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# Association Between Biofilm Phenotype and Antimicrobial Resistance in Foodborne *Listeria monocytogenes* from Northern Kazakhstan

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**Abstract**

**Background/Objectives:** *Listeria monocytogenes* is a major foodborne pathogen of public health concern because of the severe outcomes associated with invasive listeriosis, its ability to persist throughout the food chain, and its capacity to form biofilms. In this study, the occurrence, antimicrobial resistance, antimicrobial resistance genes, and biofilm-forming capacity of *L. monocytogenes* from food of animal origin in Northern Kazakhstan were investigated, and the association between the biofilm phenotype and antimicrobial resistance was also assessed. **Methods:** A total of 1561 samples were analyzed. *L. monocytogenes* was isolated and identified according to ISO 11290-1 and confirmed by MALDI–TOF MS. Antimicrobial susceptibility was determined by disk diffusion according to EUCAST recommendations. Resistance genes were detected by PCR, and biofilm formation was assessed using a crystal violet assay. **Results:** *L. monocytogenes* was detected in 34 (2.18%) samples. Resistance to at least one antimicrobial agent was observed in 18 (52.9%) isolates, with the highest resistance rate observed for trimethoprim–sulfamethoxazole (14/34, 41.2%). Multidrug resistance was detected in 8 (23.5%) isolates and was defined as resistance to at least three antimicrobial classes, including β-lactams, macrolides, and sulfonamides. The most frequently detected resistance genes were *msrA* (26.5%) and *mefA* (20.6%). All isolates formed biofilms, with 44.1% classified as strong producers and 55.9% as moderate producers. Strong biofilm formation was significantly associated with antimicrobial resistance (OR = 4.71, $p = 0.045$). **Conclusions:** *L. monocytogenes* was detected in 34 (2.18%) animal-origin food samples from Northern Kazakhstan. The isolates demonstrated antimicrobial resistance and biofilm-forming capacity, with a significant association between strong biofilm formation and antimicrobial resistance. These findings highlight the importance of continued microbiological surveillance of *L. monocytogenes* in animal-origin foods.

**Keywords:** *Listeria monocytogenes*; antimicrobial resistance; multidrug resistance; biofilm formation; antimicrobial resistance genes; food safety; animal-derived food products

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## 1. Introduction

*Listeria monocytogenes* is among the most important foodborne bacterial pathogens because of its ability to cause invasive listeriosis, a severe disease associated with high hospitalization rates. Mortality reaches 20–30% among invasive cases, particularly among pregnant women, newborns, older adults, and immunocompromised individuals [1,2]. The public health burden of this pathogen remains substantial: 2993 confirmed listeriosis cases were reported in the European Union in 2023, representing the highest annual number recorded since the establishment of EU-wide surveillance [3].

The ability of *L. monocytogenes* to persist throughout the food chain is closely linked to its exceptional physiological adaptability. This includes survival over a wide range of temperatures (0–45 °C), pH values (4.3–9.6), and salt concentrations (up to 10% NaCl), enabling the long-term contamination of foods and food-processing environments [4,5]. These traits have made *L. monocytogenes* a priority pathogen in food safety research and a key focus of the One Health approach, which promotes the integrated management of foodborne pathogens across the human, animal, and environmental sectors [6,7]. In addition to this environmental persistence, antimicrobial resistance (AMR) has become an increasing concern. Although β-lactam antibiotics, particularly ampicillin combined with aminoglycosides, remain the first-line treatment for invasive listeriosis [8], resistance to tetracyclines, macrolides, and fluoroquinolones has been reported worldwide, particularly among foodborne isolates [9–11]. At the molecular level, this resistance is associated with genes such as *tetM*, *tetS*, *ermB*, *lde*, and *mdrL*, which confer resistance through efflux and target-modification mechanisms. These genes are frequently carried on mobile genetic elements, including *Tn916*-like transposons and plasmids, which facilitate horizontal dissemination [12–15]. Combined with the extensive use of antimicrobials in food-producing animals, these mechanisms contribute to the emergence of multidrug-resistant (MDR) strains—defined as strains resistant to at least three antimicrobial classes—which may persist within the food chain and compromise treatment efficacy [16,17].

This resistance is further compounded by the biofilm-forming ability of *L. monocytogenes*. Animal-derived foods, particularly meat, fish, and dairy products, are major vehicles for transmission to humans and for food chain contamination [18]. The persistence of this pathogen in food-processing environments is largely sustained by biofilm formation on food-contact surfaces. Biofilms enhance bacterial tolerance to disinfectants and antimicrobial agents, promoting long-term survival and recurrent contamination [19,20]. Biofilm formation is influenced by environmental conditions and bacterial physiological adaptation, and its regulation involves efflux and stress-response mechanisms [21]. In *L. monocytogenes*, the stress-response regulator SigB and the virulence regulator PrfA have been shown to contribute to biofilm formation under specific environmental conditions [22,23].

The combined ability of *L. monocytogenes* to persist through biofilm formation and develop antimicrobial resistance highlights the importance of region-specific surveillance. Because the distribution, resistance patterns, and genetic characteristics of *L. monocytogenes* vary considerably across regions and food production systems, continuous regional surveillance is essential for understanding global epidemiology and strengthening international monitoring programs, such as the WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) [24]. In Kazakhstan, however, published studies have focused mainly on the microbiological safety of foods and the epizootiological surveillance of listeriosis, while the phenotypic resistance, resistance genes, and biofilm-forming capacity of foodborne *L. monocytogenes* isolates remain largely uncharacterized [25,26]. In contrast, comparable investigations have already been conducted in neighboring countries such as China and Iran [27–30], underscoring the need for similar evidence from Kazakhstan and the broader Central Asian region, where the relationship between AMR and biofilm formation in animal-derived foods remains poorly understood.

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Therefore, this study aimed to determine the occurrence, phenotypic AMR, antimicrobial resistance genes (ARGs), and biofilm-forming capacity of *L. monocytogenes* isolated from foods of animal origin in Northern Kazakhstan and to assess the association between biofilm-forming capacity and phenotypic AMR.

## 2. Results

### *2.1. Occurrence of L. monocytogenes in Foods of Animal Origin*

A total of 1561 food samples of animal origin were analyzed between January 2024 and February 2026. *L. monocytogenes* was isolated from 34 samples, corresponding to an overall positivity rate of 2.18% (34/1561) (Table 1).

**Table 1.** Distribution of *L. monocytogenes* isolates by source of isolation.

| Source of Isolation | No. of Samples | No. of Isolates | Isolation Rate (%) |
|---|---|---|---|
| Raw milk (cow and mare) | 600 | 4 | 0.67% |
| Dairy products (sour cream, butter, cheese, cottage cheese, etc.) | 120 | 0 | 0% |
| Red meat (pork, beef, horse meat, mutton, and minced meat) | 220 | 7 | 3.18% |
| Frozen semifinished products (dumplings, cutlets, etc.) | 66 | 2 | 3.03% |
| Processed meat products (sausages, frankfurters, etc.) | 67 | 0 | 0% |
| Poultry meat (chicken, turkey, and duck) | 346 | 14 | 4.05% |
| Chicken eggs | 142 | 7 | 4.93% |
| Total | 1561 | 34 | 2.18% |

The detection rate varied among food categories. *L. monocytogenes* was detected in 4/600 (0.67%) raw milk samples, 0/120 (0%) dairy product samples, 7/220 (3.18%) red meat samples, 2/66 (3.03%) frozen semifinished product samples, 0/67 (0%) processed meat product samples, 14/346 (4.05%) poultry meat samples, and 7/142 (4.93%) chicken egg samples. The highest detection rates were observed in chicken eggs (4.93%) and poultry meat (4.05%), whereas no isolates were recovered from dairy products or processed meat products.

### *2.2. Antimicrobial Susceptibility and Resistance Profiles of L. monocytogenes Isolates*

The antimicrobial susceptibility profiles of the 34 *L. monocytogenes* isolates are presented in Figure 1. All isolates were susceptible to meropenem (100%). Phenotypic resistance to at least one antimicrobial agent was detected in 52.9% (18/34) of the isolates, whereas 47.1% (16/34) were susceptible to all antibiotics tested.

The highest resistance rate was observed for trimethoprim/sulfamethoxazole, to which 41.2% (14/34) of the isolates were resistant. Resistance to ampicillin and benzylpenicillin was observed in 35.3% (12/34) of the isolates, with resistance to both agents present in 32.4% (11/34) of the isolates. Erythromycin resistance was identified in 29.4% (10/34) of the isolates.

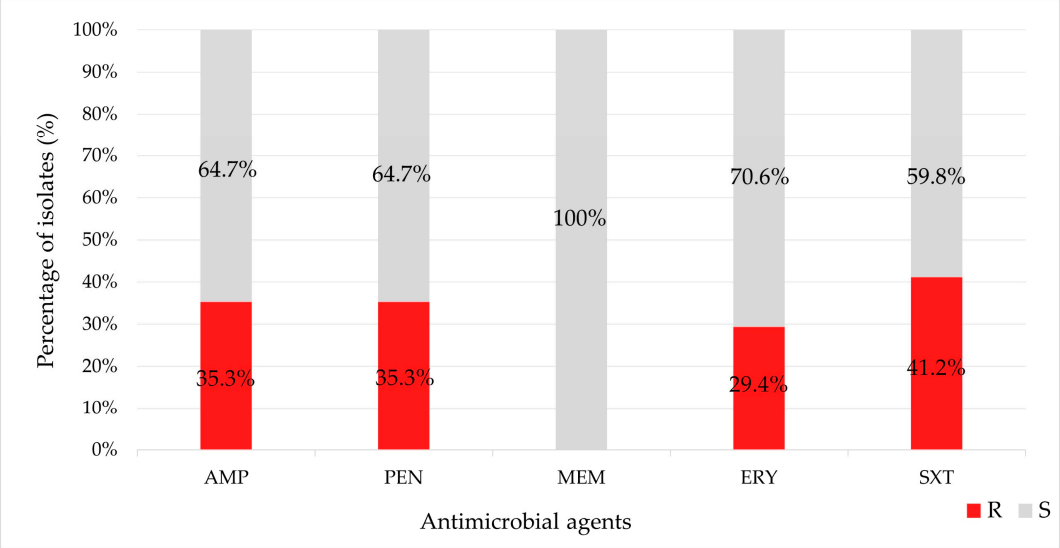


**Figure 1.** Distribution of susceptible and resistant *L. monocytogenes* isolates across the tested antimicrobial agents. AMP, ampicillin; PEN, benzylpenicillin; MEM, meropenem; ERY, erythromycin; SXT, trimethoprim/sulfamethoxazole. R, resistant isolates; S, susceptible isolates. The values are presented as percentages of the total number of isolates tested.

Several phenotypic AMR profiles were identified (Table 2). The predominant profile was AMP–PEN–ERY–SXT, representing combined resistance to β-lactam antibiotics, macrolides, and trimethoprim/sulfamethoxazole, which was identified in 23.5% (8/34) of the isolates. This profile was found exclusively among isolates recovered from red meat (beef, horse meat, and mutton) and raw milk, where it accounted for 71.4% (5/7) and 75.0% (3/4) of the isolates, respectively. No isolates with this profile were recovered from poultry meat, chicken eggs, or frozen semifinished products.

**Table 2.** Phenotypic AMR profiles of *L. monocytogenes* isolates according to the source of isolation.

| Isolate ID | Source | Resistance Patterns |
|---|---|---|
| LM01 | Frozen dumplings | ND |
| LM02 | Broiler chicken carcass | ND |
| LM03 | Broiler chicken carcass | ND |
| LM04 | Broiler chicken drumstick | ND |
| LM05 | Broiler chicken breast | ND |
| LM06 | Frozen dumplings | ND |
| LM07 | Ground turkey | ND |
| LM08 | Broiler chicken fillet | SXT |
| LM09 | Broiler chicken fillet | ND |
| LM10 | Broiler chicken breast | SXT |
| LM11 | Broiler chicken fillet | PEN–SXT |
| LM12 | Broiler chicken breast | AMP–SXT |
| LM13 | Horse meat | AMP–PEN–ERY–SXT |
| LM14 | Horse meat | AMP–PEN |
| LM15 | Horse meat | AMP–PEN–ERY–SXT |
| LM16 | Beef | AMP–PEN–ERY–SXT |
| LM17 | Beef | AMP–PEN–ERY–SXT |
| LM18 | Beef | AMP–PEN–ERY–SXT |
| LM19 | Broiler chicken drumstick | AMP–PEN–ERY |
| LM20 | Chicken egg | ND |
| LM21 | Chicken egg | ERY |

| | | |
|---|---|---|
| LM22 | Chicken egg | ND |
| LM23 | Chicken egg | ND |
| LM24 | Chicken egg | ND |
| LM25 | Chicken egg | ND |
| LM26 | Chicken egg | ND |
| LM27 | Raw cow milk | ND |
| LM28 | Raw cow milk | AMP–PEN–ERY–SXT |
| LM29 | Raw cow milk | AMP–PEN–ERY–SXT |
| LM30 | Raw cow milk | AMP–PEN–ERY–SXT |
| LM31 | Broiler chicken fillet | ND |
| LM32 | Broiler chicken fillet | SXT |
| LM33 | Broiler chicken drumstick | AMP–PEN–SXT |
| LM34 | Mutton | ERY |

Notes: LM01–LM34 represent individual *L. monocytogenes* isolates. The resistance patterns indicate the antimicrobial agents to which each isolate was resistant. AMP, ampicillin; PEN, benzylpenicillin; MEM, meropenem; ERY, erythromycin; SXT, trimethoprim/sulfamethoxazole; ND, no resistance detected.

Phenotypic AMR profiles varied according to the source of isolation. Among the poultry meat isolates, 50.0% (7/14) were susceptible to all antimicrobial agents tested. Among the resistant isolates, exclusive resistance to trimethoprim/sulfamethoxazole was the most common phenotype, accounting for 21.4% (3/14). Combined resistance phenotypes were observed in 28.6% (4/14) of the isolates and included the PEN–SXT, AMP–SXT, AMP–PEN–ERY, and AMP–PEN–SXT profiles.

MDR was detected in 8/34 (23.5%) isolates: LM13, LM15, LM16, LM17, LM18, LM28, LM29, and LM30. All MDR isolates were resistant to ampicillin, benzylpenicillin, erythromycin, and trimethoprim/sulfamethoxazole, corresponding to resistance to three antimicrobial classes: β-lactams, macrolides, and sulfonamides. Five MDR isolates were recovered from red meat and three from raw cow milk, whereas no MDR isolates were identified among poultry meat, chicken eggs, or frozen semifinished products.

### *2.3. ARGs in L. monocytogenes Isolates*

Comparisons of genotypic and phenotypic resistance profiles revealed variable concordance among the resistance genes investigated (Table 3).

**Table 3.** Genotypic–phenotypic resistance profiles and sources of *L. monocytogenes* isolates.

| Resistance Gene | Gene Class | Gene-Positive Isolates, *n* | Source of Isolation, *n* | Phenotypic Resistance Profile |
|---|---|---|---|---|
| *tetA* | Tetracycline | 3 | Poultry meat (2); frozen dumplings (1) | S (2); PEN–SXT (1) |
| *tetM* | Tetracycline | 2 | Poultry meat (2) | S (1); PEN–SXT (1) |
| *mefA* | Macrolide | 7 | Poultry meat (5); frozen dumplings (1); chicken eggs (1) | S (4); SXT (1); PEN–SXT (1); AMP–SXT (1) |
| *msrA* | Macrolide | 9 | Poultry meat (2); red meat (4); raw cow's milk (3) | SXT (1); AMP–PEN (1); AMP–PEN–ERY–SXT (6); AMP–PEN–ERY (1) |
| *ermB* | Macrolide | 1 | Red meat (1) | AMP–PEN–ERY–SXT (1) |

Notes: Values indicate the number of gene-positive isolates with the indicated phenotypic resistance profile. S, susceptible to all tested antibiotics; AMP, ampicillin; PEN, benzylpenicillin; ERY, erythromycin; SXT, trimethoprim/sulfamethoxazole.

The remaining ARGs included in the PCR screening (*tetB*, *tetK*, *tetL*, *ermA*, *strA*, *aadA*, *sul1*, *sul2*, and *dfrD*) were not detected in any of the *L. monocytogenes* isolates. None of the *mefA*-positive isolates exhibited phenotypic resistance to erythromycin, whereas erythromycin resistance was observed in 7 of the 9 *msrA*-positive isolates and in the single *ermB*-positive isolate. The *tetA* and *tetM* genes were detected in isolates with different phenotypic resistance profiles for the antimicrobial agents tested in this study. Conversely, several isolates exhibiting phenotypic resistance to β-lactams, macrolides, or trimethoprim/sulfamethoxazole did not carry any of the ARGs investigated.

Among the MDR isolates, *msrA* was the predominant resistance gene, with six of the nine *msrA*-positive isolates (66.7%) exhibiting an MDR phenotype. These isolates originated predominantly from red meat and raw cow milk and were characterized by the combined resistance profiles AMP–PEN–ERY–SXT and AMP–PEN–ERY.

### *2.4. Biofilm-Forming Capacity of L. monocytogenes Isolates*

All 34 *L. monocytogenes* isolates examined were capable of biofilm formation, with isolates distributed between strong and moderate biofilm-producing categories according to the classification proposed by Stepanović et al. [31] (Table 4). No weak or non-biofilm-producing isolates were identified.

**Table 4.** Distribution of *L. monocytogenes* isolates according to biofilm-forming ability.

| Biofilm-Forming Category | No. of Isolates (*n*) | Percentage (%) | Mean $OD_{620}$ ± SD |
|---|---|---|---|
| Strong biofilm producers | 15 | 44.1 | 0.433 ± 0.088 |
| Moderate biofilm producers | 19 | 55.9 | 0.272 ± 0.048 |
| Weak biofilm producers | 0 | 0.0 | ND |
| Nonbiofilm producers | 0 | 0.0 | ND |

Notes: Biofilm-forming ability was classified as follows: OD ≤ ODc, no biofilm formation; ODc < OD ≤ 2ODc, weak biofilm formation; 2ODc < OD ≤ 4ODc, moderate biofilm formation; OD > 4ODc, strong biofilm formation. The cutoff optical density was ODc = 0.084. The $OD_{620}$ values are presented as the mean ± SD. ND, not detected.

The distribution of biofilm-forming intensity varied by source of isolation (Figure 2). Among the isolates from red meat (n = 7), 57.1% (4/7) were classified as moderate biofilm producers, whereas 42.9% (3/7) were strong biofilm producers. A similar pattern was observed among the isolates from poultry meat (n = 14), with 64.3% (9/14) classified as moderate and 35.7% (5/14) as strong biofilm producers. In contrast, isolates from raw cow milk were predominantly strong biofilm producers (75.0%; 3/4), whereas only 25.0% (1/4) were classified as moderate biofilm producers.

Among the isolates recovered from chicken eggs (n = 7), strong biofilm producers also predominated, accounting for 57.1% (4/7) of the isolates, whereas 42.9% (3/7) were classified as moderate biofilm producers. All isolates recovered from frozen dumplings (n = 2) were classified as moderate biofilm producers (100%; 2/2). Despite the observed differences in the distribution of biofilm-forming phenotypes, no statistically significant association was detected between the source of isolation and biofilm-forming intensity (Fisher's exact test, $p = 0.483$).

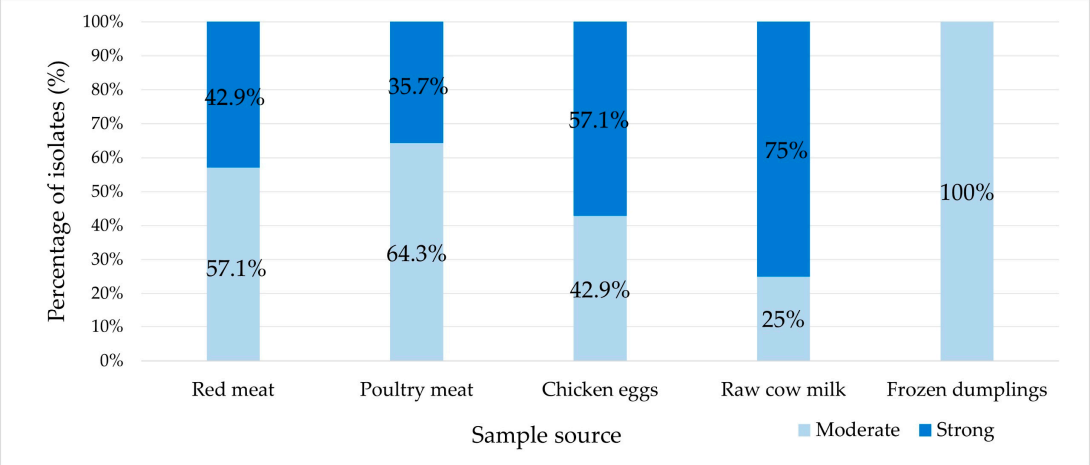


**Figure 2.** Distribution of biofilm-forming intensity among *L. monocytogenes* isolates according to the source of isolation.

### *2.5. Association Between AMR and the Biofilm-Forming Capacity of L. monocytogenes Isolates*

A greater proportion of AMR isolates was observed among strong biofilm producers (73.3%; 11/15) than among moderate biofilm producers (36.8%; 7/19) (Table 5).

**Table 5.** Association between AMR and biofilm-forming ability in *L. monocytogenes* isolates.

| Biofilm Category | AMR, *n* (%) | Non-AMR, *n* (%) | Total | OR (95% CI) | *p*-Value |
|---|---|---|---|---|---|
| Strong | 11 (73.3) | 4 (26.7) | 15 | 4.71 (1.08–20.63) | 0.045 * |
| Moderate | 7 (36.8) | 12 (63.2) | 19 | – | – |
| Total | 18 (52.9) | 16 (47.1) | 34 | – | – |

Notes: AMR, antimicrobial resistance; OR, odds ratio; CI, confidence interval. * statistically significant at $p < 0.05$ (Fisher's exact test).

Statistical analysis revealed a significant association between biofilm-forming capacity and AMR. Strong biofilm producers were 4.71 times more likely to exhibit an AMR phenotype than moderate biofilm producers were (OR = 4.71, 95% CI: 1.08–20.63; two-tailed Fisher's exact test, $p = 0.045$). To further elucidate the relationships among the biofilm-forming capacity, phenotypic AMR, MDR status, and distribution of ARGs, a comparative analysis of all *L. monocytogenes* isolates was performed (Figure 3).

A higher proportion of AMR isolates was observed among strong biofilm producers, whereas phenotypically susceptible isolates predominated among moderate biofilm producers. Nevertheless, both AMR and susceptible isolates were represented in each biofilm category. ARGs were detected in both biofilm categories, with no apparent association between ARG distribution and biofilm-forming capacity. The efflux-associated genes *msrA* and *mefA* were the most frequently detected determinants, whereas *tetA*, *tetM*, and *ermB* occurred only sporadically. MDR phenotypes were observed in both strong and moderate biofilm producers.

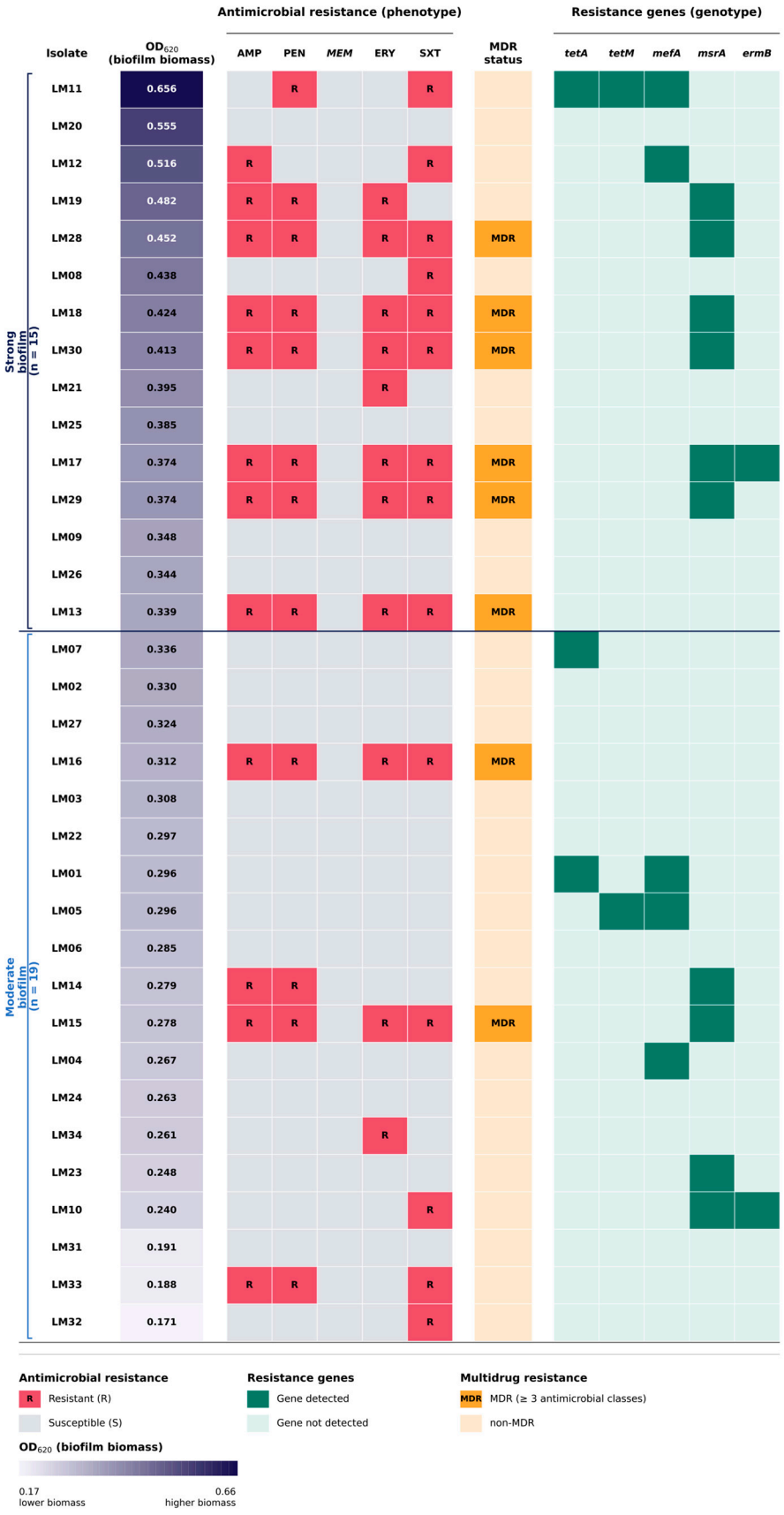


**Figure 3.** Integrated visualization of the relationships among biofilm-forming capacity, phenotypic AMR, and the distribution of ARGs in *L. monocytogenes* isolates. Isolates were ordered according to their biofilm biomass on the basis of their $OD_{620}$ values. The left panel shows the biofilm phenotype (strong or moderate) and biofilm biomass ($OD_{620}$). The middle panel presents the phenotypic

susceptibility profiles for ampicillin (AMP), benzylpenicillin (PEN), meropenem (MEM), erythromycin (ERY), and trimethoprim/sulfamethoxazole (SXT), together with the MDR status. The right panel illustrates the distribution of the ARGs *tetA*, *tetM*, *mefA*, *msrA*, and *ermB* detected among the studied isolates.

## 3. Discussion

Although *L. monocytogenes* was detected at a relatively low frequency in the analyzed food products in the present study (2.18%; 34/1561), the phenotypic characteristics of the recovered isolates suggest a potential public health concern. The coexistence of AMR and biofilm-forming capacity may increase environmental persistence and facilitate survival during food processing, underscoring the importance of continuous monitoring and effective control strategies.

The occurrence of *L. monocytogenes* in poultry-derived products in the present study was 4.05% (14/346) in poultry meat and 4.93% (7/142) in chicken eggs. By comparison, reported positivity values in the literature are 8.32% in meat products in South Korea, 7.1% in livestock and poultry meat in China, and 8.3% in the EU [32,33], while the prevalence of *L. monocytogenes* on eggshells has been reported to range from 1.8% to 6.0% [34]. These findings align with the variability in *L. monocytogenes* contamination reported across food products in different countries [35]. Differences in contamination levels among poultry-derived products are likely influenced by production systems, hygienic conditions, food processing practices, and food safety management [36]. In Taiwan, *L. monocytogenes* was detected in 11.4% of postevisceration chicken carcass rinse samples, while 16.7% positivity was reported in raw materials in a Spanish pork-processing industry [37,38]. Intensive poultry production, cross-contamination during slaughter and processing, and the psychrotrophic nature of *L. monocytogenes*—which enables survival under refrigeration—may further contribute to its occurrence in poultry and other meat products [37,39,40]. Such differences may also be related to variations in food matrices, sampling strategies, processing stages, and the persistence of *L. monocytogenes* in food-processing environments [41].

Conversely, the occurrence of *L. monocytogenes* in raw cow's milk in the present study was 0.67% (4/600), whereas no positive samples were detected among processed dairy products or sausages. This percentage was lower than the 7.69% positivity reported in raw milk samples from South Africa [42]. Global evidence also indicates substantial variation in *L. monocytogenes* occurrence across milk and dairy product supply chains [43]. The lower occurrence observed in the present study may be associated with differences in milking hygiene, environmental exposure at the farm level, and asymptomatic carriage among dairy animals [44]. These factors may contribute to the contamination of raw milk at the primary production stage, whereas subsequent processing and hygienic control may reduce the occurrence of *L. monocytogenes* in processed products [43,44].

Phenotypic antimicrobial susceptibility testing revealed that more than half of the *L. monocytogenes* isolates were resistant to at least one antimicrobial agent, indicating the presence of antimicrobial-resistant strains among the analyzed food isolates. All isolates remained susceptible to meropenem, which is consistent with the intrinsically high susceptibility of *L. monocytogenes* to carbapenems reported previously [45,46]. Resistance to trimethoprim/sulfamethoxazole was most frequently observed, followed by resistance to ampicillin, benzylpenicillin, and erythromycin. The predominance of resistance to these antimicrobial agents is consistent with findings reported for food-derived *L. monocytogenes* in other countries [47–49]. However, antimicrobial susceptibility profiles vary across regions, likely reflecting differences in antimicrobial use in food-producing animals, national antimicrobial stewardship policies, and the circulation of distinct *L. monocytogenes* lineages. Because ampicillin remains a first-line treatment for invasive listeriosis, the

detection of ampicillin- and benzylpenicillin-resistant isolates raises concerns about clinically relevant resistance and supports continued surveillance of antimicrobial resistance in food-derived *L. monocytogenes* [50].

MDR was observed predominantly among isolates recovered from beef, horse meat, and raw cow's milk, whereas poultry-derived isolates exhibited comparatively low MDR frequencies, which aligns with previous reports describing source-dependent differences in antimicrobial resistance among foodborne *L. monocytogenes* isolates [51,52]. These differences most likely reflect variations in antimicrobial exposure, animal husbandry practices, veterinary antimicrobial stewardship, surveillance programs, and the circulation of distinct *L. monocytogenes* lineages across production systems [53,54].

Molecular analysis revealed that nearly half of the *L. monocytogenes* isolates carried at least one antimicrobial resistance gene, with the efflux-associated genes *msrA* and *mefA* predominating. Similar distributions have been reported for food-derived *L. monocytogenes*, suggesting that antimicrobial efflux is a major adaptive mechanism enabling survival during antimicrobial exposure [45,55]. In contrast, genes associated with ribosomal protection and target modification were detected only sporadically, indicating that these mechanisms made a limited contribution to the resistance profiles observed in the present study.

The distribution of resistance determinants also varied among food sources. The *msrA* gene was more frequently detected in isolates from beef, horse meat, and raw cow's milk. Although the limited number of isolates precludes definitive conclusions, this pattern may reflect differences in antimicrobial selection pressure and the circulation of distinct *L. monocytogenes* lineages across production systems [56].

The relationship between genotypic resistance and phenotypic resistance was not completely concordant. Several resistance genes were detected in phenotypically susceptible isolates, whereas some phenotypically resistant isolates lacked the resistance determinants included in the present screening panel. Similar discrepancies have been reported previously and may reflect differences in gene expression, regulatory mechanisms, chromosomal mutations, or resistance determinants not included in the current screening panel [45,57]. These findings indicate that phenotypic susceptibility testing and molecular characterization provide complementary information and should be interpreted together when AMR in *L. monocytogenes* is being assessed.

All *L. monocytogenes* isolates demonstrated the ability to form biofilms, with moderate and strong biofilm producers predominating. Similar findings have been widely reported for food-derived *L. monocytogenes*, indicating that biofilm formation is a conserved survival strategy enabling persistence under food-processing conditions. The extracellular polymeric matrix protects embedded cells from disinfectants and environmental stresses while promoting surface attachment and persistence on food-contact materials, thereby facilitating the recurrent contamination of processing environments [58,59]. These findings support the implementation of sanitation strategies specifically targeting biofilm-associated *L. monocytogenes* in food-processing facilities.

A significant association was observed between biofilm-forming capacity and phenotypic antimicrobial resistance, with strong biofilm producers being more likely to exhibit an AMR phenotype than moderate producers. Similar associations have been reported for *L. monocytogenes* and other foodborne pathogens, suggesting that enhanced biofilm formation and AMR may represent complementary adaptive responses that increase bacterial persistence under environmental and antimicrobial stress, rather than reflecting a direct causal relationship [60,61]. Although MDR occurred more frequently among strong biofilm producers, this association was not statistically significant, most likely because of the limited sample size. Similarly, no clear relationship was observed between individual resistance genes and biofilm-forming capacity, indicating that biofilm

formation is regulated by complex networks involving stress responses, quorum sensing, virulence-associated factors, and global transcriptional regulators, rather than individual resistance determinants alone [58]. Similar observations have been reported previously, supporting the role of these regulatory networks in biofilm development [62].

Several limitations should be acknowledged. The relatively small number of isolates, limited geographical coverage, and targeted screening of a restricted set of antimicrobial resistance genes may have resulted in an underestimation of the diversity of resistance mechanisms among food-derived *L. monocytogenes*. In addition, sequence typing (ST) and serotyping were not performed in the present study, limiting the assessment of the molecular epidemiological diversity and serotype distribution of the isolates.

An additional limitation of the present study is that the virulence potential and clonal structure of the *L. monocytogenes* isolates were not investigated. Virulence in *L. monocytogenes* is a multifactorial trait, and the presence of individual virulence-associated genes alone does not allow reliable classification of isolates according to their virulence level. Current approaches distinguish several hypervirulence-associated clonal complexes, including CC1, CC2, CC4, and CC6, from predominantly food-associated hypovirulent clones such as CC9 and CC121 on the basis of population-genetic, epidemiological, genomic, and experimental evidence. Other geographically relevant clones, including the hypervirulence-associated CC87 reported particularly in Asia, further illustrate the diversity of virulence-associated lineages. Comprehensive characterization of the present isolate collection would therefore require molecular typing to determine STs/CCs together with analysis of virulence-associated genomic determinants, including LIPI-1, LIPI-3, LIPI-4, internalin genes, and *inlA* integrity; WGS would provide the most comprehensive framework for such an analysis. Importantly, genomic virulence profiles represent predictions of virulence potential and do not necessarily correspond directly to functional virulence, which may require complementary cell-based or in vivo assays. Such integrated characterization was beyond the predefined objectives of the present study, which focused on antimicrobial resistance, resistance-associated genes, biofilm-forming capacity, and their relationship. Nevertheless, we consider comprehensive characterization of virulence potential and population structure an important next step and plan to investigate these characteristics in this *L. monocytogenes* collection in a dedicated follow-up study.

Future studies should include larger isolate collections from broader geographical areas and incorporate serotyping, multilocus sequence typing (MLST), and whole-genome sequencing to provide a more comprehensive characterization of antimicrobial resistance, virulence determinants, molecular epidemiology, and persistence-associated traits, including resistance to disinfectants.

## 4. Materials and Methods

### *4.1. Study Design*

This study was designed as a descriptive observational study to characterize *L. monocytogenes* isolates recovered from animal-derived food products in the Kostanay Region, Northern Kazakhstan. The study was conducted from January 2024 to February 2026 and included samples collected at different stages of the food supply chain, including livestock farms, wholesale distribution centers, and retail outlets. This study involved the collection of animal-derived food products, the isolation and identification of *L. monocytogenes*, and the subsequent assessment of antimicrobial susceptibility, biofilm-forming capacity, and resistance-associated genetic determinants.

### *4.2. Sample Collection*

The *L. monocytogenes* isolates included in this study were recovered between January 2024 and February 2026 in the Kostanay Region, Kazakhstan. The isolates were obtained from animal-derived food products, including both raw materials and ready-to-eat products, collected at different stages of the food supply chain, including farms, wholesale distribution centers, and retail outlets.

A total of 1561 samples were examined, comprising 600 samples of raw cow's milk, 120 dairy products, 220 meat samples, 66 frozen semifinished products, 67 sausage products, 346 poultry meat samples, and 142 chicken egg samples.

Sample collection, transportation, and storage were performed according to ISO/TS 17728:2015 [63]. Samples were collected aseptically in sterile containers, transported at 4 ± 2 °C, and delivered to the laboratory within 24 h of collection.

### *4.3. Isolation of L. monocytogenes*

*L. monocytogenes* was isolated from animal-derived food products using a culture-based method according to ISO 11290-1:2017 [64].

Primary and secondary enrichment were performed in Half Fraser Broth and Fraser Broth (Condalab, Madrid, Spain), with sequential incubation at 30 ± 1 °C for 24 ± 2 h, followed by incubation at 37 ± 1 °C for an additional 24 ± 2 h.

Following enrichment, culture aliquots were streaked onto CHROMagar™ Listeria and CHROMagar™ Identification Listeria (CHROMagar, Paris, France) and incubated at 37 ± 1 °C for 24–48 h. Presumptive *L. monocytogenes* colonies appeared blue on CHROMagar™ Listeria and pink with an opaque halo on CHROMagar™ Identification Listeria.

### *4.4. Identification of L. monocytogenes*

Final species identification was performed using MALDI–TOF MS on a MALDI Biotyper Sirius System IVD (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). The acquired mass spectra were compared against the manufacturer's reference database, and isolates with a log(score) value ≥ 2.0 were considered reliably identified at the species level.

### *4.5. Antimicrobial Susceptibility Testing*

The antimicrobial susceptibility of *L. monocytogenes* isolates was determined using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (HiMedia Laboratories Pvt. Ltd., Mumbai, India) supplemented with 5% defibrinated horse blood and β-NAD (Servicebio, Wuhan, China; 20 mg/L), in accordance with the recommendations of the EUCAST [65].

The bacterial inoculum was adjusted to a 0.5 McFarland standard (approximately $1.5 \times 10^8$ CFU/mL) by measuring the turbidity of the bacterial suspension using a DEN-1 densitometer (SIA BIOSAN, Riga, Latvia) and adjusting the suspension until the 0.5 McFarland value was reached. Following the inoculation and placement of antimicrobial disks (HiMedia Laboratories Pvt. Ltd., Mumbai, India), the plates were incubated at 35–37 °C for 18–24 h, after which the diameters of the growth inhibition zones were measured (mm).

The antimicrobial panel included ampicillin, benzylpenicillin, meropenem, erythromycin, and trimethoprim/sulfamethoxazole. These agents were selected because EUCAST version 16.0 provides applicable disk diffusion breakpoints for their interpretation in *L. monocytogenes*. The susceptibility results were interpreted as susceptible (S) or resistant (R) according to the EUCAST breakpoints [65] using the zone diameter breakpoints provided in Table 6.

**Table 6.** EUCAST zone diameter breakpoints used for antimicrobial susceptibility interpretation of *L. monocytogenes*.

| Antibiotic | Disk Content | S (≥mm) | R (<mm) |
|---|---|---|---|
| Benzylpenicillin | 1 IU | 13 | 13 |
| Ampicillin | 2 μg | 16 | 16 |
| Meropenem | 10 μg | 26 | 26 |
| Erythromycin | 15 μg | 25 | 25 |
| Trimethoprim/sulfamethoxazole | 1.25/23.75 μg | 29 | 29 |

MDR was defined according to the international criteria proposed by Magiorakos et al. [66]. Isolates were classified as MDR when they exhibited resistance to at least one antimicrobial agent in three or more antimicrobial classes.

### *4.6. PCR Detection of ARGs*

Genomic DNA was extracted using the AmpliSens DNA-sorb-B DNA Extraction Kit (Central Research Institute of Epidemiology, Moscow, Russia) according to the manufacturer® instructions. DNA concentration and purity were determined spectrophotometrically using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA samples were stored at −20 °C until further analysis.

To identify antimicrobial resistance (AMR) determinants in *L. monocytogenes*, real-time PCR assays were performed to detect genes associated with resistance to tetracyclines (*tetA*, *tetB*, *tetK*, t*etL*, and *tetM*), macrolides (*ermA*, *ermB*, *mefA*, and *msrA*), aminoglycosides (*strA* and *aadA*), sulfonamides (*sul1* and *sul2*), and trimethoprim (*dfrD*).

Genomic DNA was extracted using the AmpliSens DNA-sorb-B DNA Extraction Kit (Central Research Institute of Epidemiology, Moscow, Russia) according to the manufacturer® instructions. Real-time PCR amplification was performed using a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Each 25 μL reaction mixture consisted of 12.5 μL of BioMaster UDG HS-qPCR Lo-ROX SYBR (2×) (Biolabmix, Novosibirsk, Russia), 2.5 μL each of the forward and reverse primers (10 pmol each; National Center for Biotechnology, Astana, Kazakhstan), 5 μL of template DNA, and 2.5 μL of nuclease-free water. The PCR cycling conditions were as follows: 50 °C for 2 min, 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, annealing at the gene-specific temperature (Table 7) for 10 s, and 72 °C for 20 s. A melting curve analysis (65–95 °C) was performed after amplification to verify product specificity.

**Table 7.** Primers used for PCR detection of ARGs in *L. monocytogenes* isolates.

| Gene | Primer Sequence (5′–3′) | Amplicon Size (bp) | Annealing Temperature (°C) | Reference |
|---|---|---|---|---|
| *tetA* | F: GCTACATCCTGCTTGCCTTC<br>R: CATAGATCGCCGTGAAGAGG | 210 | 55 | [67] |
| *tetB* | F: TTGGTTAGGGGCAAGTTTTG<br>R: GTAATGGGCCAATAACACCG | 659 | 55 | [67] |
| *tetK* | F: TCGATAGGAACAGCAGTA<br>R: CAGCAGATCCTACTCCTT | 169 | 55 | [67] |
| *tetL* | F: TCGTTAGCGTGCTGTCATTC<br>R: GTATCCCACCAATGTAGCCG | 267 | 55 | [67] |
| *tetM* | F: GTGGACAAAGGTACAACGAG<br>R: CGGTAAAGTTCGTCACACAC | 406 | 55 | [67] |
| *ermA* | F: CTTCGATAGTTTATTAATATTAGT<br>R: TCTAAAAAGCATGTAAAAGAA | 645 | 52 | [68] |

| | | | | |
|---|---|---|---|---|
| *ermB* | F: GAAAAGGTACTCAACCAAATA<br>R:AGTAACGGTACTTAAATTGTTTAC | 636 | 52 | [68] |
| *mefA* | F: AGTATCATTAATCACTAGTGC<br>R: TTCTTCTGGTACTAAAAGTGG | 345 | 52 | [67] |
| *msrA* | F:GCAAATGGTGTAGGTAAGACAACT<br>R: ATCATGTGATGTAAACAAAAT | 401 | 52 | [68] |
| *dfrD* | F: AGAGTAATCGGCAAGGATAACG<br>R: AATGGGCAATTTCACAATCC | 199 | 52 | [68] |
| *strA* | F: CTTGGTGATAACGGCAATTC<br>R: CCAATCGCAGATAGAAGGC | 348 | 50 | [67] |
| *aadA* | F: GTGGATGGCGGCCTGAAGCC<br>R: AATGCCCAGTCGGCAGCG | 525 | 50 | [67] |
| *sul1* | F: CGGCGTGGGCTACCTGAACG<br>R: GCCGATCGCGTGAAGTTCCG | 433 | 65 | [67] |
| *sul2* | F: GCGCTCAAGGCAGATGGCATT<br>R: GCGTTTGATACCGGCACCCGT | 293 | 65 | [67] |

### *4.7. Assessment of Biofilm-Forming Capacity*

The biofilm-forming capacity of *L. monocytogenes* isolates was assessed using a crystal violet microtiter plate assay. A bacterial suspension standardized to 0.5 McFarland (approximately $1.5 \times 10^8$ CFU/mL) was diluted 1:100 and inoculated into sterile 96-well polystyrene microtiter plates containing tryptic soy broth (TSB; Condalab, Madrid, Spain) supplemented with 1% glucose. The plates were incubated at 37 °C for 24 h. Sterile TSB without bacterial inoculum was used as the negative control.

Following incubation, the wells were gently washed with phosphate-buffered saline (PBS; HiMedia Laboratories Pvt. Ltd., Mumbai, India) to remove nonadherent cells. The remaining attached biofilms were fixed and stained with 0.1% crystal violet solution, after which the bound dye was eluted with 33% acetic acid. The optical density (OD) was measured at 620 nm using a Multiskan microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

All experiments were performed in triplicate. The biofilm-forming capacity of the isolates was classified according to the criteria proposed by Stepanović et al. [31]. The optical density cutoff value (ODc) was calculated using the following formula:

$$ODc = \overline{OD}_{negative\ control} + (3 \times SD_{negative\ control}), \quad (1)$$

where $\overline{OD}_{negative\ control}$—represents the mean optical density of the negative control and $SD_{negative\ control}$—represents the corresponding standard deviation.

Isolates were classified as nonbiofilm producers ($OD \leq ODc$), weak biofilm producers ($ODc < OD \leq 2 \times ODc$), moderate biofilm producers ($2 \times ODc < OD \leq 4 \times ODc$), or strong biofilm producers ($OD > 4 \times ODc$).

### *4.8. Statistical Analysis*

Statistical analyses were performed using jamovi software version 2.7.30 (The jamovi Project, Sydney, Australia). Categorical variables are expressed as absolute numbers (n) and percentages (%), whereas optical density ($OD_{620}$) values are presented as the mean ± standard deviation (mean ± SD). Fisher's exact test was used to compare categorical variables. The association between biofilm-forming capacity and the AMR phenotype was evaluated by calculating odds ratios (ORs) with corresponding 95% confidence intervals (95% CIs). Differences were considered to be statistically significant at $p < 0.05$.

## 5. Conclusions

This study provides evidence linking AMR and biofilm-forming capacity among foodborne *L. monocytogenes* isolates recovered from animal-derived foods in Northern Kazakhstan. The findings demonstrate that antimicrobial-resistant isolates with enhanced biofilm-forming capacity were present among the analyzed food samples, highlighting the relevance of these characteristics for food safety. The observed association between AMR and biofilm-forming capacity supports the importance of considering both phenotypic resistance and biofilm-forming ability in the microbiological monitoring of *L. monocytogenes* in animal-derived foods. These findings contribute to the understanding of AMR and the biofilm-associated characteristics of *L. monocytogenes* in food products from Northern Kazakhstan and emphasize the importance of integrated microbiological surveillance for food safety.

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## Abbreviations

| | |
|---|---|
| AMR | Antimicrobial Resistance |
| ARG | Antimicrobial Resistance Gene |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| MDR | Multidrug Resistance |

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