Original Article

Listeria Monocytogenes Isolated from Ready-To-at Food Products in Tehran: Prevalence and Antimicrobial Resistance

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ABSTRACT

L. monocytogenes is a significant foodborne pathogen that is associated with a range of clinical illnesses, from self-limited gastroenteritis to invasive infection, which can lead to hospitalization of immunocompromised individuals. In the present study, the incidence of L. monocytogenes in ready-to-eat (RTE) food samples from Tehran, Iran, was therefore measured. A total of 110 samples were collected from various ready-to-eat (RTE) foods in different zones of Tehran from April to September of 2022. The samples were obtained from various types of food, including Caesar salad, Olivier salad, burger, schnitzel, sushi, and sausage. The identification of isolates was facilitated by the detection of hlyA and prfA genes through a polymerase chain reaction (PCR) approach. The antimicrobial resistance profile of the isolates was assessed through the use of a disc diffusion assay and the PCR amplification of resistance genes. Among the 110 samples, 14 (12.7%) were identified as Listeria spp., and 6 (5.5%) were confirmed as L. monocytogenes by molecular methods. The prevalence of Listeria spp. was observed to be highest in schnitzel and burgers, with 30% of schnitzel samples and 25% of burger samples being positive. Among the 14 isolates, 6 samples (42%) were identified as L. monocytogenes. The highest rate of L. monocytogenes was observed in burgers, accounting for 20% of the total burger samples. In contrast, no L. monocytogenes was identified in Caesar salad, sausage, and sushi samples. The L. monocytogenes isolates demonstrated resistance to oxacillin, streptomycin, cotrimoxazole, clindamycin, and cefoxitin, and were susceptible to chloramphenicol. Furthermore, the isolates demonstrated intermediate susceptibility to fosfomycin and ampicillin. Furthermore, the isolates demonstrating resistance to erythromycin contained genes associated with resistance to the macrolide class of antibiotics, including ermA and ermB. However, the presence of cfxA and mecA genes was detected in a single isolate resistant to cefoxitin and oxacillin. The prevalence of these findings underscores significant concerns regarding the potential for listeriosis to pose a threat to consumers of ready-to-eat (RTE) food products.

Keywords: *listeria Monocytogenes*, Ready to eat, Antimicrobial Resistance, PCR Amplification, Virulence Gene.

1. Introduction

Listeria monocytogenes, a significant foodborne pathogenic bacterium, is primarily transmitted to humans via contaminated foodstuffs (1). Listeria monocytogenes is a facultative intracellular pathogen that causes self-limiting illness in immunocompetent hosts but is associated with severe disease conditions like septicemia, encephalitis, or abortion in immunocompromised hosts (2). According to the Centers for Disease Control and Prevention (CDC), approximately 1,600 cases of listeriosis are reported annually, resulting in around 260 fatalities. This bacterium commonly causes infections in susceptible populations, including pregnant women and their fetuses, young children, elderly individuals, and those with compromised immune systems. The significance of this bacterium lies in its capacity to thrive under harsh conditions, including elevated salt concentrations, broad pH ranges, low temperatures of 0-4 °C, and low water activity. The ability of Listeria to proliferate at refrigerator temperatures poses a significant concern regarding the safety of chilled ready-to-eat (RTE) food products. These foods are typically consumed without any prior heating. The significant consumer demand for RTE foods underscores the necessity for comprehensive information regarding their microbial quality (4). The conventional serotyping of Listeria using agglutination methods, a technique frequently employed in epidemiological studies, is encumbered by several limitations. These include the presence of non-typeable isolates and the substantial expense of antisera. Consequently, there has been a recent focus on expeditious genotyping of Listeria to surmount these challenges (5). These molecular techniques have been shown to be more precise, accurate, and sensitive in the initial characterization of L. monocytogenes. Furthermore, molecular methods have been shown to differentiate between virulent and non-virulent strains with respect to the pathogenesis of L. monocytogenes in humans (6). Conversely, the issue of antibiotic resistance in pathogenic bacteria is a significant global health concern, as these bacteria can be transmitted to humans through the consumption of animal products, thereby reducing the efficacy of available antibiotics (7). β-lactam antibiotics (e.g., penicillin or ampicillin) are effective antimicrobials against Gram-positive bacteria and are the optimal choice for the treatment of listeriosis, either as a standalone treatment or in combination with aminoglycosides (e.g., gentamicin). The second-line treatment consists of trimethoprim and sulfonamides (e.g., erythromycin and other antibiotics), which are effective treatments for pregnant women infected with L. monocytogenes. The pathogenicity of L. monocytogenes is identified by several virulence factors, including listeriolysin O (hlyA), an essential virulence factor causing listeriosis, and phosphatidyl-inositol-phospholipase C (plcA), which is involved in escaping from a vacuole after invading host cells. The degree of resistance varies and depends on the use of antibiotics in humans and animals, as well as on differences in geographic regions. Consequently, there is a necessity to investigate the antibiotic resistance of L. monocytogenes from foods and the environment in different areas, understand the resistance mechanisms, and take measures to prevent environmental spread (8). Despite the numerous investigations aimed at regulating the pervasiveness of Listeria spp., particularly L. monocytogenes, in various food products, there is a paucity of information regarding the presence of L. monocytogenes in ready-to-eat (RTE) foods (9). Consequently, the present study was conducted to assess the prevalence of L. monocytogenes in RTE foods in Tehran, Iran. In addition, molecular methods were employed to identify virulence genes in isolated strains, and the antibiotic resistance patterns of the isolates were elucidated.

2. Materials and Methods

2.1. Bacterial Strains

L. monocytogenes ATCC 35152 was derived from Iran, specifically from the Persian Type Culture Collection, which was utilized as a positive control in the conducted microbial tests. The standard strain was cultivated in Tryptic Soy Broth (TSB), a media developed by IBRISCO of Iran, and was incubated for 24 hours at 37°C in a shaking incubator set at 150 rotations per minute.

2.2. Samples Collection

A total of 110 samples were collected from various RTE foods in different zones of Tehran from April to September of 2022. The samples were obtained from various food items, including Caesar salad, Olivier salad, burger, schnitzel, sushi, and sausage. The samples were meticulously collected in sterile bags and stored in a refrigerated box set to 4°C. Subsequent to this, the samples were expedited to the microbial laboratory within 24 hours for further analysis.

2.3. Isolation and Listeria spp Identification

The standard method outlined in ISO 11290 was employed to detect Listeria spp. In this method, 25 grams of sample material were homogenized and transferred to 225 milliliters of half Fraser broth. This mixture contained half of the standard concentration of two supplementary materials, acriflavine hydrochloride and nalidixic acid, which were incubated for 24 hours at 30°C. In the subsequent step of selective enrichment, 100 µL of the culture was transferred to 10 mL of Fraser broth and incubated for 24 h at 37°C. The presence of Listeria spp. was indicated by the blackening of the medium due to the β-D-glucosidase activity of the bacteria. The incubation was carried out at 37°C for a duration of 24 hours. Following this, both primary and secondary cultures were streaked and plated onto PALCAM agar. Colonies manifesting as black were considered to be possible Listeria spp. and were streaked onto non-selective agar for further confirmation. The isolates were confirmed through the application of standard laboratory tests, including Gram staining, oxidase test, catalase reaction, motility test, MR/VP test, CAMP test, and B-hemolytic activity (6).

2.4. HlyA Gene-based Polymerase Chain Reaction (PCR)

Following two steps of enrichment in Fraser broth media, the total DNA content of whole bacterial cells was extracted in accordance with the manufacturer's protocol (Pouya gene Azma kit, Iran). Subsequently, the extracted DNA was subjected to HlyA gene-based PCR to ascertain the presence of L. monocytogenes. The PCR amplification was performed using a thermal cycler (Bio-Rad, USA) in a total volume of 25 µL. The PCR reaction mixture contained hlyA-R primers and 2 µL hlyA-F, along with 1 µL of template DNA (50 ng). The primer sequences are demonstrated in Table 1. The PCR conditions comprised an initial denaturation step of 94 °C, which lasted 5 min. Subsequently, 30 cycles of denaturation for 30 seconds at 98 °C were executed. The annealing temperature was subsequently adjusted for 30 seconds at 55 °C for HlyA and prfA, and between 53 and 56°C for mecA, ermA, ermB, mefA, FosA, FosB, FosC, and CfxA. The extension step was conducted for 15 seconds at 72 °C. The ultimate extension was conducted for 10 minutes at 72°C. The size of the PCR products was confirmed by 1% agarose gel electrophoresis (6,10) (Table 1).

2.5. Conventional Polymerase Chain Reaction

The pure and biochemically confirmed colonies of PALCAM agar were then subjected to a DNA extraction procedure. The PCR reaction was then performed to identify the virulence genes, following the previously described conditions. Two virulence genes, prfA and hlyA, were used to trace L. monocytogenes in food samples.

2.6. Antimicrobial Susceptibility of L. monocytogenes

The antibiotic susceptibility profile of six isolates of L. monocytogenes was evaluated using the disc-diffusion method, as outlined in the Clinical and Laboratory Standards Institute guidelines (CLSI 2021). The following discs were utilized in the study: Clindamycin (2 µg/disk), Ampicillin (10 µg/disk), Oxacillin (1 µg/disk), Fosfomycin (200 µg/disk), Chloramphenicol (30 µg/disk). Cefoxitin (30 µg/disk), Erythromycin (15 µg/disk), Cotrimoxazole (25 µg/disk), Streptomycin (10 µg/disk), and Tetracycline (30 µg/disk). The L. monocytogenes isolates were plated on Mueller-Hinton agar enriched with 5% defibrinated horse blood and were incubated at 35°C for 24 hours. The ability of the isolates to inhibit each antimicrobial disc was subsequently determined by measuring the inhibitory zone. Staphylococcus aureus ATCC 25923 and L. monocytogenes ATCC 35152 were utilized as control organisms to ensure the quality of the experiment (8).

2.7. Tracing Antimicrobial Resistance Genes by PCR

L. monocytogenes isolates were subjected to genome extraction, and the presence of macrolide resistance genes (mefA, ermB, and ermA), (cfxA) i.e., cefoxitin resistance gene, oxacillin resistance gene (mecA), and (fosA, fosB, and fosC), i.e., fosfomycin resistance genes, was investigated through PCR. The primer sets for detecting resistance genes were designed for the present research. The genomic DNA of the isolates was then subjected to PCR under conditions previously described (11).

2.8. Statistical Analysis

The statistical software SPSS (version 22) and Fisher's exact test were utilized at the 95% confidence level to compare the rate of Listeria spp. contamination among various categories of ready-to-eat (RTE) foods ($p < .05$).

3. Results

3.1. Isolation and Listeria spp Identification

A total of 110 samples were collected from six groups of ready-to-eat (RTE) food products. As shown in Table 2, 12.7% of the samples were identified as Listeria spp. The results indicated that the isolates of Listeria spp. were obtained from schnitzel, burgers, Olivier salad, and sushi. Of these food products, schnitzel and burgers exhibited the highest prevalence of Listeria spp., with 6 (42%) and 5 (35) of the 14 isolated Listeria spp., respectively. Consequently, the prevalence of Listeria spp. in schnitzel and burger samples was 30% and 25%, respectively (Table 2).

3.2. HlyA Gene-Based Polymerase Chain Reaction (PCR)

A total of 19 Fraser culture samples were found to be potentially contaminated with L. monocytogenes. Subsequently, the total bacterial load's DNA content was extracted and subjected to a PCR that targeted the HlyA gene. As illustrated in Figure 1, eight samples were found to be positive for the HlyA gene, thereby confirming their identification as L. monocytogenes (Figure 1).

3.3. Conventional Polymerase Chain Reaction (PCR)

Fourteen pure and biochemically confirmed colonies obtained from PALCAM agar were subjected to PCR analysis, of which six samples were found to be positive for the prfA and hlyA genes (see Figure 2). Six samples that were positive in the prfA and hlyA gene tests were identified by previous PCR tests of the hlyA gene on mixed and impure samples. Notably, two samples that were initially identified as positive for the hlyA gene by PCR and presumed to contain L. monocytogenes were found to lack prfA and hlyA genes, leading to their classification as Listeria spp.A molecular characterization of the 14 isolates revealed that 6 samples (42%) were identified as L. monocytogenes using molecular methods. The highest rate of L. monocytogenes was observed in burgers, with 66% of positive samples and 20% of total burger samples being positive. Schnitzel and Olivier Salad, with a rate of 5% of samples, were the second most prevalent. Conversely, Caesar salad, sausage, and sushi samples were found to be devoid of any identified L. monocytogenes (Figure 2).

3.4. L. monocytogenes Antimicrobial Susceptibility

The antibiotic susceptibility of the isolates was examined by means of a disc diffusion assay. The results (Table 3) demonstrate that all six isolates exhibited antibiotic resistance to oxacillin, streptomycin, cotrimoxazole, cefoxitin, and clindamycin. The majority of isolates (66%) demonstrated susceptibility to chloramphenicol (Table 3).

Table 1: List of primers used in this study.

Primer name	Primer sequence		Size of amplicon (bp)
hlyA	Forward	5'-AAATCATCGACGGCAACCT	489
	Reverse	5'-ATTTCGGATAAAGCGTGGTG	
prfA	Forward	5'- GATACAGAAACATCGGTTGGC	274
	Reverse	5'-GTGTAATCTTGATGCCATCAGG	
mecA	Forward	5'- GTGGAAAAGGCGAAGATGGC	199
	Reverse	5'- TCAAGTCCTGTTGCTCGTGA	
ermA	Forward	5'- GAGGTGTAATTTCGTAACTGCC	249
	Reverse	5'- TTAGCAAACCCGTATTCCAC	
ermB	Forward	5'- GCATTTAACGACGAAACTGGC	243
	Reverse	5'- ATGTCAGACGCATGGCTTTC	
mefA	Forward	5'- ACCCTATGCGGTCTTTGGA	210
	Reverse	5'- GCATTGAGAGCCGGAGAATG	
fosA	Forward	5'- ACTACACCCACTACGCGTTT	223
	Reverse	5'- GAGGTAAACACCATGTCCGC	
FosB	Forward	5'- CACAACGCCCATGGAGAGAA	273
	Reverse	5'- ACCAACGTGCTTTTGGCTTT	
FosC	Forward	5'- CCCTTTCAAAGCAGTGCCTT	171
	Reverse	5'- CGGGGCGATATTGTCACTTG	
cfxA	Forward	5'- TCTGCTATGTGGTGTATTGGC	201
	Reverse	5'- TAAAGACGGAGCTGGTTTGC	

Table 2: Prevalence of *L. monocytogenes* and *Listeria* spp. in RTE products in Tehran.

Type of food	No. of samples	No.of *Listeria* spp.	percent* of *Listeria* spp.%	percent** of *Listeria* spp.%	No. of *L. monocytogenes*	Percent* of *L. monocytogenes*
Caesar Salad	20	-	-		-	
Olivier Salad	20	2	10	14.28	1	5
Burger	20	5	25^{B}	35.7	4	20
Schnitzel	20	6	30^{A}	42.85	1	5
Sausage	20	-	-	-	-	-
Sushi* **	10	1	10^{C}	7.14	-	-
Total	110	14	12.7	100	6	5.5

Superscript letters represent statistically significant differences ($P < 0.05$) among results, based on Fisher's exact test.
* percent was calculated based on the" No of isolated bacteria / No of samples" in each type of food.
** percent was calculated based on the" No of isolated bacteria /14 (total isolated listeria spp.).
*** Sushi is produced and eaten less than other types of foods in Iran.

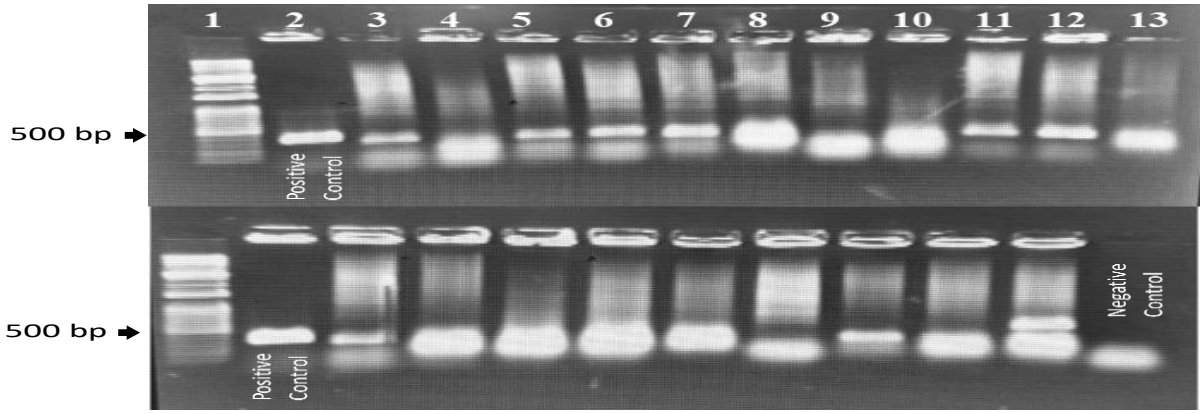

Figure 1: HlyA gene-based PCR. DNA molecular weight marker (100 bp).

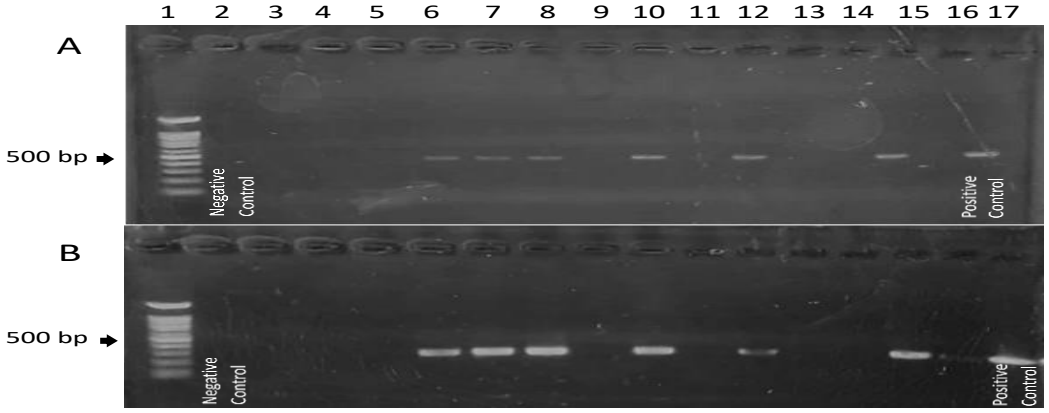

Figure 2: PCR of confirmed colonies obtained from PALCAM agar. A: HlyA gene-based PCR. B: PrfA gene-based PCR. DNA molecular weight marker (100 bp).

Table 3: *antimicrobial susceptibility* of *L. monocytogenes* isolated from RTE foods in Tehran.

Antimicrobial agents	Disc content (µg/disk)	Resistant (NO of isolates)	Intermediate (NO of isolates)	Susceptible (NO of isolates)
Tetracycline (TE)	30	4	0	2
Streptomycin (S)	10	6	0	0
Cotrimoxazole (SXT)	25	6	0	0
Erythromycin (E)	15	1	3	2
Chloramphenicol (C)	30	0	2	4
Cefoxitin (FOX)	30	6	0	0
Fosfomycin (FOS)	200	0	4	2
Oxacillin (OX)	1	6	0	0
Ampicilin (AM)	10	0	4	2
Clindamycin (CC)	2	6	0	0

3.5. Tracing Antimicrobial Resistance Genes by PCR

Among L. monocytogenes isolates that were confirmed with PCR, 33%, 16%, 16%, and 16% were positive for ermA, ermB, mecA, and cfxA, respectively. Conversely, none of the isolates were positive for mefA, fosA, fosB, and fosC (Table 4).

4. Discussion

Conventional culturing involves a two-step process: first, a selective pre-enrichment step, followed by an enrichment step and plating. The identification of suspected colonies is subsequently confirmed by conducting morphological and biochemical tests, a process that renders the method time-consuming and labor-intensive. Conversely, molecular-based methods, such as PCR, have emerged as a rapid, precise, and effective approach for tracing foodborne microorganisms (12). qPCR and PCR assays have been employed in the identification of L. monocytogenes. The selection of appropriate target DNA sequences affects the specificity and accuracy of PCR-based detection of L. monocytogenes.

Table 4: genes that were involved in antimicrobial resistance in *L. monocytogenes* isolates.

Food type	hlyA	prfA	mecA	mefA	ermA	ermB	fosA	fosB	fosC	cfxA
Schnitzel	+	+	-	-	+	-	-	-	-	-
Burger (No 5)	+	+	-	-	+	-	-	-	-	-
Burger (No 6)	+	+	-	-	-	+	-	-	-	-
Burger (No 8)	+	+	-	-	-	-	-	-	-	-
Olivier	+	+	-	-	-	-	-	-	-	-
Burger (No 13)	+	+	+	-	-	-	-	-	-	+
Positive control (L.monocytogenes)	+	+	+	-	-	-	-	-	-	-

The target genes employed in PCR tracing of L. monocytogenes include the hlyA gene, which encodes listeriolysin for the lysis of the host vacuolar membrane (13-15). This gene is crucial for the full virulence of L. monocytogenes (16). Another prevalent gene is prfA, which regulates the expression of six primary virulence genes (IAP, hemolysin gene, inLAB, Lm00733, ssrA, and ImaA) of bacteria (18). The most common target for the detection of polymerase chain reaction (PCR) is the hlyA gene, whose regions are specific to L. monocytogenes (19). However, it should be noted that some strains of serovar 4c are not equipped with the hlyA gene, which represents a limitation when utilizing this gene in PCR assays (20). In this study, we employed hlyA-based PCR in two distinct approaches. In the initial approach, the DNA of the unpurified bacterial load from the second enrichment round, suspected of containing Listeria spp., was analyzed. The second approach entailed conventional culturing to obtain a pure colony, followed by PCR-based detection of hlyA and prfA genes. A critical factor that restricts the direct tracing and evaluation of L. monocytogenes in food samples by PCR and qPCR is the presence of food matrices, which hinder PCR detection of foodborne pathogens (21). In addition, L. monocytogenes may have been present in low quantities in foods, thereby masking its detection. The subsequent multiplication during food storage could potentially generate sufficient bacterial numbers for disease initiation.Consequently, we opted to perform PCR on enriched media instead of food to obtain a more expeditious response compared to complete purification. The PCR of the hlyA gene was positive for eight samples.Subsequent to purification on PALCAM agar through biochemical tests and amplification of hlyA and prfA genes, it was also proved that L. monocytogenes existed in six of these samples.Two samples that were not confirmed by biochemical tests and subsequent PCR of pure colony DNA content were withdrawn from further tests. One potential explanation for this outcome is the potential loss of the identified isolate during the culturing process, which could be attributable to the low quantity of isolate in the sample. In a similar study, L. monocytogenes was isolated according to ISO 11290 from 200 ready-to-eat meat products from Gorgan, Iran in 2018. The isolates were confirmed through the application of both PCR and serotyping methodologies. The study found that 13% of the samples contained L. monocytogenes, with 5% of the samples being roast fish, 26% of the samples being roast chicken meat, and 8.33% of the samples being cooked beef. In this study, 16% of samples were identified as Listeria spp. (0% of Caesar Salad, 11% of Olivier Salad, 33.3% of Burgers, 35.2% of Schnitzel, 0% of Sausage, 16.6% of Sushi samples) and 7.1% of samples were identified as L. monocytogenes by molecular analysis (0% of Caesar Salad, 5.5% of Olivier Salad, 26.6% of Burgers, 5.8% of Schnitzel, 0% of Sausage, and 0% of Sushi samples). In a subsequent study, a total of 45 samples of fresh chicken and 45 samples of fresh beef were collected from Zanjan, Iran in 2018. Screening by hly-based PCR revealed a 53.33% contamination rate in beef and 46.67% in chicken meats with L. monocytogenes (23). It is noteworthy that certain food types, which do not favor the proliferation of L. monocytogenes, pose a minimal risk to consumers. These foods are characterized by low pH and/or low water activity, or they have undergone freezing and heating prior to consumption. Alternatively, foods that can reach temperatures high enough to inhibit the growth of L. monocytogenes are also considered safe (24). RTE foods, particularly those derived from meat and fish, frequently exhibit the highest prevalence of positive samples. The intrinsic high pH (pH 6–6.5) of cooked meat products renders them particularly vulnerable to L. monocytogenes proliferation. The active water content and the CO2 presence in the food package have been demonstrated to affect the growth capacity of cooked meat products (25). The study's findings further indicate that the highest rate of L. monocytogenes contamination was observed in burgers. Nemati et al. reported that isolates of L. monocytogenes from ready-to-eat (RTE) foods, including chicken meat, lamb, and fish, exhibited a predominant resistance to erythromycin, ampicillin, and penicillin. However, these isolates demonstrated a notable susceptibility to gentamicin and tetracycline (22). A subsequent study by Farhoumand et al. revealed that all isolates of L. monocytogenes from chicken and beef exhibited resistance to tetracycline, trimethoprim/sulfamethoxazole, gentamicin, and penicillin. Notably, some of the isolates exhibited susceptibility to carbenicillin and erythromycin (23). The L. monocytogenes isolates from this study demonstrated resistance to oxacillin, streptomycin, cotrimoxazole, cefoxitin, and clindamycin, while exhibiting susceptibility to chloramphenicol. Isolates demonstrated intermediate susceptibility to fosfomycin and ampicillin. The isolates demonstrating resistance to erythromycin contained genes associated with resistance to the macrolide class of antibiotics, including ermA and ermB. However, the cfxA and mecA genes were only detected in a single isolate exhibiting resistance to both cefoxitin and oxacillin. These genes were not identified in other isolates resistant to these antibiotics. Horizontal gene transfer between commensal microorganisms in food and L. monocytogenes may be a primary factor contributing to the escalating antimicrobial resistance observed in this bacterium. In addition to genetic mutation, the generation of antibiotic-resistant new strains is influenced by another factor. L. monocytogenes demonstrates a high degree of resistance to cold, heat, alkaline, acid, and osmotic stress conditions, which aid in its survival and proliferation. Such advantageous adaptations may, in turn, promote and facilitate antimicrobial resistance (26, 27).The impact of antimicrobials derived from natural sources, such as plants, algae, and microorganisms, on antibiotic-resistant strains, merits further investigation in future studies (28). L. monocytogenes infections have been associated with a wide range of clinical illnesses, from self-limited

gastroenteritis to invasive pathogenesis, which can lead to hospitalization of immunocompromised individuals. RTE foods represent a significant source of L. monocytogenes contamination, which can be mitigated through the implementation of hygienic design principles in food facility equipment, rigorous end-product verification testing, and the provision of clear labeling instructions for consumers. Contamination of ready-to-eat (RTE) products has been linked to undercooked foods, which pose severe and dangerous health risks to consumers of RTE products. Furthermore, the emergence of antibiotic resistance in L. monocytogenes isolates has the potential to contribute to severe health complications.

Acknowledgment
Not applicable

Authors' Contribution
Conceptualization: LG, RM. Sampling: RM. Methodology: LG, ZF. Validation: LG. Investigation: LG, RM. Writing original draft preparation: RM. Writing review and editing: LG. Supervision: LG, ZF.

Ethics
Not applicable

Conflict of Interest
The authors of this study have indicated that they do not have any competing interests.

Data Availability
Access to the aforementioned materials is permitted upon submission of a request to the corresponding author.

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