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Isolation and Characterization of *Listeria monocytogenes* in Selected Food Products

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Abstract: *Listeria monocytogenes* is a significant foodborne pathogen capable of causing severe illness with a high mortality rate, especially in vulnerable populations. Its ability to survive under adverse environmental conditions and contaminate a wide range of foods, including ready-to-eat products, makes it a major public health concern. In Sulaymaniyah and Halabja provinces, there is a lack of systematic data on the prevalence, virulence characteristics, and antimicrobial resistance of *L. monocytogenes* in locally consumed foods, which limits effective risk assessment and control strategies. This study aimed to determine the prevalence, virulence gene profiles, and antimicrobial resistance patterns of *L. monocytogenes* in selected dairy, vegetable, and meat products using cultural isolation, biochemical identification, and Polymers Chain Reaction based molecular confirmation. A total of 124 food samples were collected and tested, with molecular detection targeting prs, *lmo1030*, and 16S rRNA genes, and virulence profiling for *hlyA*, *prfA*, and *inlA*. Antimicrobial susceptibility was assessed against ten antibiotics using the disk diffusion method. Twelve samples (9.6%) were positive for *L. monocytogenes*, with the highest prevalence in traditional semi-hard cheese (40%), lettuce (25%), and celery (25%). The *prfA* and *inlA* genes were each detected in 41.6% of isolates, and *hlyA* in 33.3%. All isolates were resistant to ampicillin but remained susceptible to most other antibiotics. Thus, these findings provide essential baseline data that can guide targeted food safety interventions and strengthen public health protection measures in this region. Future studies should expand sampling to a wider range of food categories, include seasonal monitoring, and apply whole-genome sequencing to better understand the epidemiology and resistance mechanisms of *L. monocytogenes*.

1. Introduction

Listeria is a common foodborne bacterium that affects individuals with reduced immune function, including older adults, pregnant women, and newborns. *L. monocytogenes* is the most prevalent pathogen in the genus, despite the identification of other species with diverse origins in the last decade [1]. It is possible to isolate the common bacterium *L. monocytogenes* from both soil and water. They can also infiltrate plants and compete with the gut microbiota of herbivorous animals. It can be asymptomatically expelled into the environment through feces [2]. *Listeria monocytogenes* (*L. monocytogenes*) is the causative organism of listeriosis, a dangerous foodborne illness with a high death rate (20–30%) globally [3]. The genus *Listeria* comprises of 21 species. They are small, rod-shaped, and Gram-positive bacteria. Thus, only *Listeria ivanovii* and *L. monocytogenes* are recognized as mammalian pathogens [4]. Over 70 years ago, *L. monocytogenes* was identified as an animal pathogen [1].

A common Gram-positive, facultative anaerobic bacterium. *L. monocytogenes* is commonly found in natural settings is *L. monocytogenes*. Because of the presence of flagella, the bacterium is facultatively anaerobic and typically motile between 22°C and 28°C, but nonmotile above 30°C [5]. In 1981, it was identified as a human foodborne virus for the first time after a Canadian epidemic connected to tainted coleslaws.

Over 99 percent of human listeriosis samples are thought to be attributed to eating contaminated food, especially ready-to-eat items such as deli meat, dairy products, smoked salmon, and shellfish [6]. In susceptible people, especially those with low-level immune systems, the ingestion of tainted ready-to-eat foods may lead to listeriosis. This disease significantly affects food safety because, despite its relative rarity, these infections are associated with substantial mortality rates. Food producers that depend on these stressors for preservation find *L. monocytogenes* troublesome because they can persist in and withstand a variety of stress environments, including low pH and temperature [7]. A direct connection between the agricultural environment and human hosts is possible via the food chain. *L. monocytogenes* enters food production suppliers either through animal-derived raw materials (meat and milk) or through produce contaminated with the pathogen from feces, dirt, or from other sources due to inadequate hygiene management [8].

Although more species from a variety of origins have been identified in the past ten years, *L. monocytogenes* remains the genus's most common pathogen. Clarifying the current number of accessible species, which may include up to 21 species, is crucial because researchers have recently reported varying numbers of species for the genus. These included *L. thailandensis, L. costaricensis, L. goaensis, L. newyorkensis, L. booriae, L. riparia, L. grandensis, L. floridensis, L. cornellensis, L. aquatica, L. weihenstephanensis, L. fleischmannii, L. rocourtiae, L. marthii, L. grayi, L. ivanovii, L. welshimeri, L. seeligeri, L. innocua,* and *L. monocytogenes* [1]. *L. monocytogenes* is an important bacterium of the *Listeria* genus, which causes liisteriosis in different human and animal hosts. *L. ivanovii* is a rare human pathogen. *L. monocytogenes*, *L. ivanovii*, *L. innocua* and *L. seeligeri* are among the *Listeria species* found in cheese, milk, butter, and burger. *L. grayi* is found in milk, cheese, and burger, and *L. welshimeri* is found in milk, cheese, butter, and burger [9]. In the US, approximately 1,600 people fall ill and 260 die annually because of infection with *L. monocytogenes*. Owing to the high death rate related to listeriosis and its unfamiliar level of infection, the U.S. Food and Drug Administration remains a zero-tolerance policy for bacteria in food. Unlike most foodborne bacteria that primarily infect the gastrointestinal tract, *L. monocytogenes* can move through different regions of the human body, such as the blood-brain and placental barriers, making the illness more severe [10]. Numerous domestic animals, particularly ruminants such as goats, cattle, and sheep, contain the bacterium in their digestive tracts and commonly introduce *L. monocytogenes* into animal breeding environments [5].

There are undoubtedly benefits for the retail and restaurant sectors owing to the increase in customer expectations for ready-to-eat items. However, new microbiological concerns regarding food safety must be considered [10]. Additionally, contamination of fresh produce by *L. monocytogenes* has been reported [11]. Fresh vegetables have been linked to outbreaks of *L. monocytogenes* worldwide. Five fatalities and ten cases of listeriosis linked to chopped celery were reported in Texas [12]. Different types of substances, including raw milk, soft cheese, pasteurized dairy products, and ready-to-eat foods, have been associated with the spread of this bacterium [13].

The virulence of *L. monocytogenes* is related to the availability and coordinated expression of toxin-specific genes. The most important genes identified were *hlyA, prfA*, and *inlA*. The *hlyA* makes listeriolysin O and it is a cholesterol-dependent cytolysin that plays a basic role in facilitating the escape from phagosomes into the cytoplasm of human cells. This enabled intracellular survival and replication. The transcriptional activator, *prfA* is an important regulator of the entire *Listeria* virulence gene cluster. This cluster comprises *hlyA, actA*, and *plcA*, which orchestrate their expression in response to intracellular environmental cues [14, 15]. In addition, *inlA* encodes for internalin A. It is a surface protein that facilitates bacterial entry into cells by binding to human E-cadherin. This crucial gene participates in an organism's ability to break the intestinal barrier [14, 16].

These genes can determine the pathogenicity of *L. monocytogenes* and serve as critical biomarkers. They can also be used to evaluate the virulence potential of isolates recovered from different types of foods. Several studies have reported the detection of genes in bacteria. Dairy products, vegetables, and

meats are the common toxigenic genes of *L. monocytogenes*. The identification of these genes indicates zoonotic risk and food chain contamination [17]. Polymerase chain reaction (PCR)-based detection methods targeting these genes have become the standard in food microbiology. This is because of their high specificity and sensitivity [18, 19]. Evaluating the prevalence of these genes in foodborne strains is crucial for evaluating their virulence potential and developing risk assessment models.

Listeriosis caused by foodborne *L. monocytogenes* continues to be a severe and significant public health issue worldwide and is associated with a high fatality rate. Moreover, its ability to be maintained in unfavorable environmental conditions and its presence in ready-to-eat and minimally processed foods has raised concerns. To our knowledge, there is an absence of systematic surveillance data on the distribution of *L. monocytogenes* in locally consumed foods, and there are no published reports that have combined detection with detailed virulence gene content characterization and testing for antimicrobial resistance profiles. This gap in understanding hinders both the accurate risk assessment and the creation of pragmatic countermeasures. This study was conducted in response to the issues raised above by investigating *L. monocytogenes* in various food products seized in Halabja and Sulaymaniyah provinces. Subsequently, molecular confirmation, virulence gene profiling, antimicrobial susceptibility testing, and phylogenetic analyses of the isolates was performed. This study was designed to evaluate the prevalence, genotypic characteristics, and antimicrobial susceptibility of *L. monocytogenes* in high-risk foods circulating in the region. The hypothesis of this study was that high-risk foods in the region possessed *L. monocytogenes* and some isolates harbored major virulence genes (*hlyA*, *prfA*, and *inlA*) along with resistance to antibiotics from a clinical point of view, thus proving a potential public health threat. The results can be used to update the baselines required for data-driven interventions, enhance local food safety surveillance systems, and contribute to a more comprehensive epidemiological characterization of *L. monocytogenes* worldwide.

2. Materials and Methods

2.1. Sample Collection

This study used a cross-sectional design. A total of 124 different food products (goat milk, cow milk, soft cheese, hard cheese, yogurt, pasteurized milk, butter, sheep meat, chicken meat, beef, sausage, tuna fish, lettuce, cauliflower, cabbage, spinach, celery, juice, mayonnaise, and ketchup) were collected from Suleimani and Halabja provinces from retail sale sites, along with market streets and supermarkets from 1st December 1, 2024, 31st May 31, 2025. The samples were collected randomly to represent the city's local markets, and during sampling, information was collected in the area of products. They were then moved directly to the laboratory using an ice box.

2.2 Preparation and Isolation of Food Sample

A small portion of each food sample was collected from each part of the sample to ensure a representative analytical unit. Isolation was initially performed using *Listeria* selective enrichment broth (LSEB) to increase the growth of *Listeria* spp. The LSEB base contained trypticase soy broth in addition to 0.6% yeast extract supplemented with a Listeria selective supplement (Hi Media Laboratories). Amounts of 25 g or mL of dairy product, meat samples, vegetables, and ready to-eat foods were aseptically mixed with LSEB (225 mL) in a sterile conical flask, followed by incubation in a shaking incubator at 37°C for 24hrs at 200 rpm. About 0.1 mL of the incubated culture was cultured on "Polymyxin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol selective medium" (PALCAM) to grow *Listeria* spp. It was then incubated at 37°C for 24-48 hrs. PALCAM agar (HiMedia, India) was used for isolation and identification of Listerial colonies. This was followed by the determination of bacterial growth. Listerial colonies have black, black-green, and black halo characteristics [20, 21]. Typical colonies from the primary culture were subtly sub-cultured until a pure culture was obtained.

2.3. Identification of L. monocytogenes

2.3.1. Biochemical Tests

Suspected colonies of *L. monocytogenes* were identified using various assays, including colony properties such as color, shape, and size. In addition, Gram staining, catalase, umbrella-shaped motility

behavior, hemolysis on blood agar, fermentation of sugars (mannitol, rhamnose, xylose, glucose, and maltose), Voges Proskauer, and indole tests were analyzed [5].

2.3.2. PCR Assay for the Detection of Pathogenic L. monocytogenes

2.3.2.1. DNA Extraction

The DNA of *L. monocytogenes* was extracted using the Fla Pure Bacteria Genomic DNA Extraction Kit (Cat. No. DE703-50/Jinsha Biology/ China). Bacterial cultures of bacteria were incubated overnight in Brain Heart Infusion broth at 37 °C to make ensure that an appropriate bacterial density was reached. Approximately 2 mL of cells were pelleted by centrifugation at 11500×g for 1 min and aspirated as much supernatant as possible. DNA was extracted from the bacterial pellets according to the manufacturer´s instructions. Cell lysis, protein and other impurity removal, and nucleic acid purification extracted DNA were also evaluated for purity and concentration using an Invitrogen Qubit 4 Fluorometer (Invitrogen/Thermo fisher/USA) to determine the quality of amplification by ng/μL [22].

L. monocytogenes was confirmed at the molecular level using specific PCR primers targeting a unique gene possessing diagnostic or virulence importance in each primer set. The primer for the *prs* gene, which resulted in a 370 bp product, is specific to the *Listeria* genus, whereas the *lmo1030* gene (509 bp) is not only a confirmatory species-specific marker for *L. monocytogenes*, but also provides an indication of species-level identity. Primers targeting the highly conserved ribosomal sequence and 16S rRNA gene (475 bp) targets were also added to the screening for further molecular confirmation. Listeriolysin O (*hlyA*, 456 bp), a pore-forming toxin that is critical for the escape from host cell vacuoles and subsequent cytoplasmic multiplication13; *prfA* (695 bp), a transcriptional activator of the *Listeria* virulence gene cluster14; and Internalin A (*inlA*, 800 BP), an invasion-associated surface protein that interacts with E-cadherin on epithelial cells15, was also amplified to determine their pathogenic potential [23].

The annealing temperature for all reactions was normalized to 61°C, which was within the optimal range of each primer set derived from melting-temperature calculations, allowing simultaneous PCR runs without loss of amplification efficiency. The sequences, primer pairs, and products predicted to be amplified in each reaction are summarized in table 1. Everything is presented as 5′→3′: each target gene has both F- and R-sequences. The table also shows which of these are confirmation genes (*prs*, *lmo1030*, 16S rRNA) used for species identification or virulence genes (*hlyA*, *prfA* and *inlA*) to identify the pathogenic potential of an isolate. This molecular system, which included both diagnostic and virulence markers, reached a high specificity for *L. monocytogenes* detection, and more important epidemiological information on the pathogenic properties of food-derived isolates was obtained.

Table 1: The primers used for amplification targeted the confirmation and virulence-related genes of *L. monocytogenes*, and the annealing temperature for all primers was set at 61°C.

Reference	Target gene	Nucleotide sequence gene	Product size(bp)
[24, 25]	***PRS***	*F*- 5′ GCTGAAGAGATTGCGAAAGAAG - 3′ *R*- 5′ CAAAGAAACCTTGGATTTGCGG - 3′	370
[24, 25]	***Imo1030***	*F*- 5′ GCTTGTATTCACTTGGATTTGTCTGG - 3′ *R* - 5′ ACCATCCGCATATCTCAGCCAACT- 3′	509
[26]	***16S rRNA***	*F*- 5′ CCTACGGGAGGCAGCAGT- 3′ *R* - 5′ CGTTTACGGCGTGGACTAC-3′	475
[15, 24]	***hlyA***	*F*- 5′ GCAGTTGCAAGCGCTTGGAGTGAA-3′ *R* - 5′ GCAACGTATCCTCCAGAGTGATCG - 3′	456
[25]	***prfA***	*F*- 5′ CTCAAGCAGAAGAATTCA -3′ *R* - 5′ TCCCCAAGTAGCAGGACA-3′	695
[16, 27]	***inIA***	*F*- 5′ ACGAGTAACGGGACAAATGC-3′ *R* - 5′ CCCGACAGTGGTGCTAGATT-3′	800

2.3.2.2. Preparation of PCR Master Mix

The PCR reaction mixture (OEM/ China) was prepared in a final volume of 25 µL, containing 12.5 µL 2× GS Taq PCR Master Mix, 1.0 µL forward primer (10 pmol), 1.0 µL reverse primer (10 pmoL), 2.0 µL of template DNA, and 8.5 µL nuclease-free water [28].

2.3.2.3. Cycling Environments of the Primers

The PCR amplification (Thermo Fisher/UK) was initiated with an initial denaturation at 94 °C for 3 mins, followed by 30–35 cycles consisting of denaturation at 94°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s. The final extension was performed at 72°C for 5 mins, after which the reaction was held at 4°C [28].

2.4. Antimicrobial Susceptibility Test

The antimicrobial susceptibility of *L. monocytogenes* was determined using the disk diffusion method. Ten different antibiotic disks were used (HiMedia). Antibiotics used were as follows: 1. Penicillin G - P (10 units), 2. Amoxicillin - AMX (10 µg) 3- Clindamycin -CD (2 µg), 4- Vancomycin-VA (30 mcg), 5- Erthromycin-E 15 (15mcg), 6- Chloramphinicol-C30 (30mcg) 7-, gentamicin-GEN 10(10mcg), 8-CIPROFLOXACIN-CIP5 (5mcg),9-AMPICILIN-AMP10 (AMP10), 10-TETRACYCLIN-TE30. In short, microbial concentration was corrected to a 0.5 McFarland standard after growth of *the bacterium* on nutrient agar for 18-24 hrs at 35–37°C. Under investigation, 2 mL of 0.9 NaCl. This was followed by the inoculation of each disk on Muller Helton agar. After incubation at 35–37°C for 24 hrs, areas of inhibition were determined in millimeters [25, 29, 30].

2.5. Sequence Characterization and Phylogenetic Analysis

The amplified DNA products were sequenced using Sanger sequencing (Macrogen Co., Korea). The resulting sequences were aligned using Clustal Omega for comparative analysis of multiple sequences. To determine their similarity to known nucleotide sequences, the data were further examined using the NCBI (National Center for Biotechnology Information) BLAST parameter (https://www.ncbi.nlm.nih.gov/). A phylogenetic tree illustrating the evolutionary relationships was constructed using MEGA-X software.

2.6. Statistical Analysis

Bacterial numbers are presented as mean colony-forming units (CFU) per gram (CFU/g). *L. monocytogenes* and its virulence genes were expressed as the percentage of positive strains among the tested isolates. Antimicrobial susceptibility was presented as the mean zone of inhibition diameter (mm). The analysis was descriptive in nature, and the results are displayed in tables and figures to facilitate comparison.

3. Results

A total of 124 randomly selected food samples were collected from Sulaimaniyah and Halabja governorates. This study was conducted on twenty types of food products. With the use of PALCAM (Polymyxin Acriflavin Lithium Chloride Ceftazidime Aesculin Mannitol selective medium) the gray-green with black halo colony of *L. monocytogenes*. Of the 124 samples examined, 12 (9.5%) contained *L. monocytogenes* following confirmation of the required tests, including biochemical tests such as Gram staining, catalase test, oxidase, indole, Voges-Proskauer, motility, hemolysis on blood agar, and sugar fermentation. The ultimate validation of the identity of the presumed colonies isolated in this investigation was provided by PCR.

3.1. Identification and Prevalence of L. monocytogenes in Foods by Cultural Microscopic

First, the growth of the pathogenic colony on PALCAM agar created colonies with a surrounding gray-green colony with a black halo caused by the hydrolysis of aesculin to esculetin, which then reacts with iron salts and ferric ammonium citrate in the medium. As shown in figure 1-A, the identification was further supported by Gram staining, which revealed that the bacterial strains were rod-shaped and Gram-positive, matching the morphology of *L. monocytogenes* (Figure 1-B).

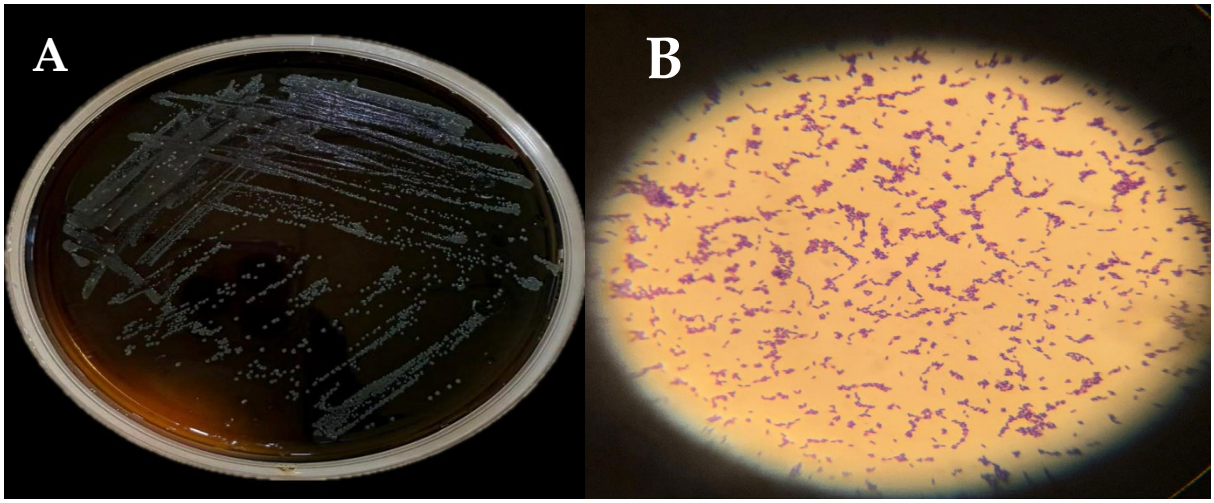

Figure 1. Cultural and microscopic identification of L. monocytogenes. (A) Colonies of *L. monocytogenes* grown on PALCAM agar after 24–48 hours of incubation at 35–37°C. Typical colonies appear gray-green with a black halo due to esculin hydrolysis. (B) Gram-stained smear of *L. monocytogenes* observed under a light microscope (1000× magnification), showing Gram-positive, short rod-shaped or coccobacillary cells arranged singly or in short chains.

3.2 Staining and Biochemical Tests

Presumptive colonies from PALCAM agar were identified as *L. monocytogenes* based on Gram staining, catalase activity, and a set of additional biochemical tests used for screening followed by molecular confirmation (Table 2). Gram-stain results showed the presence of Gram-positive, rod-shaped cells, and all isolates were catalase positive (bubble formation), but oxidase negative (no color change); a clear β-hemolysis zone was recorded on blood agar, which prompted the secretion of hemolytic enzymes by *L. monocytogenes*. A characteristic umbrella-shaped pattern was observed during motility testing at room temperature. Color changes suggested that the isolates were indole and the Voges-Proskauer test was positive. Sugar fermentation tests were positive for glucose, maltose, and rhamnose but negative for mannitol, lactose, and sucrose. These biochemical and morphological properties are in agreement with the standard diagnostic profile of *L. monocytogenes* determined by molecular detection. The results obtained from the biochemical tests were shipped with the molecular findings, which proved this identification accuracy.

Table 2. Biochemical and staining tests were done for the identification of *L. monocytogenes*.

Test	Result
Gram stain	Positive, rod-shaped
Catalase	Positive, bubble formation
Oxidase	Negative, no color change
Hemolysis on blood agar	Positive
Motility test	Positive
Indole test	Positive, color change
Voges–Proskauer test	Positive, color change
Sugar fermentation (Glucose, Maltose, Rhamnose)	Positive, color change
Sugar fermentation (Mannitol, Lactose, Sucrose)	Negative, no color change

3.3 Prevalence of L. monocytogenes in Food Samples

Upon examining *L. monocytogenes* in 124 food samples collected from different shops and markets in Sulaimaniyah and Halabja Provinces, 9.6% were contaminated with the bacterium (Table 3). Conventional semi-hard cheese ranked first with a contamination rate of 40% (6/15). This was followed by lettuce and celery, respectively. Contamination was observed in 2 out of 10 (25%) and 2 out of 8 (25%) samples, respectively. However, soft cheese and beef exhibited lower levels of contamination. One positive sample was found in each sample, corresponding to 11% and 12.5%, respectively. *L. monocytogenes* could not be isolated from goat milk, cow milk, pasteurized milk, yogurt butter sheep

meat, chicken meat sausage, tuna fish cabbage, cauliflower spinach, ketchup mayonnaise, and juice. This may be due to the hygienic methods during manufacturing or initial presence at a low level that cannot be picked up. A reason that may contribute to the pathogen being less detected in these foods is improved processing methods, decreased potential for post-processing contamination or it might be because of increased storage practices. The distribution pattern of *L. monocytogenes* was consistent with the risk-based categorization, where higher risks for *L. monocytogenes* contamination in food categories such as dairy products compared to those of minimally processed foods, particularly raw vegetables, were not correlated on a weight basis. These findings emphasize the need for better microbiological control, hygiene throughout distribution and storage, and public health interventions targeted at product specificity in study locations.

Table 3: Prevalence of *L. monocytogenes* in foods. The samples were collected from Sulaymaniyah, and Halabja regions. The highest contamination was observed in traditional semi-hard cheese, celery, and lettuce. However, no presence was detected in several other food categories.

Types of food samples	Numbers of tested samples	Positive (%)
Goat milk	5	0
Cow milk	5	0
Pasteurized milk	5	0
Soft cheese	9	1 (11)
Traditional semi- hard cheese	15	6 (40)
Yogurt	5	0
Butter	5	0
Sheep meat	5	0
Beef meat	8	1 (12.5)
Chicken meat	7	0
Sausage	5	0
Tuna fish	5	0
Cabbage	5	0
Cauliflower	5	0
Lettuce	10	2 (25)
Spinach	5	0
Celery	8	2 (25)
Ketchup	5	0
Mayonnaise	5	0
Juice	5	0
Total	124	12 (9.6)

3.4. Genus and Species Confirmation by PCR Amplification of prs, lmo1030, and 16S rRNA genes

The biochemical test results were consistent with the findings of molecular analyses. Isolates that were biochemically identified as *L. monocytogenes* were confirmed by PCR targeting the *lmo1030*, *prs*, and 16S rRNA genes. As shown in figure 2A, amplification of the *prs* gene (370 bp). It is conserved among *Listeria* species and yields clear bands in lanes 2–12. This indicated the availability of *Listeria* spp. in multiple foods. The positive control (lane 9) showed a strong band. However, no amplification was observed in the negative control (lane 10). These results confirm the specificity of the assay. The identity of *L. monocytogenes* was further confirmed by amplification of the 509 bp *lmo1030* gene (Figure 2B), which is specific only for this species. For all lanes 2-13 bands with the desired amplicon size were observed, indicating the presence of *L. monocytogenes* in these isolates. The use of a negative control (lane 8) resulted in no amplification, but there was a prominent band of the appropriate size in the positive control (lane 9), confirming assay specificity. Finally, to support species-level identification, the 16S rRNA gene (475 bp) was amplified (Figure 2C). Lanes 2 to 5 show clear, single bands of the approximate fragment size corresponding to genus- and species-level identification of bacteria. The detection of all three genetic markers in the same isolates strongly supported the presence of *L. monocytogenes* in the tested food samples. Based on our cultural and biochemical data, these findings demonstrate that contamination was significantly linked to dairy products, leafy vegetables, and raw beef, highlighting the importance of food safety measures in reference to the cross-contamination risks posed by common sources of infectious pathogens.

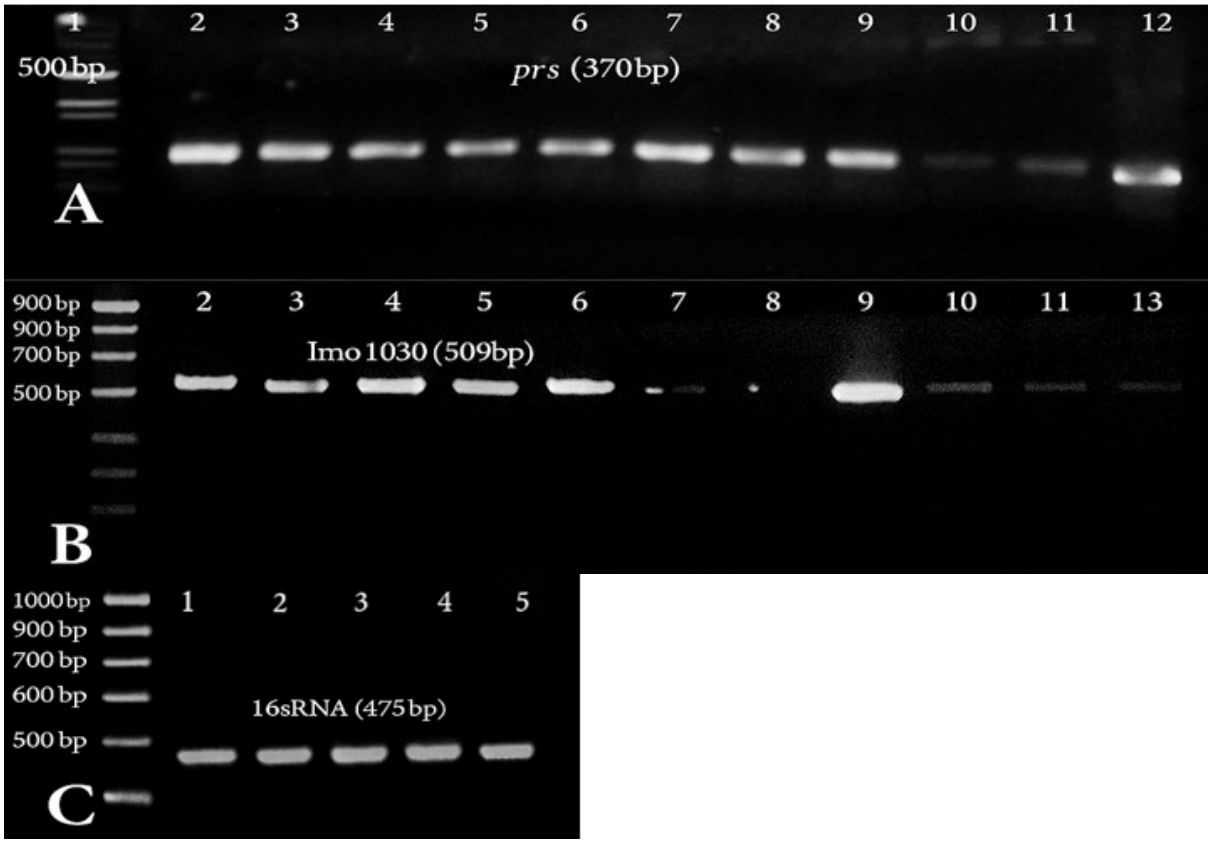

Figure 2: (A) Agarose gel electrophoresis of PCR product of *Listeria spp.* positive for food products. 1=Ladder 100bp, 2-12 positive *Listeria spp.*, apart from 9 positive control *L. monocytogenes* (ATCC 19115), 10 negative control. (B) Agarose gel electrophoresis of PCR product of *L. monocytogenes* positive for the Imo1030 gene. 1=Ladder 100 bp (Geneaid/ Taiwan), 2-13 positive *L. monocytogenes* Imo1030 genes, apart from 8 negative control, 9 positive control *L. monocytogenes* ATCC (American Type Culture Collection 19115). (C) Agarose gel electrophoresis (Thermo Fisher/UK) of PCR product of *L. monocytogenes* positive for 16sRNA gene. 1=Ladder 100 bp, 2-5 positive *L. monocytogenes* 16sRNA genes. The images were taken using digital camera (/Canon/Japan**)**.

PCR amplification targeting additional virulence-associated genes, *hlyA*, *prfA*, and *inlA*, together with additional amplification of the species-specific *lmo1030* gene, was conducted to validate the pathogenic potential of the confirmed *L. monocytogenes* isolates (Figure 3). Distinct amplicons were visible in lanes 2 to 9 of figure 3A at 456 bp for the *hlyA* gene, which encodes listeriolysin O, a critical pore-forming toxin that bacteria employ to enable their escape from the phagolysosome and intracellular survival. The amplification product of the *lmo1030* gene, which appeared at 509 bp in lanes 10 to 16 of figure 3A, reaffirmed that these isolates were *L. monocytogenes* at the species level. Figure 3B also shows clear amplicons for the *prfA* gene that appeared in lanes 2–6 at 695 bp, which encodes a master regulator of virulence that controls the expression of multiple genes critical for host cell invasion, intracellular growth, and cell-to-cell spread. Amplicons of the *inlA* gene (Figure 3B) were observed in lanes 7–13 at 800 bp, and this gene encodes a surface protein essential for bacterial internalization into non-phagocytic epithelial cells by binding to human E-cadherin. Therefore, the detection of the *hlyA*, *prfA*, and *inlA* alleles in food-derived *L. monocytogenes* isolates indicated that these isolates carried the pathogenicity determinants necessary for virulence in humans. Thus, contaminated dairy products, vegetables, and raw meat harboring these strains may compromise public health.

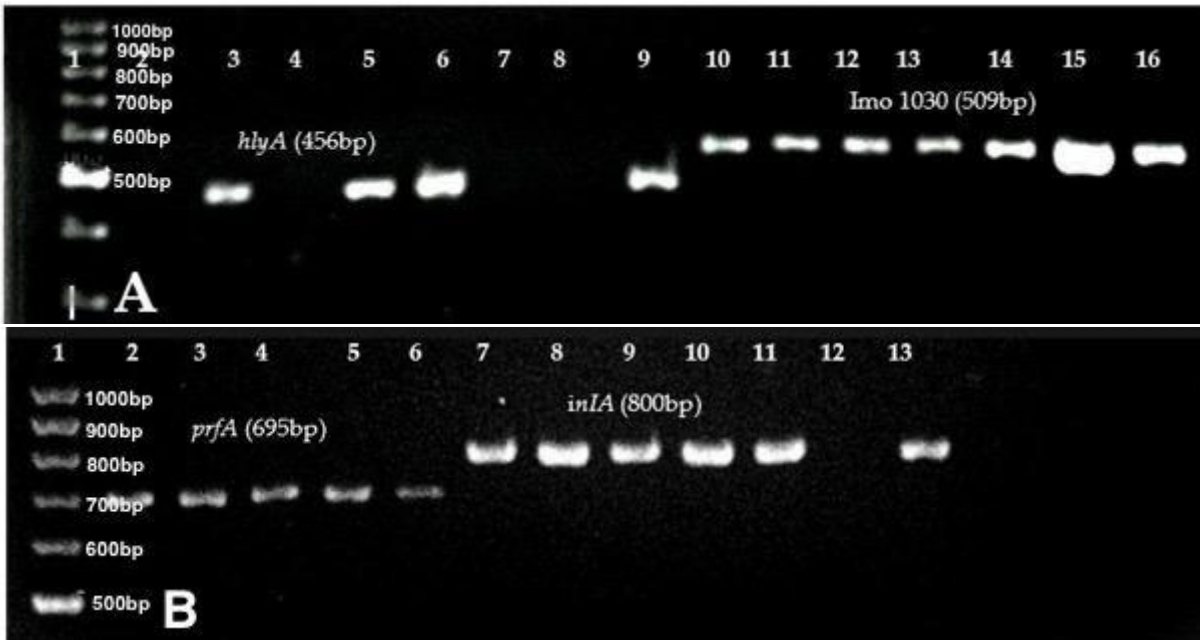

Figure 3: Agarose gel electrophoresis analysis of PCR amplification for *lmo1030* and toxigenic genes. (A) Agarose gel electrophoresis of PCR product of *L. monocytogenes* positive for *hlyA* and Imo1030 genes, 1 ladder 100 bp, 2-9 hlyA gene, 10-16 Imo1030 gene. (B) agarose gel electrophoresis of PCR product of *L. monocytogenes* positive for *inlA* and *prfA* genes. 1 ladder 100 bp, 2-6 *prfA* gene, 7-13 *inlA* gene, 12 negative control, and 13 positive controls. The images were taken using digital camera (/Canon/Japan**)**.

3.5. Distribution of Virulence Genes in the Isolated Positive Food Products

Twelve *L. monocytogenes* isolates were isolated from the tested food samples. They were analyzed for the presence of three key virulence genes: *hlyA*, *prfA*, and *inlA* (Table 4). The distribution of these virulence determinants varied according to food category. Among the seven isolates from dairy products (cheese), *hlyA* and *prfA* were each found in three isolates (42.8%). However, *inlA* was found in two isolates (28.5%). In vegetable samples (lettuce and celery), out of 4 *L. monocytogenes*-positive isolates, one isolate (25%) carried *hlyA*, one (25%) carried *prfA*, and two isolates (50%) harbored *inlA*. These results suggested a higher prevalence of cell invasion potential in plant-derived isolates. Furthermore, the single *L. monocytogenes* isolate obtained from raw beef exhibited both *prfA* and *inlA* genes but lacked *hlyA*. These results highlight a complete virulence profile with the potential for pathogenicity. In summary, *prfA* and *inlA* were most frequently detected across all food categories. Each of these was found in 5 of the 12 isolates (41.6%). However, *hlyA* was detected in 4 isolates (33.3%). The presentation of these virulence genes, particularly *prfA* (a key regulator of the listerial virulence gene cluster) and *inlA* (a major factor for epithelial cell invasion), shows the possibility of pathogenic behavior of isolates recovered from both dairy and non-dairy sources. These data also show the requirement for observant molecular surveillance of *L. monocytogenes* in food products to evaluate public health risk by testing commonly consumed foods and uncooked or minimally processed foods, including ready-to-eat or minimally processed foods.

Table 4: Gene markers and virulence gene distribution among isolated *L. monocytogenes* from different food products. Among 124 tested food products, 49 of dairy products had 7 sample containing *L. monocytogenes*, of the 33 vegetable samples contained *L. monocytogenes* and 1 of the 30 samples of raw meat and meat products contained *L. monocytogenes.*

Gene	Dairy products (cheese) (n=7)	Vegetable (Lettuce, celery) (n=4)	Raw beef meat (n=1)	Total (n= 12)
hlyA	3 (42.8%)	1 (25%)	0	4 (33.3%)
prfA	3 (42.8%)	1 (25%)	1 (100%)	5 (41.6%)
inlA	2 (28.5%)	2 (50%)	1 (100%)	5 (41.6%)

3.6. Phylogenetic Analysis of 16S rRNA and lmo1030 Genes

Subsequently, phylogenetic analysis of the two *L. monocytogenes* isolates (LM01 and LM02) in relation to the reference strains was carried out to confirm species identity and determine genetic relationships based on that level. Using 16S rRNA sequences, the dendrogram (Figure 4A) based on the three significant isolates was closely related to the powerful sequenced *L. monocytogenes* MK235117, KU208088, and PQ796064. These results indicate a high sequence conservation and a close taxonomic relationship. Clustering with *L. seeligeri* and *L. welshimeri* at adjacent nodes further validated species-level identification. However, it also distinguished isolates from non-monocytogenes species. BLAST analysis showed that LM01 had 100% coverage and 100% identity with several *L. monocytogenes* strains, including strain 13 (KY952654) and LmK1 (OM897223) (Table 5). Similarly, the phylogenetic tree derived from the *lmo1030* gene (Figure 4B) showed that both LM01 Isolate01 and LM02 Isolate02 grouped within the same clade as *L. monocytogenes* strains. These are related to serotypes 1/2a, such as strains 10-1047 and 3BS90. This clustering confirmed the presence of the virulent lineage I/II strains. High bootstrap support and low evolutionary distances across the nodes reflected strong genetic relatedness and low divergence among the isolates. This is also present, further supporting the molecular identification. These results suggest that the isolated strains are genetically consistent with globally distributed pathogenic *L. monocytogenes* lineages commonly associated with foodborne outbreaks. Overall, this combination of phylogenetic clustering and comparison of BLAST Hit% similarity between tested strains and other strains indicates pathogenic lineages that closely matched outbreak associated *L. monocytogenes* in food, with the subsequent isolate receiving support. Accordingly, the genetic homogeneity of these isolates highlights their potential importance in public health practices.

Table 5: LM01 sequence similarity values retrieved from the NCBI database. Sequence similarity (LM01) values from the NCBI. Summary of the best matches from BLAST analysis of the sequence from isolate LM01 against the NCBI nucleotide database.

Description	Query Cover (%)	Percent Identity (%)	Accession No.
L. monocytogenes strain serotype 4b 16S ribosomal RNA	100	100	MK235117.1
L. monocytogenes strain EB49 16S ribosomal RNA	100	100	MT509637.1
L. monocytogenes strain KW/FF018 16S ribosomal RNA	100	100	PQ796064.1
L. monocytogenes strain LM9 chromosome	100	100	OM281749.1
L. monocytogenes strain ATCC19112 16S ribosomal RNA	100	100	JF967618.1
L. welshimeri strainNCAIMB.01872 16S ribosomal RNA	100	100	MT759914.1
L. monocytogenes strain LM83-1 16S ribosomal RNA gene	100	100	OQ449698.1
L. monocytogenes strain LM6 16S ribosomal RNA gene	100	100	OM281746.1
L. monocytogenes strainKKP1058 16S ribosomal RNA gene	100	100	OK642584.1
L. monocytogenes strain NOL-011 16Sribosomal RNA gene	100	100	MF652072.1
L. monocytogenes strain LM22 16S ribosomal RNA gene	100	100	OR725610.1

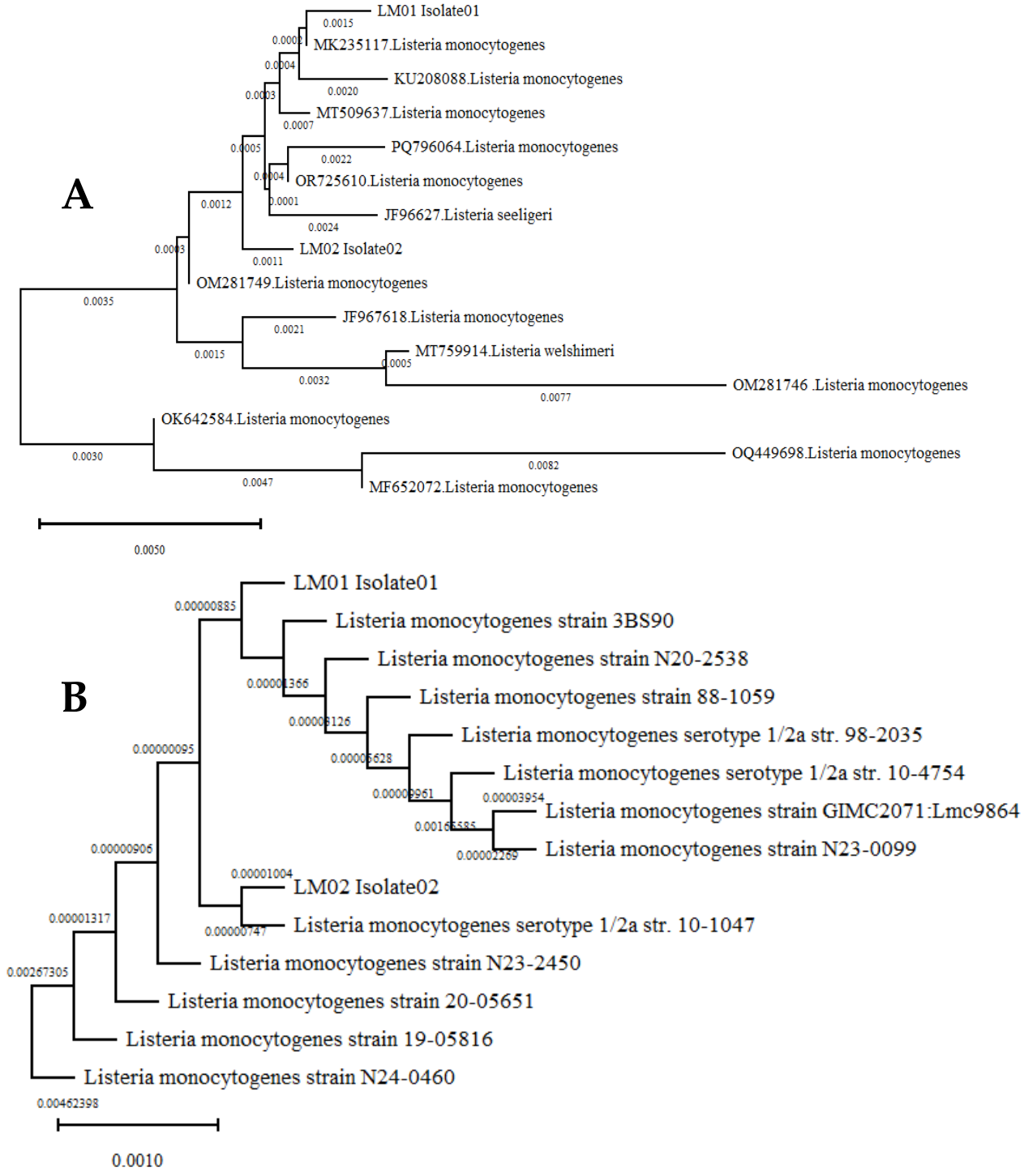

Figure 4: Maximum likelihood phylogenetic tree of *L. monocytogenes* isolates (LM01 and LM02) and closely related *Listeria* species based on 16S rRNA (A) and *lmo1030* (B) gene sequences. DNA sequences were obtained from NCBI GenBank and aligned using the MUSCLE algorithm in MEGA X. Phylogenetic analysis was conducted using the Neighbor-Joining method, with evolutionary distances calculated and represented by scale bars. Bootstrap analysis (1,000 replicates) was used to assess the robustness of the tree topology.

3.7. Antimicrobial Susceptibility Tests of L. monocytogenes Isolates

Antimicrobial susceptibility testing was performed on *L. monocytogenes*. It followed the standard disk diffusion technique on Muller-Hinton agar (Neogen, USA). The resistance and sensitivity patterns against a panel of frequently used antibiotics were determined (Figure 5 and Table 6). In the current study, all the tested isolates showed high resistance to ampicillin, with inhibition zones (0 mm). Traditionally, ampicillin is the first-line treatment for listeriosis. In contrast, the isolates were highly susceptible to a broad spectrum of antibiotics. The most important antibiotics were penicillin (28.6 mm), amoxicillin (32.6 mm), gentamicin (28.5 mm), erythromycin (31.3 mm), tetracycline (34 mm), vancomycin (27.8 mm), chloramphenicol (33 mm) and ciprofloxacin (30.8 mm). The zones of inhibition around the chloramphenicol and ciprofloxacin discs (Figure 5) clearly indicate the effectiveness of these agents in inhibiting bacterial growth. Each of these antibiotics exhibited broad inhibition zones. Importantly, clindamycin also showed susceptibility based on a 12.5 mm zone, whereas the reduced diameter presented variable or borderline sensitivity. Thus, these findings are important for foodborne disease management. Therefore, they highlighted both effective treatment options and emerging resistance trends. Consistent susceptibility to several antibiotics shows their potential utility in clinical settings. Resistance to ampicillin emphasizes the necessity of updated treatment guidelines and routine assessment of antimicrobial resistance patterns in foodborne *L. monocytogenes* strains. Overall, these data contribute to public health risk assessments. This can guide future antibiotic efforts in food safety surveillance programmes.

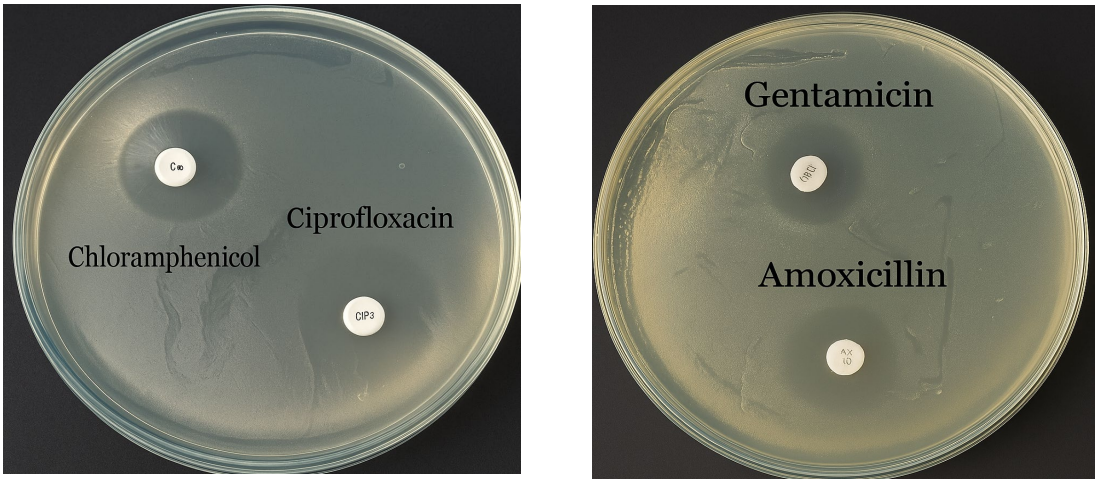

Figure 5. Antimicrobial susceptibility assay of *L. monocytogenes*. The disk diffusion technique on Muller-Hinton agar was used to perform the test. Clear zones of inhibition are observed around ciprofloxacin, chloramphenicol, gentamicin, and amoxicillin antibiotic discs indicating susceptibility of the isolate to both antibiotics.

Table 6: The antibiotic susceptibility assay of *L. monocytogenes*. The isolates were isolated from selected food products. The antimicrobial activity was evaluated using the disk diffusion method. Zone diameters were measured in millimeters (mm).

Antimicrobial Test	Zone Diameter(mm)	Interpretation
Ampicillin	0 mm	Resistant
Penicillin	29.6 mm	Susceptible
Amoxicillin	32.6mm	Susceptible
Gentamicin	28 mm	Susceptible
Erythromycin	31.3mm	Susceptible
Tetracycline	34 mm	Susceptible
Vancomycin	27.8mm	Susceptible
Chloramphenicol	33 mm	Susceptible
Ciprofloxacin	30.8 mm	Susceptible
Clindamycin	12.5 mm	Susceptible

4. Discussion

In the present study, the detection and isolation of *L. monocytogenes* was carried out using random sample sets of different types of foods. This is the first instance of isolation from the Sulaimaniyah and Halabja provinces. This provides a better understanding of the contamination, pathogenicity, and antimicrobial resistance properties of locally circulating *L. monocytogenes* in the food chain. The global presence of *L. monocytogenes*, recorded as 12 out of 124 (9.6%), reveals its ability to persist in a variety of different food matrices. This prevalence is in accordance with a number of findings from another region, including the 9.2% rate found in handmade white cheese in Turkey [31], 10.71% in goat milk from Iran [14] and some recent reports obtained from dairy products locally produced in the Egyptian market (6-10%) [32] and South African ready-to-eat foods (9.3%) [33]. Minor differences in prevalence rates among studies are often the result of methodological variations, sample types, or regional food safety procedures [34].

As previously reported [24], *L. monocytogenes* was not detected in fresh goat milk in this study. In contrast, another report found a 10.71% prevalence in 28 goat milk samples [14]. This study found no *L. monocytogenes* in raw cow milk samples, which is consistent with previous studies showing prevalence ranging from 7.1% [24] to none detected [35] in bovine raw milk. Around the world, including China, Iran, and other countries, between 1.25–20% of raw milk samples were positive in different regions of China [36, 37]. For example, a longitudinal study of raw milk contamination in three Finnish dairy farms (2013–2016) revealed repeated isolation of *L. monocytogenes* during the year, whereas the prevalence of this pathogen was significantly higher in milk filter sock samples collected during the indoor season [38].

This study found that traditional semi-hard cheese among dairy products was contaminated at the highest rate (40%), with 6/15 of the samples tested positive. This is a concerning statistic because artisanal natural cheese products often avoid strict thermal treatment and can be subject to post-craft contamination. Morocco has a lower rate of contamination (5.9%) [39]. Soft and semi-soft cheeses pose the highest risk of listeriosis, particularly when produced under artisanal conditions with inconsistent hygiene practices. In an artisanal Italian plant, where *L. monocytogenes* biofilms persist for months, recent studies have shown that repeated contamination often arises from this environment. A study in an artisanal Italian plant producing Pecorino Umbro cheese showed that although *L. monocytogenes* could be introduced during processing, counts of the pathogen decreased significantly after ripening and storage due to microbial antagonism exerted by lactic acid bacteria, as well as a decrease in pH [40].

L. monocytogenes that can contaminate milk on farms comes from a wide range of environmental sources, such as soil, silage, animal feces, and pastures, where it is found to be resistant to farm environments or contaminated water. Possible factors: poor silage fermentation, inadequate barn hygiene, or udder cleaning, although not investigated in the present study [41]. Cross-contamination also occurs postharvest during transportation and retail handling. The current study confirms previous reports on the detection *of L. monocytogenes* in vegetables, particularly lettuce and celery. This indicates that irrigation water, soil exposure, and contaminated contact surfaces are important transmission routes [42]. This is supported by the patterns of contamination in salad vegetables from North Africa, which are consistent with the current data, indicating potential ubiquitous risk determinants independent of climatic zone [43].

It is crucial to note that the majority of yogurt samples tested in this study were free from *L. monocytogenes*, which agrees with the findings of other studies [44]. Other in vitro studies have shown that *L. monocytogenes* is inhibited significantly by lactic acid bacteria such as *L. rhamnosus* and *L. paracasei*, particularly with a synchronous course through organic acids and bacteriocins [45]. In addition, the high contamination rate in cheese confirmed the ability of the pathogen to survive and grow in a more complex food matrix subjected to limited postprocessing heat treatments. Severe cases of listeriosis and outbreak investigations have been repeatedly associated with cheese, and the fatality rate in high-risk populations can reach [46]. The present findings are consistent with those of previous studies [16, 31], due to differences in prevalence between countries, this could be attributed to cheese type, types of milk used, starter cultures, and hygienic conditions during manufacturing.

Microbiological investigations detected the important virulence genes *hlyA*, *prfA* and *inlA*. They are present in many isolates. *hlyA* was found in 33.3% of isolates and both *prfA* and *inlA* were observed

in 41.6% of the isolates. These are some of the most important genes for *L. monocytogenes* pathogenesis that allow the bacterium to invade and replicate in host cells, ultimately leading to systemic spread. These data correspond with recent reports from Pakistan [5] and Italy [6]. The presence of *inlA* across meat and vegetable isolates further highlights its potential for cross-commodity contamination. In general, *prfA* was detected most widely in dairy- and plant-based foods, which may not only be an artifact of the relative proportions between food types but also indicates that highly virulent strains are present as environmental contaminants [25, 47, 48].

The results of antibiotic susceptibility testing showed that all isolates were resistant to ampicillin, the first-line therapy for listeriosis. Similarly, resistance to β-lactam antibiotics has been reported in Turkey [49], implicating intercontinental dissemination of this phenotype. The findings of clindamycin resistance in this study notably confined the antibiotic resources; however, susceptibility to gentamicin, tetracycline, ciprofloxacin, and chloramphenicol was still promising. Multidrug resistance in food isolates is being increasingly reported, and worldwide work has been carried out to determine the relationships between several parameters and Antimicrobial Resistance (AMR) gene transfer from other Gram-positive bacteria [50, 51].

Faulty silage, unclean processing water, and manual shaping/packaging of cheese are potential sources of dairy products in present study [33]. Similarly, untreated irrigation water, manure, self-manured fertilizers, and soil contact are risk factors for vegetables. These possible exposure risks at both the industrial and retail levels include poor separation of raw and ready-to-eat foods, surfaces that are not cleaned or use uncleaned equipment after cutting raw foodstuffs, and practices used by vendors to contaminate handlers. These findings underscore the importance of good agricultural and manufacturing practices for controlling contamination [52]. With the detection of virulent and AMR *L. monocytogenes* in ready-to-eat or minimally processed foods, integrated surveillance systems are of top priority. Systems of this nature should have an integrated-rapid microbiological detection that incorporates virulence profiling at the molecular level and antimicrobial resistance testing for timely alerting of high-risk contamination events.

5. Conclusions

This study confirmed the presence of *L. monocytogenes* isolates from Sulaymaniyah and Halabjah provinces, with high incidence percentages in traditional semi-hard cheese, whereas lettuce and celery samples contained lower percentages. It was clear from phenotypic features and antimicrobial resistance profiles that the isolates were typical *L. monocytogenes* strains, carried major virulence factors (*hlyA*, *prfA* and *inlA*), and exhibited strong ampicillin resistance. Phylogenetic analysis showed that the strains were closely related to globally distributed pathogenic lineages, implying a potential public health threat. These results support the importance of improving food safety regulations, hygienic production, and retail handling measures as well as the implementation of continuous molecular surveillance programs for early detection and effective control of virulent and antimicrobial-resistant *L. monocytogenes* strains along from farm to table. Future research should include the scope and seasonal coverage of sampling, whole-genome sequencing to detect genetic diversity and resistance pathways, and the determination of environmental contamination sources along the food chain.

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