototoxic drugs, and degenerative processes associated with chronic diseases [7–9]. Beyond these classical factors, recent evidence suggests that systemic elements, particularly the intestinal microbiome, may also influence auditory function [10]. Studies in animal models and preliminary human research indicate that the gut microbiome can modulate neuronal and sensory processes via immune regulation, metabolic signaling, and inflammatory pathways. In this context, it has been proposed that the effects of noise and other environmental factors on hearing may be mediated by complex systemic interactions, including a bidirectional relationship between the gut microbiome and the nervous system [10–13]. Within this framework, the concept of the gut–ear–brain axis has been proposed, suggesting that interactions among the microbiota, auditory system, and brain could influence the onset and progression of hearing loss, underscoring the importance of integrating dietary, environmental, and auditory factors [13,14].

In addition to this systemic framework, antimicrobial resistance (AMR) represents another pressing concern, driven by the excessive or inappropriate use of antibiotics [15]. AMR currently accounts for at least 700,000 deaths annually worldwide, a number projected to increase to nearly 10 million by 2050 in the absence of effective containment strategies [16]. Beyond increased mortality, AMR contributes to higher patient morbidity, greater healthcare costs, and the need to use more potent or broader-spectrum antibiotics [17], some of which have potential ototoxic effects, such as aminoglycosides [18] and certain non-aminoglycoside antimicrobials [19], thereby increasing the risk of drug-induced hearing impairment. In this context, national initiatives such as the Spanish National Plan Against Antibiotic Resistance (PRAN) 2025–2027 emphasize the urgent need for coordinated strategies to reduce antibiotic misuse, strengthen surveillance of resistance determinants—including within the human microbiome—and promote a One Health approach that links human, animal, and environmental health [20].

Despite these concerns, direct evidence linking AMR and HL is currently lacking. Investigating the intestinal resistome (the collection of antimicrobial resistance genes present in the gut microbiome) may help elucidate potential interactions among the microbiome, systemic inflammation and auditory function. Alterations in the composition and functionality of the gut microbiome have been associated with metabolic and immune processes that could indirectly influence the physiology of the auditory system [21,22]. Although the role of the gut microbiome in systemic and neurological functions is increasingly recog-

nized, no studies to date have specifically examined the gut resistome as a potential factor influencing auditory health, representing a critical research gap.

Within this context, the present study aims to evaluate the potential relationship between auditory function and the presence of antimicrobial resistance genes in the gut microbiome of young adults. This exploratory approach seeks to generate preliminary evidence supporting the hypothesis that microbial resistance patterns may be linked to auditory outcomes, thereby contributing to a better understanding of the gut–microbiome–ear axis and its implications for hearing function.

2. Results

The final study sample consisted of 22 healthy volunteers, of whom 15 were women and 7 men. The median (interquartile range) age was 22 (20–24) years for women and 22 (21–23) years for men.

2.1. Hearing Evaluation of the Sample

To determine the degree of HL, the classification system of the American Speech–Language–Hearing Association (ASHA) was used as a reference [23].

In the air conduction audiometry results, 59.1% of participants exhibited normal hearing, 36.4% showed slight HL, and 4.5% mild HL (Figure 1A). When stratified by sex, we found that 46.7% of women had normal hearing, while 53.3% exhibited some degree of HL, distributed as 46.7% slight HL and 6.7% mild HL (Figure 1B). In contrast, 85.7% of men had no hearing impairment, and only 14.3% presented slight HL (Figure 1C).

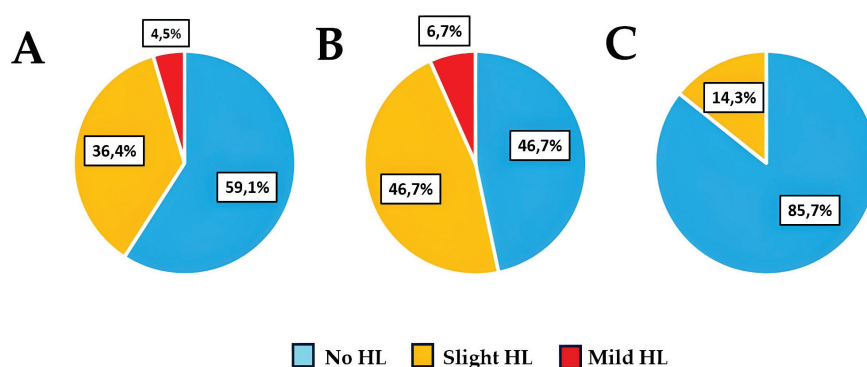


Figure 1. Air conduction audiometry results in the overall sample (A), in women (B), and in men (C). No HL: no hearing loss; Slight HL: slight hearing loss; Mild HL: mild hearing loss.

Despite the apparent differences between sexes, no statistically significant differences were observed in the air conduction audiometry results. Participants were therefore classified into two main groups based on hearing status (Table 1), with those with no hearing loss (“No HL”) and those with any degree of hearing loss (“Any degree of HL”), which included all participants with slight or mild impairment. No participants presented moderate or greater degrees of HL.

Table 1. Distribution of participants by sex and hearing loss category.

	Women	Men	Total
No HL	7 (46.7%)	6 (85.7%)	13 (59.1%)
Any degree of HL	8 (53.3%)	1 (14.3%)	9 (40.9%)

HL: hearing loss.

2.2. Analysis of the Gut Microbiome

The analysis of antibiotic resistance profiles using the cenoantibiogram technique allowed the determination of minimum inhibitory concentrations (MICs) for each fecal sample under aerobic and anaerobic conditions. These data were grouped into components based on their similar behavior. Subsequently, a principal component analysis (PCA) was performed to identify patterns and trends in microbiome behavior at the population level, considering the presence or absence of any degree of HL.

2.2.1. Comparative Analysis of Antibiotic Response

The MIC values for the antibiotic's amoxicillin, cefotaxime, imipenem, imipenem + EDTA, nalidixic acid, and ciprofloxacin were obtained using the cenoantibiogram technique under aerobic and anaerobic conditions, evaluating the influences of the HL state. The results (Figures 2 and 3) revealed distinctive patterns between the groups.

As shown in Figures 2 and 3, MIC values were generally higher in fecal samples from participants with HL compared to those without this condition. The median MIC was consistently elevated in isolates from the HL group, suggesting a general trend toward increased resistance. Significant differences between participants with and without HL were observed for several antibiotics. Under anaerobic conditions, nalidixic acid showed markedly higher median MIC values in the HL group (256 µg/mL [43.25–256.0]) compared with the group without HL (3.0 µg/mL [1.5–96.0]; $p = 0.021$). Under aerobic conditions, amoxicillin (224 µg/mL [96.0–256.0] vs. 3.0 µg/mL [3.0–48.0]; $p = 0.008$) and ciprofloxacin (0.625 µg/mL [0.2825–1.50] vs. 0.25 µg/mL [0.16–0.50]; $p = 0.046$) also exhibited significantly higher MICs among participants with HL. Although the remaining antibiotics did not reach statistical significance, their median values and dispersion tended to be greater in the HL group, suggesting a consistent pattern of elevated resistance across most agents tested.

2.2.2. Principal Component Analysis (PCA)

PCA was performed to explore the overall structure of antibiotic susceptibility patterns and its relationship between participants with and without HL. The first two principal components explained 55.67% of the total variance, with Component 1 explaining 22.17% of the variance and Component 2 explaining 33.51%. Under aerobic conditions (Figure 4), the scatter plot of the factor scores showed partial segregation between the groups. Participants with HL tended to cluster in the upper right quadrant, corresponding with high loadings on Component 1, which was mainly associated with ciprofloxacin, amoxicillin, and nalidixic acid. This distribution aligns with individual comparisons, reflecting distinct resistance profiles between groups.

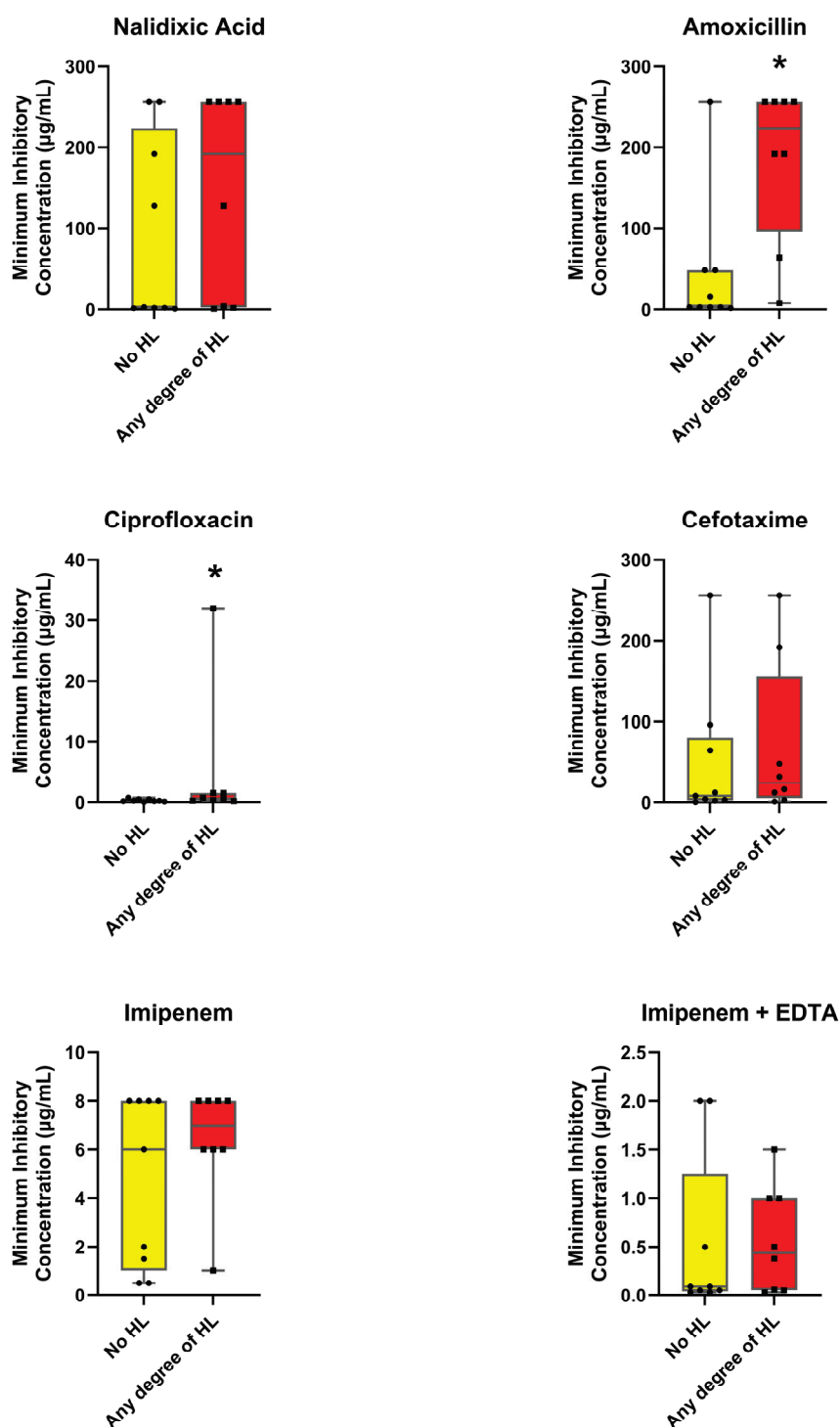


Figure 2. Comparison of minimum inhibitory concentrations of antibiotics under aerobic conditions between patients without hearing loss and those with some degree of hearing loss. Values are presented as median and interquartile range using box-and-whisker plots. * $p \leq 0.05$ indicates significant differences between groups (Mann–Whitney U-test).

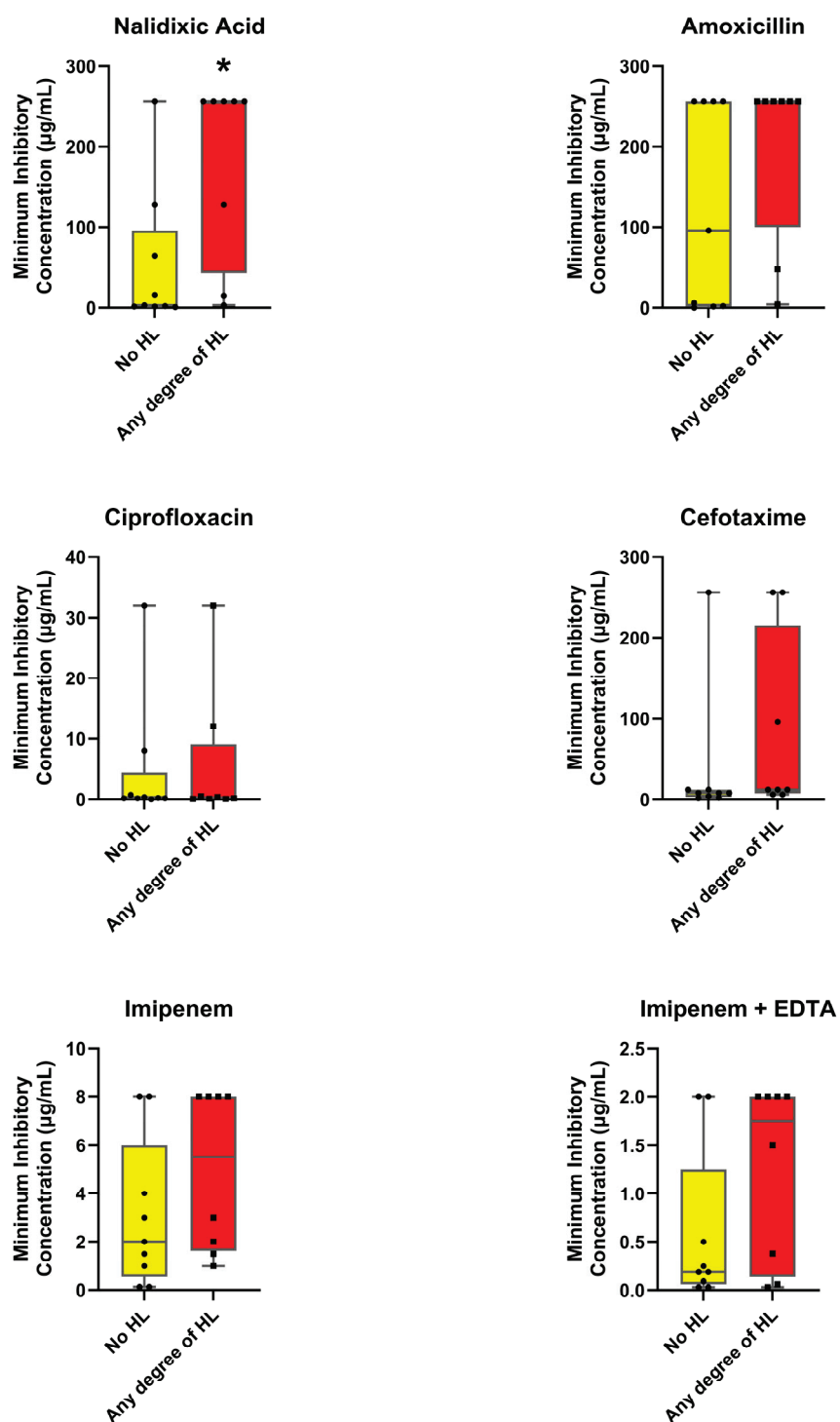


Figure 3. Comparison of minimum inhibitory concentrations of antibiotics under anaerobic conditions between patients without hearing loss and those with some degree of hearing loss. Values are presented as median and interquartile range using box-and-whisker plots. * $p \leq 0.05$ indicates significant differences between groups (Mann–Whitney U-test).

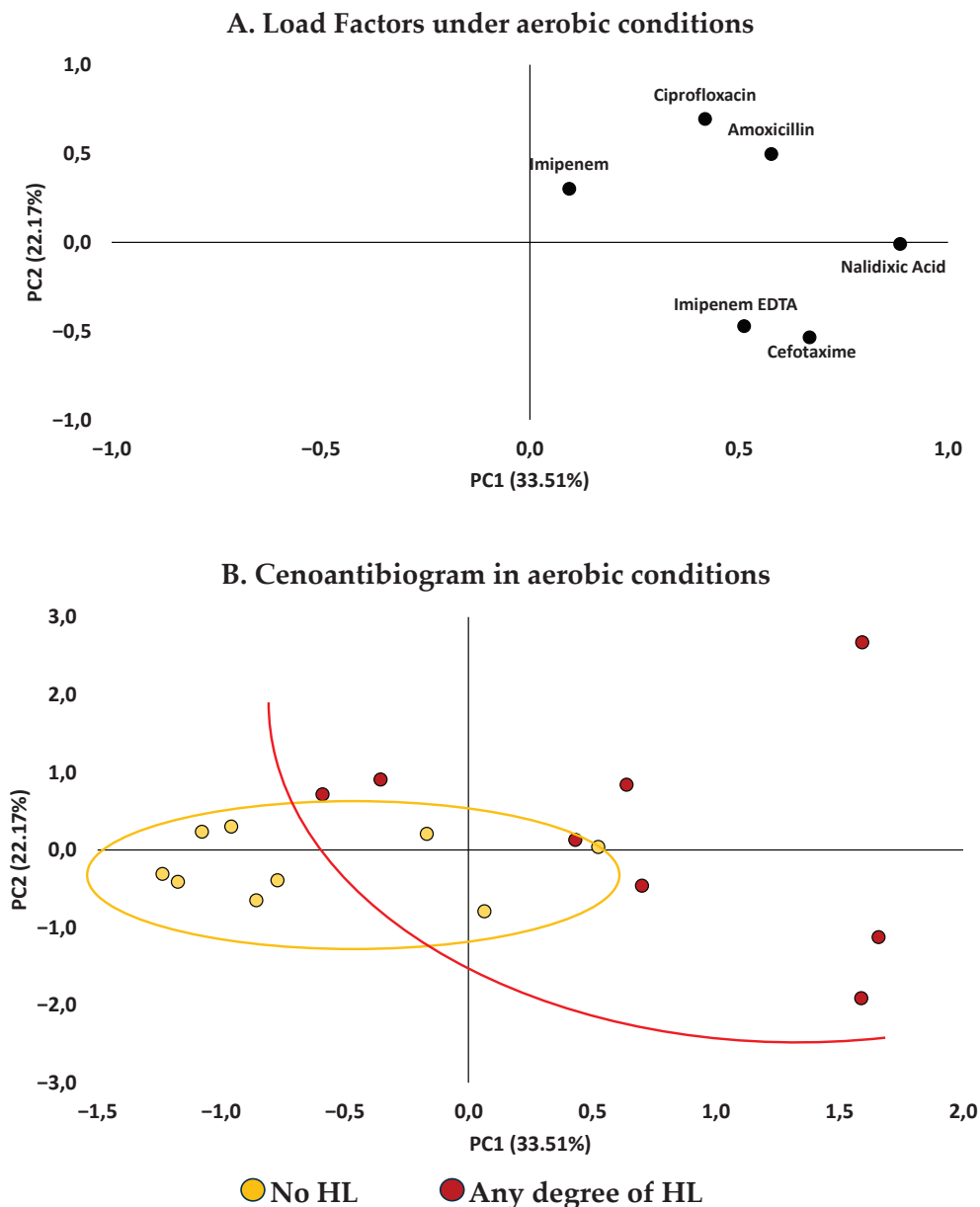


Figure 4. Antibiotic response under aerobic conditions according to hearing loss status. (A) Factor loading plot showing Component 1 (22.17%) and Component 2 (33.51%). (B) PCA biplot displaying the distribution of participants classified as “No hearing loss” and “Any degree of hearing loss.” Ellipses represent 95% confidence intervals for each group, illustrating partial clustering according to hearing status.

Under anaerobic conditions (Figure 5), the separation between groups was even more evident. Component 2 emerged as the main discriminating axis, contrasting susceptibility to imipenem and imipenem + EDTA (positive loadings) with susceptibility to cefotaxime (negative loadings). Participants with hearing loss were positioned toward higher Component 2 scores, while those without hearing loss clustered in lower-score regions. The overall distribution suggests distinct multivariate patterns of antibiotic response according to hearing status.

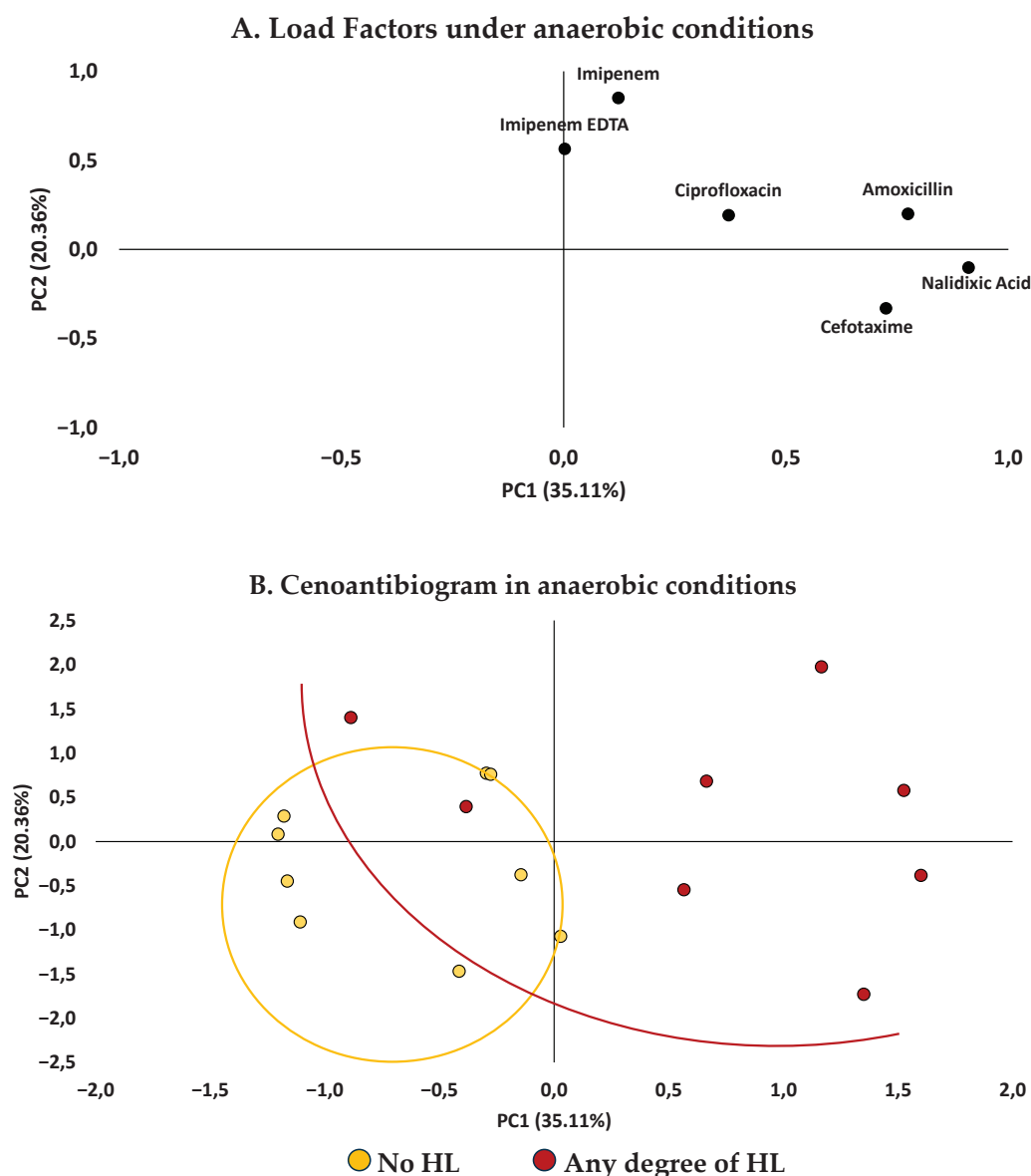


Figure 5. Antibiotic response under anaerobic conditions according to hearing loss status. (A) Factor loading plot showing Component 1 (22.17%) and Component 2 (33.51%). (B) PCA biplot displaying the distribution of participants classified as “No hearing loss” and “Any degree of hearing loss.” Ellipses represent 95% confidence intervals for each group, indicating a clearer separation of data points under anaerobic conditions.

2.2.3. Relative Abundances

To assess gut microbiota composition, total DNA was extracted from fecal samples using the NucleoSpin™ Soil DNA Kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer’s instructions and eluted in a final volume of 100 µL. The relative abundances of 15 microbial markers representing the main phyla, groups, and genera of the gut microbiota were quantified using real-time quantitative polymerase chain reaction (qPCR). The markers analyzed included *Akkermansia muciniphila*, Bacteroidota, *Candida albicans*, Clostridium cluster I, *Escherichia coli*, *Enterococcus* sp., *Faecalibacterium prausnitzii*, Bacillota (formerly Firmicutes), Gammaproteobacteria, *Lactobacillus* sp., *Methanobrevibacter smithii*, *Roseburia* sp., *Ruminococcus* spp., Clostridium cluster XIV, and Eubacteria. Relative abundances were then compared between individuals without HL (No HL) and those

with any degree of HL (Any degree of HL). The results of these analyses are presented in Figure 6.

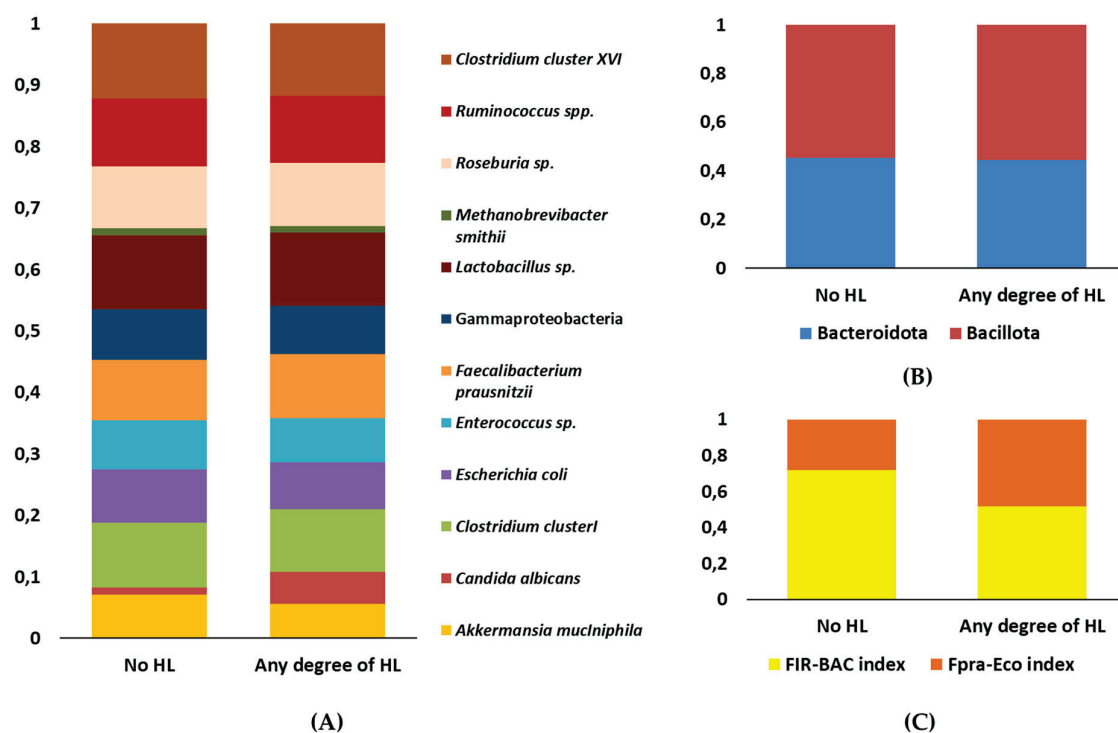


Figure 6. Gut microbiota composition and functional indices according to HL status. Antibiotic response under anaerobic conditions according to hearing loss status. The Y-axis represents relative abundance (A,B) or index value (C), while the X-axis distinguishes individuals without HL (No HL) from those with any degree of HL. Colors indicate bacterial taxa or phyla, as detailed in the legend. (A) Comparison of relative abundances at the taxon level, including genera, species, and clusters. (B) Comparison of the proportions of Bacteroidota and Bacillota phyla. (C) Comparison of Fpra-Eco and FIR-BAC indices, reflecting functional profiles of the gut microbiota.

Microbial composition analyses were performed in individuals without HL (No HL) and those with varying degrees of HL (Any degree of HL), with no notable differences observed in the overall gut microbiota composition between the groups. However, the relative abundance of *Faecalibacterium prausnitzii* was significantly higher in the HL group compared to No HL (Mann–Whitney U test, $p = 0.010$), suggesting a potential adaptive shift in the gut microbiota associated with auditory status. The Fpra-Eco index was significantly higher in the HL group, indicating an increased functional contribution of *F. prausnitzii* within the gut microbiota, whereas the FIR-BAC index did not show significant differences between groups.

To further investigate the relationship between gut microbiota composition and antibiotic resistance, analyses were focused on the antibiotics that had previously shown significant differences between HL and No HL groups. Individuals were stratified into two groups based on the median to distinguish high versus low resistance. Under these conditions, a significantly higher abundance of *Faecalibacterium prausnitzii* was observed in individuals with greater resistance to amoxicillin (Mann–Whitney U test, $p = 0.008$). In contrast, no significant differences in microbial abundances were detected when comparing groups based on resistance to ciprofloxacin and nalidixic acid.

3. Discussion

HL and AMR represent two major and expanding public health concerns worldwide, both associated with substantial impacts on quality of life and healthcare systems [1,24]. Although the present study is small and exploratory, the findings provide preliminary insights into potential links between auditory function and the intestinal resistome, which warrant further investigation.

The WHO has emphasized the growing vulnerability of young population to hearing impairment caused by recreational noise exposure, particularly through the widespread use of PLDs and attendance at loud music events [5]. Improper use of these technologies and chronic exposure to high sound levels have been recognized as preventable causes of early auditory dysfunction. In parallel, antibiotic resistance has emerged as a global health crisis, extending beyond human medicine to animal and environmental domains, and posing a significant threat to the effectiveness of current antimicrobial therapies [25].

Within this framework, the study of the intestinal resistome, the collection of antimicrobial resistance genes in the gut microbiome, has gained increasing importance as a functional biomarker of antibiotic exposure and broader lifestyle influences on microbial ecology. Alterations in the resistome may not only reflect prior antibiotic use but also mirror systemic processes, including immune and metabolic pathways, that could potentially influence extraintestinal functions [26–28].

Despite extensive research on each of these issues independently, no previous study has jointly examined the potential link between hearing loss and the intestinal resistome. Existing literature has largely focused on the direct ototoxicity of specific antibiotics, such as aminoglycosides [29] or macrolides [30], without exploring systemic or microbiome-mediated mechanisms that might contribute to auditory vulnerability. In this context, the present preliminary and exploratory study represents a pioneering approach to investigating the relationship between gut resistome profiles and auditory function in young adults, thereby contributing to the understanding of potential microbiome-mediated influences on hearing health.

3.1. Prevalence of Hearing Loss

In our cohort of 22 participants, 40.9% presented some degree of HL according to the ASHA classification [23]. When stratified by sex, 53.3% of women exhibited mild or slight HL compared to 14.3% of men. These results suggest an apparent HL prevalence between sexes, although no statistically significant difference was observed, most likely due to the limited sample size and the imbalance in sex distribution. When comparing these findings with prevalence estimates, the WHO 2020 Hearing Report [31] indicates a global prevalence of moderate or severe HL of approximately 2% among young adults aged 20–29 years, without including data on mild or slight HL, which are the most common forms in this age group and the ones primarily identified in our study. Within this age range and severity, no significant differences between sexes are reported; however, in terms of gender differences overall, global prevalence of moderate or higher levels of hearing loss is slightly higher among males than females, with 217 million males (5.6%) living with hearing loss compared with 211 million females (5.5%). Although no national data are available for the young Spanish population the 2022 CONSTANCES study conducted in France estimated HL prevalence in adults aged 18–25 years at 3.4% in women and 4.4% in men [32]. In that study, HL was defined as a pure-tone average (PTA)—the mean hearing threshold at 500, 1000, and 2000 Hz—in the better ear ≥ 20 dB [33], whereas our study employed the more sensitive ASHA classification, which considers HL from a PTA ≥ 15 dB in the better ear, allowing for earlier detection of subclinical changes. For this reason, the high prevalence observed in our sample is expected.

3.2. Relationship Between Antimicrobial Resistance and Auditory Function

In our analysis, young adults with some degree of HL showed higher MICs for nalidixic acid under aerobic conditions, for amoxicillin under anaerobic conditions, and for ciprofloxacin under anaerobic conditions, compared with participants without HL, and these differences reached statistical significance. PCA further revealed a clustering tendency among participants without HL and greater dispersion among those with HL across the antibiotics tested. These findings support the formulation of hypotheses regarding a potential association between antimicrobial resistance patterns and auditory function.

The consistently higher MICs observed in the HL group may reflect cumulative lifetime exposure to antibiotics resulting from infections of diverse origins. Although participants reporting recurrent otitis media were initially excluded from the study, it cannot be ruled out that previous episodes of infection requiring antibiotic treatment could have promoted the selection of less susceptible bacterial strains, thereby contributing to the elevated MIC values observed. Moreover, chronic or repeated exposure to certain antibiotics with potential ototoxic effects might have contributed directly to the onset or progression of HL, suggesting an indirect link between antibiotic exposure, bacterial resistance, and auditory outcomes. This relationship would not be limited to aminoglycosides [34], whose ototoxic potential is well established but unlikely relevant in our cohort given their hospital use. Other antibiotic classes, including macrolides [35], have also been associated with potential auditory toxicity, which may further support this hypothesis. It should be emphasized, however, that individual antibiotic exposure history was not assessed in this study. Therefore, these interpretations remain speculative and should be viewed as exploratory observations that warrant further investigation.

3.3. Potential Role of the Intestinal Microbiome

A complementary hypothesis is that chronic or repeated antibiotic exposure may not only promote the selection of resistant bacterial strains but could also potentially alter the intestinal ecosystem in ways reflected not only by changes in microbiome composition but also by enrichment of the gut resistome—the set of antimicrobial resistance genes harbored by gut microbes. An expanded resistome may indicate repeated antibiotic pressures and has been associated with reduced microbial diversity, shifts toward proinflammatory taxa, and decreased ecosystem stability [36–38]. The concept of the gut–brain–ear axis [10] provides a theoretical framework for understanding how intestinal dysbiosis might indirectly influence auditory function. Recent experimental studies in murine models have in fact demonstrated that perturbations in the gut microbiome can modulate systemic inflammation, oxidative stress, and the integrity of physiological barriers such as the blood–brain barrier [39] and the blood–labyrinth barrier within the cochlea, ultimately compromising cochlear homeostasis and the function of sensory hair cells responsible for mechanoelectrical transduction [13]. Although noise exposure was not assessed in our study, it is widely recognized as a potential inducer of both HL and gut microbiome alterations [40]. Animal models indicate that environmental noise may modify microbial composition and metabolic activity, including changes in short-chain fatty acids that regulate neuroinflammation and neuronal function. Communication between the gut and auditory system, mediated through the vagus nerve, sympathetic pathways, and the hypothalamic–pituitary–adrenal axis, might support bidirectional interaction influencing auditory susceptibility [10]. In line with this concept, our analyses revealed that overall gut microbiota composition did not differ markedly between individuals with and without HL; however, the abundance of *Faecalibacterium prausnitzii* was significantly higher in the HL group. This is surprising, as *F. prausnitzii* is typically associated with anti-inflammatory gut environments [41], suggesting a subtle, species-specific shift rather than broad dysbiosis. Similarly, higher resistance to

amoxicillin was associated with increased *F. prausnitzii* abundance, whereas no differences were observed for other antibiotics. These observations provide preliminary evidence that specific microbial taxa and resistome features could be linked to auditory status and antibiotic exposure, consistent with the hypothesized gut–microbiome–ear axis. These findings provide a conceptual basis for the gut–microbiome–ear axis; however, the potential contribution of the resistome to these pathways remains largely hypothetical, and further research is necessary to elucidate the underlying mechanisms and causal relationships.

3.4. Strengths, Limitations and Future Research

This study represents a pioneering effort to explore the relationship between the intestinal resistome and the auditory function, introducing an innovative approach that integrates microbiome composition, antimicrobial resistance, and auditory health. However, the small sample size and sex imbalance constitute important limitations that reduce the generalizability of the findings and constrain the statistical power to detect subtle differences between groups. Another key limitation is the lack of metagenomic analysis, which restricts the depth of characterization of the gut microbiome and resistome. In addition, key potential confounders, including diet, individual antibiotic use history, and noise exposure, were not fully controlled or quantified, although participants completed a general health and lifestyle questionnaire and individuals with prior use of ototoxic medications or chronic auditory conditions were excluded. Regarding statistical analyses, technical duplicates were performed for each assay; given the small sample size and exploratory nature of this pilot study, formal corrections for multiple testing (e.g., Bonferroni) were not applied. Furthermore, the cross-sectional design precludes the establishment of causal inferences or directionality in the observed associations between antimicrobial resistance, intestinal dysbiosis, and HL. Although causality cannot be inferred, the findings offer a conceptual framework consistent with emerging evidence linking the gut microbiome to auditory physiology. Future longitudinal and experimental studies will be required to determine the temporal and biological direction of these interactions, as well as their potential mechanistic underpinnings. Such studies should also incorporate environmental and lifestyle factors known to influence the microbiome and auditory function, including occupational and recreational noise exposure, dietary patterns, antibiotic use history and sleep quality, to provide a more comprehensive understanding of the gut–microbiome–ear axis and its implications for auditory health [42–45].

4. Materials and Methods

4.1. Study Design and Sample Selection

A descriptive, cross-sectional pilot study was conducted in a cohort of 22 healthy young adults aged 18 to 26 years, recruited at CEU San Pablo University (Madrid, Spain). Participant recruitment took place between 25 January and 30 April 2025, within the framework of the global METAEAR project.

Participants were recruited by convenience sampling according to the following eligibility criteria:

- Inclusion criteria: individuals within the specified age range.
- Exclusion criteria: subjects with acute or chronic diseases related to HL, cardiovascular disease, diabetes, and/or cancer; current use or use within the 30 days prior to sample collection of ototoxic drugs, vitamin supplements, probiotics, and/or prebiotics; history of surgical interventions of the auditory system; family history of early-onset HL; or known auditory pathologies such as otitis, otosclerosis, or Ménière’s disease.

All volunteers provided written informed consent prior to participation in the study. The study protocol was approved by the Research Ethics Committee of CEU San Pablo

University (approval code: 812/24/116 PRENUT2HEAR), in accordance with the principles of the Declaration of Helsinki.

4.2. General Description of the Study Design

Demographic, health, and lifestyle information were collected through a structured screening questionnaire to verify compliance with all inclusion and exclusion criteria. Participants underwent a comprehensive hearing assessment using pure-tone audiometry, and fecal samples were collected for microbiological and antimicrobial resistance analyses.

4.3. Sociodemographic, General Health, and Personal Questionnaire

This questionnaire was designed to characterize the demographic and clinical characteristics of the study population. The sociodemographic questionnaire collected detailed information including date and place of birth, nationality, sex, place of residence, and current educational, professional, or employment status. The general health questionnaire gathered data on the participants' medical history, particularly focusing on auditory disorders such as otitis, otosclerosis, or Ménière's disease, among others. It also considered diseases associated with HL, including cardiovascular conditions, diabetes, and/or cancer, as well as the use of medications with potential ototoxic effects or vitamin-mineral supplements. This questionnaire additionally served to confirm compliance with the inclusion and exclusion criteria established for participant selection.

4.4. Hearing Evaluation

To assess the auditory function, both subjective and objective audiological tests were performed for all volunteers. These assessments were performed by qualified audiology specialists from the Faculty of Pharmacy at CEU San Pablo University, in accordance with Spanish legislation (Royal Decree 1685/2007). The tests assessed the participants' hearing status and, in cases of HL, determined its origin (conductive, sensorineural, or mixed). All the evaluations were carried out in a soundproof booth and analyzed using the NOAH 4.0 software. Initially, otoscopic examination was performed to examine the outer ear, ear canal, tympanic membrane, and middle ear [46]. This was followed by tuning fork tests (Rinne and Weber) [47] to help differentiate the type of HL. Finally, pure-tone audiometry was conducted to determine both air and bone conduction thresholds using pure tones at frequencies of 125, 250, 500, 750, 1000, 2000, 3000, 4000, and 8000 Hz in 5 dB increments [48].

4.5. Gut Microbiome Analysis

Fresh stool samples were collected from each participant and immediately stored at -80°C until 24 h before processing. Microbiological and antimicrobial resistance analyses were subsequently performed as described below.

To assess antimicrobial resistance in stool samples, bacterial resistance to selected antimicrobials was evaluated using disk diffusion and E-test strips methods to determine the MIC, following the protocol described by Robas Mora et al. [49], under both aerobic and anaerobic conditions. Two batches of ten samples were processed. Sterile culture media were prepared including 0.45% saline solution and Mueller–Hinton agar (CondalabTM, Madrid, Spain). Approximately 2 g of each stool sample were mixed with saline under sterile conditions, homogenized using a sonicator, centrifuged, and the resulting supernatant was plated on Mueller–Hinton agar.

For each fecal sample, one biological replicate was prepared, and all antibiotic disk assays were conducted in technical duplicate to ensure reproducibility and reliability. The antibiotics tested included nalidixic acid, amoxicillin, cefotaxime, imipenem (alone and in combination with EDTA), and ciprofloxacin for both aerobic and anaerobic cultures (BioMérieuxTM, Marcy l'Etoile, France) and were selected based on their known profiles of

resistance or sensitivity in representative intestinal microorganisms. These antibiotics have been frequently used in previous studies evaluating antimicrobial resistance in human and animal gut microbiota [50–52]. Anaerobic cultures were incubated in an aerobic bag system (Merck KGaA, 64271, Darmstadt, Germany). Disks and E-test strips were placed on the inoculated plates, which were incubated following the manufacturer’s instructions. Plates were incubated at 37 °C according to the manufacturer’s instructions. Inhibition zones were measured at 24 and 48 h for aerobic cultures and at 24 h for anaerobic cultures to assess MIC values.

For the qPCR analysis, total DNA was extracted from 22 fecal samples using the NucleoSpinTM Soil DNA Kit (Macherey-Nagel, GmbH & Co., Düren, Germany) according to the manufacturer’s instructions and eluted in a final volume of 100 µL. The characterization of gut bacterial species was performed at GoodGut S.L.U. (Girona, Spain) by quantifying 15 microbial markers representing the main phyla, genera, and groups of the gut microbiota: *Akkermansia muciniphila*, Bacteroidota, *Candida albicans*, *Clostridium* cluster I, *Escherichia coli*, *Enterococcus* sp., *Faecalibacterium prausnitzii*, Bacillota (Firmicutes), Gammaproteobacteria, *Lactobacillus* sp., *Methanobrevibacter smithii*, *Roseburia* sp., *Ruminococcus* spp., *Clostridium* cluster XIV, and Eubacteria [53].

Reactions for each marker were performed individually using GoTaq[®] qPCR Master Mix (Promega Corporation, Madison, WI, USA) or GoTaq[®] qPCR Probe Master Mix (Promega Corporation, Madison, WI, USA), depending on the target. Each reaction had a final volume of 10 µL, containing 12–20 ng of genomic DNA. Primers and probes were obtained from Macrogen Inc., Seoul, South Korea. All samples were analyzed in duplicate alongside negative controls and standard curves using an AriaDx thermocycler (Agilent Technologies, Santa Clara, CA, USA), in compliance with ISO 13485 standards [53].

4.6. Statistical Analysis

Data were expressed as median and interquartile range for quantitative variables, or as frequencies for qualitative variables. Normality was checked using the non-parametric Kolmogorov–Smirnov test. Given the non-normal distribution of the data, the non-parametric Mann–Whitney U test was applied to evaluate differences between groups. For comparative analysis of microbiome resistance patterns, data derived from antibiotic resistance were integrated and dimensionally reduced into two principal components explaining most of the model variability. PCA was then performed, and the resulting components were plotted in a two-dimensional space according to hearing status (presence or absence of HL) to visualize clustering tendencies and potential group differentiation.

All statistical analyses were performed using IBM SPSS Statistics v29.0 (IBM Corp., Armonk, NY, USA).

5. Conclusions

In this cohort of young adults, a notable prevalence of mild or slight HL was observed, particularly among women. The results demonstrated a significant association between HL and higher MICs for nalidixic acid, amoxicillin, and ciprofloxacin, together with a trend toward increased MIC values and greater variability in the HL group for other antibiotics such as cefotaxime and imipenem. qPCR analyses of gut microbiota revealed that the abundance of *Faecalibacterium prausnitzii* was significantly higher in individuals with HL, and this species was also enriched in participants exhibiting greater resistance to amoxicillin.

These findings suggest that specific microbial taxa and resistome features may be linked to both auditory status and antibiotic exposure, reinforcing the emerging concept of a gut–ear–brain axis. They underscore the importance of monitoring gut microbiome

integrity and cumulative antibiotic exposure in young and potentially susceptible populations. Moreover, the observed associations provide a rationale for future longitudinal and mechanistic studies aimed at exploring microbiome-targeted strategies to preserve auditory health and mitigate the potential impact of antibiotic-induced microbiome perturbations.

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Abbreviations

The following abbreviations are used in this manuscript:

ASHA	American Speech-Language-Hearing Association
AMR	Antimicrobial Resistance
HPA	Hypothalamic–pituitary–adrenal axis
HL	Hearing loss
MIC	Minimum inhibitory concentration
PCA	Principal component analysis
PLDs	Personal Listening Devices
PTA	Pure Tone Average
qPCR	Quantitative polymerase chain reaction
WHO	World Health Organization

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Article

Plasma and Muscle Pharmacokinetics of Ceftriaxone in Nile Tilapia (*Oreochromis niloticus*) After Different Administration Routes

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Abstract: Background/Objectives: The aim of this study was to determine the plasma and muscle pharmacokinetics of ceftriaxone (25 mg/kg) in tilapia after different administration routes. **Methods:** Two hundred and sixteen fish maintained at 30 ± 1.5 °C were divided equally into three treatment groups: intravascular (IV), intraperitoneal (IP), and intramuscular (IM). Ceftriaxone concentrations were quantified using high-performance liquid chromatography, and pharmacokinetic parameters were calculated by non-compartmental analysis.

Results: The plasma total body clearance, volume of distribution at steady state, and elimination half-life ($t_{1/2\lambda z}$) were 0.22 L/h/kg, 0.85 L/kg, and 5.27 h, respectively. The $t_{1/2\lambda z}$ values were comparable among the IV, IP, and IM injection groups. The peak plasma concentration was 37.71 ± 3.12 µg/mL and 40.51 ± 2.77 µg/mL following IP and IM injection, respectively. The bioavailability was 67.04% for IP and 101.48% for IM. The peak muscle concentration was 9.49 ± 0.75 µg/g for IV, 5.71 ± 0.85 µg/g for IP, and 12.24 ± 2.41 µg/g for IM injection. The $AUC_{0-\infty \text{muscle}}/AUC_{0-\infty \text{plasma}}$ ratio was 0.23, 0.18, and 0.30 for the IV, IP, and IM groups, respectively. The $AUC_{\text{muscle}}/AUC_{\text{plasma}}$ indicates the ratio of drug penetration into the muscle, and a value less than 1 indicates that ceftriaxone penetrates into muscle tissue at a low ratio. **Conclusions:** These results indicate that ceftriaxone is well absorbed after IP and IM injections and passes into muscle tissue at a low tissue penetration. Ceftriaxone can be administered via IP and IM injection in Nile tilapia; nevertheless, its therapeutic efficacy requires evaluation.

Keywords: bioavailability; ceftriaxone; muscle; Nile tilapia; pharmacokinetic

1. Introduction

Nile tilapia, which is farmed in 83 countries around the world, reached nearly 5.2 million tons in 2023, generating a value of 11 billion dollars [1]. The tilapia stock available is characterized by rapid growth, disease resistance, and resilience to climate change [2]. However, stock density, poor environmental conditions, and stress-related bacterial infections that have become widespread due to increased tilapia farming have become a threat affecting both the fish supply and farmers' livelihoods [3]. Therefore, antibiotics are commonly utilized in aquaculture for the treatment of diseases and prevention [3]. Licensed antibiotics in aquaculture are limited, and widespread and inappropriate use of these antibiotics leads to the emergence of bacterial resistance [4]. Because bacterial resistance limits antibiotic use, new antibiotic choices are required [5].

Ceftriaxone (CTX) is a parenteral third-generation cephalosporin [6]. The bactericidal action of CTX is due to its inhibition of bacterial cell wall synthesis [7]. It is a notable antibiotic known for its strong antibacterial efficacy, broad spectrum of activity, and low adverse effects [8]. It is effective against a wide range of bacteria, including both Gram-negative and Gram-positive species, as well as anaerobic strains [6]. It is resistant to hydrolysis by various beta-lactamases [7]. CTX is effective in treating infections of the skin and soft tissue, bones and joints, abdomen, urinary tract, lower respiratory tract, meninges, and pelvic area caused by susceptible organisms in humans [9]. The European Medicines Agency (EMA) has classified third-generation cephalosporins as category B (restricted) antibiotics for veterinary use due to the risks of antimicrobial resistance in animals and public health. It is recommended for treating clinical conditions that do not have effective alternatives in a lower category [10]. Although CTX is not approved for use in animals by the EMA or the Food and Drug Administration, it is approved for use in farm and pet animals for these infections in other countries, such as China and Bangladesh [11,12]. The bacterial resistance development against antibiotics such as tetracycline and fluoroquinolone, commonly used in fish, has been reported [13]. CTX is not approved for fish, but its superior pharmacokinetic and pharmacodynamic properties make it a good alternative antibiotic in fish [5].

Parenteral antibiotic administration is not common in fish due to some significant disadvantages [14,15]. However, parenteral administration has the advantages of high bioavailability, rapid onset of action, precise dosing, and lower drug use, and has been shown to be more effective than oral administration [16,17]. Recently, parenteral antibiotic use in fish has been found to be applicable in Korean culture farms [14]. CTX is a parenteral antibiotic used in veterinary medicine; however, data about its administration in fish remains inadequate. The pharmacokinetics of CTX have been established only in brown trout, and its application is advised owing to its extended half-life [5]. Furthermore, it was determined that *Aeromonas* spp. isolated from fish exhibited sensitivity to this drug [18,19]. *Aeromonas* spp., *Vibrio* spp., and *Streptococcus* spp. bacteria have been identified as common infectious agents in tilapia [20]. CTX has a broad spectrum of activity covering these bacteria [5]. Fish are heterothermic organisms, and changes in water temperature can modify their physiology and, subsequently, their pharmacokinetics. Therefore, pharmacokinetic studies are crucial in determining the appropriate dosage regimen of the drug in any fish species [21]. Considering this information, pharmacokinetic data obtained from trout, a cold-water species, are inappropriate for use with tilapia, a warm-water species [22]. To our knowledge, there is no information on the plasma and muscle pharmacokinetics of CTX in tilapia. This study aimed to determine the plasma and muscle pharmacokinetics of CTX after intravascular (IV), intraperitoneal (IP), and intramuscular (IM) administration in Nile tilapia at a dose of 25 mg/kg.

2. Results

2.1. HPLC Method

The retention time of CTX for plasma and muscle was 8.5 min with a total run time of 12 min. The endogenous matrix from plasma and muscle did not interfere with the peak of CTX. The calibration curve varied between 0.05 µg/mL and 100 µg/mL for plasma and 0.05 µg/g and 20 µg/g for muscle, was reproducible over the concentration range studied, and showed linearity with a regression coefficient (R^2) > 0.995. For plasma and muscle, the limits of detection (LOD) and limits of quantitation (LOQ) were 0.025 and 0.05 µg/mL (g), respectively, based on a signal-to-noise ratio >3 and >6. The recovery of CTX for plasma and muscle was >91% to >87%, respectively. The intra- and inter-day coefficients of variation ranged from 3.45 to 6.04%. The intra- and inter-day bias of CTX ranged from −1.32 to 3.16%.

2.2. Animals

The IV, IP, and IM administration of ceftriaxone at a dose of 25 mg/kg to tilapia was generally well tolerated. No differences were observed in the swimming behavior of the fish during the study.

2.3. Plasma Pharmacokinetic Parameters

Figure 1 shows the plasma CTX concentration–time curve following a single dose IV, IP, and IM injection. CTX was detected in plasma for up to 36 h after IV, IP, and IM injection. The pharmacokinetic parameters of CTX following a single dose IV, IP, and IM injection are shown in Table 1. The C_{max} and T_{max} of CTX were 37.71 ± 3.12 µg/mL and 0.25 h for IP injection and 40.51 ± 2.77 µg/mL and 0.50 h for IM injection. The $C_{0.25h}$ after IV injection was 57.37 ± 4.01 µg/mL. The $t_{1/2\lambda z}$ of 5.27 h, V_{darea} of 1.67 L/kg, V_{dss} of 0.85 L/kg, and Cl_T of 0.22 L/h/kg were calculated following IV injection, respectively. The $t_{1/2\lambda z}$ values were similar in the IV, IP, and IM injection groups. The AUC_{0-36} was similar in the IV and IM injection groups but lower in the IP group. The bioavailability of CTX was 67.04% for IP injection and 101.48% for IM injection.

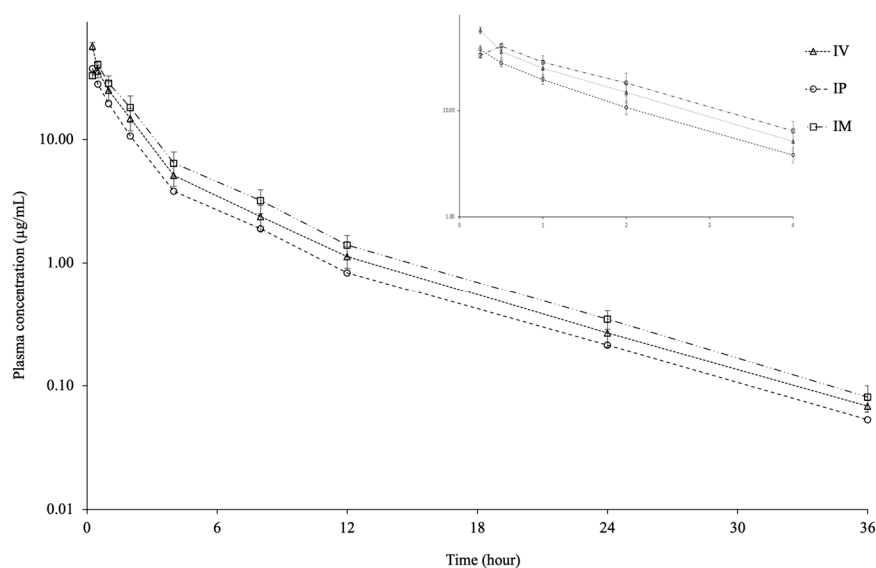


Figure 1. Semi-logarithmic plasma concentration–time curves following intravascular (IV), intraperitoneal (IP), and intramuscular (IM) administrations of ceftriaxone at a single dose of 25 mg/kg in Nile tilapia (*Oreochromis niloticus*) at 30 ± 1.5 °C (mean $\pm$ SD, $n = 6$).

Table 1. Plasma pharmacokinetic parameters following intravascular (IV), intraperitoneal (IP), and intramuscular (IM) administrations of ceftriaxone at a single dose of 25 mg/kg in Nile tilapia (*Oreochromis niloticus*) at 30 ± 1.5 °C ($n = 6$).

Parameter	IV	IP	IM
$t_{1/2\lambda_z}$ (h)	5.27	5.61	5.44
AUC_{0-36} (h * $\mu\text{g/mL}$)	113.05	75.79	114.72
$AUC_{0-\infty}$ (h * $\mu\text{g/mL}$)	113.57	76.22	115.36
AUC_{extrap} (%)	0.46	0.56	0.55
$MRT_{0-\infty}$ (h)	3.85	4.40	4.75
Cl_T (L/h/kg)	0.22	-	-
V_{darea} (L/kg)	1.67	-	-
V_{dss} (L/kg)	0.85	-	-
C_{max} ($\mu\text{g/mL}$)	-	37.71 ± 3.12	40.51 ± 2.77
$C_{0.25h}$ ($\mu\text{g/mL}$)	57.37 ± 4.01	-	-
T_{max} (h)	-	0.25	0.50
F (%)	-	67.04	101.48

$t_{1/2\lambda_z}$; terminal elimination half-life, AUC; area under the concentration-versus time curve, $AUC_{\text{extrap}\%}$; area under the plasma concentration–time curve extrapolated from t_{last} to ∞ in % of the total AUC, $MRT_{0-\infty}$; mean residence time, Cl_T ; total body clearance, V_{darea} ; apparent volume of distribution, V_{dss} ; volume of distribution at steady state, C_{max} ; peak plasma concentration, $C_{0.25h}$; plasma concentration at the first sampling time (0.25 h) after IV injection, T_{max} ; time to reach peak plasma concentration, F; bioavailability.

2.4. Muscle Pharmacokinetic Parameters

The muscle concentration-time curves and the pharmacokinetic parameters for CTX following a single dose IV, IP, and IM injection are shown in Figure 2 and Table 2. CTX was detected in muscle for up to 36 h after IV and IM injection and for up to 24 h after IP injection. The C_{max} and T_{max} of CTX were 9.49 ± 0.75 $\mu\text{g/g}$ and 0.25 h for IV injection, 5.71 ± 0.85 $\mu\text{g/g}$ and 0.50 h for IP injection, and 12.24 ± 2.41 $\mu\text{g/g}$ and 0.50 h for IM injection. The $t_{1/2\lambda_z}$ was similar in the IV and IM groups but shorter in the IP group. The AUC_{last} was obtained in the order IM > IV > IP and was different between groups. The $AUC_{0-\infty\text{muscle}}/AUC_{0-\infty\text{plasma}}$ ratio was 0.23, 0.18, and 0.30 for the IV, IP, and IM groups, respectively.

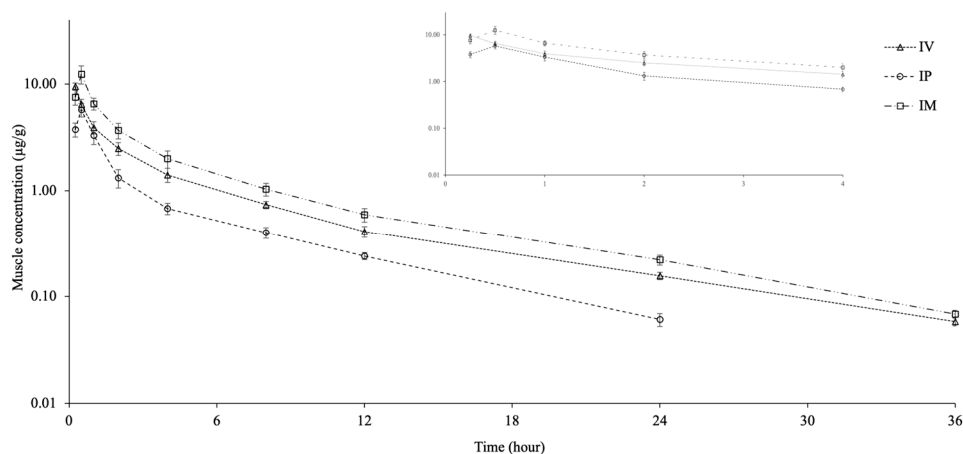


Figure 2. Semi-logarithmic muscle concentration-time curves following intravascular (IV), intraperitoneal (IP), and intramuscular (IM) administrations of ceftriaxone at a single dose of 25 mg/kg in Nile tilapia (*Oreochromis niloticus*) at 30 ± 1.5 °C (mean $\pm$ SD, $n = 6$).

Table 2. Muscle tissue pharmacokinetic parameters following intravascular (IV), intraperitoneal (IP), and intramuscular (IM) administrations of ceftriaxone at a single dose of 25 mg/kg in Nile tilapia (*Oreochromis niloticus*) at 30 ± 1.5 °C ($n = 6$).

Parameter	IV	IP	IM
$t_{1/2\lambda z}$ (h)	7.27	5.79	7.36
AUC_{0-last} (h * $\mu\text{g/g}$)	25.13	12.88	33.63
$AUC_{0-\infty}$ (h * $\mu\text{g/g}$)	25.74	13.39	34.36
AUC_{extrap} (%)	2.38	3.80	2.11
C_{max} ($\mu\text{g/g}$)	9.49 ± 0.75	5.71 ± 0.85	12.24 ± 2.41
T_{max} (h)	0.25	0.50	0.50
$AUC_{0-\infty\text{muscle}}/AUC_{0-\infty\text{plasma}}$	0.23	0.18	0.30

$t_{1/2\lambda z}$; terminal elimination half-life, AUC; area under the concentration-versus time curve, $AUC_{extrap\%}$; area under the muscle concentration–time curve extrapolated from t_{last} to ∞ in % of the total AUC, C_{max} ; peak muscle concentration, T_{max} ; time to reach peak muscle concentration.

3. Discussion

Injection of antibiotics may be preferred for valuable fish such as brood stock and aquarium fish and for fish such as olive flounders, which are generally calm and do not undergo excessive stress during injection [5,13]. In recent years, studies on the pharmacokinetics of injectable cephalosporin antibiotics in fish such as tilapia and trout have increased [5,23–26]. CTX used in veterinary medicine and appropriate for parenteral administration, is also applicable to Nile tilapia owing to its broad spectrum of activity and low adverse effects. This study demonstrates the pharmacokinetics of CTX in Nile tilapia for the first time, and this information may contribute significantly to the use of this drug in this species.

No side effects were observed after IV, IP, and IM injection of CTX at a dose of 25 mg/kg in Nile tilapia. No local or systemic adverse effects were noted following IM and IV administration of 25 mg/kg of brown trout [5]. The CTX dose of 25 mg/kg was determined considering previous studies in fish [5] and crocodiles [27].

The $t_{1/2\lambda z}$ following IV injection of CTX at a dose of 25 mg/kg in Nile tilapia at 30 ± 1.5 °C was 5.27 h. This value was shorter than the $t_{1/2\lambda z}$ value (18.22 h) obtained in brown trout (10–13 °C) at the same dose and administration route [5]. Fish are heterothermic organisms, and variations in water temperature influence their physiology and metabolism [28]. Physiological and metabolic differences among fish species may alter the V_d and Cl_T values of drugs [21]. The $t_{1/2\lambda z}$ is a hybrid parameter formed by Cl_T and V_d [29], and the fact that $t_{1/2\lambda z}$ of CTX is so variable between the two fish species may be due to the difference in these parameters.

V_{darea} is associated with body clearance, whereas V_{dss} is not influenced by clearance. In Nile tilapia, the V_{darea} of CTX (1.67 L/kg) was double that of V_{dss} (0.85 L/kg). Generally, V_{darea} is higher than V_{dss} , but a large difference indicates that a large portion of the drug is eliminated before reaching pseudo-equilibrium [30]. CTX has a hydrophilic nature and a generally low V_{dss} (0.16–0.28 L/kg) in mammals [31,32]. However, it showed a wide V_{dss} in foals and calves [33,34]. Following IV injection in Nile tilapia, CFX showed a wide V_{dss} of 0.85 L/kg, indicating extensive penetration into various body tissues and fluids. Plasma protein binding ratio and body composition influence the V_{dss} [35]. The plasma protein binding ratio of CTX in fish is unknown, but binding to 29–45% has been reported in mammals [36,37]. In fish, plasma protein concentrations and acidic drug binding are lower than in mammals [38,39]. The V_{dss} may be wide in tilapia due to differences in body components and plasma protein binding. However, the V_{dss} value in Nile tilapia (0.85 L/kg, 30 ± 1.5 °C) was approximately 10 times higher than that in brown trout

(0.09 L/kg, 10–13 °C) [5]. The water temperature influences various physiological parameters in fish, including tissue perfusion, cardiac output, and blood acid–base equilibrium [40,41]. The difference in V_{dss} between these two fish species may be due to physiological changes depending on water temperature and differences in the binding ratio to body components and plasma proteins.

There is no information on the biotransformation and excretion pathways of CTX in fish. The metabolism of CTX varies among species. While it does not undergo systemic biotransformation in humans, its metabolite has been detected in goats [42,43]. It is eliminated from the body through bile and urine [44]. The Cl_T of CTX following IV injection in Nile tilapia was 0.22 L/h/kg; this value is approximately 10 times higher than reported (0.02 L/h/kg) in brown trout [5]. Fish have biotransformation enzymes, but their content varies depending on the species, and the kidneys, bile, and gills play a role in the excretion of drugs from the body [38,45,46]. In fish, low ambient water temperature causes slow metabolic rate, biotransformation, and excretion of drugs [21,47]. The fast Cl_T of CTX in Nile tilapia may be due to rapid metabolism and excretion due to high water temperature.

CTX was well absorbed after IP and IM injection, exhibiting 67.04% and 101.48% bioavailability, respectively. There is no information on the IP bioavailability of CTX. The IM bioavailability was consistent with that previously reported (84–102%) in mammals [36,48]. It is recommended that a drug have >30% bioavailability to be useful in aquaculture [49]. CTX can be administered to Nile tilapia via IM or IP injection due to its high bioavailability. The IM bioavailability of CTX was very low (27.19%) in brown trout [5]. Water temperature variations influenced the oral bioavailability of enrofloxacin in trout. The oral bioavailability of enrofloxacin was 25% and 43% at 10 and 15 °C water temperatures, respectively [49]. The effect of temperature on IM bioavailability in fish is unknown. However, the difference in IM bioavailability in Nile tilapia and trout may be due to changes in cardiac output and tissue perfusion rate depending on temperature. The bioavailability after extravascular injection is generally less than or equal to 100% [50]. The bioavailability exceeding 100% may result from several errors, including mechanical (e.g., employing different animals in treatment groups), experimental (e.g., during sample collection, preparation, and storage), and analytical technique errors [50].

The C_{max} of CTX at a 25 mg/kg dose in Nile tilapia was 37.71 ± 3.12 µg/mL at 0.25 h for IP injection and 40.51 ± 2.77 µg/mL at 0.5 h for IM injections. These results showed that C_{max} was similar after IP and IM injection. The C_{max} obtained following IM injection of 25 mg/kg in Nile tilapia was lower than the value previously recorded (87.92 µg/mL) in brown trout [5]. This difference in C_{max} may be due to differences in drug formulation, Cl_T , V_d , and bioavailability.

The $t_{1/2\lambda_z}$ value obtained in muscle tissue after IV and IM injection was longer than plasma. Similar results have been observed in brown trout [5]. The C_{max} was obtained in the order IM > IV > IP and was different between groups. The $AUC_{tissue}/AUC_{plasma}$ indicates the ratio of drug penetration into the tissue, and a value >1 indicates that it penetrates into the tissue at a higher ratio than plasma. The tissue penetration ratio of CTX is generally low. The $AUC_{0-\infty muscle}/AUC_{0-\infty plasma}$ ratio in the IV, IP, and IM groups was 0.23, 0.18, and 0.30, respectively, indicating low muscle penetration of CTX. This value was 0.02 and 0.04 for IV and IM applications in brown trout, respectively [5]. These results indicate that CTX penetration into muscle tissue is approximately 10 times greater in Nile tilapia than in brown trout. The difference in muscle tissue transition between the two fish species may be due to changes in tissue blood flow depending on water temperature.

The amount of drug in edible tissues of fish above the maximum residue limits (MRLs) causes undesirable effects and the development of bacterial resistance [21]. Therefore, it is very important to pay attention to the MRL value in edible tissues. To our knowledge, the

MRL value of CTX has not been determined in food-valued animals. Muscle concentration of CTX decreased below the LOQ value (0.05 µg/g) at 36, 24, and 36 h after IV, IP, and IM injection, respectively. However, to determine the withdrawal time in fish, regulatory bodies responsible for the protection of human health need to determine the MRL values for CTX in muscle tissue, and tissue depletion studies need to be conducted [5].

Beta-lactams are a time-dependent class of antibiotics, with drug concentration having little effect on the overall rate and amount of bacterial death. Instead, it is critical to keep the free drug concentration above the organism's MIC for a portion of the dosing period [51]. For successful beta-lactam therapy and to prevent the development of resistance, the percentage of a drug that remains above the MIC throughout the dosing interval to provide maximum bactericidal effect ($T > MIC$) is crucial. For therapeutic efficacy, a $T > MIC$ value is desired to be >50% [52]. The MIC value of CTX for bacteria isolated from fish has not been determined. Therefore, the evaluation was made based on the susceptible breakpoint MIC values reported for CTX by CLSI for *Enterobacteriaceae* spp. (1 µg/mL) and *Streptococcus* spp. (0.5 µg/mL) [53]. Because plasma protein binding was not determined in this study, free plasma concentration was calculated by averaging plasma protein binding ratios previously reported (29–45%) in mammals [36,37]. The $T > MIC$ 50% value provided 24 h for bacteria with MIC of 0.5 µg/mL and 16 h for bacteria with MIC of 1 µg/mL across all administration routes.

This research acknowledges several limitations. Critically, the absence of data regarding CTX's plasma protein binding ratio, metabolic pathways, and excretion routes represents a significant gap in understanding its systemic disposition. Furthermore, the study's failure to establish the MIC and demonstrate therapeutic efficacy against susceptible bacterial strains limits the translational applicability of the findings. The use of healthy animals, rather than those with induced or naturally occurring infections, further restricts the assessment of CTX's clinical relevance. The study also lacks pharmacokinetic data following multiple-dose administrations, which is crucial for determining optimal dosing regimens and predicting potential accumulation effects. Importantly, the influence of water temperature on CTX's pharmacokinetic profile was not investigated. Water temperature can significantly impact drug stability, absorption, and distribution, potentially altering its bioavailability and therapeutic efficacy. Moreover, the investigation did not include an assessment of residue levels and withdrawal time in tissues. Determining tissue residue levels is essential for assessing potential human exposure and ensuring compliance with regulatory limits.

4. Materials and Methods

4.1. Chemicals

The CTX analytical standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol was brought from J.T. Baker (Gliwice, Poland). Trifluoroacetic acid was obtained from Merck (Darmstadt, Germany). The injectable formulation of CTX (Forsef, 1000 mg, Bilim Pharmaceuticals, Istanbul, Türkiye) was used for drug administration to fish.

4.2. Animals

The Institutional Animal Care and Use Committee at Mindanao State University granted ethical approval for the study (approval no: 2019/05). The experimental procedures were conducted at the Naawan campus, located in Misamis Oriental, Philippines. Nile tilapia (*Oreochromis niloticus*) exhibiting no external injuries or signs of disease, and displaying normal morphological and behavioral characteristics, were classified as clinically healthy. A total of 216 such individuals, each weighing between 100 and 120 g, were

utilized in the study. These fish were randomly distributed across eight plastic fiberglass tanks, each with a volume of 400 L and housing 27 fish. To adjust to the environment, the fish endured 10 days in their tanks before the experiments. Throughout the experimental period, the water temperature was kept at 30 ± 1.5 °C, and a natural light/dark cycle was maintained using ambient daylight. The fish were fed with the commercial feed (Tilapia Grower, Tateh Aquafeeds, Quezon City, Philippines) twice a day until they were satiated.

4.3. Experimental Design

Two hundred and sixteen fish were randomly divided into three treatment groups: IV, IP, and IM, each including seventy-two animals. Each group ($n = 72$) was randomly divided into twelve subgroups of six fish, and one subgroup ($n = 6$) was used at each sampling time. IV, IP, and IM injections were performed from the caudal vessel, peritoneal cavity, and right epaxial muscle, respectively. CTX was administered at a dose of 25 mg/kg in all three administration groups. For drug administration to fish, the commercial formulation of CTX was diluted to 20 mg/mL with sterile water immediately before drug administration. Therefore, the fish received doses of CTX ranging from 0.125 to 0.150 mL based on their weight. Drug administration and blood collection were performed with an insulin syringe (1 mL, 26-gauge 1/2-inch needle) under tricaine methanesulfonate anesthesia (MS-222, 150 mg/L). Blood samples (1 mL) were taken from the caudal vessel at 0 (control), 0.25, 0.5, 1, 2, 4, 8, 12, 24, 36, 48, and 72 h. Following blood collection, the fish were promptly euthanized through immersion in a high dose of MS-222 (250 mg/L) solution, after which muscle (left epaxial muscle) samples were obtained at each specified time point. Blood samples were immediately placed in tubes containing lithium heparin, mixed by inversion multiple times, and stored in cold boxes. Blood samples were centrifuged ($4000 \times g$ for 10 min) within one hour, and plasma samples were obtained. Plasma and muscle tissues were kept at -80 °C until CTX analysis.

4.4. Ceftriaxone Analysis

CTX analysis from plasma and muscle samples was performed by HPLC using the previously mentioned method [5]. The HPLC system (Shimadzu, Tokyo, Japan) was equipped with a model SPD-20A UV detector, a model SIL 20A auto-sampler, a model LC-20AT pump, a model DGU-20A degasser, and a model CTO-10A column oven. The chromatographic separation of CTX from plasma and muscle samples was carried out at 274 nm wavelength and using a C18 column (4.6×250 mm²; 5 μ m, GL Sciences, Tokyo, Japan). The column temperature was kept at 35 °C via a column oven. The mobile phase consisted of methanol (35%) and trifluoroacetic acid (0.1% in ultrapure water) with a flow rate of 1 mL/min. The analysis of data was performed with Shimadzu Corp.'s LC Solution software (Version 1.25 SP5). The sample was thawed at room temperature for plasma and muscle analyses. One gram of muscle tissue was weighed, transferred to homogenization tubes, and homogenized. One hundred μ L of plasma and 100 mg of muscle tissue were transferred to 2 mL microcentrifuge tubes, and 200 μ L of methanol was added. The samples were vortexed for 30 s and then centrifuged at $10,000 \times g$ for 10 min. The supernatant was transferred to autosampler vials, and 20 μ L was injected into the HPLC system.

4.5. Pharmacokinetic Analysis

Since repeated blood collection from fish was not possible (blood volume limitations), different fish were used at each sampling time, and CTX concentrations in plasma and muscle tissue were calculated for six animals in each group. However, as distinct animals were used at each sampling time, pharmacokinetic calculations were not performed individually for each animal but were instead based on the mean concentrations of six animals at each sampling point [22,54]. The use of curves that only show the mean may obscure the

variability in concentrations between animals. Plasma and muscle concentrations of CTX were reported as mean $\pm$ standard deviation (SD).

The plasma and muscle CTX concentration versus time profile from fish in the IV, IP, and IM groups was subjected to pharmacokinetic analysis using Winnonlin software version 6.1.0.173 (Pharsight Corp., Mountain View, CA, USA). Pharmacokinetic data were calculated by non-compartmental analysis. The following pharmacokinetic parameters were determined for plasma: peak concentration (C_{max}), plasma concentration at the first sampling time (0.25 h) after IV injection ($C_{0.25h}$), time to reach C_{max} (T_{max}), area under the concentration-versus time curve from 0 to last h and 0 to infinity (AUC_{0-last} , $AUC_{0-\infty}$), area under the plasma concentration–time curve extrapolated from last to ∞ in % of the total AUC ($AUC_{extrap\%}$), terminal elimination half-life ($t_{1/2\lambda_z}$), apparent volume of distribution (V_d), volume of distribution at steady state (V_{dss}), total body clearance (Cl_T), and mean residence time (MRT). The following parameters were calculated for the muscle: C_{max} , T_{max} , AUC, $AUC_{extrap\%}$, and $t_{1/2\lambda_z}$. The AUC was calculated using the linear-log trapezoidal method for IV application and the linear-up log-down method for IP and IM application. The C_{max} , $C_{0.25h}$, and T_{max} were determined from the individual plasma and muscle concentration–time profiles. The bioavailability (F) was determined using $(AUC_{IP,IM}/AUC_{IV}) \times 100$.

5. Conclusions

This study presents the plasma and muscle pharmacokinetics of CTX in Nile tilapia for the first time. CTX demonstrates rapid absorption and substantial systemic exposure following IP and IM administration at a 25 mg/kg dose. CTX exhibited larger V_d and shorter $t_{1/2\lambda_z}$ compared with cold-water species such as brown trout, with Cl_T reflecting temperature-dependent metabolic and excretory processes. Bioavailability was good after IP and IM injection, while muscle penetration remained limited ($AUC_{muscle/plasma}$ ratios of 0.18–0.30), indicating low tissue accumulation similar to other cephalosporins. Overall, CTX was determined to be pharmacokinetically appropriate for parenteral use in tilapia. However, further research addressing the identified limitations is necessary to fully elucidate its therapeutic utility and ensure its safe and effective use in this economically important fish species.

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Institutional Review Board Statement: The Institutional Animal Care and Use Committee at Mindanao State University granted ethical approval for the study (approval no: 2019/05, approval date: 18 June 2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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

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Article

Antimicrobial Resistance in Bacterial Strains of Agricultural Interest: Predictions Based on Genomic Data

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Abstract

Background: Plant growth promoting bacteria (PGPB) are non-pathogenic bacteria that enhance plant growth through several mechanisms such as nutrient mobilization, phytohormones production, defense against phytopathogens, and alleviation of plant stress. Hence, these bacteria are used as ecologic biofertilizers to diminish the use of agrochemicals. Nevertheless, some PGPR strains can harbor antibiotic resistance determinants and the possibility of spreading them upon releasing these bacteria is an environmental concern. **Objectives:** The objectives of this work are as follows: (1) evaluating the antibiotic resistance in a collection of PGPB, and (2) prospecting antibiotic resistance genes in the genomes of PGPB in order to predict the risk for antibiotic resistance dissemination. **Methods:** The resistance towards 12 antibiotics in a collection of 20 PGPB (10 Gram-positive and 10 Gram-negative strains) has been evaluated using disk diffusion in agar, broth microdilution, and agar dilution tests. In addition, the whole genomes of six strains have been sequenced in order to find the correlation between the resistance levels and AMR genes by using bioinformatic tools. **Results:** The results indicated a wide range of halo diameters, but in general Gram-negatives showed higher resistance compared to Gram-positives. The four most resistant strains and the two more susceptible strains were selected for further analysis and sequencing the whole genomes. The resistant strains were identified as *Achromobacter spanius* N6, *Leclercia adecarboxylata* H17, *Priestia aryabhattai* strain MHA1, and *Bacillus cereus* N25. The susceptible strains were identified as *Pantoea* sp. S3 and *Priestia megaterium* MS4. Mining antibiotic resistance genes in the genomes confirmed the existence of resistance determinants responsible for the phenotypic behavior, indicating the potential of genomics for predicting antibiotic resistance in PGPB. However, there was not an exact correspondence between the presence of the genes and the level of resistance, suggesting the existence of additional regulatory mechanisms. **Conclusions:** The information obtained by genomics must be complemented experimentally by tests for antibiotic resistance determination. In this regard, it is necessary to develop a global antibiotic resistance database for PGPB, due to the difficulty of interpretation of the antibiotic susceptibility tests after comparing the experimental results with those tabulated for clinical species.

Keywords: antimicrobial resistance (AMR); plant growth promoting bacteria (PGPB); minimal inhibitory concentration (MIC); genomics; genome mining; PATRIC; CARD

1. Introduction

Antibiotic resistance in bacteria, particularly pathogenic species, represents one of the greatest challenges humans will face in the coming decades. Its effects are already becoming evident through the reduced efficacy of traditional antibiotics and, consequently, a decreasing availability of effective treatments, coupled with increasing difficulty in discovering new antibiotics [1]. Additionally, it is estimated that in 2021, approximately 4.71 million people died due to antimicrobial resistance (AMR), and that by 2050, this number could rise to around 8.71 million deaths per year [2].

Bacteria have developed a wide range of mechanisms to avoid the action of antibiotics, including efflux pumps, enzymatic inactivation, target modification, sequestration/chemical modification of the antibiotics, reduced permeability, biofilm formation, etc. [3–5]. The coexistence of several of these mechanisms within a single genome account for the multidrug resistance profiles commonly observed in clinical settings.

Although resistance can emerge through mutation, its global dissemination primarily relies on horizontal gene transfer (HGT). Plasmid-mediated conjugation is the dominant pathway: a meta-analysis of 113 studies reported transfer frequencies spanning 12 orders of magnitude, with peaks in manure-fertilized agricultural soils and livestock effluents [6]. In biofilms, cell proximity and the extracellular matrix facilitate the conjugation even of non-conjugative plasmids, via co-integrated plasmids or auxiliary systems [7]. Natural transformation and bacteriophage-mediated transduction also contribute to gene exchange, although with a more restricted taxonomic scope. These processes give rise to a genetic mosaic that links environmental, animal, and human reservoirs, driven by co-selection involving antimicrobials, heavy metals, and biocides. The spread of broad-host-range plasmids, such as IncP-1, poses an emerging threat under the One Health framework [8].

PGPB (plant growth promoting bacteria) enhance plant development through both direct and indirect mechanisms, being considered as ecological biotechnological tools [9,10]. Direct mechanisms include nutrient solubilization, phytohormone production, and the stimulation of lateral root formation to improve nutrient uptake [11]. Indirectly, they help mitigate abiotic stress and exert biocontrol over phytopathogens [12]. Genera such as *Bacillus*, *Pseudomonas*, *Azospirillum*, and *Rhizobium* include non-pathogenic PGPB strains frequently used as natural biofertilizers [13–15]. However, the environmental release of such bacterial inoculants may present a risk if they act as potential vectors of antibiotic resistance genes (ARGs). A meta-analysis of 125 studies showed that around 85% of phosphate-solubilizing bacteria isolated from soil harbored at least one ARG, with enzymatic deactivation (48.7%) and active efflux (25.2%) as the most prevalent resistance mechanisms [16]. Recent examples include *Serratia rubidaea* ED1, isolated from agricultural soil and resistant to seven antibiotics with multiple efflux pumps, and *Delftia tsuruhatensis* [17]. The use of commercial bioinoculants (which may contain 10^8 – 10^9 CFU/mL) represents a potential risk for horizontal ARG transfer within agricultural ecosystems [16]. For this reason, international entities advocate the implementation of phenotypic and genomic screening prior to the approval of such microbial fertilizers.

Standardized methods for determining antibiotic resistance (as established by institutions such as CLSI in the US or EUCAST in Europe) rely on comparing experimental results to breakpoint tables that define whether a strain is considered susceptible or resistant to a given antibiotic. However, these tables (published and updated yearly) are based on data from clinical isolates. Therefore, most of the time, when testing antibiotic susceptibility in agricultural strains, there is no dedicated reference database, since most antibiotics lack established breakpoints for beneficial soil bacteria such as PGPB. Consequently, susceptibility results are interpreted using criteria defined for clinically relevant species, which may lead to misinterpretation. Moreover, EU Regulation 2019/1009 outlines the requirements for mi-

crobal biostimulants, such as a minimum viable concentration and the absence of specific pathogens, but does not address antimicrobial resistance profiles, leaving a regulatory gap. This situation hinders comparison across studies, limits the quality control of biofertilizers, and constitutes a barrier to their safe integration into sustainable agricultural systems.

Genomic analysis has become a key tool for identifying and contextualizing resistance genes in environmental bacteria [18]. Databases such as PATRIC (Pathosystems Resource Integration Center) provide access to thousands of fully annotated bacterial genomes, along with tools for comparative analysis, mobile element visualization, and the identification of resistance and virulence factors [19]. Likewise, the CARD (Comprehensive Antibiotic Resistance Database) combines highly curated annotations with protein homology-based prediction models, facilitating the detection of both known and emerging resistance genes [20].

Our department holds a big collection of PGPB strains which have been isolated during the past 10 years, including both Gram-positive and Gram-negative bacteria with good plant growth promoting activities. However, the antibiotic resistance profile of these microorganisms has not been evaluated. In this context, the present work aims to (a) determine the antibiotic susceptibility profiles of 20 selected PGPB based on experimental testing; (b) identify resistance genes within their genomes using experimental databases and sequence similarity analyses; and (c) establish potential correlations with the experimental data obtained in order to help to predict the risk of disseminating antimicrobial resistance determinants upon releasing particular PGPB.

2. Results

2.1. Collection of Bacterial Strains and Antibiotics Selected for the Study

In the department of Microbiology and Parasitology of the Faculty of Pharmacy, there was already a pre-existing collection of PGPB strains isolated from agricultural soils. Ten Gram-positive strains and ten Gram-negative strains were selected for this study (Table 1). The selected organisms were those with properties considered optimal for promoting plant growth as previously published [21–25]. The strains were retrieved from frozen cultures stored at -80°C in glycerol and plated on TSA Petri dishes, followed by incubation at 28°C for 24 h.

Table 1. Strains retrieved from the collection with the best plant growth promoting activities. Aux: production of auxins; PS: phosphate solubilization; KS: potassium solubilization; Bio: formation of biofilms; N_2 -fix: nitrogen fixation; Sid: production of siderophores; ACC: ACC-deaminase activity; Amy: amylase activity; Cel: cellulase activity; Pect: pectinase activity; Prot: protease activity; DNase: DNase activity; Chit: chitinase activity.

Gram-Negatives		
Strain	PGP properties and extracellular activities	Reference
H4	Aux, PS, KS, Sid, N_2 -fix, Amy, Prot	[22]
H17	KS, Sid, Aux, Bio, N_2 -fix, Prot	[22]
MH3	KS, Bio, Aux, Amy, Cel, Prot	[22]
N1	Sid, ACC, Bio, N_2 -fix	[23]
N3	PS, Sid, Aux, Bio, N_2 -fix, Cel, Chit	[23]
N6	Sid, Bio, N_2 -fix	[23]
N11	PS, Sid, Aux, Bio, N_2 -fix, Prot	[23]
N23	PS, Sid, Aux, Bio, N_2 -fix, Cel	[23]
N30	PS, Sid, Aux, ACC, N_2 -fix, Prot	[23]
S3	Bio, N_2 -fix, Aux, PS, KS, Sid, Cel	[21]

Table 1. *Cont.*

Gram-Positives		
Strain	PGP properties and extracellular activities	Reference
MS4	KS, Aux, N ₂ -fix, Bio, Amy, Prot	[23]
MHA1	KS, Sid, Aux, N ₂ -fix, Bio, Amy, Prot	[23]
MH6	Sid, Aux, N ₂ -fix, Amy, Prot	[23]
MH9B	Aux, Sid, Cel, Prot	[23]
MR4	Bio, N ₂ -fix, Sid, Aux, Prot, Amy, Pect, DNAase	[23]
MS5	KS, Sid, Aux, N ₂ -fix, Amy, Prot	[21]
N14	PS, Sid, Aux, Bio, N ₂ -fix, Prot	[23]
N18	PS, Sid, Aux, Bio, N ₂ -fix, Prot, Pect, Cel	[23]
N25	PS, Sid, Aux, Bio, N ₂ -fix, Prot, Cel, Chit	[23]
N32	PS, Sid, Aux, N ₂ -fix, Prot, Pect, Cel	[23]

The antibiotics used in this study are those recommended by the French regulation on biofertilizers ANSES (2020) [26] as the current Spanish and European legislations do not include microbial resistance criteria in the regulation of biofertilizers. These antibiotics are listed in Table 2.

Table 2. Selected antibiotics for the determination of susceptibility or resistance in the bacterial samples.

Gram-Positives		Gram-Negatives	
Antibiotic	Amount on the Disk (micrograms)	Antibiotic	Amount on the Disk (micrograms)
Ampicillin	10	Ampicillin	10
Clindamycin	2	Ciprofloxacin	5
Chloramphenicol	30	Colistin sulphate	10
Erythromycin	15	Streptomycin	25
Streptomycin	25	Fosfomicin	50
Gentamicin	10	Gentamicin	10
Kanamycin	30	Kanamycin	30
Tetracycline	30	Tetracycline	30
Vancomycin	30		

2.2. Determination of Antimicrobial Susceptibility by the Disk Diffusion Method on Agar

In the first step, the susceptibility was tested by the agar diffusion test (Supplementary Information Figure S1). Data of halo diameters for the selected strains are shown in Table 3 (Gram-negatives) and Table 4 (Gram-positives). Results for ampicillin revealed fully resistant strains (both within Gram-negative and Gram-positive collections) giving no inhibition halo ($\phi = 0$). Among the susceptible strains, the halo diameters in Gram-negatives were in general lower (9–14 mm) than in Gram-positive (13–17 mm), indicating the higher ampicillin resistance in the Gram-negatives. For gentamicin, no strain gave full resistance. The halo diameters for Gram-negative (18–26 mm) and Gram-positive strains (17–30 mm) were comparable, although slightly lower for Gram-negatives. Regarding kanamycin, the strain N30 was resistant. The rest of both collections gave diameter halos comprised between 11 and 25 mm for Gram-negatives, and 14–33 mm for Gram-positives, indicating a higher susceptibility of Gram-positives. For streptomycin, the Gram-negative strain H17 was resistant ($\phi = 0$). Again, the halo diameters of the Gram-negative collection were in general lower (11–22 mm) than those of Gram-positive (9–27), indicating the higher resistance of Gram-negatives. For tetracycline, the strains H4 and N30 (both Gram-negatives) did not produce any inhibition halo, and were resistant. In the Gram-positive collection, all strains gave inhibition halos with ϕ values between 8 and 25 mm, indicating high variability in the level of resistance, probably due to different resistance mechanisms and/or gene doses of genes associated with antibiotic resistance. From all these data, the

first conclusion is that in general the Gram-negative strains showed a higher resistance to several antibiotics as compared to Gram-positives.

Table 3. Inhibition zone diameters around antibiotic disks, measured in millimeters, for Gram-negative bacteria. Amp: Ampicillin; Cipro: Ciprofloxacin; Colis: Colistin; Fosfo; Fosfomycin; Genta: Gentamycin; Strepto: Streptomycin; Tetra: Tetracycline.

Strain	Amp	Cipro	Colis	Fosfo	Genta	Kana	Strepto	Tetra
H4	0	23	13	9	18	22	13	0
H17	0	0	0	19	21	19	0	17
MH3	9	24	11	12	26	27	20	11
N1	0	8	16	0	19	13	13	11
N3	9	30	0	37	19	11	18	14
N6	0	0	14	0	16	20	11	15
N11	0	20	12	16	19	19	15	10
N23	11	32	14	22	20	21	19	18
N30	12	31	10	23	21	0	21	0
S3	11	31	13	26	22	25	22	18

Table 4. Inhibition zone diameters around antibiotic disks, measured in millimeters, for Gram-positive bacteria. Amp: Ampicillin; Chloram: Chloramphenicol; Clinda: Clindamycin; Erythro: Erythromycin; Genta: Gentamycin; Kana; Kanamycin; Strepto: Streptomycin; Tetra: Tetracycline; Vanco: Vancomycin.

Strain	Amp	Chloram	Clinda	Erythro	Genta	Kana	Strepto	Tetra	Vanco
MHA1	0	0	0	0	17	17	9	8	0
MH6	15	29	14	39	29	33	26	27	7
MH9B	14	21	31	28	30	28	20	12	15
MR4	16	29	29	30	27	30	19	15	16
MS4	13	26	17	19	26	30	25	24	17
MS5	17	17	14	34	28	32	27	25	17
N14	14	27	13	29	25	29	23	16	24
N18	14	27	19	14	20	20	20	18	0
N25	0	17	6	12	19	14	16	14	8
N32	10	18	11	19	20	22	18	15	0

Furthermore, there were antibiotics specifically used for Gram-negatives: Ciprofloxacin, colistin, and Fosfomycin. Two strains, H17 and N1, showed resistance against Ciprofloxacin, whereas the rest displayed halos comprised between 8 and 32 mm; H17 and N3 were resistant towards colistin ($\phi = 0$), and the rest produced halos between 10 and 16 mm. For Fosfomycin, N1 and N6 were fully resistant ($\phi = 0$), and the rest formed halos with ϕ between 9 and 37 mm, reflecting high variability.

Regarding the Gram-positives, Chloramphenicol, Clindamycin, Erythromycin, and Vancomycin were the specific antibiotics used. For Chloramphenicol, MHA1 showed full resistance ($\phi = 0$), and the rest ranged between ϕ 17 and 29 mm, showing variable sensibility. As for Clindamycin, ϕ ranged between 6 and 31 mm (0 mm for MHA1). Erythromycin sensibility was prevalent, with ϕ being 12–39 mm, once again excluding the full resistance of MHA1. Finally, most strains were resistant to Vancomycin, with a ϕ of 7–24 mm, except for MHA1, N18, and N32, which did not develop inhibition halos.

Figure 1 shows a box plot diagram of the halo diameters for the five antibiotics assayed in both the Gram-negative and the Gram-positive collections, namely, Ampicillin, Gentamicin, Kanamycin, Streptomycin, and Tetracycline. As we can see, this representation reinforces the previous conclusion: the median value of the halo diameter was lower for Gram-negative strains, indicating the higher resistance of Gram-negative bacteria. On the other hand, there were antibiotics for which the diameter halos showed very

low variability (for instance, gentamicin and colistin in Gram-negatives), whereas for other antibiotics such as Ciprofloxacin, Erythromycin, Fosfomycin, and Vancomycin, large variability was found. These results could suggest the existence of multiple resistance mechanisms, an effect of gene dosage or regulatory mechanisms controlling the expression of the resistance determinants.

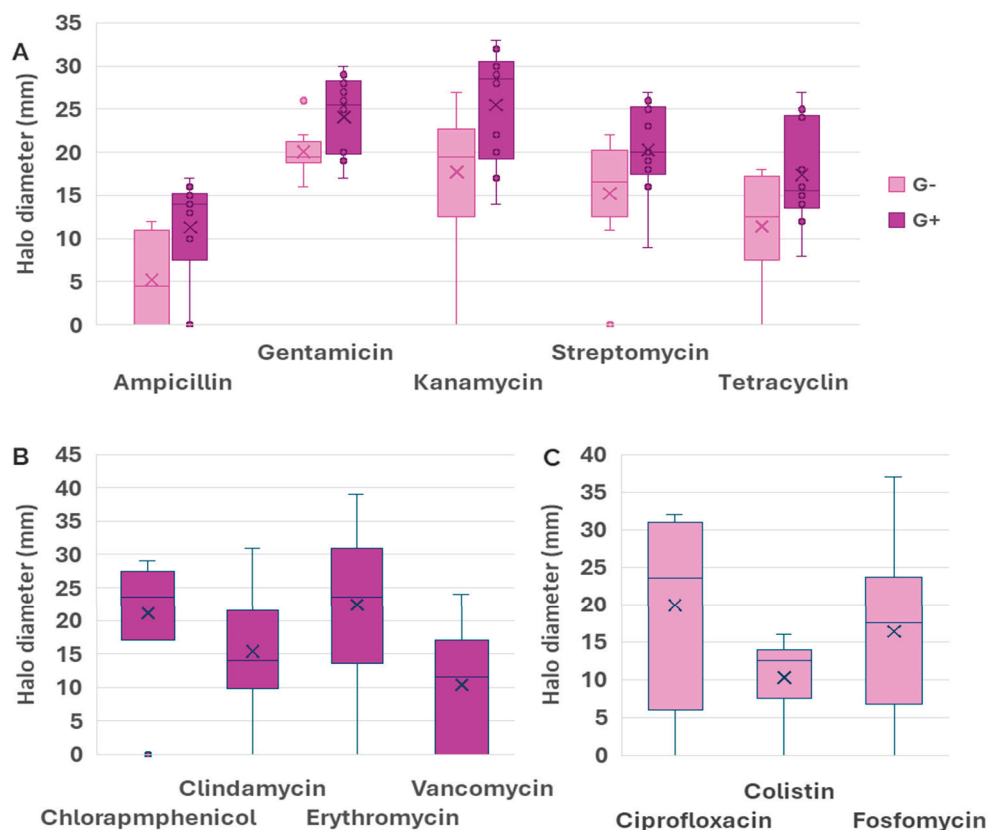


Figure 1. Box-plot comparing the average values and the range intervals of halo diameters for antibiotics used in both Gram-positive and Gram-negative strains (A), exclusive antibiotics for Gram-positives (B), and exclusive antibiotics for Gram-negatives (C). Box indicates the quartile, and the median is indicated by a line, whilst the average is shown as a cross. Bars indicate the full range of data and circles indicate outliers, whereas crosses represent the median of the data and the horizontal lines represent the average. Data for Gram-negatives are shown in pink color whereas purple was used for Gram-positives.

2.3. Selection of Six Strains for Further Studies

In the next step, we have determined the minimal inhibitory concentration (MIC) to antibiotics of six selected strains, for which we picked three strains among the Gram-positives and three from the Gram-negatives, selecting the most resistant and sensitive strains. Since the sensibility or the resistance depended on every specific antibiotic, we needed a criterion to select the “most sensitive strains” and “the most resistant strains” regarding the whole set of antibiotics. Among Gram-negatives, N6 and H17 were selected, as they were the most antibiotic-resistant bacteria when considering the complete spectrum. As for the most sensitive strains, S3 was selected over N3, as its halo diameters were larger. Among the Gram-positive bacteria, MHA1 was clearly the most resistant strain, followed by N25, and both strains were selected. In contrast, the most susceptible strains were MH6, followed by MR4, MS5, and MS4. However, MS4 was selected for the study over the other candidates due to the difficulty of growing MH6. Finally, the selected strains were N6, H17, S3, N25, MHA1, and MS4.

2.4. Determination of Minimal Inhibitory Concentration

The determination of the MIC was carried out by the microdilution test for all the antibiotics, except for Fosfomycin. For this particular antibiotic, the EUCAST (European Committee on Antimicrobial Susceptibility Testing, <https://www.eucast.org/>, accessed on 19 November 2025) recommends the agar dilution test in the presence of 25 mM glucose-6-phosphate [27].

The main problem at the time of assigning the categories of resistant/sensitive has been the lack of information for the species found in agriculture. It should be taken into account that breakpoints are tabulated for clinical strains, not for strains isolated from agricultural or environmental samples. In fact, the tables published yearly by both the Clinical Laboratory Standard Institute (CLSI) and EUCAST are elaborated for clinically relevant species, and it is not possible to find data for species such as *Leclercia adecarboxilata* or *Achromobacter spanius*. In this regard, the interpretation of the results to classify the strains into the resistant (R) or susceptible (S) categories has been performed following the breakpoints for *Bacillus* spp. for Gram-positives, and for *Pseudomonas* and Enterobacteriaceae for Gram-negatives [27]. When no data were available, the EUCAST guidance “When there are no breakpoints in breakpoint tables” was followed [28].

The data of MIC for MHA1 were comprised between 128 and 512 mg L⁻¹, indicating the high resistance towards many antibiotics (Table 5). By contrast, the values of MIC for MS4 were very low, indicating the susceptibility towards all antibiotics but ampicillin. By its part, N25 showed high MIC for ampicillin and clindamycin and was susceptible or showed an intermediate susceptibility towards the rest of the antibiotics (Table 6). Among Gram-negatives, the strain showing the highest values of MIC was N6, whereas the most susceptible strain was S3 (Table 6). N6 showed high MIC for all antibiotics, except for ciprofloxacin and kanamycin. The resistance towards streptomycin could not be compared with any tabulated data. *Leclercia adecarboxylata* H17 showed high values of MIC for ampicillin and Fosfomycin, and was susceptible to gentamicin and kanamycin. Concerning S3, it was susceptible to most antibiotics, except for ampicillin and ciprofloxacin.

Table 5. Minimum inhibitory concentration (MIC) of selected Gram-positive bacteria for different antibiotics. In the lower row are breakpoints for Gram-positives when there are no data for the corresponding species according to EUCAST, 2024. n.d., there are no data. Amp: Ampicillin; Clinda: Clindamycin; Genta: Gentamycin; Kana: Kanamycin; Strepto: Streptomycin; Tetra: Tetracycline.

Strain	Amp (mg L ⁻¹)	Clinda (mg L ⁻¹)	Genta (mg L ⁻¹)	Kana (mg L ⁻¹)	Strepto (mg L ⁻¹)	Tetra (mg L ⁻¹)
MHA1	>512 (R)	>512 (R)	128	128	>512	256 (R)
MS4	>512 (R)	<0.5 (S)	8	4	8	<0.5 (S)
N25	>512 (R)	>512 (R)	16	8	32	2 (S)
Breakpoints for <i>Bacillus</i> spp. (except <i>B. anthracis</i>) [27]	n.d.	1	n.d.	n.d.	n.d.	n.d.
Breakpoints for Gram-positives when there are no other data [28]	0.5	0.5	n.d.	n.d.	n.d.	2

Figure 2 shows a heatmap depicting the correlation between halo diameters and the MIC. The correlations were good in general, with values between 1 and 0.7 for most cases. In general, we can see that there are similar correlation indices in Gram-positive and Gram-negative strains. For Gram-positives, the lowest correlation was found for N25 in tetracycline (0.47) and MS4 for ampicillin and clindamycin (0.57 in both cases). In Gram-negatives, the lowest correlation was found for N6 in tetracycline (0.20).

Table 6. Minimum inhibitory concentration of selected Gram-negative bacteria for different antibiotics. In the lower row are breakpoints for Gram-negatives when there are no data for the corresponding species according to EUCAST. n.d., there are no data. Amp: Ampicillin; Cipro: Ciprofloxacin; Fosfo; Fosfomycin; Genta: Gentamycin; Kana: Kanamycin; Strepto: Streptomycin; Tetra: Tetracycline.

Strain	Amp (mg L ⁻¹)	Cipro (mg L ⁻¹)	Fosfo (mg L ⁻¹)	Genta (mg L ⁻¹)	Kana (mg L ⁻¹)	Strepto (mg L ⁻¹)	Tetra (mg L ⁻¹)
H17	16 (R)	128 (R)	8 (S)	8 (R)	64 (S)	64	4 (R)
N6	>512 (R)	2 (R)	>512 (R)	64 (R)	64 (S)	64	4 (R)
S3	128 (R)	1 (R)	4 (S)	4 (S)	16 (S)	32	1 (S)
Breakpoints for <i>Pseudomonas</i> spp.	n.d.	0.5	64	4	64	n.d.	2
Breakpoints for Enterobacterales [27]	8	0.25	8	2	n.d.	n.d.	4
Breakpoints for Gram-negatives when there are no other data [28]	8	0.25	n.d.	n.d.	n.d.	n.d.	2

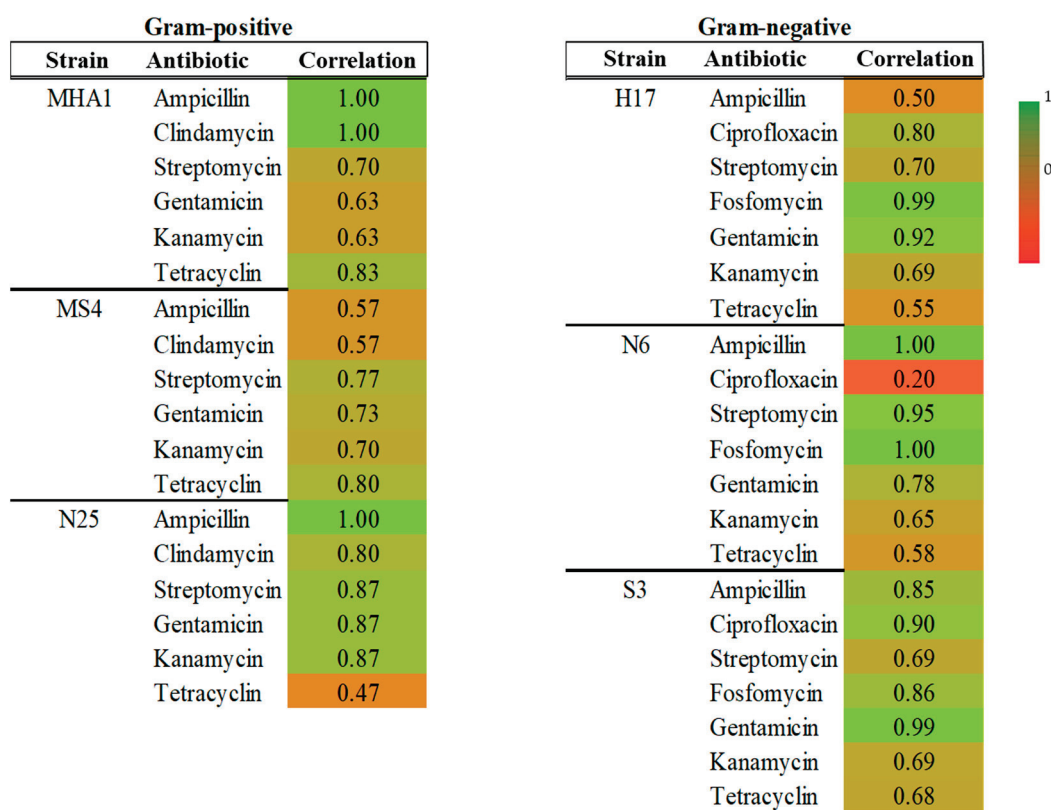


Figure 2. Heatmap showing correlation between MIC values and halo diameters for each strain and antibiotic in Gram-positive and Gram-negative groups.

In our study, we have multiple variables, such as 20 different bacteria (10 Gram-negative and 10 Gram-positive) and 5 common antibiotics for both types of bacteria, together with specific antibiotics, determination of halo diameters, and determination of MIC by two different methods (microdilution and agar dilution). In this regard, the multifactorial nature of the data can be simplified by reducing the number of variables to two principal components. The principal components analysis (PCA, Figure 3) indicates that both axes together accounted for 83.7% of the variability, with 64.5% of variability associated with dimension 1 and 19.2% of variability associated with dimension 2. Thus, the reduction in variables did not much affect the representativity of data and the conclusions. On the other hand, the PCA allows visual representation of the distance between samples and also to visualize how the samples can be grouped. We can see that Gram-positive strains were more disperse according to component 1, while Gram-negative strains occupied a

central position in the graphic and spanned more regarding component 2. This would suggest different resistance mechanisms in both types of bacteria, since the data of MIC and halos are grouped in a different way in Gram-positives and Gram-negatives. In addition, we can observe superposition of the circles englobing both types of bacteria, which could also suggest the existence of common antimicrobial resistance mechanisms [29].

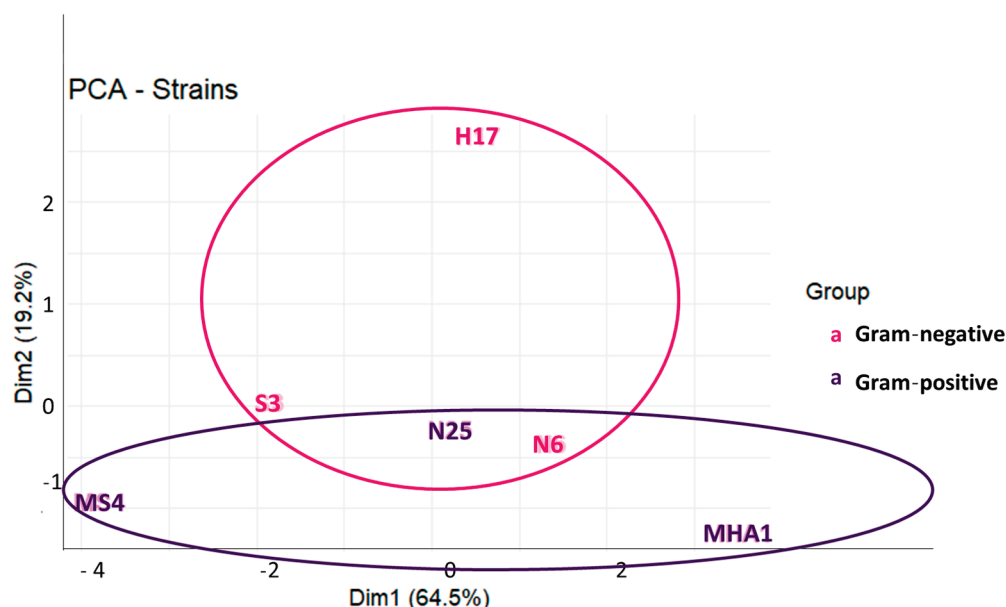


Figure 3. Principal components analysis performed on a dataset composed of the values of MIC and halo diameters for common antibiotics used in both Gram-positive and Gram-negative strains.

2.5. Whole Genome Sequences and Main Features of the Assemblies

The whole genomes of the six bacteria were sequenced and a study of the assembled genomes was conducted. The main features of the sequences are shown in Table 7. The genomes had sizes comprised between 4.9 and 6.6 Mb, without significant differences between Gram-positive and Gram-negative strains. The number of contigs oscillated between 72 and 219, being little shorter in Gram-negatives. The G + C percentages clearly reflected differences between both types (34–37% in Gram-positives and 54–64% in Gram-negatives). The number of coding sequences oscillated between 4577 and 6185. There were great differences between the number of RNA genes, being much higher in Gram-positives (127–128) than in Gram-negatives (65–97). Also, the number of rRNA and tRNA were higher in Gram-positives. Finally, there were 10–20 contigs related to plasmids in the strains MHA1 and MS4 (genus *Priestia*, as we will comment on next), whereas in N25 and in Gram-negatives, the number of contigs associated with plasmids was comprised between 0 and 3.

Table 7. Summary statistics of genome assemblies.

Genomic Features	MHA1	MS4	N25	H17	N6	S3
Total length (bp) ^a	5,891,127	6,043,838	5,805,119	4,949,670	6,641,484	5,276,853
Number of contigs (≥ 100 bp) ^a	200	219	92	74	72	170
Largest contig (bp) ^a	1,407,233	1,236,212	842,001	1,093,983	1,263,707	1,548,608
N50 (bp) ^a	1,005,957	1,008,741	330,999	404,426	412,829	414,608
L50 ^a	3	3	5	3	5	3
N75 (bp) ^a	227,051	228,602	211,237	288,132	235,369	229,884
L75 ^a	6	6	11	7	10	8

Table 7. Cont.

Genomic Features	MHA1	MS4	N25	H17	N6	S3
DNA G + C content (mol%) ^a	37.46	37.44	34.79	56.41	64.25	54.40
Ns per 100 kb ^a	0	0	0	0	0	0
Mean coverage	74.97×	73.40×	78.81×	62.05×	47.05×	41.72×
Number of CDS ^b	5958	6185	5729	4577	5963	4955
RNA genes ^b	182	162	127	97	65	88
rRNA ^b	27	27	19	14	4	13
tRNA ^b	154	134	107	82	60	74
tmRNA ^b	1	1	1	1	1	1
Number of contigs as plasmid ^c	10	20	1	1	0	3
CRISPR repeat ^d	0	7	5	3	5	2

^a Data from QUAST v. 5.3.0 software [29]. ^b Data from Prokka v.1.14.5 [30]. ^c Data from Deeplasmid software, version August 22, 2024 [31]. ^d Data from CRISPRCasFinder, version 4.2 [32].

2.6. Identification of the Selected Strains

Table 8 shows the identification of the strains based on the sequence of the 16S rRNA extracted from the WGS. Among the Gram-positive strains, MS4 and MHA1 were identified as *Priestia megaterium* and *P. aryabhattai*, whereas N25 corresponded to *Bacillus cereus*. Among Gram-negatives, N6 was identified as *Achromobacter spanius*, H17 as *Leclercia adecarboxylata*, and S3 corresponded to *Pantoea* sp. In this case, the comparison of the complete rRNA16S sequence gave the highest percentage of similarity with *P. conspicua* (99.64%) and also with *P. agglomerans*. (99.64%). However, after digital DNA–DNA hybridization (dDDH) comparing the whole genome, the maximum identity (69.8%) was observed with *P. pleuroti* (isolated from the fungus *Pleurotus ostreatus*) [33]. Moreover, this low identity after the comparison of the whole genome (below 70%) allowed us to suspect that it can belong to a novel species within the genus and this is the reason for us to not assign the strain to a particular species. Further studies will be performed to confirm this possibility. For the rest of isolates, the same species was assigned upon 16S rRNA sequence and dDDH.

Table 8. Identification of the six selected strains based on the sequence of the 16S rRNA gene sequence and dDDH. The strain S3 is identified by the bioproject since a percentage of dDDH below 70% could indicate a novel species, which is an aspect that is under study.

Strain	16S rRNA (bp)	Most Similar Taxon	Identity (%)	dDDH Value	Accession Number
N6	1527	<i>Achromobacter spanius</i>	100%	89.3% (<i>A. spanius</i>)	GCA_977062005
H17	1438	<i>Leclercia adecarboxylata</i>	99.71%	74.2% (<i>Leclercia tamurae</i>) 44.8% (<i>L. adecarboxylata</i>)	GCA_977061975
S3	1443	<i>Pantoea conspicua</i>	99.64%	69.8% (<i>Pantoea pleuroti</i>) 29.7% (<i>P. conspicua</i>)	PRJEB102903
N25	1318	<i>Bacillus cereus</i>	100%	72.5% (<i>B. cereus</i>)	GCA_977062015
MHA1	1459	<i>Priestia aryabhattai</i>	100%	73.1% (<i>P. aryabhattai</i>)	GCA_977061995
MS4	1560	<i>Priestia megaterium</i>	99.86%	92.0% (<i>P. megaterium</i>)	GCA_977061985

dDDH values $\geq 70\%$ indicate same species. dDDH values $< 70\%$ indicate possible new species [34].

2.7. Mining Antibiotic Resistance Genes in the WGS

In order to work at the prediction level, genes for antibiotic resistance have been prospected in the whole genomes of the six bacteria using the bioinformatics tools PATRIC [19] and CARD [20]. As shown in Tables 9 and 10, the genomes of Gram-negative bacteria contain greater numbers of resistance genes against the tested antibiotics (17–26) compared to those of Gram-positive bacteria (10–17). These results were in accordance with the experimental determinations of halos and MICs.

Table 9. Antimicrobial resistance genes found in the genomes of Gram-positive strains, number of copies, and localization. ARG: number of antibiotic resistance genes; N: total number of ARG found by PATRIC; n: number of genes found by PATRIC related to the antibiotics used in this work. Tet., Tetracycline; Strep., Streptomycin; Chloram., Chloramphenicol.

Strain	ARG N/n	Gene	Number of Copies	Localization	Confers Resistance Towards
MHA1	49/10	<i>gyrA, gyrB</i>	2 copies each	Chromosome	Ciprofloxacin
		<i>s10p</i>	1 copy	Chromosome	Tetracycline
		<i>s12p</i>	1 copy	Chromosome	Streptomycin
		<i>gidB</i>	1 copy	Plasmid	Streptomycin
		<i>murA</i>	1 copy	Chromosome	Fosfomycin
		<i>fosA</i>	1 copy	Chromosome	Fosfomycin
MS4	49/10	<i>gyrA, gyrB</i>	2 copies each	Chromosome	Ciprofloxacin
		<i>s10p</i>	1 copy	Chromosome	Tetracycline
		<i>s12p</i>	1 copy	Chromosome	Streptomycin
		<i>gidB</i>	1 copy	Chromosome	Streptomycin
		<i>murA</i>	2 copies	Chromosome	Fosfomycin
		<i>fosB</i>	1 copy	Chromosome	Fosfomycin
N25	52/17	<i>gyrA, gyrB</i>	1 copy each	Chromosome	Ciprofloxacin
		<i>s10p</i>	1 copy	Chromosome	Tetracycline
		<i>s12p</i>	1 copy	Chromosome	Streptomycin
		<i>gidB</i>	1 copy	Chromosome	Streptomycin
		<i>murA</i>	2 copies	Chromosome	Fosfomycin
		<i>fosB</i>	1 copy	Chromosome	Fosfomycin
		<i>BcII</i> family	1 copy	Chromosome	Ampicillin
		<i>CatA15/A16</i>	1 copy	Chromosome	Chloramphenicol
		<i>YkkA, YkkC, YkkD</i>	1 copy each	Chromosome	Tet., Strep., Chloram.
		<i>VanXY</i> -unclassified	2 copies	1(chr) + 1(plas)	Vancomycin
		<i>VanA/I/Pt</i> -type	1 copy	Plasmid	Vancomycin
		<i>VanF/M</i> -type	2 copies	1(chr) + 1(plas)	Vancomycin
		<i>VanR</i> -unclassified	1 copy	Plasmid	Vancomycin

Among the genes common to both groups are *gyrA* and *gyrB*, which are involved in resistance to ciprofloxacin through target modification. This antibiotic inhibits DNA gyrase, an essential enzyme for DNA replication. Gram-positives and Gram-negatives also shared the determinants *s12p* and *gidB*, related to resistance to aminoglycosides such as streptomycin, which act on the ribosomal small subunit. The determinant *s10p* is related to the resistance towards tetracycline. In this case, mutations in the ribosomal proteins prevent the antibiotic from interfering with protein synthesis. Another gene found in both Gram-positives and Gram-negatives was *murA*. Mutations in this gene confer resistance towards Fosfomycin by affecting the protein MurA involved in the first steps of peptidoglycan synthesis (before the action of beta-lactamases) [35].

On the other hand, some resistance genes appear exclusively in one group. For example, the resistance towards Fosfomycin mediated by *fosA* and *fosB* was found only in Gram-positives. FosA, present in MHA1, is a class of metalloenzymes able to disrupt the epoxide ring of the Fosfomycin drug, preventing its action [36], whereas FosB, present in MS4, codifies an enzyme catalyzing the binding of L-cysteine or bacillithiol to Fosfomycin, thus inhibiting its bactericidal effect [37]. The strain N25 was the only Gram-positive showing genes for resistance towards chloramphenicol and vancomycin. There are correlations with the diameter of halos (17 and 8 mm, respectively, Table 3). The resistance towards chloramphenicol was represented by the gene *catA* codifying the chloramphenicol acetyl-transferase, which modifies the conformation of chloramphenicol by acetylation and prevents its binding to the ribosomal large subunit [38]. In addition, the genes *VanXY*, *VanA/I/Pt*-type, and *VanF/M*-type—associated with resistance to vancomycin (an antibiotic that inhibits the polymerization of peptidoglycan in the bacterial cell wall)—were only found in the Gram-positive strain N25. It is noteworthy that the determinants for vancomycin and Fosfomycin resistance are shared in the same plasmid in enterococci [39]. The strain

N25 also carried the gene *BcII* conferring resistance towards ampicillin, as it was confirmed experimentally by the absence of a halo (Table 4) and the high MIC ($>512 \text{ mg L}^{-1}$) (Table 5).

Table 10. Determinants for antimicrobial resistance found in Gram-negative strains, number of copies, and localization. ARG: number of antibiotic resistance genes; N: total number of ARG found by PATRIC; n: number of genes found by PATRIC related to the antibiotics used in this work. Amp., Ampicillin; Cipro., Ciprofloxacin; Chloram., Chloramphenicol.; Genta., Gentamicin; Tet., Tetracycline; (chr), chromosome; (plas), plasmid.

Strain	ARG (N/n)	Gene	Number of Copies	Localization	Confers Resistance Towards
H17	55/26	<i>BlaEC</i>	1 copy	Chromosome	Ampicillin
		<i>marA, marB</i>	1 copy each	Chromosome	Activator protein (regulation)
		<i>AcrA, AcrB, AcrD, AcrE,</i>	1 copy each	Chromosome	Amp., Cipro., Chloram., Genta., Tet.
		<i>AcrF, AcrZ</i>	1 copy each	Chromosome	Amp., Cipro., Chloram., Genta., Tet.
		<i>TolC</i>	1 copy	Chromosome	Export channel, several antibiotics
		<i>TolC/OmpH</i>	1 copy	Chromosome	Export channel, several antibiotics
		<i>MdtA, MdtB</i>	1 copy each	Chromosome	Multidrug efflux pump
		<i>MdtC</i>	2 copies	Chromosome	Multidrug efflux pump
		<i>MdtL</i>	1 copy	Chromosome	Multidrug efflux pump
		<i>H-NS</i>	1 copy	Chromosome	Hist.-like nucleoid-structur., Colistin
		<i>gyrA, gyrB</i>	1 copy each	Chromosome	Ciprofloxacin
		<i>MacA, MacB</i>	1 copy each	Chromosome	Erythromycin
		<i>s12p</i>	1 copy	Chromosome	Streptomycin
		<i>gidB</i>	1 copy	Chromosome	Streptomycin
		<i>murA</i>	1 copy	Chromosome	Fosfomycin
		<i>MdfA/Cmr</i>	1 copy	Chromosome	Multidrug efflux pump
		<i>EmrD</i>	1 copy	Chromosome	Chloramphenicol
N6	50/17	<i>MexAB-OprM</i>	2 copies	Chromosome	Multidrug efflux pump
		<i>MexCD-OprJ</i>	3 copies	Chromosome	Multidrug efflux pump
		<i>MexHI-OpmD</i>	3 copies	Chromosome	Multidrug efflux pump
		<i>OprM</i>	1 copy	Chromosome	Export channel, several antibiotics
		<i>gyrA, gyrB</i>	1 copy each	Chromosome	Ciprofloxacin
		<i>MacA, MacB</i>	1 copy each	Chromosome	Erythromycin
		<i>s12p</i>	1 copy	Chromosome	Streptomycin
		<i>gidB</i>	1 copy	Chromosome	Streptomycin
		<i>murA</i>	2 copies	Chromosome	Fosfomycin
S3	54/26	<i>MarA</i>	1 copy	Chromosome	Activator protein (regulation)
		<i>AcrA, AcrB, AcrD, AcrZ</i>	1 copy each	Chromosome	Multidrug efflux pump
		<i>TolC</i>	1 copy	Chromosome	Export channel, several antibiotics
		<i>MdtA, MdtB, MdtC</i>	2 copies each	Chromosome	Multidrug efflux pump
		<i>H-NS</i>	5 copies	3 (chr), 2 (plas)	Hist.-like nucleoid-structur., Colistin
		<i>gyrA, gyrB</i>	1 copy each	Chromosome	Ciprofloxacin
		<i>s12p</i>	1 copy	Chromosome	Streptomycin
		<i>gidB</i>	1 copy	Chromosome	Streptomycin
		<i>murA</i>	1 copy	Chromosome	Fosfomycin
		<i>MdfA/Cmr</i>	2 copies	Chromosome	Multidrug efflux pump
		<i>OprB</i>	1 copy	Chromosome	Export channel, several antibiotics
		<i>MexEF-OprN</i>	1 copy	Chromosome	Multidrug efflux pump

Regarding the localization of these genes, all of them were found in the chromosomes of the bacterial strains, with the exception of the gene *gidB* from MHA1 conferring resistance towards streptomycin and a complete copy of the operon *Van*, found in plasmids. Concerning the number of copies, we call attention to the resistance against ciprofloxacin (two copies of *gyrAB*) in MHA1 and MS4 and two copies of some vancomycin resistance genes in N25.

Analogously, there were genes specifically found only in the Gram-negative strains. For example, β -lactamase genes from the *BlaEC* family, which hydrolyze the β -lactam ring of antibiotics such as ampicillin, were detected exclusively in the genome of H17 (according to the absence of an inhibition halo (Table 3)).

The most singular finding concerning Gram-negatives (Table 10) was the ubiquitous presence of multiple multidrug efflux pumps belonging to three superfamilies: the resistance–nodulation–cell division (RND) antibiotic efflux pump superfamily, the Major

Facilitator Superfamily (MFS), and the ABC (ATP-binding cassette) transporters. These tripartite efflux pumps are connected to channels in the outer membrane for extruding several antibiotics and/or drugs. Concerning the RND superfamily, *AcrAB/TolC* and *TolC/OpmH* were found in the strains H17 and S3 (Enterobacteriaceae) [40]. For its part, MdtABC-TolC is a multidrug efflux system of Gram-negative bacteria, including *E. coli* and *Salmonella* [41] which were found in H17 and S3. MdtA is a membrane fusion protein; TolC is the outer membrane channel; MdtBC forms a drug transporter. In the absence of MdtB, the MdtAC-TolC has a narrower drug specificity. All these proteins are part of RND-type efflux pumps that actively expel various classes of antibiotics (e.g., tetracyclines, macrolides, chloramphenicol) in Gram-negatives, thereby reducing their intracellular concentrations [41].

The genes *MexAB/OprM*, *MexCD/OprI*, *MexHI-OpmD*, and *MexEF-OprN* also belong to the RND superfamily and were found in N6 and S3. OprM is an outer membrane factor protein found in *Pseudomonas aeruginosa* and *Burkholderia vietnamiensis*. It is part of the MexAB-OprM, MexVW-OprM, MexXY-OprM, and the AmrAB-OprM complexes and confers resistance against aminoglycoside antibiotic, phenicol antibiotic, tetracycline antibiotic, carbapenem, fluoroquinolone antibiotic, macrolide antibiotic, penicillin beta-lactam, cephalosporin, and disinfecting agents and antiseptics [42]. According to CARD, the prevalence of this sequence in *Pseudomonas aeruginosa* is 98.47% in the chromosome and 0.58% in plasmids, whereas in the PGPB *Pseudomonas fluorescens*, the prevalence was 2.78%, not being found in plasmids (<https://card.mcmaster.ca/ontology/36518>, accessed on 11th November, 2025). In our case, it was found in the chromosome of N6, with four copies, three of them associated with *MexAB/OprM* genes and one independent copy.

Concerning the Major Facilitator Superfamily (MFS), the genes *MdfA/Cmr* codify a multidrug resistant transporter conferring resistance towards multiple antibiotics [43]. In our case, these genes were found in H17 and S3. In addition, *EmrD* codifies a multidrug transporter from the Major Facilitator Superfamily (MFS) primarily found in *Escherichia coli*. EmrD couples the efflux of amphipathic compounds with proton import across the plasma membrane. This pump confers resistance towards phenicol antibiotics (chloramphenicol), disinfecting agents and antiseptics [44].

With regard to tripartite efflux pumps that belong to the family of the ABC transporters, the genes *macAB* conferring resistance towards erythromycin [45] were detected in the strains N6 and H17.

We also found the operon *marAB* conferring resistance towards multiple antibiotics [46]. We found both genes in the strain H17 and only *marA* in S3. MarA is an activator protein encoded by the *marRAB* locus. The *mar* locus is reported to mediate resistance primarily by up-regulating the efflux of some antibiotics, disinfectants, and organic solvents via the AcrAB-TolC efflux pump and down-regulating influx through Outer Membrane Protein F (OmpF) [47].

Finally, the gene H-NS is a Histone-like nucleoid-structuring protein found in *Acinetobacter baumannii* that plays a regulatory role in antibiotic resistance. Inactivation of this gene increases the resistance towards colistin [48]. This gene was found in H17 (one chromosomal copy) and S3 (five copies; three in the chromosome and two in plasmids), but not in N6, confirming the inverse relationship between the presence of this gene and the resistance towards colistin ($\phi = 13\text{--}14$ mm for S3 and H17, and $\phi = 0$ in N6, Table 3). Except for the two copies of H-NS in plasmids of S3, all the rest of the genes had a chromosomal localization.

3. Discussion

The increasing use of plant growth promoting bacteria, such as those reviewed in this study, and despite their agronomics benefits as biofertilizers, poses a potential risk due to their capacity to contain and disseminate antibiotic resistance genes [16]. In this

regard, there is an increasing number of publications calling attention to this environmental problem [49,50].

At the experimental level, we determined the diameters of both halos and MICs. In our study, we have observed, in general, a higher resistance in Gram-negative strains, visualized in the box plot diagram. Particularly high was the resistance to ampicillin for which the smaller halos were found in Gram-negatives (four strains gave no inhibition halos), followed by colistin and tetracycline. Overall, we found good correlation between halo diameters and MIC values (r between 0.7 and 1), although in some cases there were lower correlations. It is possible that the availability of the antibiotic (in liquid or solid media) or additional regulatory mechanisms could explain the lack of correlation between the two techniques. In addition, sometimes the bacteria could adopt a sessile way of life, forming biofilms, which is a mechanism of resistance and elevates the values of MIC for antibiotics since the bacteria are protected from it while immersed in the polysaccharide matrix [51].

Gram-negative strains are particularly concerning in terms of antibiotic resistance. Their intrinsic resistance is often higher due to the outer membrane acting as a permeability barrier, combined with the frequent presence of efflux pumps and the ability to acquire resistance genes via plasmids. These features make them more prone to develop multidrug resistance and complicate treatment options, as reported in various clinical and environmental studies [52]. Regarding Gram-positives, the resistance towards vancomycin was outstanding, which was present in at least three strains.

The protocols for determining both, susceptibility tests with disks and MICs, are perfectly known and established. However, one problem at the time of applying these tests to PGPB has been the lack of tabulated breakpoints for these bacteria. In this regard, it has been necessary to compare our results with general breakpoints for a particular genus, for example, *Bacillus*, or even for a complete family, for instance Enterobacteriaceae or Pseudomonadaceae. It is presumed that, in the short term, national or supranational regulations will assess the problem of AMR associated with PGPB and will establish a paradigm of antibiotics for Gram-positive and Gram-negative strains, as it occurs in France. Hence, the development of a specific database with breakpoints of halo diameters and MIC based on PGPB experimental determinations is needed.

Predicting whether PGPR contains AMR determinants is essential before being applied to agriculture. An approach used is the analysis of the whole community antibiotic resistance phenotypic profile (cenoantibiogram) [53–55]. In this context, in addition, the usage of omics techniques can be extremely useful for this matter, as they show directly the determinants contained in each strain [18]. In addition, the presence of resistance genes can be approached at a metagenomic level in soil DNA by shotgun sequencing without previous cultivation [56]. These studies can shed light on the prevalence of some ARG determinants, in particular microbial communities in soils, and constitute rapid tools for improving AMR surveillance, especially upon the application of machine learning-based AMR prediction models that allow the detection of ARG in mobile genetic elements [56].

Upon the isolation of PGPB, determining the antibiotic susceptibility and prospecting the relevant genes in the genomes is becoming usual. Some recent studies concerning PGPB include not only the beneficial properties of these microorganisms, but the evaluation of their antibiotic sensitivity [57–59]. In Gram-negative strains, efflux pumps and β -lactamases in the strain *Pantoea agglomerans* RSO7, isolated from the rhizosphere of halophytes in coastal environments, were found [25]. As for Gram-positive strains [50], resistance genes in *Bacillus* spp. from soil were identified. These findings highlight the relevance of genome mining tools for ARG detection in both Gram-negative and Gram-positive PGPR.

In the present study, across the analyzed genomes, we detected resistance determinants associated with well-established mechanisms: target modification genes (e.g., *gyrA*, *gyrB*, *S10p*, *S12p*, *MurA*); antibiotic inactivation enzymes such as β -lactamases (BlaEC) and FosB; genes conferring resistance via absence (*gidB*); regulators modulating the expression of antibiotic resistance genes (*marA*, *marB*); transcription factors (H-NS); etc. These findings reflect the molecular diversity of resistance strategies encoded in both Gram-positive and Gram-negative PGPR and illustrate the value of comprehensive genome mining approaches to map them. In Gram-negative strains, the existence of multiple efflux pumps belonging to three superfamilies, RND, ABC transporters, and MFS, was noteworthy. Within the tripartite RND pumps are AcrAB–TolC in Enterobacteriaceae, MexAB–OprM, MexCD–OprJ, and MexXY–OprM in *Pseudomonas aeruginosa* and MtrCDE in *Neisseria gonorrhoeae* [40]. Genes AcrAB–TolC were found in H17 and S3, whereas MexAB–OprM, MexCD–OprJ, and MexXY–OprM were found in N6. The genes MdfA/Cmr and EmrD belonging to the Major Facilitator Superfamily (MFS) were also found in Enterobacteriaceae H17 and S3 and the genes *macAB* encoding a tripartite efflux pump that belong to the family of the ABC transporters were found in H17. In general, the presence of multidrug efflux pumps belonging to different families (ABC transporters, RDN efflux pumps, and MFS superfamilies) in Gram-negative strains could help explain their high levels of multidrug resistance.

The critical role of the mobile genetic elements (MGEs), such as insertion sequences, transposons, cassettes/integrations, plasmids, and bacteriophages, in the dissemination of antibiotic resistance determinants via horizontal gene transfer (HGT) makes necessary the localization of genes in the genome [60,61]. In our case, all the genes but two were located in the chromosomes of the bacteria. The genes *VanA/I/Pt*-type of the Gram-positive *Priestia megaterium* N25 and two copies of the gene H-NS of the Gram-negative S3 are in plasmids. We have not detected genes in other mobile elements. In this regard, we call attention to the fact that short reads sequencing (Illumina) and assembly were performed, which limit the detection of AMR-associated MGE. Third generation sequencing platforms generating long reads like Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT), together with predictive machine learning models, can provide more accurate tools for linking ARGs to mobile genetic elements [56]. The low number of ARGs found in mobile elements could be a guarantee for the lower dispersion of these resistances in the environment upon using these PGPB.

Another aspect of the foremost relevance is the number of copies, ranging from one copy in most of the genes to the maximum number of five copies for H-NS in the strain S3. However, H-NS is not properly a resistance mechanism but a master regulator of the expression of other genes [48]. The strain N6 was the one with the highest number of copies of several genes including two to three copies of the multidrug efflux pumps *MexAB-OprM*, *MexCD-OprJ*, and *MexHI-OpmD*, as well as two copies of *MurA* conferring resistance towards Fosfomycin.

Nevertheless, a certain discrepancy is observed between the number of identified resistance genes and the actual phenotypic resistance when the susceptibility is determined experimentally. For example, the strain S3 appeared as the most susceptible strain among the Gram-negatives studied and it was classified as susceptible in most assays. However, the analysis of the genome reported a similar total number of resistance genes (54) to more resistant strains like N6 (50) or H17 (55). This suggests that other factors such as expression levels, epigenetic regulation, or synergistic effects among resistance genes may contribute to the final resistance profile. For instance, strain MHA1, which showed strong phenotypic resistance, did not possess the highest number of resistance genes. In addition, other environmental factors such as the presence of heavy metals, co-selection of different resistance mechanisms, or the biofilm/planktonic can affect the actual level of resistance as

determined experimentally [18,51,62]. This highlights the need to complement genomic analysis with experimental data when evaluating candidate biofertilizer strains.

Finally, establishing a database of breakpoints for PGPB will allow us to have a scientific criterion to define the regulatory bases for safe biofertilizers in the context of a One Health perspective, trying to avoid the spreading of AMR due to the agricultural practices related to inoculation with beneficial microorganisms [63].

Limitations of the Work and Future Perspectives

One of the main hindrances of this work, and also one of its main conclusions, is the lack of breakpoints (for both MIC and halo diameters) for environmental bacteria, which reveals the need for establishing an updated database for these species. Regarding this point, it is important to note the low availability of data concerning the antibiotic resistances of PGPB and highlight the necessity for creating a global database of antibiotic resistance in strains of agricultural or environmental application, instead of using the breakpoints for clinical species.

Another limitation is the scarce number of strains used in this work. It is necessary to increase the determination of antibiotic susceptibility at the experimental level together with WGS to find correlations between both approaches and also with the localization in MGE.

Finally, regarding the functional validation, it would be adequate to perform expression analysis and evaluate the influence of environmental conditions in the expression levels. We plan to deepen our study and perform new analyses in the future.

4. Materials and Methods

4.1. Determination of Antimicrobial Susceptibility by the Disk Diffusion Method on Agar

The procedure was carried out following CLSI guidelines [64], using 12 cm square plates containing Mueller-Hinton (MH) agar. In addition, each bacterial strain was cultured in liquid medium (TSB) for 24 h at 28 °C and 200 rpm. Plates were inoculated with bacterial suspension with optical density equivalent to MacFarland 0.5 standard (approx. 1.5×10^8 CFU/mL) using sterile swabs rotating the plate during the process to ensure uniform coverage of the surface. Disks of the antibiotics (as in Table 2) were placed on the inoculated plate with sterile twickers. Plates were incubated for 24 h at 28 °C. After incubation, the diameters of the inhibition zones around each antibiotic disk were measured in millimeters using a ruler (Supplementary Information Figure S1). The assignment of the categories (resistant, intermediate, or susceptible) was performed after consulting the diameter of the halo in the tables elaborated every year by EUCAST [27]. When no data were available for a particular antibiotic and/or bacterial genera, the criterion of the EUCAST guidance on “When there are no breakpoints in breakpoint tables” was followed [28].

4.2. Determination of Minimal Inhibitory Concentration by the Broth Microdilution Method

To corroborate the antibiotic susceptibility or resistance of each organism, the MIC was determined for each strain and each available antibiotic. This was carried out by the broth microdilution test [65] in 96-well microplates (8 rows $\times$ 12 columns). Liquid cultures of the microorganisms were inoculated into MH liquid medium supplemented with decreasing concentrations of antibiotics ranging from 512 μ g/mL to 0.5 μ g/mL. Antibiotic stock solutions were prepared at an initial concentration of 1024 μ g/mL and sterilized by filtration through a filter of 0.22 μ m pore size. The first dilution (512 μ g/mL) was prepared by mixing equal volumes of MH liquid medium and the stock solution of the antibiotic. Then, serial half dilutions were performed up to final concentration 0.5 μ g/mL, keeping a column for MH medium without antibiotic. Each well was filled with 0.25 mL

of MH containing the appropriate antibiotic concentration, along with 5 µL of bacterial culture. A row was kept without inoculation as a control for sterility of the medium. After inoculation, the plates were incubated for 24 h at 28 °C, with the lids properly sealed to prevent desiccation and contamination.

The MIC for each microorganism–antibiotic combination was determined by visually inspecting each row of wells and identifying the first clear well, which corresponded to the lowest antibiotic concentration capable of inhibiting visible bacterial growth (Supplementary Information Figure S2).

4.3. Determination of the Minimal Inhibitory Concentration for Fosfomycin

Due to the fact that Fosfomycin uptake into bacterial cells requires co-transport with glucose-6-phosphate, susceptibility testing for Fosfomycin was performed using Mueller-Hinton agar medium supplemented with 25 mM glucose-6-phosphate, as recommended over the standard method used for the other antibiotics [66]. The supplemented medium was poured into 12 cm square plates, each containing decreasing concentrations of Fosfomycin, with the goal of obtaining 12 different concentrations, starting at 512 µg/mL and halving successively down to a final concentration of 0.5 µg/mL. The last plate was reserved for Mueller-Hinton medium without antibiotic.

Each series of plates was inoculated with three different bacterial strains (in triplicate). Plates were then incubated for 24 h at 28 °C, after which the maximum concentration at which each microorganism was still able to grow was determined by visual inspection (Supplementary Information Figure S3).

4.4. Isolation of Genomic DNA and Whole Genome Sequencing (WGS)

For bacterial DNA sequencing, the commercial provider MicrobesNG was selected, and their proprietary workflow was followed. This protocol was based on a cell harvesting and isolation procedure, after which the samples were shipped for further processing by the company.

From a single colony of each bacterial strain, fresh TSA agar plates were inoculated and incubated at 28 °C for 24 h. After incubation, between half and two-thirds of the plate surface was scraped and suspended in tubes containing 5–10 mL of PBS buffer, until the suspension appeared visibly turbid. The optical density (OD) of each tube was then measured at 600 nm using a spectrophotometer. If necessary, the samples were diluted in cuvettes to achieve a reliable OD reading within the optimal range of 0.8 to 1.2. Once this reading was obtained, the required volume to reach an OD of 10 was extrapolated, and this volume was centrifuged to obtain a cell pellet with sufficient biomass to ensure reliable downstream sequencing.

Finally, the pellet was resuspended in an inactivation buffer provided by the sequencing company and transferred to a pre-labeled tube (also provided), after which the samples were shipped to the company's laboratories for Illumina shotgun metagenomic sequencing.

Illumina reads for each strain genome were trimmed using Trimmomatic [67], and the quality was determined using an in-house script. Assembly was performed using SPAdes version 3.7 [68]. The assembly metrics and other basic statistics were obtained using QUAST v. 5.3.0 software [29], CRISPRCasFinder [32], and Deeplasmid version of August, 22nd, 2024 [31] software. Finally, all genomes were annotated with Prokka version 1.14.5 [30]. The whole genomes were deposited in the DDBJ/EMBL/GenBank databases with the following accession numbers: GCA_977061975 (H17), GCA_977061995 (MHA1), GCA_977061985 (MS4), GCA_977062005 (N6), GCA_977062015 (N25), and PRJEB102903 (S3).

4.5. Identification of the Selected Strains

The identification of the strains used in this study was carried out using the 16S rRNA gene sequence obtained by the genome sequences using the EZBioCloud web server [69]. The identification was accurate by a digital DNA–DNA hybridization (dDDH) with the most related species using the GGDC web server [70].

4.6. Mining Antibiotic Resistance Determinants in the Genomes

In order to obtain a summary of the antibiotic resistance determinants from every strain's genome, we used a tool from BV-BRC (Bacterial and Viral Bioinformatics Resource Center) which annotated the genomes using the obtained paired contigs and by comparing them to those genomes stored in specialized databases for antibiotic resistance and specialty genes for species similar to those predicted before for each individual strain. Those databases include mainly PATRIC [19] and CARD [20].

4.7. Statistical Analyses

The determination of halo diameters was performed in triplicate. Resulting data are the means of three determinations. The values of MIC were also assessed in triplicate. When there were discrepancies between one of the three determinations, the highest value of MIC was considered. Box plots and the heatmap were elaborated using Microsoft Excel (Microsoft Corporation, Redmond (Washington), USA, 2024), the latter using normalized data, while PCA was performed by using R version 4.4.1 (R Core Team, Vienna, Austria, 2024).

5. Conclusions

The determination of the susceptibility to 12 antibiotics in 20 PGPB demonstrated a higher level of resistance in Gram-negatives compared to Gram-positives. Furthermore, a good correlation was found between the data of halo diameters and MIC values. Genome mining performed in the WGS of four resistant and two susceptible strains showed the existence of common and different resistance mechanisms among the Gram-positive and Gram-negative PGPB. Gram-positive PGPB harbored genes of resistance by target modification, acquiring resistance to antibiotics affecting protein synthesis and DNA replication. Gram-negative PGPB harbored an elevated number of multidrug resistance efflux pumps of different families (RDN, ABC, MFS) that expel antibiotics from the cell. In summary, genomic analysis confirms and expands the phenotypic observations, revealing both shared and specific resistance genes and mechanisms. In addition, only two of the genes were found in mobile genetic elements (plasmids), which limits the possible horizontal gene transfer. However, since the genotype-to-phenotype relationship is not always direct, further experimental validation will be essential before approving any strain as a safe microbial inoculant. In this regard, the establishment of a global database with the susceptibility of PGPB is compulsory in order to have reference data based on agricultural or environmental strains, instead of comparing the experimental data with those tabulated for clinical species.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/antibiotics15010014/s1>, Figure S1: Antimicrobial susceptibility test with disks. Figure S2: Determination of MIC using the broth microdilution test. Figure S3: Determination of the MIC for Fosfomycin.

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Abbreviations

The following abbreviations are used in this manuscript:

ABC	ATP-Binding Cassette Transporters
AMR	Antimicrobial Resistance
ARG	Antibiotic Resistance Genes
CARD	Comprehensive Antibiotic Resistance Database
CLSI	Clinical Laboratory Standards Institute
dDDH	Digital DNA–DNA Hybridization
EUCAST	European Committee for Antimicrobial Susceptibility Testing
HGT	Horizontal Gene Transfer
MDR	Multidrug Resistance
MFS	Major Facilitator Superfamily of Transporters
MGE	Mobile Genetic Elements
PATRIC	Pathosystems Resource Integration Center
PGPB	Plant Growth Promoting Bacteria
RND	Resistance–Nodulation–Cell Division Efflux Pumps Family
WGS	Whole Genome Sequencing

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Article

Genomic Evidence Supporting a One Health Perspective on *Staphylococcus aureus* Bovine Mastitis

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Abstract

Background/Objectives: *Staphylococcus aureus* is a multifaceted pathogen responsible for diseases in humans and in several animal species, including dairy cows. This study aimed to characterize and compare the genetic diversity, lineage distribution, and antimicrobial resistance profiles of *S. aureus* isolates from bovine milk with human-derived reference genomes to investigate host adaptation and inter-species transmission. **Methods:** Genomic analyses were performed on *S. aureus* isolates from quarter milk samples of dairy cows together with human-derived sequences from public databases. Whole-genome sequencing and multi-locus sequence typing (MLST) were used to determine sequence type (ST) distribution, and the presence of key antibiotic resistance genes and mobile genetic elements (MGEs) was assessed. Comparative genomics was applied to evaluate gene content, phylogenetic relationships, and lineage–host associations. **Results:** The dataset encompassed bovine-adapted lineages (CC97, CC133, CC151) and human-associated lineages (CC1, CC5, CC8, CC30, CC45), as well as livestock-associated ST398 in bovine samples and human-adapted ST5 and ST6 in animals. ST8 was the only ST shared between animal and human isolates and showed differing resistance profiles, with animal ST8 carrying resistance determinants absent from human ST8. Bovine-adapted strains were characterized by recurrent loss of human-associated virulence genes and acquisition of bovine-associated mobile genetic elements, and *blaZ* and *mecA* were rarely detected in bovine-adapted CC97 but were frequently present in human CC5 and CC8. Overall, animal isolates carried fewer resistance genes than human isolates. **Conclusions:** *S. aureus* from dairy cows and humans displayed substantial genetic diversity, with evidence of host-associated lineages and dynamic changes in gene and mobile element content. These findings support the need for integrated One Health surveillance to track shared and host-adapted lineages and their antibiotic resistance determinants.

Keywords: *Staphylococcus aureus*; mastitis; One Health; whole genome sequencing; antimicrobial resistance

1. Introduction

Staphylococcus aureus is known for inducing various diseases in both animals and humans. In humans, it can induce skin infections, pneumonia, and more severe diseases such as endocarditis and toxic shock syndrome [1]. The rise in antibiotic-resistant clones, especially methicillin-resistant *Staphylococcus aureus* (MRSA), has intensified worries about public health and zoonotic transmission for this pathogen. In dairy cows, it is a principal causative agent of mastitis, resulting in a significant negative economic impact on the dairy industry [2]. Despite the extensive literature on the epidemiology and control of intramammary infections due to *S. aureus* [3–6], these infections are still recorded in many countries, and particularly in Italy [7–9].

The potential role of milk as a source of food-poisoning due to *S. aureus* enterotoxins is well known [10], as is its role in the case of livestock-associated methicillin resistance [11]. However, the potential zoonotic role of *S. aureus* commonly associated with intramammary infections in dairy cows is still unclear. This pathogen has a vast array of virulence factors and antimicrobial resistance genes [12–17] and its genetic apparatus suggests that the potential zoonotic capabilities should not be ignored.

The genetic diversity of *S. aureus* is evident in its multi-locus sequence typing (MLST), which classifies the bacterium into various sequence types (STs) and clonal complexes (CCs) [18]. Several STs are notably shared across humans and animals, revealing a complex global health interaction and making a One Health approach relevant. ST398 is primarily linked to livestock, mainly pigs, although it has also been detected in humans, particularly those with close contact to these animals [19]. Likewise, ST5 and ST6 have been identified in both meat products and human clinical isolates, indicating that foodborne transmission pathways may contribute to the epidemiology of *S. aureus* infections [20]. Besides cattle, wildlife has also been involved in the transmission dynamics of *S. aureus* with ST49 and ST581 being the main STs sharing genetic similarities with human isolates [21,22]. The existence of these shared STs prompts enquiries into the biological mechanisms that enable their persistence among various species. Bovine-adapted *S. aureus* lineages include CC97, CC133, and CC151 while human-adapted lineages include CC1, CC5, CC8, CC30, and CC45 [5]. *S. aureus* host spillover followed by adaptation to a new host is typically associated with the acquisition of a new set of genes specific for survival in the new host losing virulence to the former host species [23]. Recent comparative genomic reports indicate that bovine-adapted strains swiftly discard genes associated with human infections, thereby likely enhancing their fitness in bovine hosts [5,23,24]. Moreover, cows serve as a reservoir for antimicrobial-resistant (AMR) *S. aureus*, which could be transmitted to people and persist within them [12,13]. Few studies have tried to understand this association by correlating AMR and lineage. Few STs are known to be positive for *blaZ* or *mecA* among the *S. aureus* CC97 isolated from cows, which comprises many STs (ST97, ST115, and ST352). Moreover, ST5 (CC5), ST8 (CC8), and their variants are human-adapted lineages that have been isolated from cows and were positive for *blaZ* and *mecA* [25–27].

Despite its significance in human and veterinary medicine, research is scarce on the gene profile, strain-to-strain relationships, disease–host relationships, and host–pathogen interactions of *S. aureus*. We implemented genome-wide analyses in the present study to gain a more thorough understanding of these issues by comparing *S. aureus* strains isolated from the milk samples of dairy cows and human-derived sequences from public databases.

2. Results

2.1. Genomic Features of the Analyzed Strains

We performed Nanopore Whole Genome Sequencing on 50 *S. aureus* strains isolated from bovine milk. All the sequences were deposited in NCBI under the Bioproject PR-

JNA1243330. Table 1 summarizes the main descriptors of the sequenced strains. The distribution of *agr* types, SCCmec types, and sequence types (STs) among human (H₊) and animal (A₊) isolates reveals distinct population structures characterized by specific host-associated predominance. Regarding the *agr* locus, human isolates are predominantly *agr* group 1 (66%), followed by group 2 (30%), and group 3 (4%). Animal isolates show a similar pattern with slightly less dominance of group 1 (64%), group 2 (26%), and a higher proportion of group 3 (10%). The SCCmec typing, highlights the absence of methicillin resistant strains in the A₊ cohort (98%), with the detection of a single strain found positive for *mecA* gene and clustered as SCCmec type V:Vc. In contrast 36% of H₊ strains carried SCCmec IV:IVj type, 12% II:IIa and IV (other subtypes found comprise SCCmec types IV:IVa (6%), I:Ia (6%), as well as IV, V, and III (each 2%)).

Table 1. Characteristics and accession numbers of the 50 *S. aureus* strains sequenced.

Sample Name	Genome	Biosample	Comp ^a	Cont ^b	CDS ^c	Cov ^d	Genome Length (Mbp)	Contigs	GC (%)	ST	Agr_Type
A_SAU10	JBRUVB000000000	SAMN47618783	100.0	0.07	2474	135	2.71	6	32.9	151	gp1
A_SAU107	CP186908	SAMN47618826	100.0	0.16	2571	259	2.73	1	32.8	71	gp2
A_SAU109	CP186909	SAMN47618827	100.0	0.05	2511	322	2.74	1	32.8	71	gp1
A_SAU13	JBRUV000000000	SAMN47618784	100.0	0.09	2506	290	2.74	2	32.9	71	gp1
A_SAU16	JBRUV000000000	SAMN47618785	100.0	0.17	2575	404	2.79	2	33.2	133	gp1
A_SAU19	JBRUV000000000	SAMN47618786	100.0	0.08	2556	471	2.76	2	32.9	352	gp1
A_SAU20	CP186883	SAMN47618787	99.98	0.47	2682	347	2.78	1	32.8	479	gp2
A_SAU23	CP186884	SAMN47618788	100.0	0.21	2509	100	2.68	1	32.8	504	gp2
A_SAU24 ^e	JBRUV000000000	SAMN47618789	100.0	0.15	2555	361	2.8	2	33.0	398	gp1
A_SAU28	CP186885	SAMN47618790	100.0	0.12	2545	73	2.79	1	32.8	8	gp1
A_SAU3	JBRUV000000000	SAMN47618778	100.0	0.12	2535	209	2.79	2	32.7	8	gp1
A_SAU34	JBRUV000000000	SAMN47618791	100.0	0.13	2584	91	2.82	2	32.7	109	gp1
A_SAU35	CP186886	SAMN47618792	100.0	0.2	2730	249	2.89	1	32.8	6	gp2
A_SAU36	JBRUVH000000000	SAMN47618793	100.0	0.11	2422	363	2.7	2	32.9	6	gp1
A_SAU39	CP186887	SAMN47618794	100.0	0.1	2415	116	2.69	1	32.9	6103	gp1
A_SAU4	CP186882	SAMN47618779	100.0	0.08	2533	146	2.77	1	32.8	151	gp1
A_SAU40	JBRUVI000000000	SAMN47618795	100.0	0.69	2641	40	2.77	2	32.8	8	gp2
A_SAU43	JBRUVJ000000000	SAMN47618796	100.0	0.13	2599	418	2.84	2	32.7	700	gp1
A_SAU48	JBRUVK000000000	SAMN47618797	100.0	0.64	2709	380	2.88	2	32.9	8	gp3
A_SAU5	JBRUVY000000000	SAMN47618780	100.0	0.22	2684	166	2.88	2	33.4	6	gp3
A_SAU59	CP186888	SAMN47618798	100.0	0.1	2482	290	2.74	1	32.9	700	gp1
A_SAU6	JBRUVZ000000000	SAMN47618781	100.0	0.12	2534	165	2.79	2	32.8	8	gp1
A_SAU61	JBRUVL000000000	SAMN47618799	100.0	0.13	2588	240	2.82	2	32.7	8	gp1
A_SAU62	JBRUVM000000000	SAMN47618800	100.0	0.12	2532	116	2.79	2	32.8	522	gp1
A_SAU64	CP186889	SAMN47618801	100.0	0.38	2564	202	2.78	1	32.9	151	gp1
A_SAU65	CP186890	SAMN47618802	100.0	0.29	2617	265	2.77	1	32.8	701	gp2
A_SAU66	CP186891	SAMN47618803	100.0	0.22	2587	197	2.79	1	32.9	7823	gp1
A_SAU67	CP186892	SAMN47618804	100.0	0.08	2481	212	2.74	1	32.8	701	gp1
A_SAU68	CP186893	SAMN47618805	100.0	0.22	2586	203	2.74	1	32.9	130	gp1
A_SAU69	CP186894	SAMN47618806	100.0	0.26	2524	294	2.78	1	32.9	8	gp3
A_SAU7	JBRUVA000000000	SAMN47618782	100.0	0.53	2705	355	2.9	3	33.3	700	gp3
A_SAU71	CP186895	SAMN47618807	100.0	0.27	2660	326	2.84	1	32.9	151	gp3
A_SAU73	CP186896	SAMN47618808	100.0	0.18	2618	182	2.77	1	32.8	1074	gp2
A_SAU74	CP186897	SAMN47618809	100.0	0.18	2556	266	2.72	1	32.8	6103	gp2
A_SAU75	CP186898	SAMN47618810	100.0	0.26	2489	237	2.74	1	32.8	352	gp1
A_SAU76	JBRUVN000000000	SAMN47618811	100.0	0.08	2624	229	2.84	12	32.8	352	gp1
A_SAU78	JBRUVQ000000000	SAMN47618812	100.0	0.08	2545	290	2.77	2	32.9	700	gp1
A_SAU80	CP186899	SAMN47618813	100.0	0.13	2590	181	2.74	1	32.8	504	gp2
A_SAU81	CP186900	SAMN47618814	100.0	0.06	2536	256	2.76	1	32.9	352	gp1
A_SAU83	CP186901	SAMN47618815	100.0	0.18	2513	309	2.69	1	32.8	151	gp2
A_SAU85	JBRUV000000000	SAMN47618816	100.0	0.08	2558	118	2.78	2	32.9	352	gp1
A_SAU86	JBRUVQ000000000	SAMN47618817	100.0	0.08	2558	232	2.78	2	32.9	352	gp1
A_SAU87	CP186902	SAMN47618818	100.0	0.29	2615	182	2.77	1	32.8	151	gp2
A_SAU88	JBRUVR000000000	SAMN47618819	100.0	0.27	2828	201	2.98	4	32.9	1094	gp1
A_SAU89	CP186903	SAMN47618820	100.0	0.13	2559	260	2.8	1	32.8	8	gp1
A_SAU90	CP186904	SAMN47618821	100.0	0.17	2620	179	2.77	1	32.8	151	gp2
A_SAU91	JBRUVS000000000	SAMN47618822	100.0	0.27	2793	230	2.95	3	32.9	1094	gp1
A_SAU92	CP186905	SAMN47618823	100.0	0.29	2837	186	2.97	1	33.0	1094	gp1
A_SAU94	CP186906	SAMN47618824	100.0	0.19	2509	263	2.68	1	32.8	705	gp2
A_SAU96	CP186907	SAMN47618825	100.0	0.06	2544	315	2.76	1	32.9	352	gp1

^a Comp = Completeness; ^b Cont = Contamination; ^c CDS = Coding Sequences; ^d Cov = Coverage; ^e A_SAU24: this is the only isolate which was positive for *mecA* gene and clustered as SCCmec type V.

Sequence type analysis further emphasizes the circulation of distinct clones in humans and animals. ST22, a lineage globally recognized as a hospital-associated MRSA strain linked to epidemic spread and high virulence, is the most represented ST in H₊ strains

(38%). Other human-associated STs include ST5 (14%), ST8 (10%), and additional less frequent STs such as 228 (6%), 105 (4%), 1 (4%). In A_ isolates, ST8 is the most frequent (16%), closely followed by ST352 and ST151 (14%), ST700 (8%), and other STs such as 71, 1094, 6, 6103, 701, 504.

2.2. Pangenome Analysis

Figure 1 (see also Supplemental Figures S1–S4) illustrates the pangenome generated with the ANVI'O pipeline.

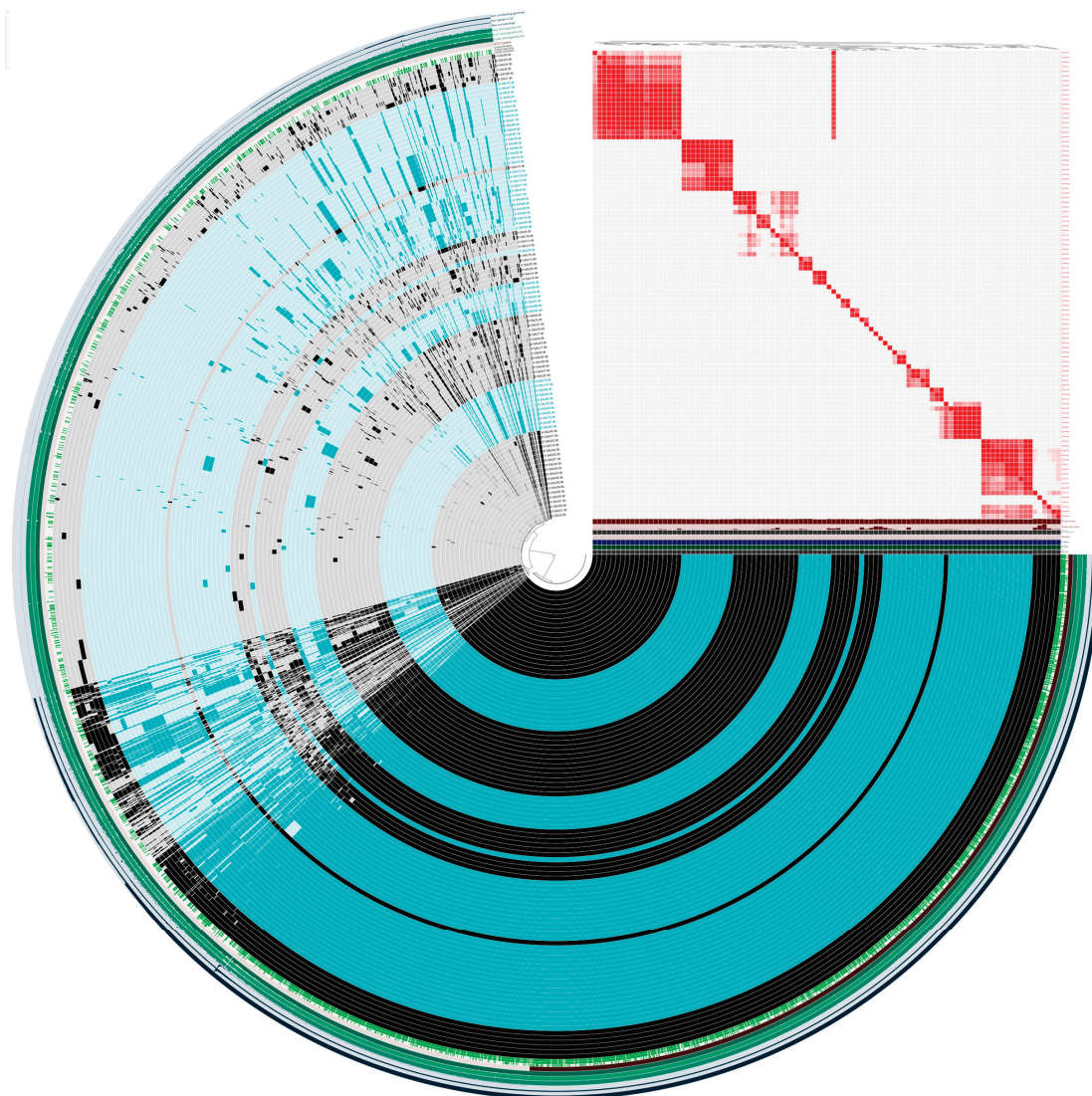


Figure 1. Pangenome structure and host-associated genomic clusters. Circular representation of the pangenome generated with the Anvi'o pipeline, in which each ring corresponds to a single genome and each radial sector to a gene cluster, with dark segments indicating gene presence and light segments gene absence. Strains of animal origin are highlighted in blue and strains of human origin in black, allowing visual identification of host-associated clusters of genomes with similar gene content. The outermost colored rings depict categorical genome metadata, where distinct shades of green correspond to different annotation or bin categories assigned in the Anvi'o interface (for example, separate genome groups or annotation sources) rather than to a quantitative gradient. The inset matrix displays pairwise average nucleotide identity (ANI) based on gene presence–absence at a 99% similarity threshold, where red blocks denote highly similar genomes and lighter regions indicate greater genomic distance, revealing clades of closely related strains that mirror the host-associated structure of the pangenome.

The pangenome comprised 7200 gene clusters, including 1729 core genes (found in at least 99% of strains), 157 soft-core genes (present in 95–99% of strains), 1352 shell genes (in 15–95% of strain), and 3962 cloud genes found in less than 15% of strains. The dominance of cloud genes, which represent more than half of the pangenome, together with a sizeable shell fraction, is consistent with an open pangenome and extensive genomic flexibility. The separation between core and soft-core components highlights a conserved backbone of housekeeping and essential functions, accompanied by a subset of quasi-essential genes whose incomplete distribution suggests ongoing gene gain and loss dynamics. This soft-core fraction likely reflects adaptation to specific ecological contexts or hosts, where certain functions provide a selective advantage without being strictly required across all lineages.

The accessory genome (shell and cloud) accounts for the majority of gene clusters and underscores marked variability among strains, with many genes present in only a limited number of strains, thus indicating a high genomic plasticity and suggesting that horizontal gene transfer and niche-specific selection are important drivers of species evolution.

Clustering patterns based on gene presence–absence and average nucleotide identity (ANI) revealed groups of highly similar genomes, often corresponding to strains of shared animal or human origin. These clusters indicate that host-associated lineages tend to retain more similar gene clusters, supporting the contribution of host adaptation to the shaping of the accessory genome.

The combination of a stable core and a highly dynamic accessory genome likely facilitates persistence in different hosts and environments, and may underpin differences in virulence, colonization ability, or antimicrobial resistance potential between animal- and human-derived strains.

2.3. Phylogeny

Whole genome phylogenetic reconstruction (Figure 2) revealed substantial genetic diversity, with animal- and human-derived strains distributed across distinct branches and, in several cases, clustering within shared clades. A complex evolutionary pattern is indicated by the existence of both host-specific lineages and mixed clades. This pattern is marked by separate host-associated divergence in some lineages, while other lineages exhibit indications of genetic mixing or recent zoonotic trade.

A difference in accessory gene acquisition emerged between the two populations; human-derived isolates harbored significantly more resistance genes (mean 17.9 per genome) compared to animal-derived strains (mean 13.8 per genome; Welch's *t*-test, $p < 0.0001$). This pattern suggests that human-associated lineages have accumulated antimicrobial resistance determinants, consistent with exposure to antimicrobial selective pressure in clinical and community settings. Conversely, animal-derived strains displayed a higher mean count of virulence-associated genes (77.02 vs. 74.64; Welch's *t*-test, $p = 0.0043$), suggesting that preservation of a broader virulence factor repertoire may be maintained through ecological or infection-related selection in animal hosts.

These results indicate that whilst a conserved phylogenetic backbone links the species, differential acquisition and dissemination of resistance and virulence genes reflects distinct selective pressures ongoing in animal and human hosts, with implications for pathogenic potential and treatment challenges in each population.

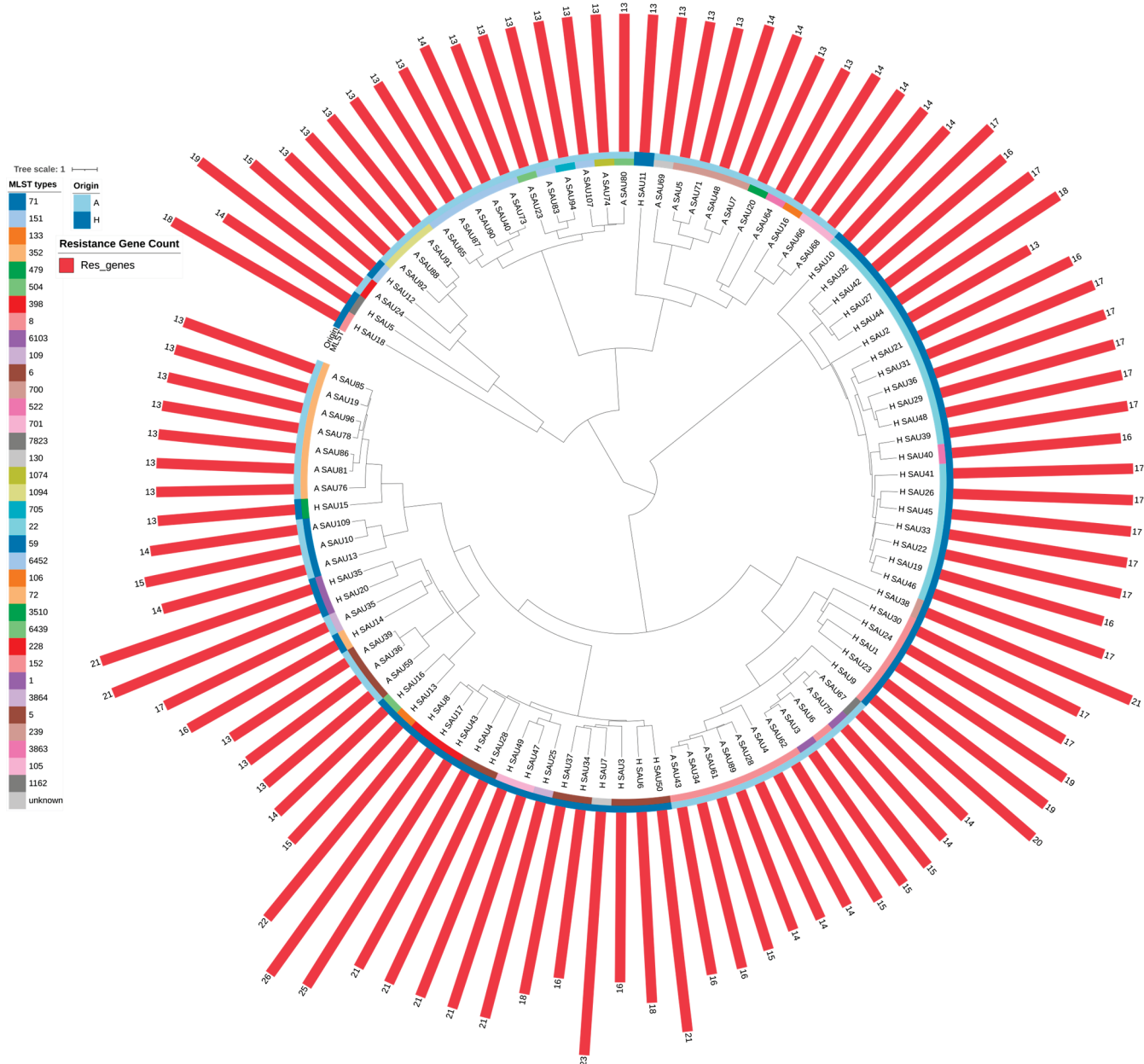


Figure 2. Phylogenetic relationships and antimicrobial resistance gene profiles across animal and human strains. A circular phylogenetic tree in which each leaf represents a single isolate, with animal-derived strains color-coded in blue and human-derived strains in black. Branch lengths reflect evolutionary distance, and the clustering of strains along the tree reveals both host-specific lineages (forming monophyletic clades of the same origin) and mixed clades containing both animal and human isolates, indicating a complex evolutionary history with varying degrees of genetic isolation and admixture between populations. Radial bars extending from each tip represent the number of antimicrobial resistance genes per genome, detected using the CARD database and scaled to allow visual comparison of resistance gene burden across isolates. Red bars are scaled to highlight variation in resistance gene content, with human-derived strains typically showing greater bar length than animal-derived strains, reflecting the significantly higher mean resistance gene count in clinical versus zoonotic isolates.

2.4. Antibiotic Resistance of *S. aureus* in Human (H_SAU) and Animal (A_SAU) Strains

Figure 3 summarizes the predicted phenotypic antibiotic resistance detected in the two groups (H_SAU and A_SAU).

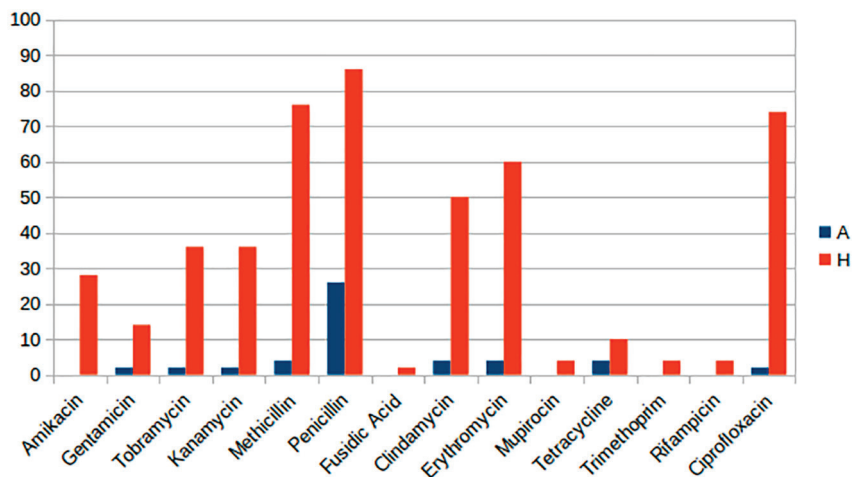


Figure 3. Predicted antibiotic resistance expressed as proportion (%) in H_SAU and A_SAU strains.

Predicted antibiotic resistance distribution reveals substantial disparities, with increased resistance in H_SAU across all examined antibiotic classes. Specifically, amikacin resistance is absent in animal strains, whereas it is present in 28% of human strains. Resistance to aminoglycosides (gentamicin, tobramycin, and kanamycin) in human strains is 14%, 36%, and 36%, respectively, although in animal strains, it ranges from 2% to 4%. Significant disparities are noted with methicillin (4% A vs. 76% H), penicillin (26% A vs. 86% H), erythromycin (4% A vs. 60% H), and ciprofloxacin (2% A vs. 74% H). Certain antibiotics, including fusidic acid, mupirocin, trimethoprim, and rifampicin, exhibit no resistance in animal strains, but human strains demonstrate resistance rates ranging from 2% to 4%.

In the scatter plot (Figure 4A), most of the animal samples did not show any resistance or exhibit resistance to a maximum of two antibiotics. The plot shows a clear separation between animal- and human-derived strains, with animal isolates ($n = 50$) exhibiting a very low resistance burden (mean 0.34 resistant antibiotics per strain; median 0; range 0–3) and human isolates ($n = 50$) displaying a substantially higher load (mean 5 resistant antibiotics per strain; median 5; range 0–10). This pattern indicates that multiple resistance phenotypes are largely confined to the human population, where individual isolates frequently combine resistance to several classes, including beta-lactams (methicillin and penicillin), aminoglycosides (amikacin, gentamicin, tobramycin, kanamycin), macrolides and lincosamides (erythromycin, clindamycin), fluoroquinolones (ciprofloxacin), and occasionally rifampicin, mupirocin or tetracycline, whereas animal isolates are typically fully susceptible or resistant only to penicillin, with sporadic resistance to tetracycline. No animal isolates can be defined as MRSA.

The violin plot (Figure 4B) represents the distribution of the number of resistances for each group. Animal strains show a highly compressed distribution, with a median of 0 resistant antibiotics per isolate (interquartile range [IQR] 0–0; range 0–3), whereas human strains exhibit a much broader and right-shifted distribution, with a median of 5 resistances per isolate (IQR 2–7; range 0–10). This difference in distributions is statistically significant (e.g., Welch's t -test or a non-parametric alternative; $p < 0.0001$), reflecting both the higher central tendency and the greater dispersion of resistance counts among human isolates compared with the tightly clustered, mostly fully susceptible profiles observed in animal isolates.

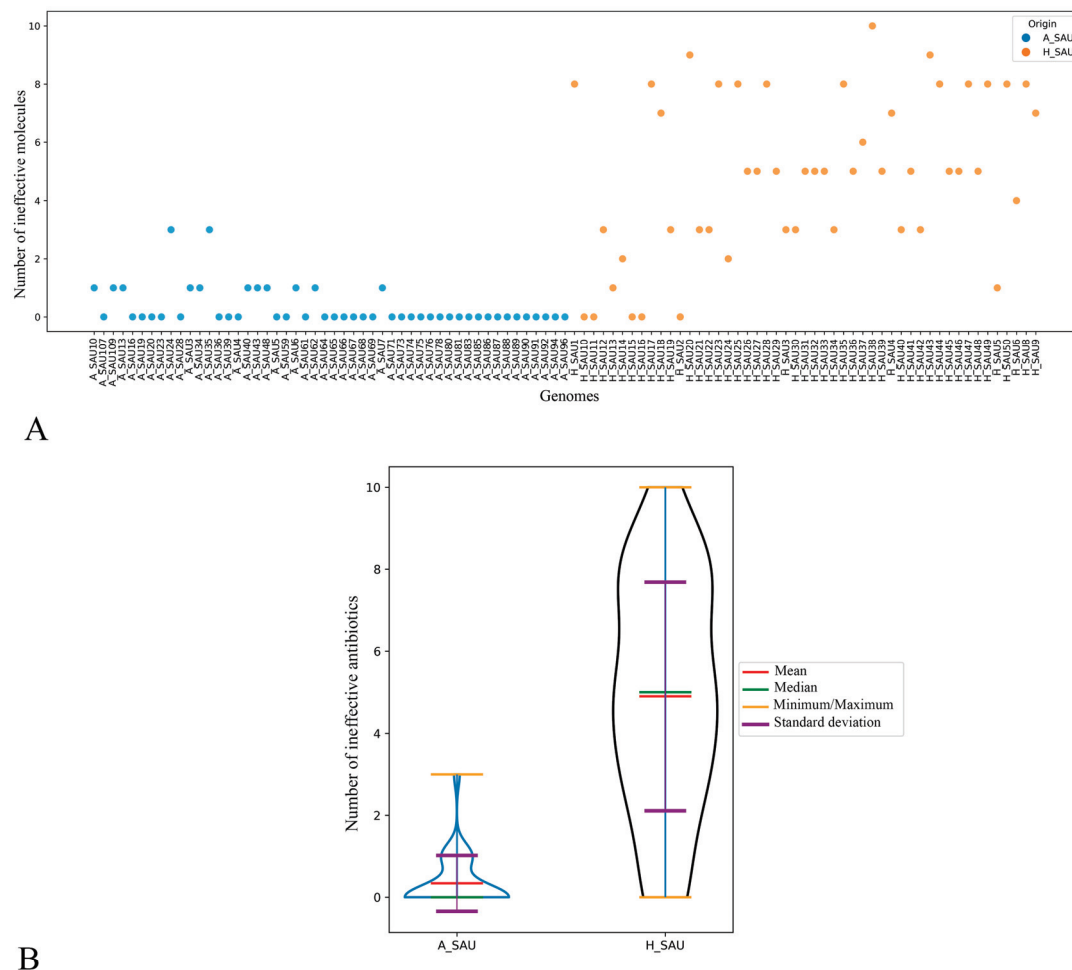


Figure 4. Comparative Burden of Ineffective Antibiotics. Scatter plot (**A**) and violin plot (**B**). The scatter plot (**A**) illustrates the number of antibiotics each genome is resistant to, grouped by origin (blue animal strains, orange human isolates). The violin plot (**B**) represents the distribution of the number of resistances for each group. Each “violin” represents the frequency distribution of the values (resistance to antibiotics) observed in the two groups. Within each violin, the mean (red line), median (green line), range (yellow lines for minimum and maximum) and standard deviation (purple line) are indicated.

The heatmap (Figure 5) clusters the STs of the two groups based on the level of antibiotic resistance. Some STs assigned to the A_SAU group show no resistance, while most of the human samples are resistant to multiple antibiotics (as shown in the previous figures). The only ST common to the two groups is ST8, which however shows different levels of resistance between the two groups. Indeed, ST8 from animal isolates carried resistance determinants that were not detected in the human ST8 isolates, specifically *PC1_blaZ*, *AAC6_Ie_APH2_Ia*, and *ermC*. Conversely, resistance genes identified exclusively in human ST8 isolates were *tet(K)*, *aad(6)*, *SAT-4*, and *APH(3')-IIIa*. These differences may be useful to identify the source of the ST8 isolates (e.g., in an outbreak epidemiological investigation).

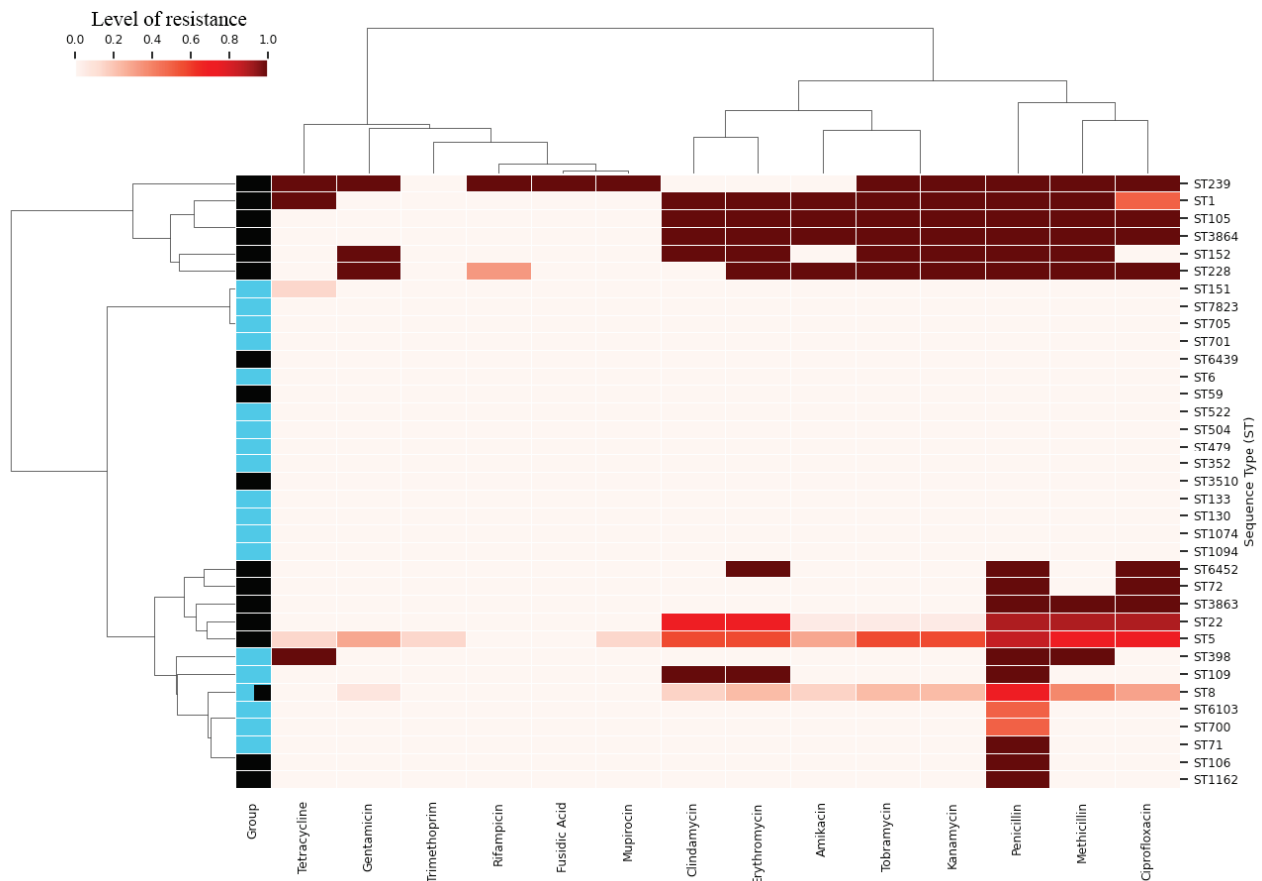


Figure 5. Heatmap and Hierarchical Clustering of Antimicrobial Resistance Profiles. Hierarchical clustering and heatmap analysis highlighting the level of resistance to multiple antibiotics across different *S. aureus* STs. Shades indicate quantitative resistance, revealing group-specific patterns and multidrug-resistant clones between animal derived strains (blue) and human derived isolates (black).

Among the antibiotics analyzed, penicillin is the one towards which most STs show resistance, followed by methicillin, ciprofloxacin, erythromycin and clindamycin (Figure 6).

The Minimum Spanning Tree (MST, Figure 7) highlights clusters of related strains based on the number of antibiotic resistance genes (ARGs). Two main clusters can be observed: one in the top left, composed almost exclusively of animal samples, and one in the bottom, predominantly human. The human cluster appears more fragmented, with several sub-clusters. This could indicate a greater diversity of genetic resistance profiles in the human STs, perhaps due to different selective pressure. The presence of nodes of opposite color in the two clusters (A_ or H_), suggest that STs that share similar resistance profiles in both animal and human samples. These nodes are particularly interesting because they could represent interspecific transmission events or the emergence of clones adapted to both hosts.

The Principal Component Analysis (PCA) results (Figure 8A) show that the two principal components together explain approximately 72.64% of the total variance in the resistance gene data, with PC1 alone accounting for 59% and PC2 for 13.64%. The PC1 principal component represents the main axis of variation, while PC2 adds a complementary dimension. Analyzing the contributions of the ARGs to the principal components, it is observed that PC1 is characterized by methicillin (0.439), erythromycin (0.414), ciprofloxacin (0.403), clindamycin (0.349), penicillin (0.389) and kanamycin/tobramycin (approximately 0.30) ARGs. These results indicate that variability in resistance to these antibiotics is the main factor distinguishing the strains along PC1. PC2, with positive contributions

from kanamycin, tobramycin, amikacin and gentamicin ARGs, may reflect a secondary dimension of variation.

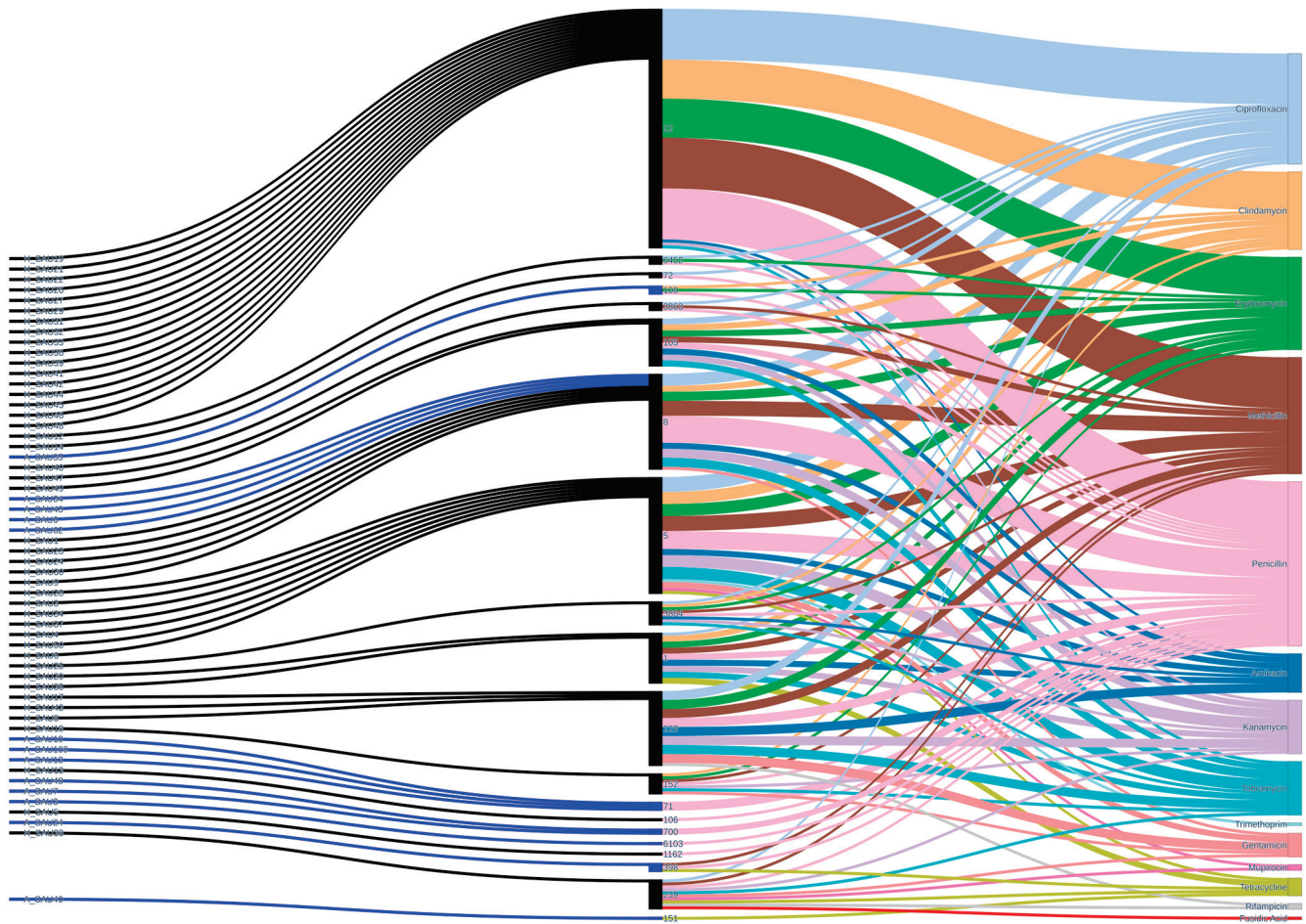


Figure 6. Sankey diagram illustrates the relationships between *S. aureus* isolates (**left**), sequence types (**center**), and their corresponding antibiotic resistance phenotypes (**right**). Animal-derived isolates are depicted in blue and human-derived isolates in black. The width of each ribbon is proportional to the number of isolates sharing a specific sequence type or resistance profile. This visualization reveals the diversity and overlaps of resistance patterns across host origins and facilitates the identification of multidrug-resistant lineages prevalent in both animal and human populations.

The analysis of ARGs combinations by means of the UpSet plot (Figure 8B) allows to monitor the evolution of resistance profiles and to detect the appearance of new multiresistance patterns. The results show that the most frequent combinations involve several ARGs: amikacin, tobramycin, kanamycin, methicillin, penicillin, clindamycin, erythromycin and ciprofloxacin. The recurrence of these combinations in many strains suggests the presence of co-selected resistance mechanisms. Aminoglycosides (amikacin, tobramycin and kanamycin) ARGs are frequently associated, which is consistent with the presence of aminoglycoside-modifying enzymes (AMEs) that confer cross-resistance to multiple molecules of this class. The concomitant presence of resistance to beta-lactams (methicillin and penicillin) indicates the spread of strains by multiple mechanisms, such as beta-lactamase production or alterations of the PBP (penicillin-binding protein) target.

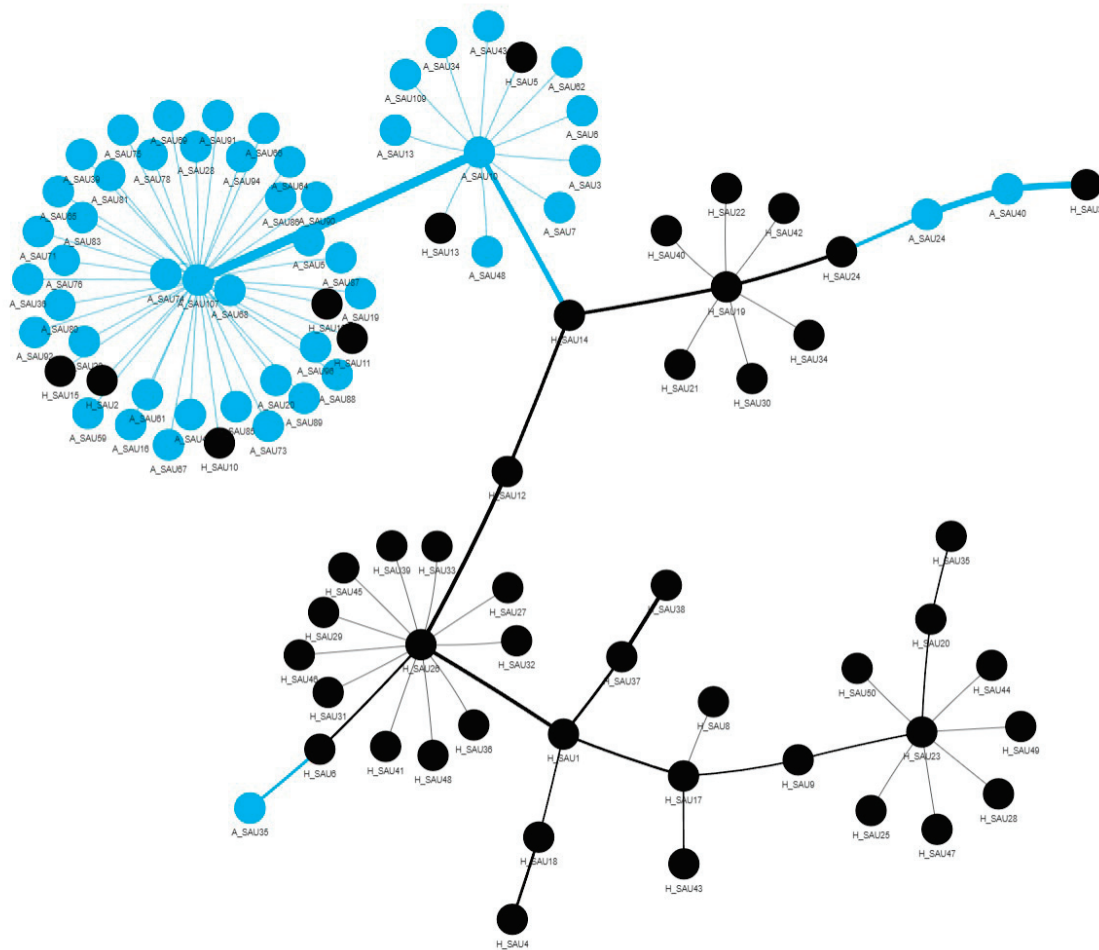


Figure 7. Minimum spanning network showing the genetic distance and relatedness among *S. aureus* isolates. Node colors distinguish human-(black) and animal-(blue) derived genomes, with clusters and bridges reflecting transmission or genetic divergence events.

Increased resistance in human-derived isolates, particularly to first-line agents such as penicillin, methicillin, erythromycin, clindamycin and ciprofloxacin, has direct implications for empirical therapy and the risk of treatment failure in clinical settings. The lower resistance burden in bovine isolates indicates that dairy herds may act as reservoirs of resistant *S. aureus*, with potential spillover into humans through occupational exposure or the food chain, reinforcing the need for coordinated antimicrobial stewardship under a One Health framework.

2.5. Resistance Genes and Virulence Factors

Figure 9 shows the relationships among the bacterial strains analyzed, their STs, the ARGs identified, and the associated molecular mechanisms. The first level, consisting of the strains, shows significant diversity, with numerous isolates distributed across different STs, suggesting considerable genetic heterogeneity and the possible presence of clones. The strain → ST transition highlights some particularly well-represented STs, which act as hubs in the network (i.e., ST22, ST8, ST352, ST151). This pattern may reflect the presence of clones that have spread across different contexts, probably due to selective advantages conferred by resistance or virulence. Some STs are associated with a broad spectrum of ARGs, while others show a more limited carriage (ST7823). The presence of multiple ARGs in some STs suggests the co-localization of mobile genetic elements, such as plasmids or transposons, which facilitate their horizontal spread. The last level, resistance gene → mechanism, represents the functional categorization of the identified genes. Here, some

dominant mechanisms clearly emerge, such as enzymatic modification of the antibiotic, target modification, active efflux and target protection.

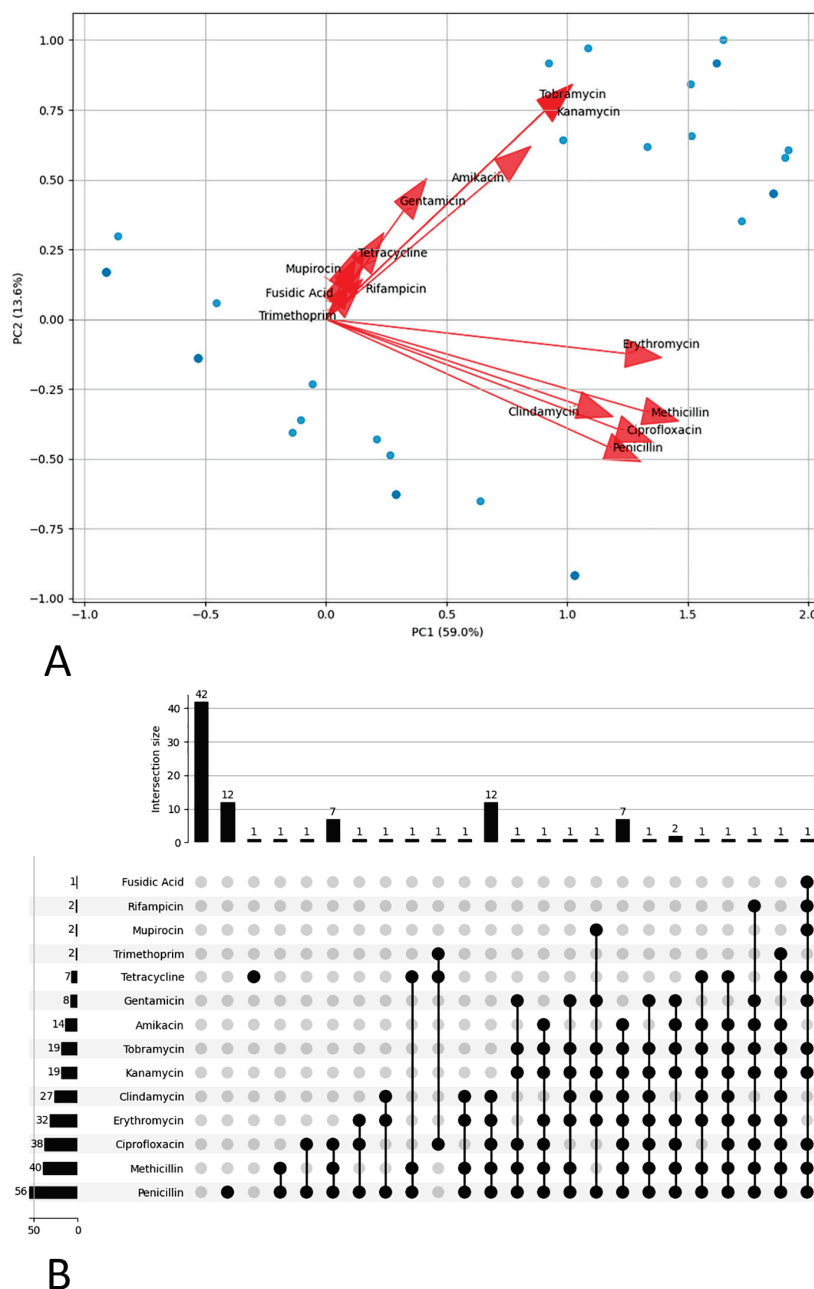


Figure 8. Distribution of Antimicrobial Resistance. (A) Principal component biplot depicting variation in resistance phenotypes across isolates, with antibiotic vectors indicating discriminatory power. (B) UpSet plot summarizing the intersection size for resistance to different antibiotics.

Figure 10 shows the distribution and association of virulence factors in the strains analyzed. Starting from the isolates, the graph shows a structure similar to that observed for resistance, with numerous samples converging on a relatively limited number of STs. This pattern suggests that, even for virulence, there are dominant clones that serve as the main carriers of certain virulence factors within the population. The strain → ST transition again highlights some ST, which bring together numerous strains (ST22, ST8), suggesting a complex clonal structure and the possible presence of hypervirulent clones. Some STs are associated with a broad spectrum of virulence factors, which may facilitate adaptation to different hosts or ecological niches. Other STs, on the other hand, show a

“specialization” towards certain categories (penicillin and tetracycline ARGs), indicating a possible narrower ecological niche or infection strategy (ST109, ST152, ST106). The presence of numerous connections between STs and virulence categories suggests that genetic plasticity affects not only resistance but also pathogenic potential. The figure shows that some categories of virulence factors are particularly well represented, such as adhesins and toxins, while others, such as immune evasion factors or degradative enzymes, are less frequent or more specific to certain STs.

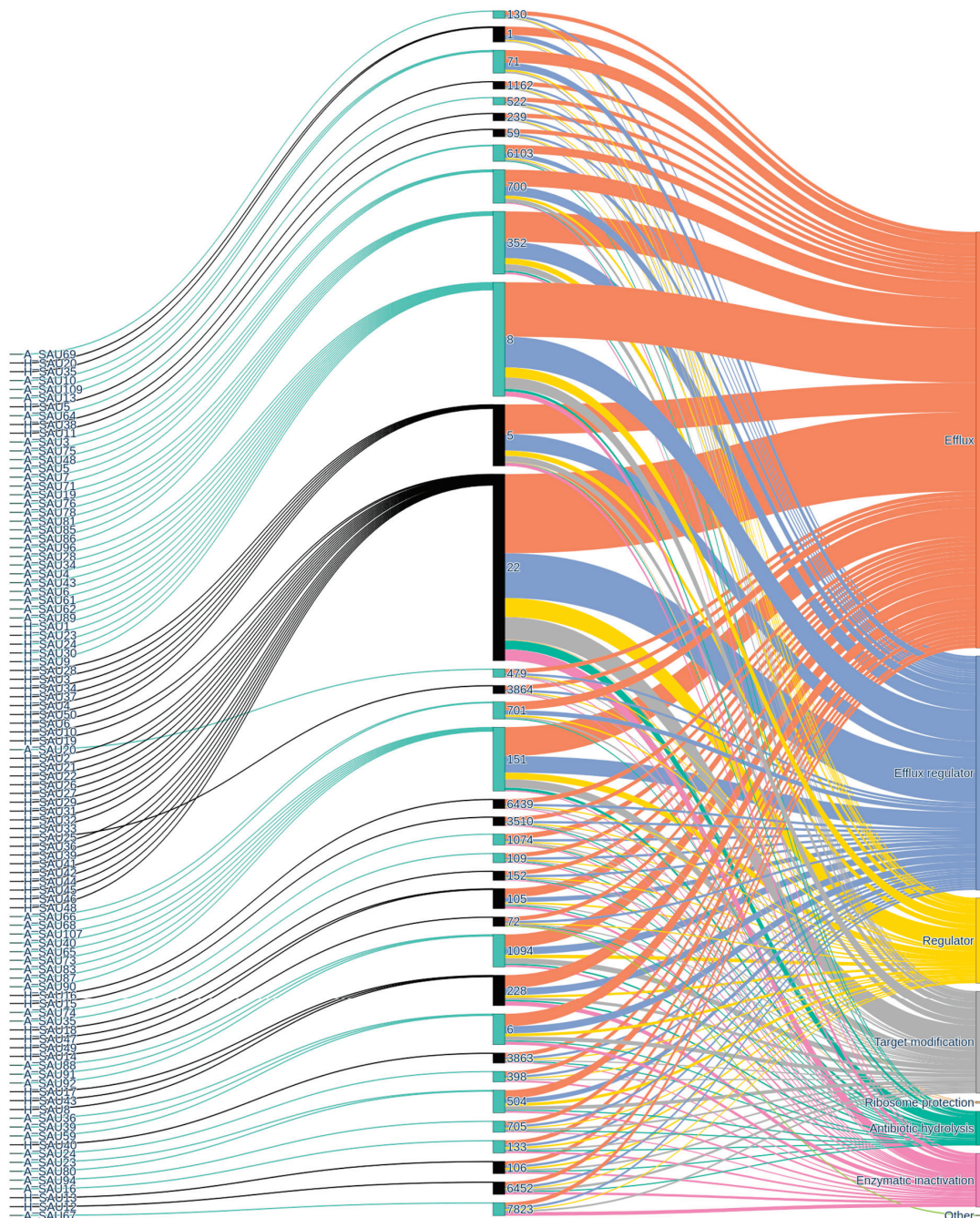


Figure 9. Sankey diagram traces the flow from *S. aureus* isolates (**left**), segregated by animal (light green) and human (black) origin, through sequence type (**center**), to specific antibiotic resistance genes (**right**). Colored link highlight connections between host source, genetic background, and resistance, offering a comprehensive view of the spread and diversity of antimicrobial resistance within and between host reservoirs, and identifying linkages between sequence types and multidrug-resistant profiles.

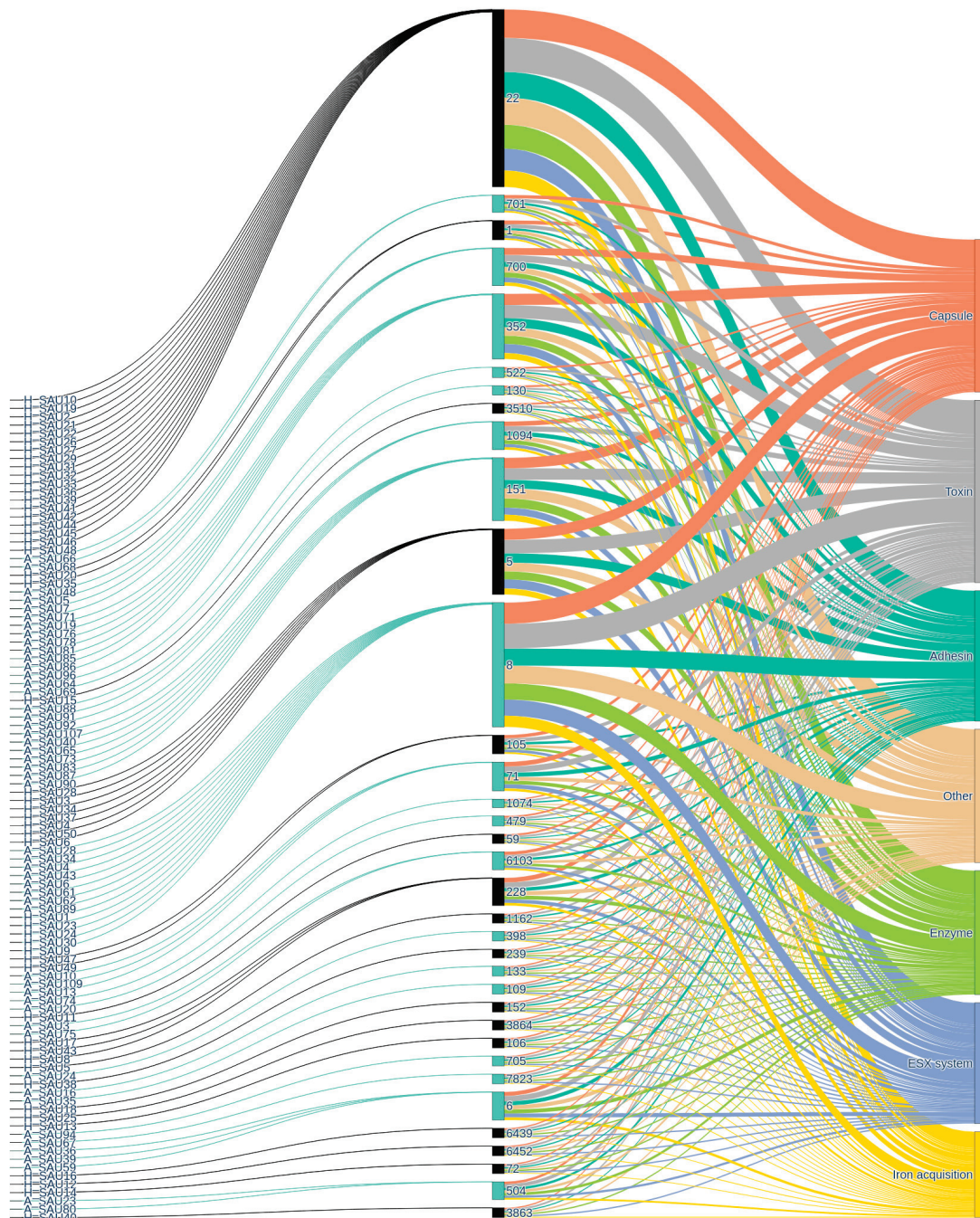


Figure 10. Distribution of virulence factors across *S. aureus* ST and functional classes. Sankey diagram illustrating the flow from *S. aureus* isolates, divided in animal (blue) and human (black) origin (**left**), through ST (**center**), to categorized virulence factor classes (**right**). This visualization highlights the diverse distribution of virulence genes related to capsule formation, toxins, adhesins, enzymes, secretion systems, iron acquisition, and other functions.

To better determine the relationship between resistance and virulence genes, a network analysis was performed focusing on co-occurrence (Figure 11). The arcs connecting the nodes are colored orange if the co-occurrence is exclusive to the A_ strains, purple if exclusive to the H_ strains, and lilac if both groups share the co-occurrence. The size of the nodes is proportional to the degree of connection (i.e., the number of genes with which each resistance or virulence gene co-occurs), while the thickness of the arcs reflects the frequency of co-occurrence. The network is characterized by most resistance genes connected to many virulence factors, and vice versa. This suggests that, in the strains analyzed, resistance

genes are not isolated events but are accompanied by several virulence genes, highlighting the genetic plasticity of *S. aureus*. Most co-occurrences are shared between the two groups of strains (lilac), while only a minority of arcs are exclusive to strains A₋ (orange) or H₋ (purple). This suggests that the structure of the co-occurrence network is largely conserved between the two groups.

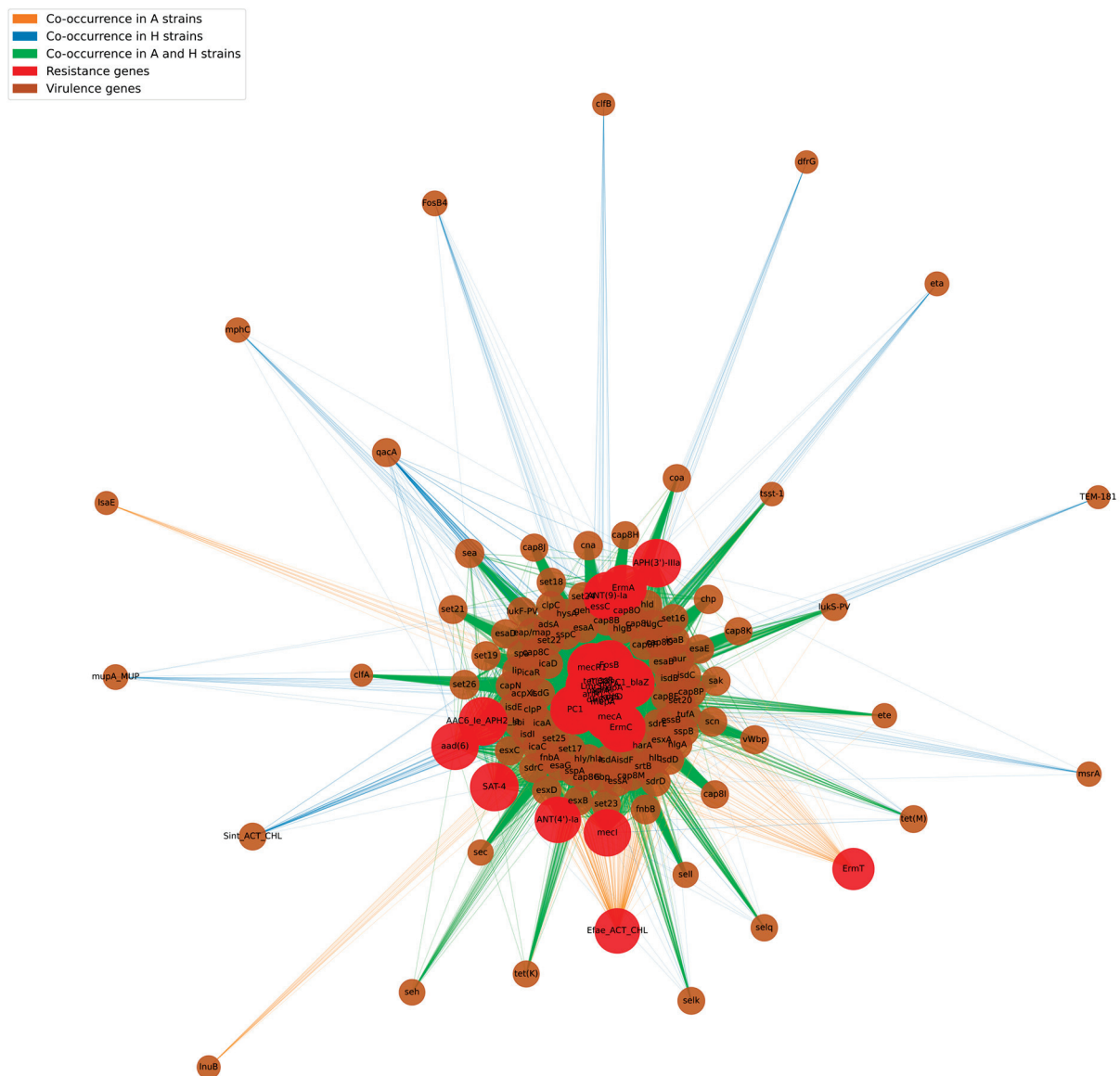


Figure 11. Network of resistance and virulence gene co-occurrence in *S. aureus*. Network graph depicts the co-occurrence patterns of antimicrobial resistance (red nodes) and virulence genes (brown nodes) in *S. aureus* isolates. Edge colors differentiate co-occurrence within animal strains (orange), human strains (blue), or both (green), as indicated in the legend. Node size is proportional to gene connectivity, highlighting core resistance determinants and the diversity of accessory virulence factors across host backgrounds. This representation provides insight into the potential for co-selection and dissemination of clinically relevant gene combinations.

3. Discussion

Staphylococcus aureus has emerged as a paradigm of host adaptation, clonal evolution, and global distribution among pathogens with zoonotic and anthroponozoonotic potential. The results of our comparative genomic analysis of staphylococcal strains isolated from milk samples and human clinical sources expand current knowledge on host-specialized

lineages, genetic diversification, and the mechanisms underlying host jumps. We critically discuss the implications of these models, referring to comparative genomics, population-level investigations, phylogenetics, antimicrobial resistance (AMR), and virulence traits.

The clonal diversity of *S. aureus* in both humans and cattle reflects a global trend that determines the epidemiology in each host. Our data, in line with other investigations, confirm that CC97, CC133, and CC151 are the main complexes adapted to cattle, consistent with the results of molecular and genomic typing studies demonstrating their prevalence in dairy herds in Europe, North America, Asia, and Africa [1,28,29]. This is reflected in human populations by dominant clonal complexes such as CC1, CC5, CC8, CC30, and CC45 [29,30]. Several studies report the presence of “human” lineages (particularly CC8 and CC5) in cattle and, conversely, the detection of “zootechnical” lineages such as ST398 in human carriers, especially among individuals who work closely with livestock [29,31–33]. Our results support these findings, with evidence of sporadic host shifts and complex patterns of clonal overlap, although true sustained host adaptation appears relatively rare for most lineages. Bovine-adapted lineages are characterized by the acquisition of specific phages and pathogenicity traits, supporting genetic events that facilitate adaptation to the bovine mammary niche. Human-adapted strains often contain prophages that modify β -hemolysin and encode immune evasion factors particular to humans [34]. It is crucial to acknowledge that transitions from human to bovine, exemplified by CC8, involve the loss of human-specific phage elements concomitant with the acquisition of novel SCC cassettes, occasionally encoding proteins featuring the new LPXTG motif. This phenomenon appears to facilitate rapid adaptation, as evidenced by our research and by comparative microarray-based genomics conducted in China and Switzerland, respectively [32,33,35].

Principal component analysis (PCA), and minimal spanning tree (MST) are examples of multivariate statistical studies that help us understand the population structure. PCA shows that resistance to important clinical antibiotics like methicillin, erythromycin, and ciprofloxacin is the main factor that separates strains. Resistance to macrolides and lincosamides (erythromycin and clindamycin) is prevalent in the most common combinations, probably mediated by ribosomal target modifications or efflux pumps. Co-resistance of beta-lactams and aminoglycosides is frequently associated with plasmids with multiple gene cassettes, while resistance to macrolides and lincosamides may arise from *erm* or *mef* genes, often co-localized with other resistance genes. Moreover, the influence of beta-lactam antibiotics (methicillin and penicillin) and macrolides (erythromycin and clindamycin) ARGs on PC1 underlines the importance of closely monitoring resistance to these classes, which represent the first choice for empirical therapy. In addition, the relevant contribution of ciprofloxacin highlights the need to also consider resistance to fluoroquinolones [36,37]. The MST shows distinct clusters that are mostly based on the host’s origin, but there are also important bridging nodes that signal genetic overlaps and possibly cross-transmission or shared environmental reservoirs. This supports the idea that pathogens’ evolution and ecology are connected in ways that make it harder to develop controls measures to manage them separately. Therefore, we need to think about diversity in pathogen genomics in terms of ecology and evolution.

Recent studies that used global phylogenetic reconstruction show that clonal complexes switch hosts at different rates and in different ways. The dairy bovine population acts more like a “sink” than a “source” for host hops, save for certain lineages like CC425 [38]. Modeling shows that ecological factors including farming practices, antibiotic use, and herd management, as well as microevolutionary adaptation, all affect how populations of cattle and people change over time [38–40]. In our strains, the presence of compact clades consisting exclusively of animal or human strains suggests that, despite the possibility of transmission between species, there are still selective or ecological barriers that favor the

specialization of certain evolutionary lines. The presence of mixed clades, on the other hand, suggests that in some circumstances these barriers can be overcome, allowing the transmission of strains between animals and humans.

S. aureus can swap hosts and spread from animals to people because it has a lot of genetic variation and can adapt to new environments. Finding different but overlapping clonal populations shows that some strains may be better suited to certain hosts, but ecological barriers are not always strong enough to stop genetic exchange and adaptation. This ability to adapt is made worse by the continual antibiotic selective pressures that cause multi-drug resistance phenotypes to appear, especially in the clinical setting for humans. The fact that animal strains have a lower but still significant level of resistance means that there is still a risk of reservoirs existing in livestock populations, which could be sources of resistant diseases that could spread to people. Resistance genes such as *tet(38)*, *dfrC*, *norA*, *norC*, *sepA*, *mepA*, *mgrA*, *sdrM*, *arlS*, *arlR*, *mepR*, *kdpD*, *LmrS* are associated with a wide range of virulence factors (*set22*, *cap8G*, *fnbB*, *clpC*, *hly/hla*, *ebp*, *esaD*, *sspA*, *icaR*, etc.). This breadth of co-occurrences suggests that most strains possess multiple resistance and virulence determinants simultaneously. The central structure of the network, in which resistance nodes are most represented (such as *tet(38)*, *norA*, *norC*, *dfrC*, *sepA*, *mepA*, *mgrA*), shows a high degree of connection, indicating that these genes play a key role in the bacterial population analyzed. They could represent “core” genes, whose presence is almost ubiquitous, and which act as a platform for the acquisition or co-localization of additional virulence genes. Because of the complicated genetic networks and the fact that resistance and virulence genes often happen at the same time, interventions that only look at resistance may miss important parts of how pathogens spread and how they cause disease.

However, this study has some limitations that should be considered when interpreting the results. First, the sample size and restriction to dairy herds from a single geographic region limit the generalizability of the findings to other production systems and epidemiological contexts. Second, human comparator genomes were selected from Italian public database entries and lack detailed and harmonized metadata on clinical presentation, which constrains inferences on directionality and frequency of cross-species transmission. Finally, the analysis is based on genomic prediction of resistance and virulence rather than systematic phenotypic characterization, so functional consequences of specific gene constellations should be confirmed in future experimental and longitudinal studies.

These results show how important it is to have integrated surveillance systems that can follow the evolution of resistance in *S. aureus* and the ways it spreads across both human and animal populations. This supports the One Health concept for addressing antibiotic resistance and zoonotic diseases caused by *S. aureus*. Reducing AMR requires coordinated efforts of human health care, veterinary medicine, farming, and environmental management. To stop the emergence of resistant and virulent *S. aureus* strains, it is important to use antibiotics wisely in both clinical and agricultural contexts, improve genomic surveillance, and take targeted steps to prevent infections. Further studies on the molecular mechanisms that allow hosts to adapt and pathogens to become resistant could aid in the development of new interventions to reduce the spread of this pathogen.

4. Materials and Methods

4.1. Bacterial Isolation and Identification

Isolates were collected in the period January 2024–May 2025 from the dairy herds in Lombardy region applying a mastitis control program and delivering samples to ARAL (Associazione Regionale Allevatori Lombardia) laboratories. Only *S. aureus* isolated from quarter milk samples of cows with subclinical mastitis were considered. Diagnosis was

performed following current diagnostic protocols applied in ARAL laboratories and following standardized procedures [4,41]. A positive *S. aureus* mastitis was defined when the bacteria was found in pure culture or when no more than two different bacteria species were observed. For each herd, a single isolate was considered. When more than one cow was positive within a herd, the selection criteria were: presence of a pure culture and highest somatic cell count.

The bacteriological analysis of the single-quarter milk samples was conducted in accordance with the guidelines established by the National Mastitis Council (NMC). Milk samples were inoculated onto Tryptic Soy Agar (TSA, Microbiol, Uta, Cagliari, Italy) supplemented with 5% defibrinated sheep blood, and Baird-Parker agar plates (Thermo Fisher, Milan, Italy), subsequently incubated aerobically at 37 °C for 24 h. Colonies presumptively identified as staphylococci based on golden-yellow pigmentation and hemolytic activity, underwent Gram staining. After staining, confirmatory tests include catalase, coagulase and biochemical tests performed on Vitek[®] system (BioMerieux, Lyon, France).

4.2. DNA Extraction

Isolated colonies from fresh overnight culture of *S. aureus* on TSA agar were re-suspended in 500 µL of PBS (Phosphate-Buffered Saline, Euroclone, Milan, Italy) and centrifuged at 10,000 × g for 1 min at room temperature. DNA was extracted using the Quick-DNA[™] HMW MagBead kit (Zymo Research, Irvine, CA, USA) in accordance with the manufacturer's guidelines.

The quantity and quality of the extracted DNA were evaluated using UV-Vis spectrophotometry with the NanoReady Touch Micro Volume Reader (Aurogene, Rome, Italy), confirming that the A_{260}/A_{280} and A_{260}/A_{230} ratios fell within the range of 1.8 to 2, respectively.

4.3. Sequencing and Bioinformatics Analyses

Genomic libraries were constructed using 200 ng of input DNA per sample, which underwent tagmentation using transposase with the Rapid Barcoding Sequencing kit (SQK-RBK114.96, Oxford Nanopore Technologies, Oxford, UK). Subsequently, twelve strains were loaded on a single flowcell (FLO-MIN114, version R10.4.1) and sequenced using the third-generation MinION Mk1C sequencer (Oxford Nanopore Technologies, Oxford, UK) for up to 72 h.

Dorado (v0.8.2, [42]) was used for basecalling, adapter trimming, and demultiplexing. Summary data about the quality and amount of reads were generated using NanoPlot (v1.44.0, [43]). Filtration was conducted using FiltLong (version 0.2.1, [44]). Subsequently, the genomes were de novo assembled using Flye (v2.8.1-b1676, [45]), and the resulting contigs were polished and corrected via Medaka (v2.0.1, [46]). The assessment of genomic completeness and contamination was conducted using CheckM2 (v1.2.4, [47]). Structural and functional genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP, [48]), which identifies and annotates coding sequences, rRNAs, and tRNAs. Pan-genome analyses, including identification of core and accessory genes and construction of the pangenome matrix, were carried out with PGAP2 [49] and complementary tools such as Anvi'o [50]. Average nucleotide identity was assessed using ANIClustermatrix tool [51] and the phylogenetic tree was visualized with iTOL (v.6) [52].

The genomes of the isolated bacteria were compared with those in the database (Pathogen Detection, GenBank, <https://www.ncbi.nlm.nih.gov/pathogens/> accessed on December 2024) to analyze pathogenic features, including clonality and the presence of antibiotic resistance genes. Fifty genomes of *S. aureus*, obtained from human isolates, were randomly chosen from samples collected in Italy (Supplemental Table S1).

4.4. Statistical Analysis

A Chi-squared test was used for categorical variables (e.g., resistant vs. susceptible strains, animal vs. human), and Welch's *t*-test for continuous variables (e.g., number of resistance or virulence genes per isolate); a *p*-value < 0.05 was deemed statistically significant for all analyses. Analyses (Chi-squared test and inspection of variable distributions by histograms, Q–Q plots, and boxplots prior to parametric testing) were originally performed in Excel™ (Microsoft Corp., Redmond, WA, USA).

Principal component analysis (PCA) was performed on the antibiotic resistance profiles using scikit-learn's PCA implementation in Python (v3.13), retaining two principal components. Each isolate was represented by its numeric resistance values across antibiotics; features were centered (default behavior of PCA in scikit-learn) but not scaled to unit variance, and principal components were obtained from the eigendecomposition of the covariance matrix, with loadings computed as component coefficients multiplied by the square root of the explained variance and visualized in a biplot.

For clustering, pairwise dissimilarities between isolates were computed on binary (0/1) resistance profiles using the Jaccard distance, and agglomerative hierarchical clustering with average linkage was applied to generate a dendrogram of isolates. The same Jaccard distance matrix was used to construct a complete weighted graph in Python (Linux environment), from which a minimum spanning tree (MST) was obtained using a standard minimum-spanning-tree algorithm and visualized with node colors indicating isolate origin.

5. Conclusions

The results of this study are broadly consistent with current knowledge on *Staphylococcus aureus*, while providing additional resolution on host association and genomic diversity. The data indicate strong, although not absolute, host adaptation, together with a dynamic capacity for host switching mediated by mobile genetic elements (MGEs) and patterns of antimicrobial resistance. The analyses further identify lineage-specific adaptation events and reveal complex transmission pathways involving both animal and human populations, underscoring the importance of integrated, genomics-informed surveillance.

Despite these advances, important gaps remain in understanding the relationship between genomic variation and pathogenic potential. In particular, the mechanisms that constrain or facilitate host jumps, as well as the ecological determinants that shape local and global distribution patterns, are still only partially resolved. Addressing these gaps will require a comprehensive approach that integrates comparative genomics, population-level epidemiology, experimental studies of pathogenesis, and multi-host ecological investigations. Such an integrated framework is essential to improve the control of a pathogen that exhibits adaptable and dynamic host interactions in both bovine and human populations, and to inform coordinated monitoring systems that are globally connected, genomics-based, and explicitly ecology-aware.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/antibiotics15010098/s1>. Table S1: Characteristics and accession numbers of the 50 H_ *S. aureus* strains. Figure S1: Preprocessing quality metrics and feature-level consistency of Animal-derived *Staphylococcus aureus* genomes. Figure S2: Pan-genome structure, gene cluster dynamics, and paralogy patterns across A_strains. Figure S3: Preprocessing quality metrics and feature composition of 50 H_ derived strains via PGAP2. Figure S4: Pan-genome architecture and gene cluster dynamics of 50 H_ *S. aureus* genomes analyzed with PGAP2.

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Article

Prospective Study of the Relative Abundance of Antimicrobial Resistance Genes in *Escherichia coli* O157:H7 Obtained from Chicken Carcasses from Local Markets in Lima, Peru

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Abstract

Objective: This study addresses antimicrobial resistance (AMR), a growing public health threat, by evaluating the role of chicken carcasses as possible vehicle for the spread of *Escherichia coli* O157:H7 and antimicrobial resistance genes (ARGs), with the aim of analyzing the association between bacterial load and the relative abundance of ARGs in samples obtained from an open and an enclosed market in Lima, Peru. **Methods:** SYBR Green qPCR was used to analyze 28 chicken carcasses from two local markets in the Lima metropolitan area (Enclosed market $n = 13$, and Open Market $n = 15$), detecting *Escherichia coli* O157:H7 and ARGs like *bla*_{CTX-M}, *bla*_{TEM}, and *strA*. **Results:** The bacterial load was higher in the enclosed market (5.062 log CFU/mL) than in the open market (3.875 log CFU/mL). *E. coli* O157:H7 was detected in 76.9% and 86.6% of samples, with average loads of 1.676 and 1.251 log CFU/mL, respectively. The relative abundance of *bla*_{CTX-M} and *bla*_{TEM} showed greater dispersion in the open market, whereas *strA* was more homogeneous in both markets. Significant positive correlation was found between *E. coli* load and ARGs abundance, stronger in the enclosed market ($r = 0.904\text{--}0.945$) and moderate to high in the open market ($r = 0.794\text{--}0.920$). **Conclusions:** The results demonstrate a significant correlation between *E. coli* O157:H7 load and ARGs, highlighting the need for a comprehensive approach within the framework of the “OneHealth” initiative.

Keywords: antimicrobial resistance; chicken meat; qPCR; bacterial pathogens; resistance genes

1. Introduction

A poorly explored sector in the development of antimicrobial resistance (AMR) is the unregulated use of antibiotics in livestock production, particularly in animals and their derived products intended for human consumption [1]. In recent decades, a significant increase in the prevalence of AMR in animal-derived food has been documented [2]. This phenomenon is particularly relevant for meat and its derivatives, which, in addition to being an essential source of proteins, vitamins, and minerals in the human diet, have exerted increasing pressure on production systems [3]. The sustained rise in meat demand has driven the unregulated use of antibiotics in animals, both for therapeutic purposes and as antibiotic growth promoters (AGPs), a practice still common in low- and middle-income countries. This has led to the evolution and spread of high levels of bacterial resistance, which must be addressed within the framework of the “One Health” approach [4].

The transfer of AMR bacteria from animal-based food products to humans, either through the consumption of contaminated food or through handling and marketing, represents a serious food safety issue [5]. Recent studies confirm the hypothesis that foodborne bacteria transfer antimicrobial resistance genes (ARGs) to the human gut microbiota [6,7]. Due to the high demand for meat products intended for human consumption, AGPs have been used indiscriminately, progressively contributing to the acquisition of AMR within the animal microbiota [8]. According to official data from the Ministry of Agrarian Development and Irrigation (2024), the average per capita consumption of chicken meat in Peru reached 52 kg in 2023 [9].

In this context, the commercialization of fresh chicken in local markets represents a high risk of bacterial contamination, as the handling and hygiene conditions vary considerably across different sales environments. In many cases, the lack of proper cold chain management and prolonged exposure of the meat favor cross-contamination, increasing the likelihood of pathogen transmission to consumers [10]. Additionally, in intensive farming systems, poultry vendors often offer chickens raised under suboptimal conditions, resorting to the indiscriminate use of antibiotics to maximize productivity and growth. This practice increases the risk of consumers being exposed to pathogenic bacteria carrying ARGs [11]. Given this scenario, it is necessary to investigate the presence of them directly associated with pathogenic bacteria in foods like chicken, a widely consumed product in the Peruvian population that, due to its production, marketing, and handling chain, constitutes a potential transmission vehicle to humans.

In this regard, we propose the quantification of the bacterial pathogen *Escherichia coli* O157:H7, recognized as one of the most relevant variants of enterohemorrhagic *E. coli* and a producer of Shiga toxins, which is an important causative agent of foodborne diseases (FBD). Its presence and load in chicken meat constitute a risk to public health, as this product could act as a vehicle of transmission to consumers and has been implicated in foodborne outbreaks worldwide [12]. This reinforces the need for constant surveillance of high-turnover poultry products such as chicken.

Additionally, this risk takes on greater significance when strains simultaneously carry antimicrobial resistance genes, such as *bla*_{TEM} and *bla*_{CTX-M} (related to resistance to β -lactam antibiotics) and *strA* (associated with resistance to aminoglycosides such as streptomycin), whose relative burden or abundance is also sought to be determined, mainly favoured by the indiscriminate use of antimicrobials during intensive poultry farming. The combination of virulence, resistance and high bacterial load not only increases the spread of these pathogens but also limits the available treatment options, making the monitoring and quantification of both bacteria and antibiotic resistance genes an essential component of food safety [13].

In order to achieve this, real-time quantitative PCR (qPCR) will be used, a sensitive and accurate technique that allows for the simultaneous identification and quantification of both pathogens and ARGs. Unlike conventional methods based on bacterial enrichment and culturing, which are often labor-intensive and limit the detection of certain foodborne microorganisms, qPCR offers a more efficient alternative for pathogen detection, generating key scientific evidence for epidemiological surveillance and antibiotic use control in poultry production [14]. Furthermore, the distribution of both resistance genes and the target pathogen will be assessed in samples of chicken carcasses sold in two marketing contexts: open markets (i.e., street vendors or informal stalls) and enclosed markets (i.e., permanent retail establishments). In both types of markets, processing and sanitation practices differ significantly, potentially increasing the risk of bacterial contamination and ARG abundance, influenced by environmental factors. Consequently, the aim of this study is to detect and quantify, using total DNA, both bacterial pathogens and antimicrobial resistance

genes in chicken carcasses to analyze their relative abundance distribution across different marketing contexts, and to provide relevant scientific evidence to improve food safety regarding the AMR challenge.

2. Results

2.1. Organoleptic Characteristics of the Samples

The organoleptic characteristics of each sample were recorded, including colour, odour, texture, and visible fat content, which allowed for a comparison of the apparent quality of the product between the two local markets prior to the total dressing of the carcass. In terms of colour, a yellowish tone predominated in the enclosed market (46.1%) and a greyish/yellow tone in the open market (64.3%). A fresh odour was more common in the enclosed market (61.5%), while a rancid odour predominated in the open market (73.3%). A rough texture predominated in both markets, especially in the open market (80%). Figure 1 shows the relative frequency of each characteristic by market, highlighting the predominant patterns in the samples analyzed.

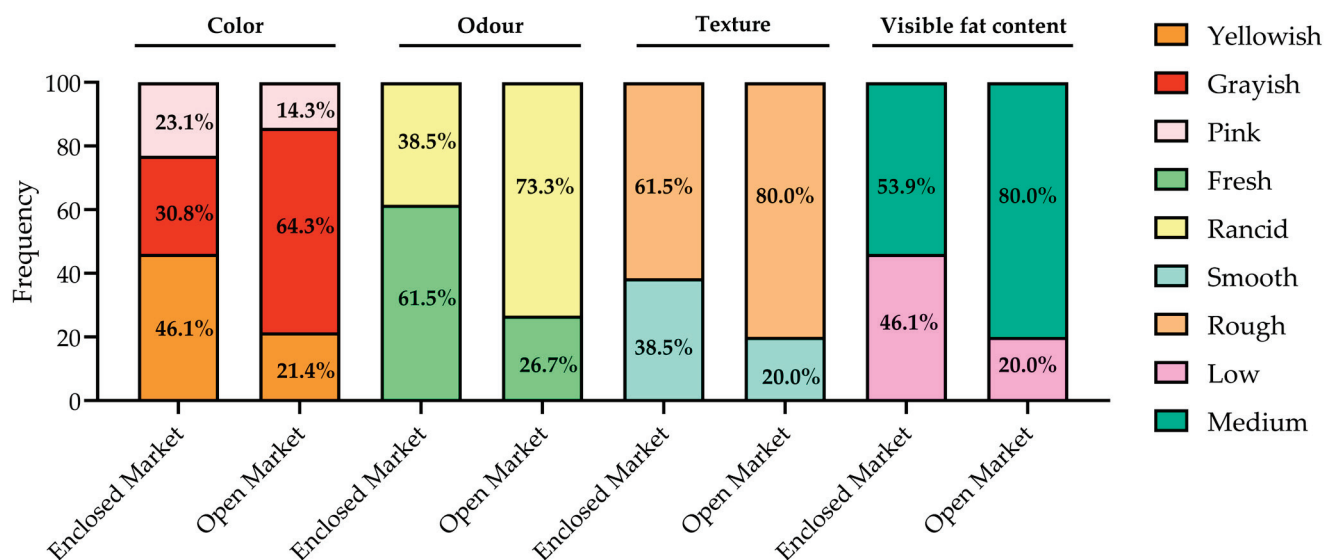


Figure 1. Frequency of organoleptic characteristics (color, odour, texture, and visible fat content) in chicken carcasses by local markets. Bars represent the frequency of each organoleptic characteristic for chicken carcasses from the enclosed market ($n = 13$) and open market ($n = 15$), highlighting differences between them.

2.2. Analytical Performance of 16S rRNA and *Escherichia coli* O157:H7 qPCR Assays

Standard curves were established for the quantification of the total bacterial population by qPCR using the control strain ATCC 25922 and primers targeting the 16S rRNA gene. This step was performed solely to construct and validate the 16S rRNA standard curve, ensuring precise and consistent amplification before quantifying the total bacterial population in chicken carcass samples. The assay was previously performed in the laboratory using available strains from the genera *Raoultella*, *Salmonella*, *Pseudomonas*, and *Klebsiella*, showing no significant variations in 16S rRNA gene amplification (Figure 2a).

The Ct values obtained from the serial dilutions allowed a linear regression curve to be generated ($y = -3.2863x + 40.3701$; $R^2 = 0.99$), with a lower detection limit of Ct 38.8 and an upper limit of 11.3, calculating an amplification efficiency of 95.3%. Figure 2 shows the behaviour of each dilution with respect to bacterial log(CFU/mL).

For the absolute quantification of *Escherichia coli* O157:H7, the *fliC* gene was used, obtaining the following standard curve parameters (Figure 3): slope -3.316 , intercept

37.969, efficiency 100.25%, coefficient of determination $R^2 = 0.9984$, lower detection limit Ct 38.0, and upper limit 17.63. The variability of the unknown samples ranged from 0.007 to 0.445. The specificity of the amplified products was verified using melting curves. The positive and negative controls included in each run corroborated the validity of the tests, while the linearity of the curves and the consistency of the Ct values in the serial dilutions confirmed the reproducibility of the methods and the high reliability of the quantification of the total bacterial population and *E. coli* O157:H7 in chicken carcasses.

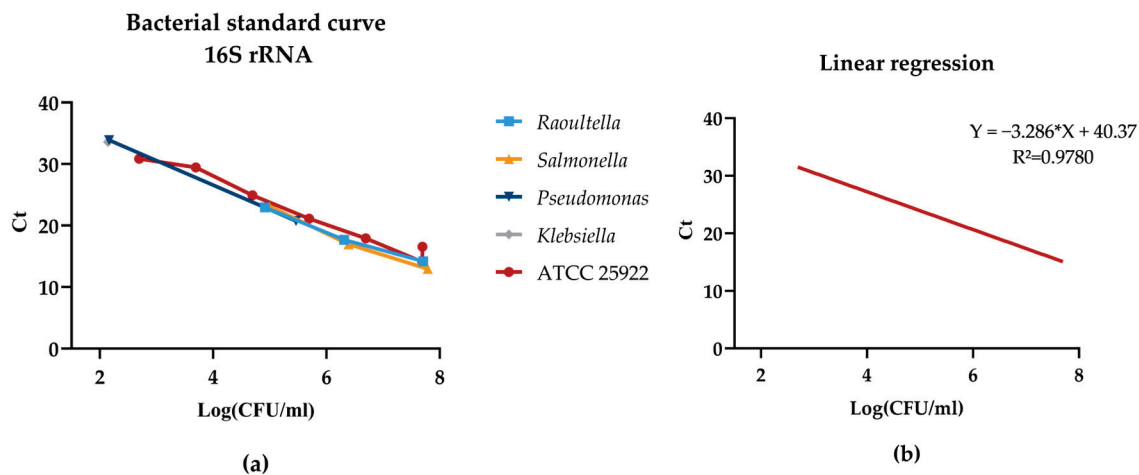


Figure 2. Standard curve for the absolute quantification of the bacterial population. Figure (a) shows the evaluation of representative strains of the genera *Raoultella*, *Salmonella*, *Pseudomonas*, *Klebsiella*, and *Escherichia coli* ATCC 25922, demonstrating the correspondence between serial dilutions and Ct values. (b) Shows the linear regression analysis for *Escherichia coli* ATCC 25922, including its linear equation and R^2 value, used to calculate reaction efficiency and estimate the bacterial population load.

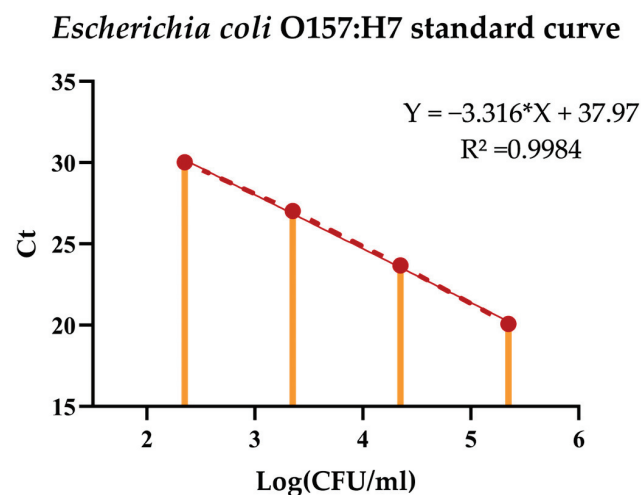


Figure 3. Standard curve for the absolute quantification of *Escherichia coli* O157:H7. The points corresponding to each dilution relative to their Ct values are shown, as well as the standard curve equation and R^2 value used to calculate efficiency and estimate the bacterial load of *Escherichia coli* O157:H7.

2.3. Detection and Quantification of Total Bacterial Load and *Escherichia coli* O157:H7

The absolute load of the total bacterial population (16S rRNA) and *E. coli* O157:H7 (*fliC*) was quantified from the chicken carcasses collected. Descriptive statistics for positive samples are presented in Tables S1 and S2, available in the Supplementary Material.

A high bacterial load was observed in both markets, with an average of 5.062 Log CFU/mL in the enclosed market and 3.875 Log CFU/mL in the open market, the latter showing the greatest variability. With regard to *E. coli* O157:H7, the open market had a higher percentage of positive samples (86.6%), but with a slightly lower concentration (1.251 Log CFU/mL) than the enclosed market (1.676 Log CFU/mL).

Overall, the results indicate that the total bacterial population remained high in both markets, although with greater variability in the open market (Figure 4).

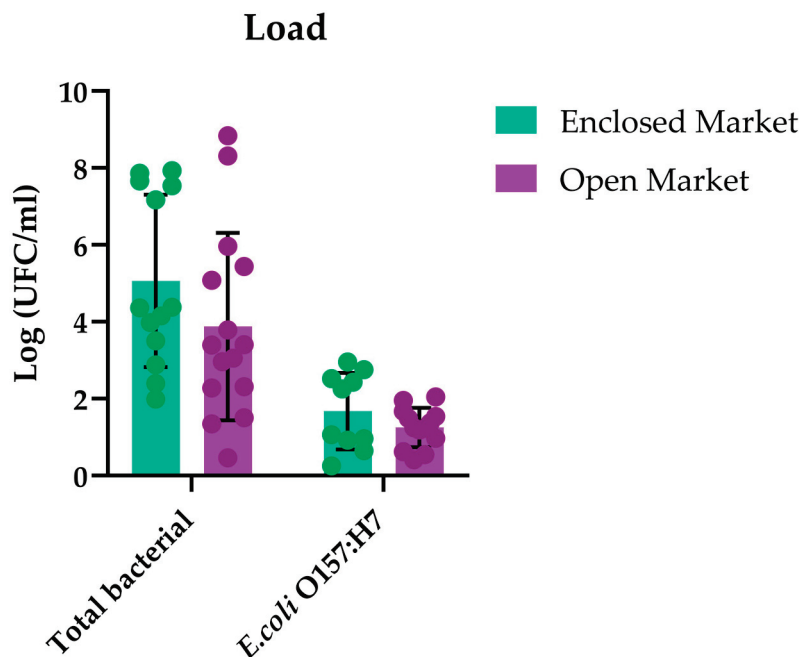


Figure 4. Comparison of total bacterial load and *Escherichia coli* O157:H7 load in analyzed samples.

Additionally, the graphical amplification analyses and melting curves corresponding to representative samples from each market are shown. In Figure 5, panel (a1,a2) presents the results obtained for the 16S rRNA, showing the detection of the total bacterial population together with a single peak in the dissociation curve, with a T_m value of 86.53 ± 0.23 °C, confirming the specificity of the reaction. Panel (b1,b2) shows the results of the amplification of the *fliC* gene of *Escherichia coli* O157:H7, whose specific product showed a T_m of 83.69 ± 0.27 °C, also with a single peak in the dissociation curve, corroborating the absence of non-specific products.

2.4. Distribution of ARGs in Chicken Carcasses

The average relative abundance of each AMR gene was estimated (Table S3), and the differences between chicken carcasses from the enclosed and open markets were compared (Figure 6). The *bla*_{CTX-M} gene showed a higher average relative abundance in samples from the open market (0.107 Log₁₀(AR)) compared to the enclosed one (−1.205 Log₁₀(AR)). Likewise, *bla*_{TEM} and *strA* presented greater values in open-market samples (1.199 and 0.847, respectively) than in those from the enclosed market (−0.464 and −0.072).

These results show a consistent trend towards higher relative expression of AMR genes in carcasses from the open market. Full details of relative abundance values and their variability are presented in the Supplementary Material, which complements the general comparison summarized in the main tables and figures.

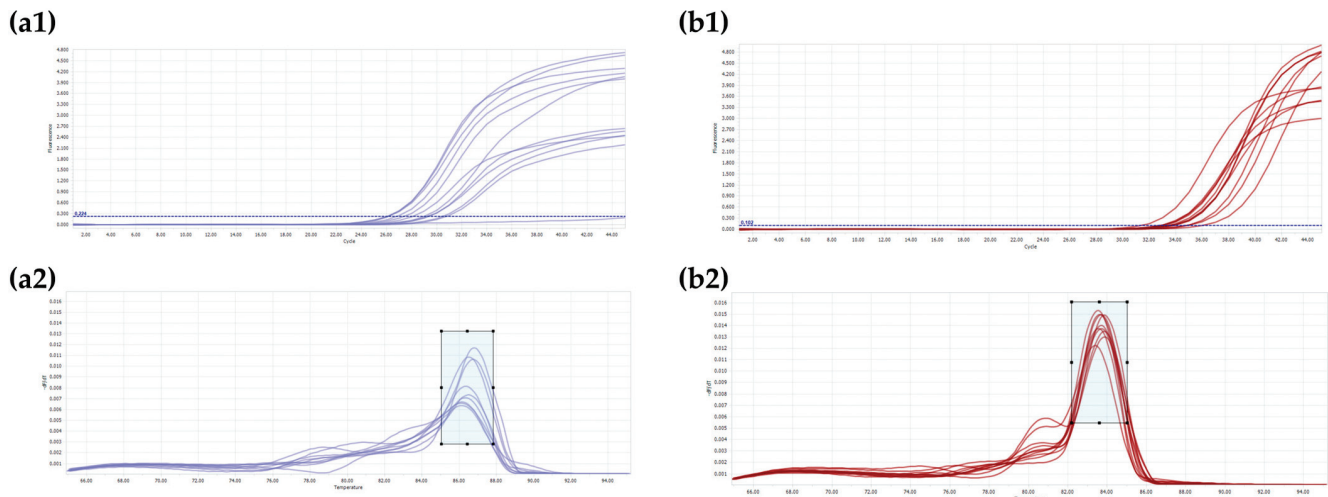


Figure 5. Representative chicken samples amplified by qPCR. Panel (a1,a2) shows the amplification of the 16S rRNA in selected samples from both markets, accompanied by their melting curves, which support the specificity of the products generated. Panel (b1,b2) shows the detection of the *fliC* gene of *Escherichia coli* O157:H7, together with the corresponding dissociation curve, which confirms the absence of non-specific amplifications and the validity of the results obtained.

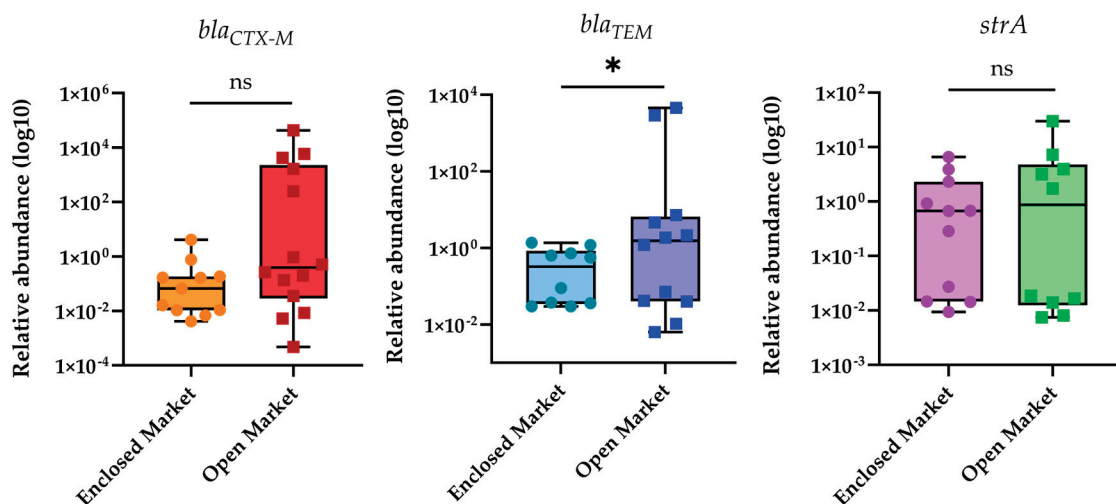


Figure 6. Comparison of the relative abundance of resistance genes in chicken carcasses at the enclosed and open markets. The graphs show the differentiation in relative abundance, represented by boxplots. Boxplots represent the distribution of data, with individual data points shown as dots. The black line within each box indicates the median. Statistical analysis was performed using Student's *t*-test, with *p*-values of *bla*_{CTX-M} = 0.08, *bla*_{TEM} = 0.03, and *strA* = 0.26. (*) indicates statistical significance at *p* < 0.05, while "ns" denotes a non-significant result.

Furthermore, Figure 7 shows the representation of the amplifications obtained by qPCR for each of the AMR genes analysed (*bla*_{CTX-M}, *bla*_{TEM}, and *strA*). Complementarily, the associated melting curves present a single defined peak, confirming the specificity of the amplicons.

2.5. Relational Analysis of Bacterial Load and Antimicrobial Resistance Genes

In the enclosed market, the *E. coli* O157:H7 load showed significant correlations with *bla*_{CTX-M} (*r* = 0.927), *bla*_{TEM} (*r* = 0.945) and *strA* (*r* = 0.904). Likewise, a strong association was found between *bla*_{CTX-M} and *bla*_{TEM} (*r* = 0.982, *p* < 0.05), indicating possible co-selection of these genes.

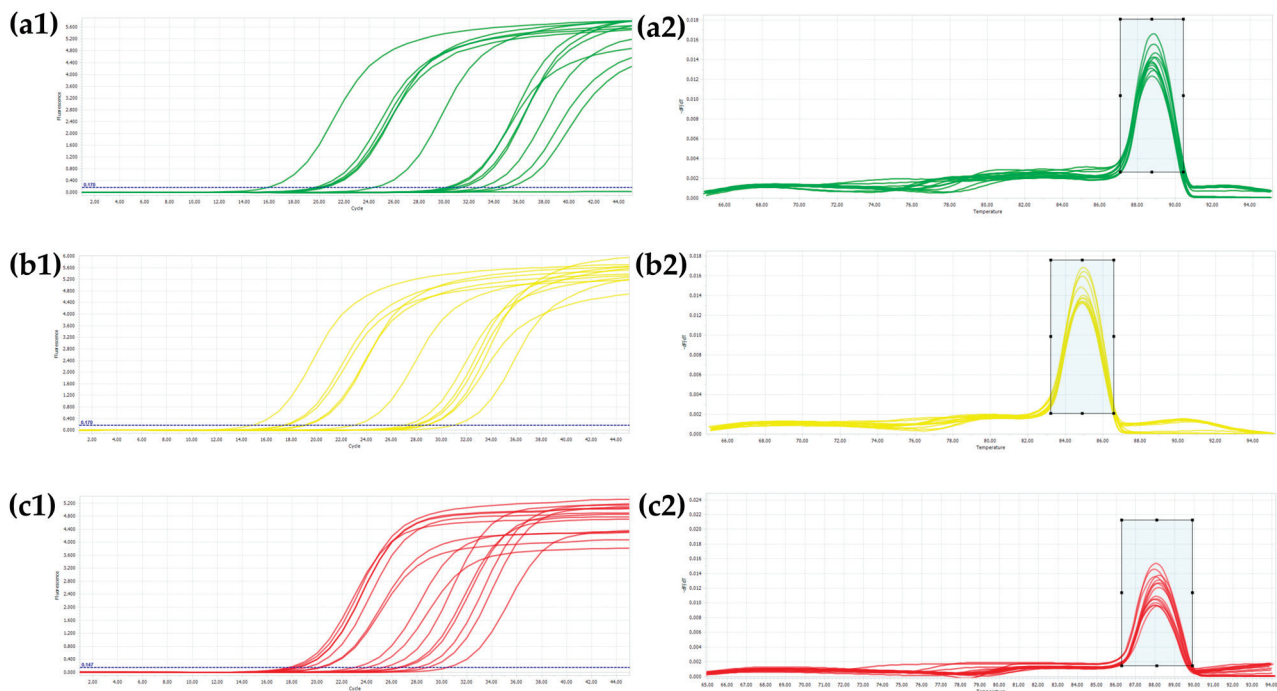


Figure 7. ARG qPCR amplifications and melting curves. (a1,a2) The T_m value of the specific amplicons of the *bla*_{CTX-M} gene was 88.78 ± 0.13 °C. (b1,b2) The T_m value for the detection of the *bla*_{TEM} gene was 84.82 ± 0.12 °C. (c1,c2) The T_m value for the detection of the *strA* gene was 87.90 ± 0.21 °C.

Similar patterns were observed in the open market: positive correlations between *E. coli* O157:H7 load and *bla*_{CTX-M} ($r = 0.794$), *bla*_{TEM} ($r = 0.844$) and *strA* ($r = 0.920$). In this case, the correlation between *bla*_{CTX-M} and *bla*_{TEM} was even higher ($r = 0.995$, $p < 0.05$), reinforcing the hypothesis of genetic co-occurrence and possible joint transfer.

Taken together, the results show that a higher burden of *E. coli* O157:H7 is consistently associated with a higher abundance of AMR genes, suggesting that these pathogens may act as key reservoirs in the spread of bacterial resistance in chicken meat.

3. Discussion

In the present study, a total of 28 chicken carcasses from the enclosed and open markets were analyzed. Total DNA was extracted from each sample, and qPCR was employed as a detection method for both bacterial pathogens and ARGs. This approach aligns with the “One Health” framework, which emphasizes the close relationship between human health, animal welfare, and environmental balance. In this context, poultry products can serve as a potential channel for the transmission of antibiotic-resistant bacteria to consumers [15]. The application of molecular tools in this research aims to optimize monitoring and surveillance processes of AMR, as such resistance can go undetected using conventional microbiological methodologies. It has been reported that various microorganisms present in food matrices and the environment, when found in low concentrations (CFU/mL) or in a viable but non-culturable (VBNC) state, may not be detected using conventional culture techniques, which can lead to false-negative results and pose a potential risk of exposure to public health [16].

Initially, the organoleptic characteristics of the samples collected from each market were evaluated (Figure 1). It was observed that the enclosed market had a higher proportion of carcasses in acceptable conditions of freshness, whereas the open market showed signs of deterioration, suggesting a direct impact of environmental conditions and product handling in each setting. This contrast is consistent with [17], which identified a higher frequency

of bacterial contamination in meat sold in informal and open markets, where exposure to the environment favours both sensory deterioration and microbiological risk. Likewise, other studies reinforce the idea that informality and lack of sanitary control at points of sale contribute not only to the physical deterioration of meat but also to the proliferation of pathogens and the spread of antimicrobial resistance genes [18]. These conditions not only result in greater physical deterioration of food, as initially evident in organoleptic characteristics, but also favour the proliferation of bacterial pathogens, thereby increasing the risk of exposure to contaminated food.

Similar studies have been reported in other countries. For example, in China, poultry products were analyzed in seven provinces, specifically in outdoor stalls. It was determined that establishments without a continuous refrigeration chain and with constant product handling favored bacterial proliferation and, therefore, posed a significant risk to food safety [19].

The difference between the two sampling points suggests variations in the handling or storage of chicken carcasses, as well as possible differences in the initial microbial load of the product (Figure 4). The higher bacterial load observed in the enclosed market may be attributed to environmental and management conditions, as limited air circulation and higher humidity inside the facility could favor microbial proliferation. In contrast, the open market, characterized by greater air flow and outdoor exposure, may experience reduced surface bacterial accumulation. Based on the results presented in Table S1, the average absolute quantification of the bacterial population was 5.06 log CFU/mL for the enclosed market and 3.87 log CFU/mL for the open market. This was higher than the 3.3 log CFU/mL previously reported in a study using the carcass rinsing method and conventional microbiological methodologies in two slaughterhouses in the Huancavelica region, Peru [20].

The *E. coli* O157:H7 bacterial load calculated in Table S2 for both markets was considered moderate to low, with an average range of 1.25–1.67 log CFU/mL. According to NTS No. 071, approved by R.M 591-2008/MINSA/DIGESA-V-01, which establishes the permitted microbiological limit for *E. coli* in raw chopped and ground meat ($50\text{--}5 \times 10^2 \sim 0.7\text{--}1.70$ log CFU/mL), the results obtained reflect a low frequency of bacterial load of this pathogen in most samples (Figure 4). However, even at low bacterial loads, the detection of *E. coli* O157:H7 is of public health importance due to its low infectious dose and potential to disseminate extended-spectrum β -lactamase (ESBL)-producing enterobacteria. In line with this, Cortez-Sandoval et al. (2022) [21] identified the presence of resistance genes such as *bla*_{CTX-M-1}, *bla*_{CTX-M-2}, *bla*_{CTX-M-9}, *bla*_{TEM}, and *SHV* in *E. coli* strains obtained from chicken meat samples sold in supply markets in the Santiago de Surco district of Metropolitan Lima. Despite the low bacterial counts, these isolates carried antimicrobial resistance genes, emphasizing the need for further studies in Peru to explore the association between bacterial pathogens and AMR genes [21]. The slightly higher values observed in this study compared with those reported previously may be related to differences in sampling conditions, analytical methods (qPCR vs. culture-based), or environmental exposure at the retail level. Despite the moderate bacterial counts, these findings highlight the epidemiological relevance of continuous monitoring of pathogenic *E. coli* and associated AMR genes in food products in Peru.

The detection frequency of *E. coli* O157:H7 was 76.9% and 86.6% of the total samples analyzed in the enclosed and open markets, respectively. This high occurrence, consistent with other qPCR-based reports, poses a public health concern given the low infectious dose of this pathogen. Contamination may occur during slaughter, handling, or sale due to inadequate hygiene or temperature control, underscoring the need for improved sanitary practices in the poultry supply chain. For instance, Alhadlaq et al. (2023) [22] reported

that imported meat could act as a carrier of *E. coli* O157:H7. In their study conducted in Saudi Arabia, meat products accounted for 15.71% of all imported foods, within which the presence of this pathogen was confirmed [22].

The relative abundance of the evaluated ARGs—*bla*_{CTX-M}, *bla*_{TEM}, and *strA*—was calculated using the 16S rRNA gene as a normalizer, establishing the minimum and maximum values for each quantified gene (Table S3). It was observed that the relative abundance of *bla*_{CTX-M} had a lower standard deviation in the enclosed market (0.9) compared to the open market (2.3), suggesting greater dispersion of relative abundance in open environments. Similarly, for *bla*_{TEM}, the relative abundance showed greater variability in the open market.

According to the literature, the diversity in resistance gene abundance is influenced by external environmental conditions, with open spaces being more prone to heterogeneity. In a comparative study of open and enclosed poultry farms in China, open farms were found to harbour a greater variety and diversity of AMR genes [23]. This supports the present findings, indicating that open environments promote greater dispersion and heterogeneity of resistance genes, reflected in the variability of relative abundance in the markets analyzed.

Additionally, the differences in variability between the two markets (Figure 6) showed statistically significant behaviour ($p < 0.05$) for *bla*_{TEM}, while for *bla*_{CTX-M} and *strA*, the differential pattern was non-significant, reinforcing the influence of structural conditions of the chicken meat retail environment on the distribution of AMR genes.

On the other hand, the relative quantification of *strA*, belonging to the aminoglycoside resistance family frequently used in poultry farming, did not show high variability between the two markets. This suggests a more homogeneous distribution, possibly due to the widespread and systematic use of aminoglycosides in intensive poultry farming, with a higher prevalence and abundance as previously reported [24]. As a result, no statistically significant differences were observed in the relative abundance of *strA* between the two markets.

The correlation between *E. coli* O157:H7 bacterial load and the relative abundance of AMR genes was evaluated using Pearson's correlation coefficient. The results showed a significant positive association ($r > 0.5$) in both markets, indicating that an increase in bacterial load is directly related to a greater abundance of the AMR genes analyzed.

As observed in the correlation for the enclosed market (Figure 8a), there was a strong positive correlation ($r > 0.9$) between bacterial load and the relative abundance of AMR genes. In addition, strong correlations were evident among the resistance genes themselves, suggesting possible co-abundance and co-occurrence of these determinants in bacteria present in chicken carcasses. This pattern can be explained by co-selection mechanisms described in recent resistome studies, which report high connectivity between β -lactam and aminoglycoside resistance genes, favoured by mobile genetic elements [25].

In the open market (Figure 8b), a similarly significant associative pattern was observed, though with slightly lower correlation strength. Pearson coefficients ranged from 0.794 to 0.920 between ARGs and *E. coli* O157:H7 load. This reduced association strength may be attributed to environmental exposure typical of open-air spaces, as exemplified by the open market. This variability is also reflected in Figure 8c, where the distribution of relative abundance of resistance genes shows greater dispersion across most of the collected samples. Nonetheless, despite this heterogeneity, the association between variables remains statistically significant and positive, reinforcing the potential relationship between pathogen presence and the relative expression of the ARGs evaluated in this study.

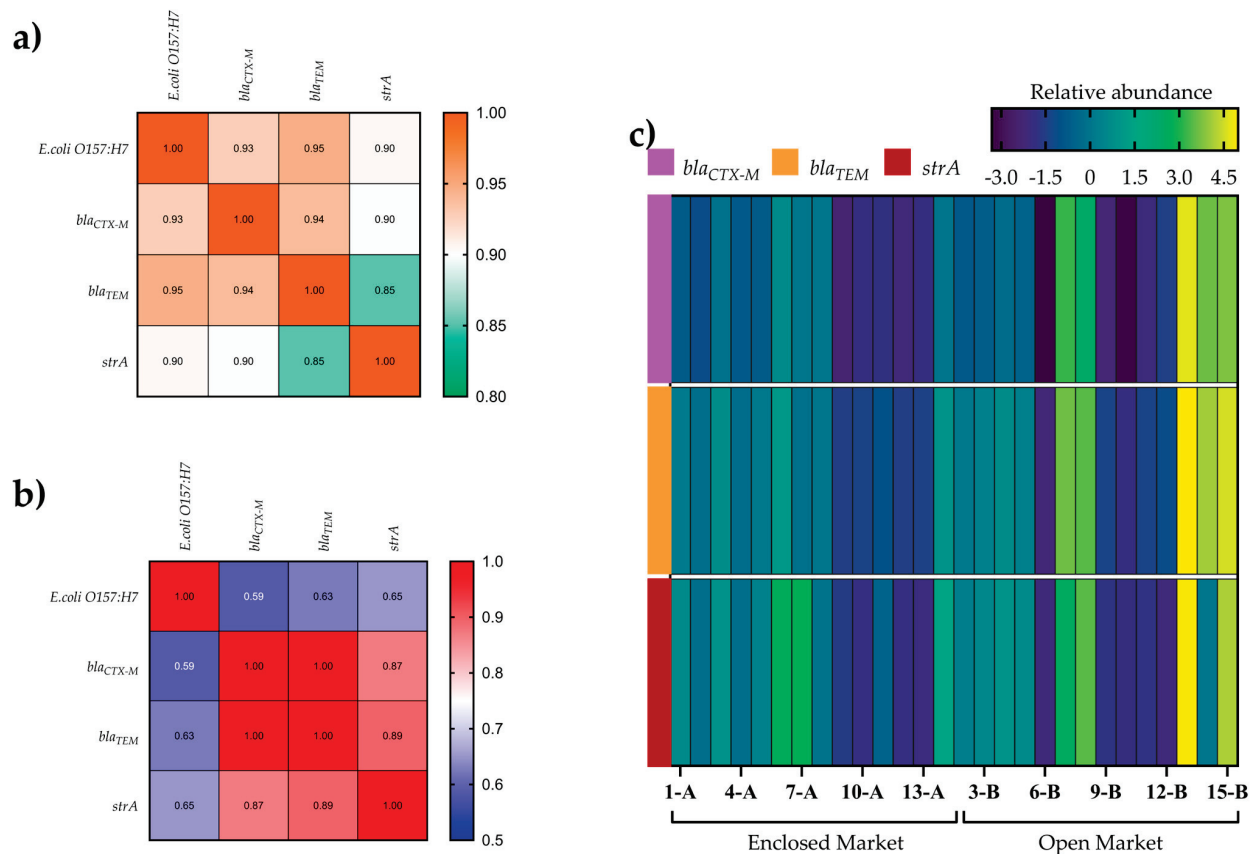


Figure 8. Positive correlations for the enclosed (a) and open (b) markets. Heat map (c) showing the distribution of the relative abundance of the antimicrobial resistance genes evaluated, based on the total number of chicken carcass samples collected in each market analyzed.

This study had limitations due to resource and time constraints. Antibiotic use practices on poultry farms were self-reported by vendors and not validated by independent inspections. Regarding the identification of *E. coli* O157:H7, although a validated molecular marker was used, it is recommended to complement this with virulence genes such as *eae*, *stx1*, and *stx2* to categorize pathogenicity. Furthermore, the analysis focused only on *bla*_{CTX-M}, *bla*_{TEM}, and *strA*, without exploring other AMR gene families such as *qnr*, *sul*, or *tet*. While qPCR detection was sensitive and specific, future studies should broaden ARG evaluation and assess phenotypic correlations to provide a more comprehensive understanding of the resistome and its relationship with pathogen load. The observed correlations should be regarded as indicative trends rather than definitive relationships, as the small sample size limits statistical power. Expanding the dataset in future studies is recommended to strengthen the validity of these associations.

4. Materials and Methods

4.1. Study Area and Sample Collection

A total of 28 chicken carcasses, each weighing approximately 400–500 g, were analyzed. Samples were randomly collected between 4 November 2024 and 6 December 2024 from two local markets, an enclosed market and an open market, located in the district of Comas (Lima, Peru). These markets were selected for their representativeness in terms of typical sales flow and daily product availability. The sample size was estimated using the proportional stratified sampling formula:

$$n_h = n \times \frac{N_h}{N}$$

assuming a 95% confidence level and a 0.05 sampling error [26]. Although the ideal sample size was calculated as 35 chicken carcasses, a total of 28 samples were obtained (13 from the enclosed market and 15 from the open market), ensuring proportional representation according to market availability and the exploratory nature of the study.

Each carcass was placed in an individual sterile bag, labelled with the origin and date of purchase, and transported in a thermal container with cold gel packs, maintaining a temperature of approximately 4 °C until arrival at the Laboratorio de Bioquímica y Biología Molecular, Facultad de Ciencias Naturales y Matemática, Universidad Nacional Federico Villarreal, Lima, Peru, where they were processed immediately. In the laboratory, the organoleptic characteristics (color, odor, texture, and visible fat) were evaluated through direct observation according to the criteria established in Annex 2 of the *Reglamento Sanitario de Funcionamiento de Mercados de Abasto* (Ministerial Resolution No. 282-2003-SA/DM, Ministry of Health, Peru) [27], in order to record the general condition of the samples prior to molecular analysis.

4.2. DNA Extraction from Chicken Carcasses

Total DNA extraction was performed following a modified whole-carcass rinsing method [28]. Each whole chicken carcass was rinsed with 225 mL of peptone water for 30 min in an orbital shaker to release the bacterial DNA present. Subsequently, DNA was extracted using the Qiagen DNA extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. DNA concentration was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) with the Qubit dsDNA HS Assay Kit, ensuring the recovery of high-quality DNA for subsequent qPCR analyses.

4.3. Optimization of qPCR Conditions

Quantitative real-time polymerase chain reaction (qPCR) was used to quantify the total bacterial load (16S rRNA), *Escherichia coli* O157:H7, and antimicrobial resistance genes (ARGs) in DNA extracted from chicken carcasses. Amplification was performed using a LightCycler 96 thermocycler (Roche, Mannheim, Germany). Each 20 µL reaction contained 12.5 µL of 2× SYBR Green I Master Mix, 0.5 µL of each primer, 0.3 µL of Taq polymerase, 3 µL of DNA, and nuclease-free water, following the protocol of Suria et al. (2012) [29] with minor modifications. The qPCR programme included a pre-incubation at 95 °C for 180 s, then 40 cycles of denaturation at 95 °C for 15 s, annealing at 57–60 °C for 15 s, and extension at 72 °C for 15 s. A pre-melting step at 70 °C for 180 s and a melting curve (95 °C for 10 s, 65 °C for 60 s, 90 °C for 1 s) were included to verify specificity. All reactions were performed in three biological replicates. Standard curves with 1:10 serial dilutions of bacterial DNA extracted from standardized reference strains were used to quantify total bacterial load (using strain ATCC 25922) and *E. coli* O157:H7.

Table 1 lists the primers used for 16S rRNA detection [30] and for the specific identification of *E. coli* O157:H7 through the *fliC H7* gene, which have been previously validated in beef and chicken by other studies [30,31]. *E. coli* O157:H7 positive DNA obtained from the Clinical and Bacteriology Laboratory of the National Institute of Health (INS) in Lima, Peru, was used as a positive control.

4.4. qPCR Assay Efficiency and Validation

qPCR efficiency was evaluated using standard curves generated from serial dilutions of bacterial DNA (10^{-1} to 10^{-5}), prepared from suspensions adjusted according to 0.5 McFarland scale to obtain a defined bacterial density. Threshold cycle (Ct) values were

plotted against the logarithm of colony-forming units (CFU/mL). Amplification efficiency (E) was calculated using the formula [32]:

$$E = 10^{(-1/\text{slope})} - 1$$

Efficiencies between 90% and 110% and correlation coefficients (R^2) ≥ 0.95 were considered acceptable. Specificity was verified by melting curves, ensuring a single peak per reaction. Each assay included positive and negative controls, and the 16S rRNA gene was used as an internal control to standardize quantification of bacterial load and ARGs (*bla*_{TEM}, *bla*_{CTX-M}, *strA*), ensuring accuracy and reproducibility.

Table 1. Primers used for the detection of bacterial species, *Escherichia coli* O157:H7 and 16S rRNA genes in qPCR assays.

Primer	Sequence (5'-3')	Amplicon Size (bp)	Identified in	References
<i>fliC</i>	F ATAATCTACGCCGCAACT	284	<i>Escherichia coli</i> O157:H7	[29]
	R GACTCCATCCAGGACGAAA			
16s rRNA	F GTG CCA GCM GCC GCG GTA A	900	<i>Escherichia coli</i> str. K-12	[30]
	R TAC GGY TAC CTT GTT ACG ACTT			

4.5. Relative Abundance of ARGs

Relative quantification of the resistance genes *bla*_{TEM}, *bla*_{CTX-M}, and *strA* was performed by qPCR using specific primers. Specificity was confirmed by melting curves. The relative abundance of each ARG was calculated using the 16S rRNA gene as a bacterial load reference, applying the ΔC_t method [33]:

$$\text{Relative abundance} = 2^{-(C_{t\text{ARGs}} - C_{t16s\text{ rRNA}})}$$

All reactions were performed in triplicate. The resulting data were used to compare ARG abundance across samples and marketing contexts. Table 2 lists the primers used.

Table 2. Primers for antimicrobial resistance genes.

Primer	Sequence (5'-3')	Amplicon Size (bp)	References
<i>bla</i> _{CTX-M}	F CGCTTTGCGATGTGCAG	550	[34]
	R ACCGCGATATCGTTGGT		
<i>bla</i> _{TEM}	F ATCAGCAATAAACCAGC	516	[34]
	R CCCCAGAAGAACGTTTTC		
<i>strA</i>	F CCTGGTGATAACGGCAATTC	548	[35]
	R CCAATCGCAGATAGAAGGC		

4.6. Statistical Analysis

Data were compiled in a database and analyzed using SPSS v25 and GraphPad Prism v10. For group comparisons of ARGs, parametric (*t*-test) or non-parametric (Mann–Whitney) tests were applied depending on data distribution. Associations were assessed using Pearson's correlation. A difference was considered statistically significant at $p < 0.05$. Results are presented as mean $\pm$ standard deviation or median with interquartile range, as appropriate.

5. Conclusions

Under the evaluated conditions, this comparative exploratory study, based on total DNA extraction from chicken carcasses, enabled the identification and quantification of *Escherichia coli* O157:H7 using SYBR Green-based real-time qPCR. To date, this represents one of the first reports in Peru to apply this quantitative molecular technique to chicken samples within the context of bacterial antibiotic resistance. Furthermore, the study successfully determined and quantified the antimicrobial resistance genes *bla*_{CTX-M}, *bla*_{TEM}, and *strA* with high efficiency and specificity. Differences in the relative abundance of these genes between the two markets were observed, associated with environmental exposure conditions of the carcasses, with more pronounced differences in the open market, characterized by its ambulatory nature. These findings confirm that qPCR is a reliable and robust tool for context-specific monitoring of pathogens and antimicrobial resistance genes within the food supply chain.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antibiotics15020191/s1>. Table S1: Absolute quantification of total bacterial load in chicken carcasses; Table S2: Absolute quantification of *Escherichia coli* O157:H7 bacterial load; Table S3: Quantification of the relative abundance of ARGs.

Author Contributions: D.D.-V.: data curation, formal analysis, investigation, validation, visualization, writing—original draft, and writing—review and editing; O.N.: data curation, formal analysis, investigation, validation, visualization, writing—original draft, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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

Abbreviations

The following abbreviations are used in this manuscript:

AMR	Antimicrobial resistance
qPCR	Quantitative real-time polymerase chain reaction
FBD	Foodborne diseases
AGPs	Antibiotic growth promoters
ARGs	Antimicrobial resistance genes
CFU	Colony Forming Units

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Article

Antimicrobial Resistance Profiles of Gram-Negative Bacteria Isolated from Saker Falcons (*Falco cherrug*) in Western Romania

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Abstract

Background/Objectives: The Saker Falcon (*Falco cherrug*) is an endangered raptor species of ecological and conservation relevance. Despite its status, data regarding its microbiota and the prevalence of antimicrobial resistance (AMR) remain scarce, especially in Eastern Europe. This single-facility study aims to investigate the phenotypic and genotypic AMR profiles of Gram-negative bacteria isolated from captive Saker Falcons in Western Romania. **Methods:** Freshly voided fecal droppings were collected non-invasively from 40 clinically healthy Saker Falcons. Bacterial identification was performed using selective media and the VITEK[®] 2 system. Antimicrobial susceptibility testing (AST) was conducted on a representative subset of 12 isolates. Selected resistance-associated genes were screened by conventional PCR. **Results:** *Escherichia coli* was the most prevalent 60% (n = 24/40), followed by *Hafnia alvei* 10% (n = 4/40) and *Pseudomonas* spp. 10% (n = 4/40). AST revealed phenotypic resistance among *Enterobacteriaceae* primarily to ampicillin 20% (n = 2/10), tetracycline 20% (n = 2/10), fluoroquinolones and sulfonamides 10% (n = 1/10), while susceptibility to imipenem 90% (n = 9/10) and gentamicin 90% (n = 9/10) remained high. The targeted resistance-associated genes were detected in selected phenotypically resistant isolates. PCR screening detected *blaZ* and *ampC* in 62.5% (n = 5/8) of tested isolates, *blaOXA-61* in 37.5% (n = 3/8), *blaOXA-51* in 25% (n = 2/8), *tetK* in 37.5% (n = 3/8), and *gyrA* in 12.5% (n = 1/8). The isolate used as the negative control, pansusceptible in AST, was confirmed negative for all targeted genes. **Conclusions:** This single-facility study provides baseline data on AMR traits in Gram-negative bacteria associated with Saker Falcons in Western Romania. Given the limited scale and isolate-based design of the study, the findings should be interpreted cautiously, but they support further investigation of wildlife-associated AMR within a One Health context.

Keywords: antimicrobial resistance; *Enterobacteriaceae*; *Falco cherrug*; One Health; Romania

1. Introduction

The Saker Falcon (*Falco cherrug* Gray, 1834), a large diurnal raptor of the family *Falconidae*, is widely distributed across the Palearctic map, from Eastern Europe and the Middle East to Central and East Asia. Across Eastern and Central Europe, breeding populations have been reported in Hungary, Romania, Bulgaria, Ukraine, and Turkey, as illustrated in Figure 1 [1].

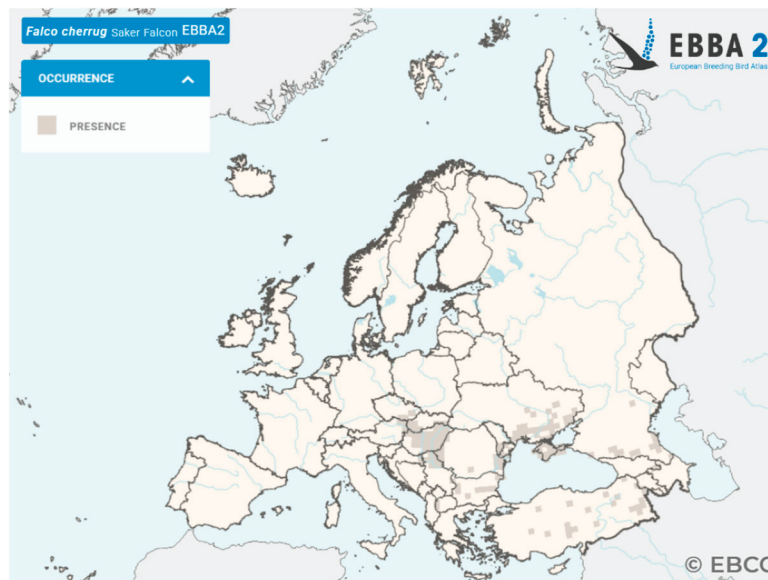


Figure 1. Distribution and occurrence of the Saker Falcon (*Falco cherrug*) in Europe according to the European Breeding Bird Atlas 2 (EBBA2) [1].

Recently, the Saker Falcon population in Western Romania has shown a significant recovery, primarily due to conservation efforts involving the installation of artificial nest boxes on high-voltage electricity pylons [2]. According to the European Red List of Birds, the breeding population is estimated at 430–630 pairs, with the largest populations occurring in Hungary, Ukraine, and Slovakia, whereas small and declining populations persist in Romania, Bulgaria, and Serbia [3].

In Central Europe, Hungary represents an important source population for the regional expansion of this species, including toward Western Romania, and breeding success has been shown to depend strongly on habitat quality and the availability of safe nesting sites, including artificial structures [4–6].

Despite local recovery trends, the species remains affected by important conservation pressures in other parts of its range, including habitat loss and illegal trapping [7].

Its populations have declined during recent decades due to habitat loss, prey depletion, electrocution, and illegal trapping, leading to its classification as endangered by the International Union for Conservation of Nature (IUCN) [8,9]. The species is of ecological and cultural significance, particularly through its long association with falconry, where birds are frequently bred, traded, and maintained in captivity [10]. Studies on Saker Falcons have described wide foraging movements and have established clinical reference values useful to avian medicine [11,12]. Captive falcons used in falconry or rehabilitation may be exposed to diverse microbial communities, parasites, and environmental contaminants, and surveys from Europe and the Arabian Peninsula have reported parasitic, fungal and bacterial infections, as well as heavy metal exposure in these birds [13–15]. Opportunistic pathogens such as *Aspergillosis* and *Caryospora cherrughi* infection have been documented in *F. cherrug* and hybrid falcons [14,16], while microbiological screening has revealed the

presence of opportunistic and potentially pathogenic bacteria, including *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, often in asymptomatic carriers [17]. Physiological and environmental stressors, including nutritional imbalance, contaminants, and human handling, may further impair immunity and facilitate bacterial colonization [18,19].

Recent advances in genetic research have provided insights into the evolutionary and molecular features of *Falco cherrug* [20,21], yet relatively little is known about its microbiota and the occurrence of antimicrobial resistance (AMR) in associated bacterial populations. Raptors are recognized as important sentinels of environmental health and potential reservoirs of antimicrobial-resistant microorganisms. Their diet, which includes small mammals and birds from agricultural landscapes, and their interaction with human activities place them at the interface of wildlife, domestic animals, and human health. Consequently, the detection of resistant Gram-negative bacteria in raptors is increasingly regarded as an early warning signal within the One Health framework [4,22,23].

Among Gram-negative bacteria, members of the *Enterobacteriaceae* family and related genera are of particular concern, as they harbor genes conferring resistance to β -lactams, tetracyclines, fluoroquinolones, and other clinically relevant antimicrobial classes. Monitoring these pathogens in wild and captive raptors can help understand the environmental dissemination of resistance determinants. Previous studies have emphasized the need for integrated surveillance linking wildlife microbiology and veterinary diagnostics to broader AMR control strategies [13,14,24,25].

The present study investigates the phenotypic and genotypic AMR profiles of Gram-negative isolates recovered from fecal samples of Saker Falcons maintained in captivity. By combining phenotypic and molecular approaches, this work aims to provide baseline data on antimicrobial resistance in *Falco cherrug* and to contribute to the broader discussion of wildlife-associated AMR within a One Health framework.

2. Results

2.1. Bacterial Isolation and Identification

A total of 40 bacterial isolates recovered from fresh fecal droppings collected non-invasively from clinically healthy Saker Falcons (*Falco cherrug*) were subjected to bacteriological examination. Gram-negative bacteria were successfully isolated and identified using the VITEK[®] 2 automated system.

The most prevalent species was *Escherichia coli*, identified in 24 out of 40 samples, representing an isolation rate of 60.0%. Other members of the *Enterobacteriaceae* family were detected at lower frequencies, including *Hafnia alvei* 10.0% (n = 4/40), *Pantoea* spp. 10.0% (n = 4/40), and *Escherichia hermannii* 5.0% (n = 2/40). Less frequently isolated pathogens included *Serratia fonticola* and *Klebsiella aerogenes*, each detected in single samples 2.5% (n = 1/40). Additionally, *Pseudomonas* species (*P. fluorescens* and *P. putida*) were identified in 10.0% (n = 4/40) of the samples combined, as detailed in Figure 2.

The automated identification process demonstrated high reliability. The probability of identity for the vast majority of isolates ranged from 95% to 99%, corresponding to an “Excellent Confidence” level according to the VITEK[®] 2 system specifications. Only two isolates (*S. fonticola* and *K. aerogenes*) were identified with a “Good Confidence” level (91%), which is considered sufficient for diagnostic accuracy in veterinary clinical microbiology.

The detailed identification data for each of the 40 isolates, including VITEK[®] 2 probability levels and isolation media, are provided in Table S1.

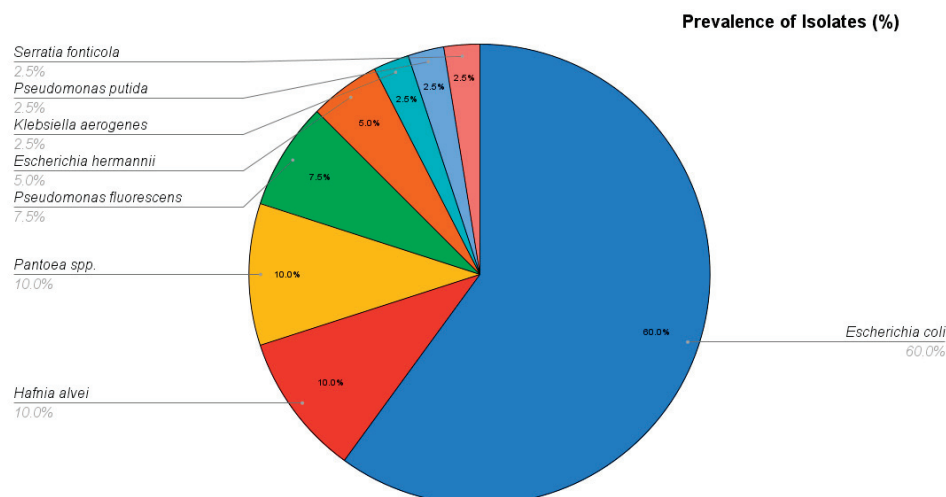


Figure 2. Prevalence and identification of Gram-negative bacteria isolated from Saker Falcon.

2.2. Phenotypic Antimicrobial Resistance Profiles

From the total bacterial collection, a representative subset of 12 isolates was selected for detailed AST. This subset contains 10 isolates belonging to the *Enterobacteriaceae* family (predominantly *Escherichia coli*, alongside *Hafnia alvei* and *Pantoea spp.*) and 2 isolates of *Pseudomonas spp.* (*P. fluorescens*).

Among *Enterobacteriaceae*, resistance was predominantly associated with β -lactam antibiotics. Ampicillin resistance was detected in 20% ($n = 2/10$) of the isolates, whereas 60% ($n = 6/10$) were classified as susceptible. Ticarcillin/clavulanic acid showed high activity, with 80% ($n = 8/10$) of isolates categorized as susceptible and only 10% ($n = 1/10$) as resistant. The first-generation cephalosporin cefalotin showed limited efficacy, with 10% ($n = 1/10$) resistant, 30% ($n = 3/10$) intermediate, and 20% ($n = 2/10$) susceptible isolates. For cefalexin, 70% ($n = 7/10$) of isolates were classified as susceptible, while 20% ($n = 2/10$) exhibited phenotypic resistance. In contrast, third- and fourth-generation cephalosporins, including cefoperazone, ceftiofur, and cefquinome, demonstrated improved efficacy, with 60% ($n = 6/10$) of *Enterobacteriaceae* isolates characterized as susceptible.

Carbapenem susceptibility remained high among *Enterobacteriaceae*, as 90% ($n = 9/10$) of isolates were susceptible to imipenem. Aminoglycoside testing revealed 70% susceptibility to neomycin and 90% ($n = 9/10$) to gentamicin. Resistance to quinolones and fluoroquinolones (flumequine, enrofloxacin, and marbofloxacin) was low, with each agent showing 10% ($n = 1/10$) resistance and 50% ($n = 5/10$) susceptibility among *Enterobacteriaceae* isolates. Tetracycline showed moderate activity, with 40% ($n = 4/10$) of isolates classified as susceptible and 20% ($n = 2/10$) as resistant. For sulfonamides, trimethoprim/sulfamethoxazole exhibited 80% ($n = 8/10$) susceptibility and 10% ($n = 1/10$) resistance among isolates.

According to the predefined multidrug resistance (MDR) criterion (≥ 3 antimicrobial classes), MDR was observed in *Hafnia alvei* and *Escherichia hermannii* isolates, which exhibited simultaneous resistance to multiple antimicrobial classes, including penicillins, cephalosporins, and tetracyclines. This multidrug-resistant profile highlights the clinical relevance of the bacterial flora carried by these raptors.

The *Pseudomonas spp.* ($n = 2$) and *Klebsiella aerogenes* ($n = 1$) isolates displayed susceptibility profiles consistent with their known intrinsic resistance characteristics. For *K. aerogenes*, the isolate was classified as resistant to ampicillin and first-generation cephalosporins, a result validated by the VITEK[®] 2 Therapeutic Resistance Monitoring (TRM) system due to the species' constitutive chromosomal *ampC* expression. In the case of *Pseudomonas spp.*, certain antibiotics yielded 'Insufficient Evidence' (IE) messages; this aligns with EUCAST [26]

observations where clinical breakpoints are not always established for non-aeruginosa *Pseudomonas* spp., although their phenotypes remain consistent with intrinsic mechanisms, such as efflux pumps and *AmpC* β -lactamases. These findings reflect inherent biological traits rather than acquired resistance. Nevertheless, both isolates were resistant 100% ($n = 2/2$) to ticarcillin/clavulanic acid and showed intermediate susceptibility 100% ($n = 2/2$) to imipenem.

Overall, the phenotypic resistance data indicate a higher frequency of resistance to older β -lactam antibiotics among *Enterobacteriaceae*, whereas carbapenems retained high efficacy against both *Enterobacteriaceae* and *Pseudomonas* spp. Distinct resistance patterns between enteric fermenters and non-fermenting species are detailed in Tables 1 and 2 and provide the basis for the subsequent analysis of antimicrobial resistance genes.

Table 1. Antimicrobial susceptibility testing results for bacterial isolates.

	AMP	AMC	TIC/CLA	CEX	CFL	CFP	CTF	CFQ	IPM	GEN	NEO	FLU	ENR	MAR	TET	SXT
<i>Hafnia alvei</i> FC-V01	R	R	S	R	R	S	S	S	S	S	S	S	S	S	R	S
<i>E. hermannii</i> FC-T09	R	R	I	R	S	S	S	S	S	S	S	R	R	R	R	R
<i>K. aerogenes</i> FC-V15	IR	IR	IR	IR	IR	IR	IR	IR	IR	IR	S	IR	IR	IR	IR	IR
<i>E. coli</i> FC-V17	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S
<i>E. coli</i> FC-T21	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S
<i>P. agglomerans</i> FC-V25	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
<i>Ps. fluorescens</i> FC-V29	IE	IE	R	IE	-	-	-	-	I	IE	-	-	-	-	IE	IE
<i>E. coli</i> FC-T33	S	-	S	S	-	-	-	-	S	S	-	-	-	-	IE	S
<i>E. coli</i> FC-T34	S	S	S	S	I	S	S	S	S	S	S	S	S	S	S	S
<i>E. coli</i> FC-V37	S	S	S	S	-	-	-	-	S	S	-	-	-	-	IE	S
<i>E. coli</i> FC-V38	S	-	S	S	-	-	-	-	S	S	-	-	-	-	IE	S
<i>Ps. fluorescens</i> FC-V32	IE	IE	R	IE	-	-	-	-	I	IE	-	-	-	-	IE	IE

Note: S = Susceptible (green); I = Intermediate (yellow); R = Resistant (red); IR= Intrinsic Resistance (gray); IE = Insufficient Evidence that species is good target for therapy (blue); AMP—ampicillin; AMC—amoxicillin-clavulanate; TIC/CLA—ticarcillin-clavulanate; CEX—cefalexin; CFL—cefalotin; CFP—cefoperazone; CTF—ceftiofur; CFQ—cefquinome; IPM—imipenem; GEN—gentamicin; NEO—neomycin; FLU—flumequine; ENR—enrofloxacin; MAR—marbofloxacin; TET—tetracycline; SXT—trimethoprim-sulfamethoxazole; “-” indicates antibiotic not tested or no interpretative result available.

Table 2. Antimicrobial resistance patterns across antibiotic classes in *Enterobacteriaceae* vs. *Pseudomonas* spp. isolates.

Antimicrobial Class	Antibiotic Agent	<i>Enterobacteriaceae</i> (n = 10)			<i>Pseudomonas</i> spp. (n = 2)		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Penicillins	Ampicillin	20		60			
	Amoxicillin/Clavulanic Acid	20		50			
	Ticarcillin/Clavulanic Acid		10	80	100		
Cephalosporins	Cefalexin	20		70			

Table 2. Cont.

Antimicrobial Class	Antibiotic Agent	<i>Enterobacteriaceae</i> (n = 10)			<i>Pseudomonas</i> spp. (n = 2)		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Carbapenems	Cefalotin	10	30	20			
	Cefoperazone			60			
	Ceftiofur			60			
	Cefquinome			60			
	Imipenem			90		100	
	Gentamicin			90			
Aminoglycosides	Neomycin			70			
	Flumequine	10		50			
Chinolone	Enrofloxacin	10		50			
	Marbofloxacin	10		50			
Tetracyclines	Tetracycline	20		40			
Sulfonamides	Trimethoprim/Sulfamethoxazole	10		80			
Others	Florfenicol (Amphenicol Class)						
	Polymyxin B (Polypeptide Class)						

Note: S = Susceptible; I = Intermediate; R = Resistant; Percentages were calculated based on the number of isolates for which AST interpretation was available. “IE” (insufficient evidence) and “IR” (intrinsic resistance) results were excluded from percentage calculations.

2.3. Genotypic Antimicrobial Resistance Profiles

Isolates exhibiting phenotypic resistance in AST were subjected to molecular confirmation. The number of isolates screened for each gene corresponded to the respective resistance phenotype observed in VITEK® 2 testing.

The tetracycline resistance gene *tetK* was investigated for isolates phenotypically resistant to tetracycline. The isolates with tetracycline resistance yielded the expected 360 bp amplicon, indicating that the phenotypic resistance profiles are genotypically supported by the detection of the specific resistance determinants.

Notably, isolate FC-T34, classified as intermediately susceptible in the phenotypic AST, produced a distinct PCR amplicon corresponding to the targeted resistance gene (Figure 3a, lane 5), indicating that the resistance determinant was present despite the absence of a fully resistant phenotype.

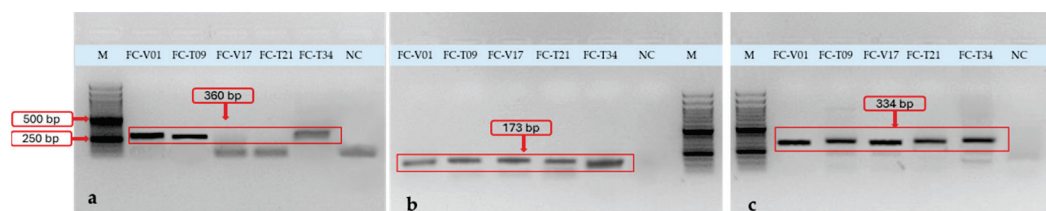


Figure 3. Agarose gel electrophoresis of PCR products showing amplification of selected antimicrobial resistance genes in Gram-negative isolates from Saker Falcons (*Falco cherrug*). Lane M: 50 bp DNA Ladder RTU (Simply). (a) Lanes FC-V01, FC-T09, FC-T34: amplification of the *tetK* gene, showing distinct bands at approximately 360 bp; (b) Lanes FC-V01, FC-T09, FC-V17, FC-T21, FC-T34: detection of the *blaZ* gene with an expected amplicon size of 173 bp; (c) Lanes FC-V01, FC-T09, FC-V17, FC-T21, FC-T34: amplification of the *ampC* gene with bands corresponding to ~334 bp. Lane NC: internal negative control (DNA from pansusceptible isolate), showing absence of amplification. Band sizes were estimated by comparison with the 50 bp molecular weight marker.

β -lactam resistance determinants were analyzed in isolates displaying reduced susceptibility to penicillins and/or early-generation cephalosporins. Amplification of *blaZ* (173 bp) and *ampC* (334 bp) was detected in all five resistant isolates. When calculated relative to the total number of isolates subjected to AST, *blaZ* and *ampC* were present in 62.5% ($n = 5/8$) of tested isolates. Class D β -lactamase genes were identified at lower frequencies.

The *blaOXA-61* gene (280 bp) was detected in three isolates (37.5%, $n = 3/8$), while *blaOXA-51* (353 bp) was identified in two isolates (25%, $n = 2/8$). Importantly, despite the presence of *blaOXA* genes, none of these isolates exhibited carbapenem resistance, consistent with the high imipenem susceptibility observed phenotypically.

Fluoroquinolone-associated resistance was investigated by amplification of the quinolone resistance-determining region of *gyrA*. A specific 626 bp amplicon was detected in one isolate (12.5%, $n = 1/8$) exhibiting reduced susceptibility to fluoroquinolones. No amplification was observed in phenotypically susceptible isolates.

Overall, the distribution of resistance genes among tested isolates was as follows: *tetK* (37.5%), *blaZ* (62.5%), *ampC* (62.5%), *blaOXA-61* (37.5%), *blaOXA-51* (25.0%), and *gyrA* (12.5%) (Figures 3 and 4, see also Figures S1 and S2 for original gel images in Supplementary Materials). The Quinolone Resistance-Determining Regions (QRDR) fragment of *gyrA* was successfully amplified in the phenotypically non-susceptible isolate. However, PCR amplification alone does not confirm the presence of resistance-associated mutations; sequencing is required for definitive confirmation.

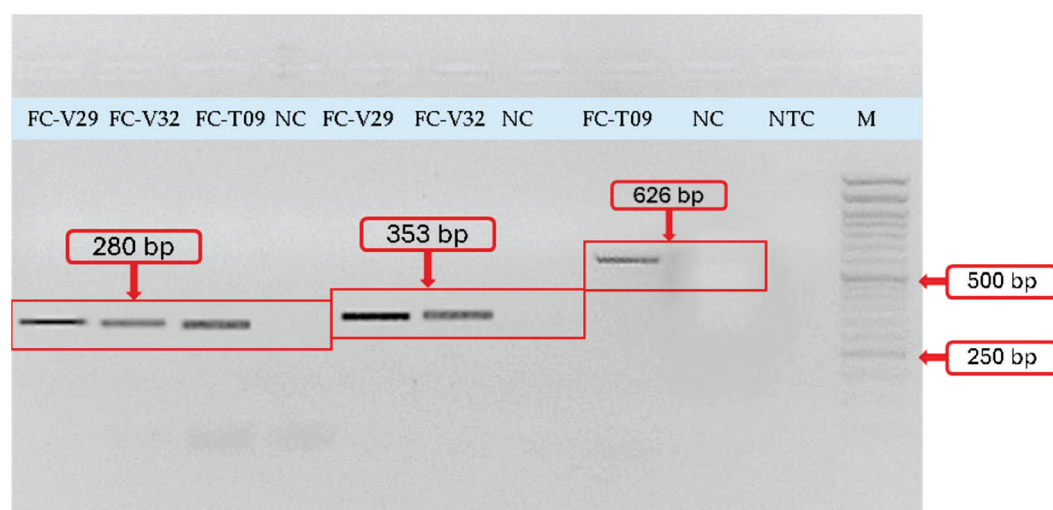


Figure 4. Agarose gel electrophoresis showing amplification of fluoroquinolone- and OXA-type β -lactamase-associated genes in isolates from Saker Falcons (*Falco cherrug*). Lane M: 50 bp DNA Ladder RTU (Simply). Lanes FC-V29, FC-V32, FC-T09: *blaOXA-61* amplicon (~280 bp). Lanes FC-V29, FC-V32: *blaOXA-51* amplicons (~353 bp). Lane FC-T09: amplification of the *FQgyrA* fragment with an expected size of 626 bp. Lane NC: internal negative control (pansusceptible isolate DNA), showing no amplification. Lane NTC: non-template control, confirming absence of reagent contamination. Fragment sizes were determined relative to the 50 bp ladder.

The integration of phenotypic and genotypic data revealed that the observed resistance profiles were consistent with the presence of the identified resistance determinants. Table 3 summarizes these findings for the most representative isolates, highlighting the complex resistome of Gram-negative bacteria carried by captive Saker Falcons. Notably, multi-resistant isolates such as *Hafnia alvei* and *Escherichia hermannii* harbored multiple gene clusters, including *blaZ*, *ampC*, and *tetK*, which explains their resistance to penicillins, cephalosporins, and tetracyclines.

Table 3. Phenotypic and genotypic antimicrobial resistance profiles of bacterial isolates from *Falco cherrug*.

Bacterial Isolate	Sample ID	Phenotype			Gene Detected
		Resistance (R)	Susceptibility (S)	Intermediate (I)	
<i>Hafnia alvei</i>	FC-V01	AMP, AMC, CEX, CFL, TET	TIC/CLA, CFP, CTF, CFQ, IPM, GEN, NEO, FLU, ENR, MAR, SXT	-	<i>tetK</i> <i>blaZ</i> <i>ampC</i>
<i>Ps. fluorescens</i>	FC-V29	TIC/CLA	-	IPM	<i>blaOXA-61</i> <i>blaOXA-51</i>
<i>Ps. fluorescens</i>	FC-V32	TIC/CLA	-	IPM	<i>blaOXA-61</i> <i>blaOXA-51</i>
<i>E. hermannii</i>	FC-T09	AMP, AMC, CEX, FLU, ENR, MAR, TET, SXT	CFL, CFP, CTF, CFQ, IPM, GEN, NEO	TIC/CLA	<i>tetK</i> <i>blaZ</i> <i>ampC</i> <i>blaOXA-61 gyrA</i>
<i>E. coli</i>	FC-V17	-	AMP, AMC, TIC/CLA, CEX, CFP, CTF, CFQ, IPM, GEN, NEO, FLU, ENR, MAR, TET, SXT	CFL	<i>blaZ</i> , <i>ampC</i>
<i>E. coli</i>	FC-T21	-	AMP, AMC, TIC/CLA, CEX, CFP, CTF, CFQ, IPM, GEN, NEO, FLU, ENR, MAR, TET, SXT	CFL	<i>blaZ</i> <i>ampC</i>
<i>E. coli</i>	FC-T34	-	AMP, AMC, TIC/CLA, CEX, CFP, CTF, CFQ, IPM, GEN, NEO, FLU, ENR, MAR, TET, SXT	CFL	<i>tetK</i> <i>blaZ</i> <i>ampC</i>
<i>E. coli</i>	FC-T33	-	AMP, TIC/CLA, CEX, IPM, GEN, SXT	-	-

Note: AMP: Ampicillin; AMC: Amoxicillin/Clavulanic acid; TIC/CLA: Ticarcillin/Clavulanic acid; CEX: Cephalexin; CFL: Cephalothin; CFP: Cefoperazone; CTF: Ceftiofur; CFQ: Cefquinome; IPM: Imipenem; GEN: Gentamicin; NEO: Neomycin; FLU: Flumequine; ENR: Enrofloxacin; MAR: Marbofloxacin; TET: Tetracycline; SXT: Sulfamethoxazole/Trimethoprim.

3. Discussion

The present study highlights the phenotypic and genotypic AMR profiles of Gram-negative bacteria isolated from the endangered Saker Falcon (*Falco cherrug*) in Western Romania. To our knowledge, this is one of the first investigations targeting this specific raptor population in the region, contributing valuable data to the limited body of literature regarding the microbiota of captive falcons in Eastern Europe. The recovery of AMR isolates from these sentinel birds underscores the growing concern of environmental pollution with resistance determinants, highlighting the relevance of raptors within the One Health framework.

3.1. Bacterial Prevalence and Spectrum

Our findings confirm the presence of *Enterobacteriaceae* in the gastrointestinal tract of clinically healthy captive Saker Falcons, particularly *Escherichia coli*, which exhibits multiple AMR profiles. The high prevalence of *E. coli* (60%, $n = 24/40$) found in our samples aligns with previous research on birds of prey, where this bacterium is frequently reported as a dominant component of the cloacal microbiota in both wild and captive individuals [27,28].

In a similar study conducted on captive falcons in Saudi Arabia and Egypt, Salah-Eldein et al. (2025) [29] reported an *E. coli* prevalence of 11.77% ($n = 4/34$) in fecal samples and 21.43% ($n = 6/28$) in oropharyngeal swabs. Similarly, Vidal et al. (2017) [30] identified *E. coli* as the most frequent isolate at 35% ($n = 160/457$) in diseased free-living raptors in Spain. The detection of these bacteria in our study, even in the absence of clinical symptoms, suggests that Saker Falcons may act as asymptomatic reservoirs.

The detection of MDR profiles of *Hafnia alvei* and *Escherichia hermannii* is noteworthy, as these isolates exhibited resistance to at least three antimicrobial categories. This finding suggests that captive Saker Falcons may harbor Gram-negative bacteria with increased resilience to multiple antimicrobial classes in Western Romania. Similar MDR profiles have been documented in other wildlife species from the same region, such as fallow deer [31], supporting the broader relevance of wildlife-associated antimicrobial resistance in this area. However, the present study was not designed to investigate transmission routes or the dissemination of resistance genes between domestic and wild animal populations, and such interpretations should therefore be made with caution.

The detection of multiple Gram-negative taxa in our study, such as *Hafnia alvei* (10%, $n = 4/40$) and *Pantoea* spp. (10%, $n = 4/40$), further indicates that the cultivable bacterial population recovered from the fecal material was taxonomically heterogeneous. This observation is consistent with the expected diversity of enteric bacteria associated with carnivorous birds, whose microbiota may include opportunistic or potentially pathogenic taxa without necessarily being associated with clinical disease [32].

3.2. Phenotypic Antimicrobial Resistance

The phenotypic resistance patterns observed in this study provide baseline data on resistant Gram-negative isolates recovered from captive Saker Falcons in Romania. Our findings of a 20% ($n = 2/10$) resistance rate to penicillins and the same to tetracycline among *Enterobacteriaceae* are in line with broader European reports showing that these antibiotic classes are among those most frequently associated with resistance in wild bird isolates [33].

A notable finding was the 10% ($n = 1/10$) resistance to quinolones. As fluoroquinolones are considered “critically important antimicrobials” and are frequently used in avian medicine, the presence of this phenotype is relevant from both veterinary and public health perspectives [29,34]. However, because no data on previous antimicrobial treatments or specific exposure sources were available, the origin of this resistance could not be determined in the present study. Compared to diseased raptors in Spain, where resistance to amoxicillin was observed at higher levels [30], the isolates examined in our study showed lower resistance frequencies, although AMR traits remained detectable within the tested subset. The high sensitivity to imipenem (90%, $n = 9/10$) suggests that carbapenem-resistant *Enterobacteriaceae* (CRE) were not prominent among the isolates tested in this specific falcon population, unlike in other European wild bird species [28].

3.3. Genotypic Characterization

The genotypic analysis in this study focused on a specific set of markers to elucidate the mechanisms underlying the observed resistance in Saker Falcon isolates. We investigated the presence of *tetK* (tetracycline resistance), *blaZ* (β -lactamase associated with penicillin resistance), *ampC* (cephalosporinase), *blaOXA-61*, *blaOXA-51* (class D β -lactamases), and the *gyrA* region associated with fluoroquinolone resistance.

Although *tetK* and *blaZ* are more commonly reported in Gram-positive bacteria, these genes have also been identified in environmental and wildlife-associated Gram-negative isolates, likely through horizontal gene transfer. Their inclusion in the present study aimed

to explore potential cross-species dissemination of resistance determinants in a mixed ecological environment, such as captive wildlife facilities [35].

The investigation of the *blaZ* and *ampC* genes is particularly relevant given the 20% phenotypic resistance to ampicillin observed in our *Enterobacteriaceae* isolates. While *blaZ* is classically associated with staphylococcal penicillin resistance, its screening in the context of raptor microbiota is crucial, as these birds often harbor a mix of Gram-positive and Gram-negative bacteria, facilitating potential horizontal gene transfer [29,34]. The focus on *ampC* and Class D β -lactamases (*blaOXA-61* and *blaOXA-51*) reflects a proactive approach to extended-spectrum resistance monitoring. Although these OXA-type carbapenems are more frequently reported in *Acinetobacter* or *Campylobacter* species, their surveillance in apex predators like the Saker Falcon is relevant for monitoring the occurrence of such determinants in wildlife-associated bacterial isolates [28,33].

Regarding tetracycline resistance, we targeted the *tetK* gene, an efflux pump mechanism. In European wild bird populations, tetracycline resistance is often mediated by various *tet* genes, and the detection of such markers in the Western Romanian population aligns with the high prevalence of tetracycline resistance reported in broader European systematic reviews [33].

The detection of the resistance gene in isolate FC-T34, which exhibited only intermediate susceptibility in the phenotypic AST, highlights a known genotype–phenotype discordance frequently reported in antimicrobial resistance studies. The presence of resistance determinants does not necessarily translate into a fully resistant phenotype, as gene expression may be modulated by regulatory elements, transcriptional activity, or environmental conditions influencing gene activation. Moreover, certain resistance genes may remain weakly expressed or require additional chromosomal mutations to achieve clinically relevant levels of resistance. Similar observations have been discussed in detail by Martínez et al. (2015) [36] in their analysis of environmental resistomes, by Munita and Arias (2016) [37] in their review of AMR mechanisms, and by Van Belkum and Dunne (2013) [38] in the context of genotype–phenotype discrepancies in antimicrobial susceptibility testing.

A key finding of our study is the detection of the *gyrA* region in the *E. hermannii* isolate, which is consistent with its phenotypic resistance to flumequine, enrofloxacin, and marbofloxacin. This confirms that fluoroquinolone resistance in these raptors is likely mediated by chromosomal mutations in the QRDR [30]. Further sequencing of the QRDRs is necessary to confirm the presence of specific mutations associated with fluoroquinolone resistance.

3.4. Ecological and Conservation Implications

The Saker Falcon is an endangered species with a fragile population status in Romania and the broader Pan-Pannonian region. The health status of these birds is crucial for conservation efforts. Fântână et al. (2025) [39] emphasized the importance of monitoring parameters for the remaining breeding pairs in Southern Romania. Infectious diseases and the carriage of MDR bacteria represent cryptic threats that could compromise rehabilitation success or breeding programs. As noted by Giacobello et al. (2016) [28], wildlife rescue centers and captive breeding facilities must implement strict hygiene protocols to prevent the amplification of resistant strains. Although the present study did not include parallel sampling from humans, domestic animals, or environmental matrices, the detection of resistant Gram-negative bacteria in captive raptors remains relevant to the One Health perspective, as these birds occupy an interface between wildlife, husbandry-associated environments, and human handling. In this context, our findings should be interpreted

as baseline wildlife-associated AMR data rather than as direct evidence of transmission across sectors.

3.5. Limitations and Future Directions

This study has limitations, primarily the restriction to a single facility, which may not fully reflect the AMR status of wild Saker Falcon populations in Romania. However, given the species' rarity, these data provide a valuable regional baseline. Future research should aim to compare captive and wild populations and should incorporate broader molecular approaches, including metagenomic analysis, the investigation of mobile genetic elements, and amplicon sequencing, in order to better understand the dissemination of AMR at the human–wildlife interface. Furthermore, our findings highlight the value of routine microbiological surveillance in falconry facilities, serving not only as a diagnostic tool but also as a proactive management strategy. Integrating health screening with genomic profiling in captive breeding programs may help improve the evaluation of individuals intended for reinforcement or falconry, while also supporting monitoring for potential pathogen transmission [32,40,41]. Such a multidisciplinary approach may help maintain captive populations in good health and genetic robustness, and reduce the risk of introducing resistant pathogens into the wild during reintroduction programs or the movement of birds.

4. Materials and Methods

4.1. Sampling

Freshly voided fecal droppings were collected non-invasively from 40 Saker Falcons housed in a single falconry facility, positioned near Timișoara, Romania. Sampling was performed using Copan® sterile transport tubes with eSwab LQ Amies (urethral flocked applicator, customized for MLS; eSwab 1 mL minitip progressive FLOQSwab BP80 MLS) (Copan Diagnostics, Brescia, Italy). Samples were transported under refrigerated conditions (4 °C) and processed within 24 h of collection. All falcons included in this study were clinically healthy at the time of sampling. The study was therefore designed as an isolate-based analysis, and the bacterial isolate was considered the analytical unit.

4.2. Bacterial Isolation and Culture Conditions

For bacterial isolation, the collected fecal material was inoculated onto both Violet Red Bile Glucose (VRBG) agar (Oxoid, Basingstoke, UK) and Tryptone Bile X-Glucuronide (TBX) agar (Oxoid, Basingstoke, UK). These media are recommended for selective isolation of *Enterobacteriaceae* and *Escherichia coli*, respectively, according to ISO standards (no. 16649-2:2001 and no. 21528-2:2017) [42,43]. Plates were incubated aerobically at 37 °C for 24 h. After incubation, multiple representative colonies showing distinct morphologies were selected to recover phenotypically distinct Gram-negative isolates for further characterization. A total of 40 bacterial isolates were successfully recovered and stabilized: 16 isolates from TBX plates and 24 isolates from VRBG plates. The isolates were codified based on the selective media used for recovery: those labelled with the prefix 'V' (e.g., FC-V01) originated from VRBG agar, while those labelled with 'T' (e.g., FC-T05) were recovered from TBX agar.

4.3. Bacterial Identification

Isolates were identified using the VITEK® 2 automated system (bioMérieux, Marcy-l'Étoile, France), performed with VITEK® 2 GN ID cards (REF 21341), designed for the identification of *Enterobacteriaceae* and selected non-fermenting Gram-negative organisms. Individual identification reports were generated for each of the 40 isolates. The

VITEK® 2 system is widely used in both clinical and veterinary microbiology, providing rapid and standardized results.

4.4. Antimicrobial Susceptibility Testing

AST was performed using the VITEK® 2 AST system (bioMérieux, Marcy-l'Étoile, France). From the total collection of Gram-negative isolates, a representative subset of 12 isolates was selected for AST in order to capture phenotypic and taxonomic diversity observed on primary culture plates while avoiding redundant testing of colonies with similar morphology and identification profiles. This subset-based approach was intended to provide baseline information on resistance profiles among distinct cultivable isolates and not to estimate isolate-level prevalence across the entire collection. Each isolate was tested using VITEK® 2 AST-GN96 cards (REF 421849), validated for Gram-negative bacteria of veterinary relevance, according to the manufacturer's instructions. MDR was defined as resistance to at least three antimicrobial classes. Interpretation of susceptibility profiles was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [26,44].

4.5. Molecular Confirmation by PCR

Molecular confirmation of phenotypically detected antimicrobial resistance was performed by conventional PCR targeting selected resistance determinants in isolates exhibiting reduced susceptibility in the VITEK® 2 antimicrobial susceptibility testing. Genomic DNA was extracted from overnight bacterial cultures by thermal lysis. Briefly, a single colony was suspended in 1 mL sterile nuclease-free water and centrifuged at $12,000 \times g$ for 5 min. The pellet was resuspended in 200 µL nuclease-free water, incubated at 95 °C for 10 min to ensure complete cell lysis, and centrifuged again at $12,000 \times g$ for 5 min. The supernatant containing genomic DNA was collected and stored at −20 °C until further use.

DNA concentration and purity were assessed using a NanoDrop™ 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only DNA samples with A260/A280 ratios between 1.8 and 2.0 and concentrations ≥ 25 ng/µL were used for amplification. PCR assays were performed to confirm the presence of resistance genes corresponding to the observed phenotypic profiles.

The following genes were investigated: *tetK* (tetracycline resistance), *blaZ* (β-lactamase associated with penicillin resistance), *ampC* (cephalosporinase), *blaOXA-61* and *blaOXA-51* (class D β-lactamases), and *gyrA* (fluoroquinolone resistance-associated region).

Amplification reactions were carried out in a final volume of 25 µL containing 12.5 µL GoTaq® Green Master Mix (Promega, Madison, WI, USA), 1 µL of each primer (20 pmol), 1 µL template DNA (~25–50 ng), and nuclease-free water to volume. PCR amplification was performed using an Eppendorf Mastercycler® Pro S (Eppendorf, Hamburg, Germany) under the following cycling conditions: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at gene-specific temperatures (Table 4) for 30 s, and extension at 72 °C for 45 s; followed by a final extension at 72 °C for 5 min.

Each PCR run included an internal negative control consisting of DNA extracted from an isolate phenotypically susceptible to all tested antibiotics. PCR screening was restricted to isolates showing phenotypic resistance or reduced susceptibility to the corresponding antimicrobial classes and was therefore intended as targeted molecular confirmation rather than comprehensive resistome profiling.

PCR products were resolved by electrophoresis on 1.5% agarose gels prepared in 1× TAE buffer and stained with ethidium bromide. Electrophoresis was conducted at 90–100 V for approximately 60 min. A 50 bp DNA Ladder RTU (Simply, Cat. No. SD012-R500) was used as a molecular size marker, enabling precise sizing of amplicons ranging from

150 to 700 bp. Amplified fragments were visualized under UV illumination using a Gel Doc™ XR imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Data acquisition and precise band size determination, relative to the molecular weight marker, were performed using Quantity One® 1-D Analysis Software (version 4.6.5). Primer sequences, annealing temperatures, and expected amplicon sizes are presented in Table 4. The negative control for resistance genes consisted of DNA extracted from a bacterial isolate identified by the VITEK® 2 automated system as pan-susceptible to all antimicrobial agents.

Table 4. Primer sequences used for PCR amplification of AMR genes in Gram-negative isolates from *Falco cherrug*.

Target Gene	Primer	Primer Sequence (5'–3')	Amplicon Size (bp)	Annealing Temperature	Reference
<i>tetK</i>	<i>tetK</i> F	GTAGCGACAATAGGTAATAGT	360	49 °C	[45]
	<i>tetK</i> R	GTAGTGACAATAAACCTCCTA			
<i>blaZ</i>	<i>blaZ</i> F	ACTTCAACACCTGCTGCTTTC	173	50 °C	[45]
	<i>blaZ</i> R	TGACCACTTTTATCAGCAACC			
<i>ampC</i>	<i>ampC</i> F	ATGATGAAAAAATCGTTATGC	334	50 °C	[46]
	<i>ampC</i> R	TTGCAGCTTTTCAAGAATGC			
<i>blaOXA-61</i>	<i>blaOXA-61</i> F	GAGTATAATACAAGCGGCAC	280	56 °C	[47]
	<i>blaOXA-61</i> R	CCAATTCCTTCTTGCCACTTC			
<i>blaOXA-51</i>	<i>blaOXA-51</i> F	TAATGCTTTGATCGGCCTTG	353	60 °C	[48]
	<i>blaOXA-51</i> R	TGGATTGCACTTCATCTTGG			
<i>FQgyrA</i>	<i>FQgyrA</i> F	ACGTACTGTCGGTAACAGTG	626	55 °C	[49]
	<i>FQgyrA</i> R	TTAATGATTGCCGCCGTCGG			

5. Conclusions

In conclusion, Saker Falcons in Romania harbored Gram-negative bacteria with phenotypic and selected genotypic traits of antimicrobial resistance, including resistance to antimicrobial classes relevant to both human and veterinary medicine. Although derived from a single-facility baseline investigation, these findings could be a primary source from Romania that supports further investigation of raptors extending the studies of wildlife-associated AMR.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antibiotics15040400/s1>, Table S1: Identification and origin of the 40 bacterial isolates analyzed by the VITEK® 2 automated system; Figure S1: Uncropped Figure 3; Figure S2: Uncropped Figure 4.

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Abbreviations

The following abbreviations are used in this manuscript:

AMC	Amoxicillin–clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial Resistance
AST	Antimicrobial Susceptibility Testing
bp	Base pairs
CEX	Cefalexin
CFL	Cefalotin
CFP	Cefoperazone
CFQ	Cefquinome
CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem-resistant <i>Enterobacteriaceae</i>
CTF	Ceftiofur
DNA	Deoxyribonucleic Acid
ENR	Enrofloxacin
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FLU	Flumequine
GEN	Gentamicin
I	Intermediate (susceptibility)
IE	Insufficient Evidence (that species is a good target for therapy)
IPM	Imipenem
MAR	Marbofloxacin
MDR	Multidrug-Resistant
NEO	Neomycin
PCR	Polymerase Chain Reaction
R	Resistant
S	Susceptible
SXT	Trimethoprim–sulfamethoxazole
TAE	Tris-Acetate-EDTA (buffer)
TBX	Tryptone Bile X-glucuronide (agar)
TET	Tetracycline
TIC/CLA	Ticarcillin–clavulanic acid
TRM	Therapeutic Resistance Monitoring
UV	Ultraviolet
V	Volts
VRBG	Violet Red Bile Glucose (agar)

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