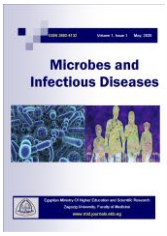
Microbes and Infectious Diseases 2024; 5(4): 1590-1605



Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Evaluating the risk of listeriosis through genotypic profiling of potentially hazardous strains isolated from local food market

Lamiaa I. Fahmy, Heba M. Amin *

Department of Microbiology and Immunology, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 6th October City, Giza 12451, Egypt

ARTICLE INFO

Article history:
Received 24 May 2024
Received in revised form 12 July 2024
Accepted 16 July 2024

Keywords:
Listeria monocytogenes
virulence factors
antimicrobial resistance
frozen food
Egypt

ABSTRACT

Background: Listeriosis is a serious food-borne disease caused by *Listeria monocytogenes* pathogen that causes fatal systemic infections in the elderly and immunocompromised people. *Listeria monocytogenes* can be transferred from contaminated ready-to-eat or frozen foods. This study aimed to investigate the most prevalent serotypes of *Listeria monocytogenes* isolates in frozen food in Egypt, their phenotypic and genotypic antimicrobial resistance, biofilm formation, as well as virulence encoding genes. **Methods:** A total of 331 frozen food product samples were randomly chosen from various marketplaces and cultured for the presence of *Listeria monocytogenes* . The antibiogram of the isolated *Listeria monocytogenes* was assessed using the Kirby–Bauer disk diffusion method. Polymerase chain reaction (PCR) was performed to detect four antimicrobial resistance encoding genes and seven virulence genes. A multiplex PCR assay was performed. **Results:** *Listeria monocytogenes* was found in 14.2% (47/331) of the examined samples, and the most predominant serotypes belonged to molecular sero-groups 1/2a-3a. *Listeria monocytogenes* bacteria showed high phenotypic resistance rates to ampicillin (91%), cefotaxime (87%) and clindamycin (66%). Strong and moderate biofilm producers accounted for 21.4% and 11.9% of isolates, respectively. The most commonly detected genetic markers of resistance were strB and tetA genes (70%). The virulence genes: plcB, prs-prfA, iap and hlyA genes, were detected in 90%,87%, 87% and 30% of the isolates, respectively, while plc A, prfA and flA genes were detected in 83%, 97% and 90% of the isolates, respectively. **Conclusion:** *Listeria monocytogenes* food pathogen exists with considerable rates in frozen products, which may seriously threaten public health.

Introduction

Foodborne pathogen *Listeria monocytogenes (L.monocytogenes)* is a facultative intracellular Gram-positive bacterium that frequently occurs in various kinds of foods. However, environmental sources, including sludge, water, sewage and soil, may contain it [1,2].

Listeria monocytogenes possesses the remarkable ability to survive in temperatures between -7°C and 45°C, with an optimum growth temperature of approximately 37°C [3]. Moreover, *L. monocytogenes* can resist extreme environmental circumstances, such as high alkalinity or acidity; therefore, it persists for a longer period under unfavourable settings than other non-spore-forming foodborne bacterial pathogens [1]. In addition, it can colonize the intestinal tissue in the presence of gut microbiota [4]. Accordingly, in most instances,

DOI: 10.21608/MID.2024.291528.1967
* *Corresponding author:* Hebatallah Magdy Aly Amin
E-mail address: *htmagdy@msa.edu.eg*

infection with *L. monocytogenes* is commonly caused by ingesting contaminated foods. Previous epidemiological studies have revealed outbreaks associated with consuming *L. monocytogenes*-contaminated dairy products and processed meat such as sausage, fish products, salad, and ice cream cake. In addition, studies have proposed that contaminated animal carcasses during the slaughtering process are sources of *L. monocytogenes*-contaminated meat [5,6].

The disease caused by *L. monocytogenes*, known as listeriosis, is classified into invasive listeriosis and non-invasive febrile gastroenteritis [7]. Immunosuppressed patients, neonates, older adults and pregnant women are at high risk of getting invasive listeriosis disease [8,9]. Moreover, invasive listeriosis poses a significant threat of invading the central nervous system and causing meningitis, meningoencephalitis and septicemia in old ages and infants. However, non-invasive listeriosis occurs in healthy people, causing gastroenteritis, atypical meningitis and septicemia [10,11]. The pathogenesis of *L. monocytogenes* infection is mediated by several driving virulence factors and involves multiple stages, including adhesion and invasion of host cells, internalization by host cells, vacuole lysis and diffusion to the adjacent cell [12]. Moreover, biofilm formation gives extra protection to Listeria from harsh environmental conditions such as disinfectant treatment, rendering it a threatening source of food contamination [13-15].

Recent years have seen the emergence of resistance to various antimicrobial classes among *L. monocytogenes* bacteria due to overuse use of various antimicrobial agents which resulted in acquiring target gene mutations and resistance-encoding genes on mobile genetic elements [16]. Resistance to single or multiple antimicrobials has recently been reported for *L. monocytogenes* isolates from different food and environmental sources [17,18]. Additionally, Listeria species can exchange resistance determinants of various antimicrobial classes with other bacterial species, through self-transferable plasmids, and transposons [19].

In an effort to overview the occurrence of Listeria in different Egyptian food products, this study aimed to investigate the prevalence of isolated L. monocytogenes from various frozen food products in the Egyptian market, as well as characterize underlying genetic determinants of virulence and antimicrobial resistance

2.Materials and Methods

2.1. Isolation of *L. monocytogenes* from food sources.

This study included *L. monocytogenes* isolates from 331 frozen food samples collected randomly from different unknown trade source through different Egyptian markets in Cairo City, Egypt. The food samples included frozen vegetables and fruits products: 45 okra, 16 carrots, 20 green beans, 57 artichokes, 36 molokhia, eight spinach, 11 green peas, 11 grape leaves, two broad beans, four broccoli, two cauliflowers, six mixed vegetables, 29 green salad, 61 strawberry, 14 grape, two peach and seven pomegranate samples. The contamination detection by *L. monocytogenes* bacteria was performed according to the methodology described in the ISO 11290-1 method (ISO 11290-1, Technical committee ISO iTC 34, Food products) [20]. Processing of food products for isolation and identification of *Listeria* was performed as previously described in **Mohamed and Abdelmonem** 2018 [21].

2.2. Determination of antimicrobial susceptibility profile of isolates

In vitro susceptibilities of 47 *L. monocytogenes* isolates to diverse classes of antimicrobials were tested by the Kirby–Bauer disk diffusion method on Mueller-Hinton agar (Merck, Darmstadt Germany) according to guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2020)[22]. The plates were incubated at 37°C for 24 hours. The antimicrobials representing different antimicrobial classes (Oxoid, Hampshire, UK) were selected for testing and fit with the target resistance genes to be investigated. The tested antimicrobials were ampicillin (10 µg), amoxicillin-clavulanic acid (20 µg/10 µg), imipenem (10 µg), erythromycin (15 µg), clindamycin (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), tetracycline (30 µg), cefotaxime (30 µg) and ampicillin-sulbactam (20 µg). The susceptibility was investigated by measurement of the inhibition zone around the antimicrobial disc, and the results were analyzed according to the breakpoints of CLSI [22]. *Staphylococcus aureus* 25923 and *E. coli* ATCC 25922 were used as reference strains.

2.3. Phenotypic detection of biofilm formation

The biofilm production assay was performed using a previously established method with minor modifications [23]. Bacterial cells were cultured overnight in 10 mL Luria broth (LB) (Oxoid, Hampshire, UK) at 37 °C In a 96-well flat-bottomed polystyrene tissue culture plate, aliquots of 100 µL from overnight cultures (equal to 0.5 McFarland standard (1.5 x108 CFU) were added to 130 µL of LB broth media supplemented with 0.8% glucose. The last 24 wells were inoculated with sterile broth as a negative control. The plates were covered with a lid and incubated aerobically at 37°C for 24 hours before each well's optical density (OD) was measured at λ = 620 nm. The contents of each well were then pipetted out, and the wells were thoroughly washed three times with 0.2 mL of phosphate buffer saline (pH 7.2) to eliminate any floating bacteria. The plates were then decanted and dried. Wells were then stained for 15 minutes with 130 µL of 1% crystal violet. The excess stain was washed away with running distilled water. Following an hour of air drying, the dye attached to the adherent cells was dissolved in 200 µL of 33% (v/v) glacial acetic acid per well [24]. The optical density (OD) of the stained adherent bacteria and control wells was measured at λ = 540 nm. The following tests were performed in triplicate. The following equation determined the degree of biofilm formation: SBF = (AB)- CW) G, where SBF is the Specific Biofilm Formation index, G is the OD620 nm of cells growing in suspension culture, AB is the OD540nm of stained adherent bacteria, and CW is the OD540 nm of stained control wells containing solely bacterium-free media [25]. And the findings were as follows: strong for ≥ 1.1, moderate for 0.7-1.09, and weak for 0.35-0.69.

2.4. Genotypic study of the isolated listeria strains

2.4.1. *L. monocytogenes* genomic DNA Extraction

The bacterial cells from an overnight culture were harvested by centrifugation at 10,000 ×g for 10 min. After centrifugation, genomic DNA was extracted from cell pellets using the GeneJET Genomic DNA Purification Kit (Thermofisher, Waltham, USA) according to the manufacturer's procedure. The purified DNA was eluted in 100 µL sterile distilled water and stored in aliquots at -20°C until use in PCR assays [26].

2.4.2. PCR oligonucleotide primers

PCR oligonucleotide primers used in this study are listed in **table(1)** [26-33]. The primers were synthesized by Invitrogen (Waltham, USA) and the lyophilized powders were dissolved in the required volume of nuclease-free water to achieve a stock concentration of 100 pmole/µl. The concentration of each primer was adjusted to 10 pmole/µl.

2.4.3. Molecular serotype identification by multiplex PCR

Bacterial colonies were grown in Hichrom media plates (Sigma, UK), emulsified in 50 µl of a 0.25% sodium dodecyl sulfate-0.05 N NaOH solution, and incubated at 99°C for 15 min. Then 100 µl of H2O was added to the mixture, 2 µl of which was used for the PCR. Amplification reactions were performed in a 96-well plate thermocycler (Abgene Ltd., UK) in a final volume of 100 µl containing 2 U of Taq DNA polymerase (Roche, Boehringer), 0.2 mM deoxynucleoside triphosphates, and 50 mM Tris-HCl-10 mM KCl-50 mM (NH4)2SO4-2 mM MgCl2, pH 8.3. The five primer sets were added at the final concentrations: 1 µM for lmo0737, ORF2819, and ORF2110; 1.5 µM for lmo1118 **(Table 1)**. PCR was performed with an initial denaturation step at 94°C for 3 min; 35 cycles of 94°C for 0.40 min, 53°C for 1.15 min, and 72°C for 1.15 min; and one final cycle of 72°C for 7 min in a thermocycler (I cycler), Five microliters of the reaction mixture was mixed with 3 µl of loading buffer and separated on a 2% agarose gel in a TBE buffer (90 mM Trizma base, 90 mM boric acid, 2 mM EDTA, pH 8.3). The PCR product was visualized by ethidium bromide staining and photographed directly under a UV light source [26].

2.4.4.PCR detection of the virulence and resistance-encoding genes

The 47 *L. monocytogenes* isolates were screened for virulence genes iap, prs-prfA, plcA, plcB, flaA, prfA and hly [27-30]. The isolates were also investigated for the presence of four antimicrobial resistance genes, including ampC, strB, tetA and parE, which encode for resistance to ampicillin, Streptomycin, tetracycline and ciprofloxacin respectively [31-33]. PCR reactions were carried out in a total volume of 25 µl containing 2 µl of extracted DNA, 12.5 µl of GoTaq® Green Master 2× Ready Mix (Promega, Wisconsin, USA), one µl of each primer (equivalent to 10 pmol concentration) and the volume was completed to 25 µl by nuclease-free water. The PCR

amplification programs included initial denaturation for 5 min at 95°C, then 35 cycles of denaturing at 95°C for 30 seconds, annealing for 30 seconds and extension at 72°C, followed by a final extension at 72°C for 7 min. The suitable annealing temperature for each pair of primers and the time for the extension step for each PCR amplicon are mentioned in Table 1. DNA fragments of PCR products were detected through TAE agarose gel (0.8 to 1 %) (Bioline, UK) electrophoresis in 1× TAE buffer containing ethidium bromide (Sigma-Aldrich®, Darmstadt, Germany) for DNA visualization by placing the gel on a UV light source and photographed directly. A suitable DNA molecular weight marker (Thermo Scientific, Waltham, USA) was used to size the PCR products.

2.5. Statistical analysis

The findings were presented as descriptive statistics in percentages and relative frequencies using GraphPad Prism software (GraphPad version 8.0.2. http:// www.graphpad.com/prism/prism.htm). The virulence and antimicrobial susceptibility profiles were analyzed using the Dice similarity coefficients and a dendrogram was constructed using the unweighted pair group method with arithmetic averages (UPGMA) clustering method available at http://insilico.ehu.es/dice_upgma.

3. Results

3.1. Prevalence of isolates

47 samples out of 331 (14.2%) were positive for the presence of *L. monocytogenes* (17 okra, 1 carrot, 6 green beans,9 artichoke, 8 molokia, 3 spinach, 1 green peas, 1 strawberry,1 grape leaf)

3.2. Antimicrobial susceptibility profiles of *L. monocytogenes* isolates

Most of the isolates were significantly resistant to the examined antimicrobial agents, including ampicillin, cefotaxime and clindamycin, with frequencies of 91%, 87% and 66%, respectively. The isolates showed higher sensitivity rates to imipenem and ampicillin-sulbactam with frequencies of 96% and 94%, respectively. The antimicrobial susceptibility testing results are presented in **table (2)**.

3.3. Detection of biofilm formation

The phenotypic biofilm-forming capability of 42 *L. monocytogenes* food isolates was assessed using the crystal violet (CV) assay. The results revealed that 9/42 (21.4%) of *L. monocytogenes* isolates were strong biofilm producers and 5/42(11.9%) were moderate biofilm producers, while the rest of the isolates, 28/42 (66.6%) were weak biofilm producers. The results were presented in **figure (1)**. Strong biofilm isolates showed resistance to different antimicrobial agents as presented in **table (3)**.

3.4. Molecular serogroups of *L. monocytogenes* isolates

Based on multiplex PCR serotyping, the most predominant serovars belonged to molecular serogroups 1/2a-3a (amplification of only the lmo0737 DNA fragment).

3.5. Frequencies of antimicrobial resistance genes among *L. monocytogenes* isolates

In the present study, *L. monocytogenes* isolates showed a high incidence of antimicrobial resistance genes. A high percentage of the strB gene (streptomycin resistance) and tetA gene (tetracycline resistance) were detected in *L. monocytogenes*, each with a rate of 70%. The ampC gene (ampicillin resistance) and parE gene (ciprofloxacin resistance) showed lower resistance rates of 30%. For the clindamycin resistance gene, 34% of isolates examined in the present study were susceptible **(Table 4, Figure 2).**

3.6. Frequencies of virulence genes among *L. monocytogenes* isolates

Regarding the virulence genes, PCR-based experiments revealed that all tested isolates carried more than one virulence factor encoding gene. The plcB, prs-prfA and iap genes were detected in 90%, 87% and 87% of isolates, respectively. The genes plcA, prfA and flA genes were detected in 83%, 97% and 90% of the isolates, respectively. The listeriolysin O (hlyA) gene was detected in 30 % of isolates **(Table 5, Figure 2)**. Genotypic profiles of *L. monocytogenes* isolates harboring multiple antimicrobial resistance and virulence encoding genes are presented in **table (6)** and **figure (3)**.

Table 1. Sequences of PCR oligonucleotide primers used in this study.

Target gene & function	Gene Locus*	Sequence (5' – 3')	Amplicon size (bp)	Ta/extension time	Source
Serotyping genes					
lmo0737 serovars 1/2a, 1/2c, 3a, and 3c	*lmo0737*	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCATTC	691	53°C/75 s	[26]
lmo1118 serovars 1/2c and 3c	*lmo1118*	F: AGGGGTCTTAAATCCTGGAA R: CGGCTTGTTCGGCATACTTA	906		
ORF2819** Indicate serovars 1/2b, 3b, 4b, 4d, and 4e	ORF2819	F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG	471		
ORF2110** Indicate serovars 4b, 4d, and 4e	ORF2110	F: AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597		
prs ribose-phosphate pyrophosphokinase	lmo0199	F: GCTGAAGAGATTGCGAAAGAAG R: CAAAGAAACCTTGGATTTGCGG	370		
Virulence factors encoding genes					
iap: extracellular protein p60, invasion-associated secreted endopeptidase	lmo0582	F: CAAGCTGCACCTGTTGCAG R: TGACAGCGTGTGTAGTAGCA	171	57°C/30 s	[27]
prs-prfA: encodes a product homologous to the posttranslocation molecular chaperone protein PrsA	*lmo2219*	F: CGAAACTGCTGGTGCAACTA R: AACCAATGGGATCCACAAGA	203	58°C/30 s	[28]
plcB: inositol 1,4,5-trisphosphate and diacylglycerol formation	lmo0205	F: GCATGATATTGACAGCAAATTA R: TGAAATACTTTGCTCCTGTT	246	55°C/30 s	[29]
plcA: secreted phosphatidylinositol-specific phospholipase C	lmo0201	F: CAGCATACTGACGAGGTGTG R: GATGTCCGCTCTACCTGA	233	57°C/30 s	
flaA: flagellin gene	lmo0690	F: TTACTAGATCAAACTGCTCC R: AAGAAAAGCCCCTCGTCC	125	55°C/30 s	[30]
prfA: a protein that activates transcription of the listeriolysin gene	lmo0200	F: AACCAATGGGATCCACAAG R: ATTCTGCTAACAGCTGAGC	232	59°C/30 s	[29]
hlyA: listeriolysin o precursor	lmo0202	F: AAACTGAAGCAAAGGATGCA R: CTAATGTATTTACTGCGTTGTTA	162	57°C/30	
Antimicrobial resistance encoding genes					
tetA: tetracycline resistance	lmo0839	F: GGCCTCAATTTCCTGACG R: AAGCAGGATGTAGCCTG	372	52°C / 30 s	[31]
strB: aminoglycoside 3'-phosphotransferase 2	Gene ID: 2830393	F: ATCGTCAAGGGATTGAAACC R: GGATCGTAGAACATATTGGC	509	52°C/40 s	[32]
ampC: beta-lactamase	lmo0540	F: TTCTATCAAACTGGCACC R: CCTTTTATGTACCCAGA	550	50°C/40 s	[33]
parE: DNA topoisomerase 4 subunit B	lmo1286	F: AGGCGAAAGACATTTGAGT R: TACGGTCAGTTTCATCACG	470	52°C / 30 s	
*ORF2819 and ORF2110, identified in the partial sequence of *L. monocytogenes* 4b strain CLIP 80459 [26]					

Table 2. Antimicrobial resistance frequencies of *L. monocytogenes* isolates.

Antimicrobial agent	Resistant No (%)*	Sensitive No (%)*
Amoxicillin-clavulanic acid	8 (17%)	39 (83%)
Ampicillin	43 (91%)	4 (9%)
Ampicillin-sulbactam	3 (6%)	44 (94%)
Cefotaxime	41 (87%)	6 (13%)
Ciprofloxacin	8 (17%)	39 (83%)
Clindamycin	31 (66%)	16 (34%)
Erythromycin	5 (11%)	42 (89%)
Gentamicin	15 (32%)	32 (68%)
Imipenem	2 (4%)	45 (96%)
Tetracycline	7 (15%)	40 (85%)

*Percentages correlated to the total number of isolates (47 isolates)

Table 3. Correlation between strong biofilm forming isolates and antimicrobial resistance.

Isolates no.	Biofilm Index	Resistance pattern
7	2.3	Resistant to tetracycline, cefotaxime, gentamicin and clindamycin
17	1.4	Resistant to cefotaxime
21	1.5	Resistant to erythromycin, clindamycin, tetracycline and cefotaxime
24	1.2	Resistant to clindamycin and cefotaxime
27	1.4	Resistant to amoxicillin, clindamycin and cefotaxime
28	2	Resistant to tetracycline and cefotaxime
35	1.102	Resistant to clindamycin and cefotaxime
37	1.165	Resistant to cefotaxime
42	1.03	Resistant to tetracycline, cefotaxime and gentamicin

Table 4. Frequencies of antimicrobial resistance encoding genes among isolates.

Resistance gene	Number of positive isolates (%)*
strB	33 (70%)
tetA	33 (70%)
ampC	14 (30%)
parE	14 (30%)

*Percentages correlated to the total number of isolates (47 isolates).

Table 5. Prevalence of examined virulence traits encoding genes among *L. monocytogenes* isolates.

Virulence genes	No. (%)*
plcB	42 (90)
prs-prfA	41 (87)
iap	41 (87)
plcA	39 (83)
prfA	46 (97)
flaA	42 (90)
hlyA	14 (30)

*Percentages correlated to the total number of isolates (47 isolates).

Table 6. Genotypic profiles of *L. monocytogenes* isolates harbouring antimicrobial resistance and virulence traits encoding genes.

Pattern	Genotypic profile	Total no. isolates (%)*	Resistance genes	Virulence genes
			No.	No.
I	*tetA, plcB, plcA, iap, hlyA, prfA, flaA*	2 (4.25)	1	6
II	*tetA, plcB, plcA, iap, hlyA, prfA, flaA, prs-prfA*	2 (4.25)	1	7
III	*ampC, strB, tetA, plcB, plcA, iap, prfA, flaA, hlyA*	2 (4.25)	3	7
IV	*pare, ampC, strB, plcB, plcA, iap, hlyA, prfA, flaA, prs-prfA*	1 (2.21)	3	7
V	*pare, strB*	2 (4.25)	2	0
VI	*plcB, plcA, iap, prs-prfA, flaA, hlyA*	4 (8. 5)	0	6
VII	*plcB, prs-prfA, prfA, flaA, hlyA*	2 (4.25)	0	5
VIII	*plcB, plcA,iap, hlyA, prfA, flaA*	12 (25.5)	0	6
IX	*plcA, iap, prs-prfA, prfA, flaA, hlyA*	2 (4.25)	0	6
X	*plcB, plcA, hlyA, prfA, flaA*	2 (4.25)	0	6
XI	*plcA, iap, prs-prfA, prfA, hlyA*	2 (4.25)	0	5
XII	*plcB, iap, prs-prfA, prfA, flaA, hlyA*	2 (4.25)	0	6

*Percentages correlated to the total number of isolates (47 isolates).

Figure 1. Isolates are classified into strong, moderate, and weak biofilm-forming.

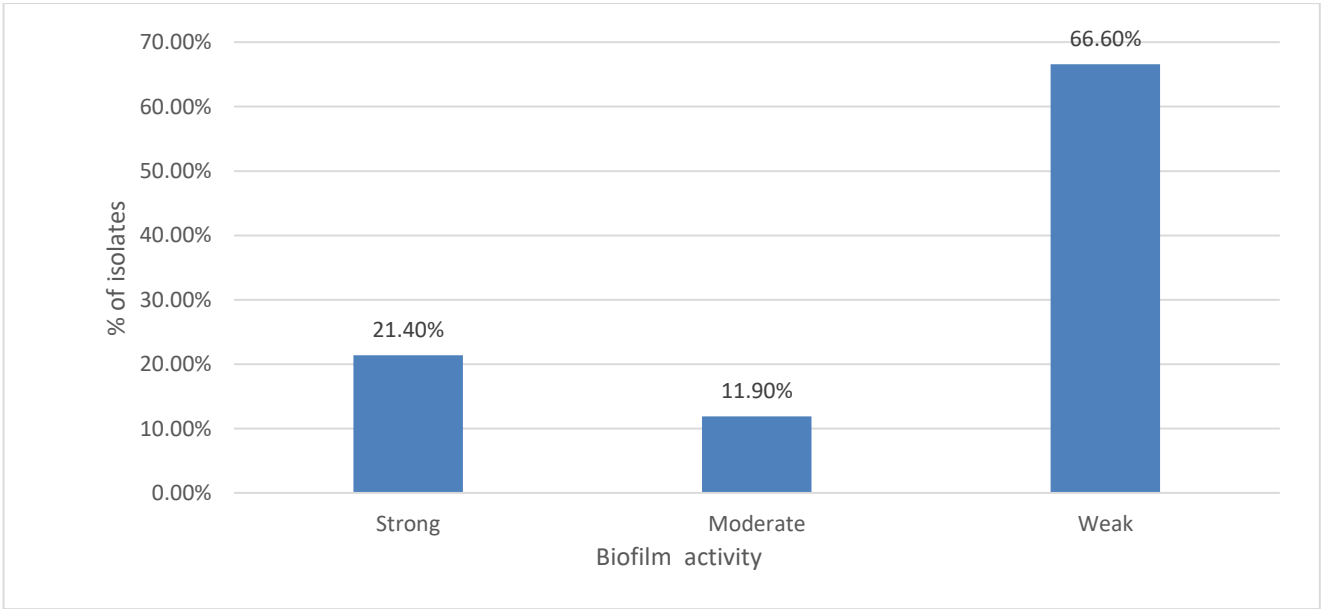


Figure 2. Frequencies of detected resistance genes (a) and virulence genes(b) among *L. monocytogenes* isolates.

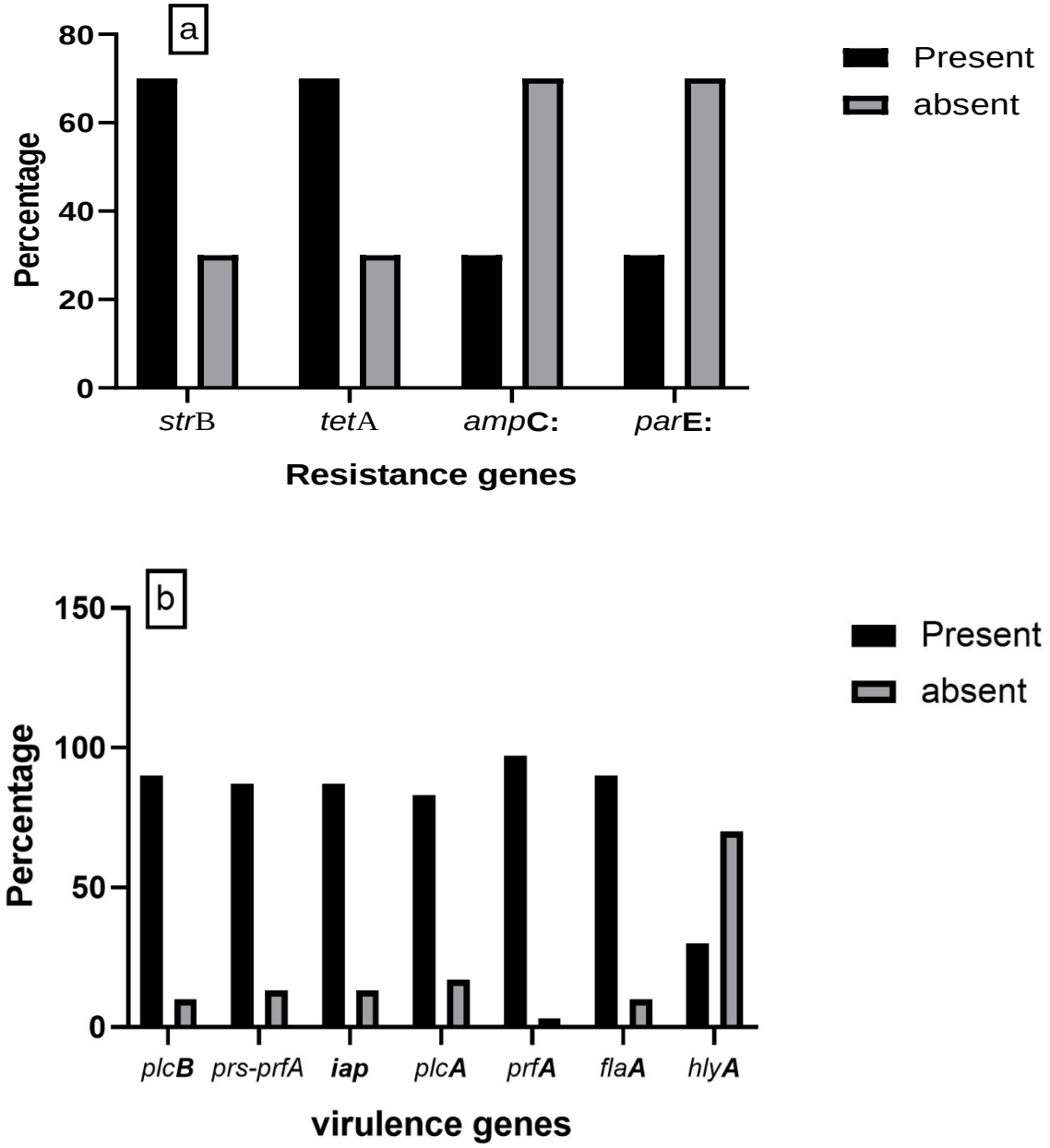
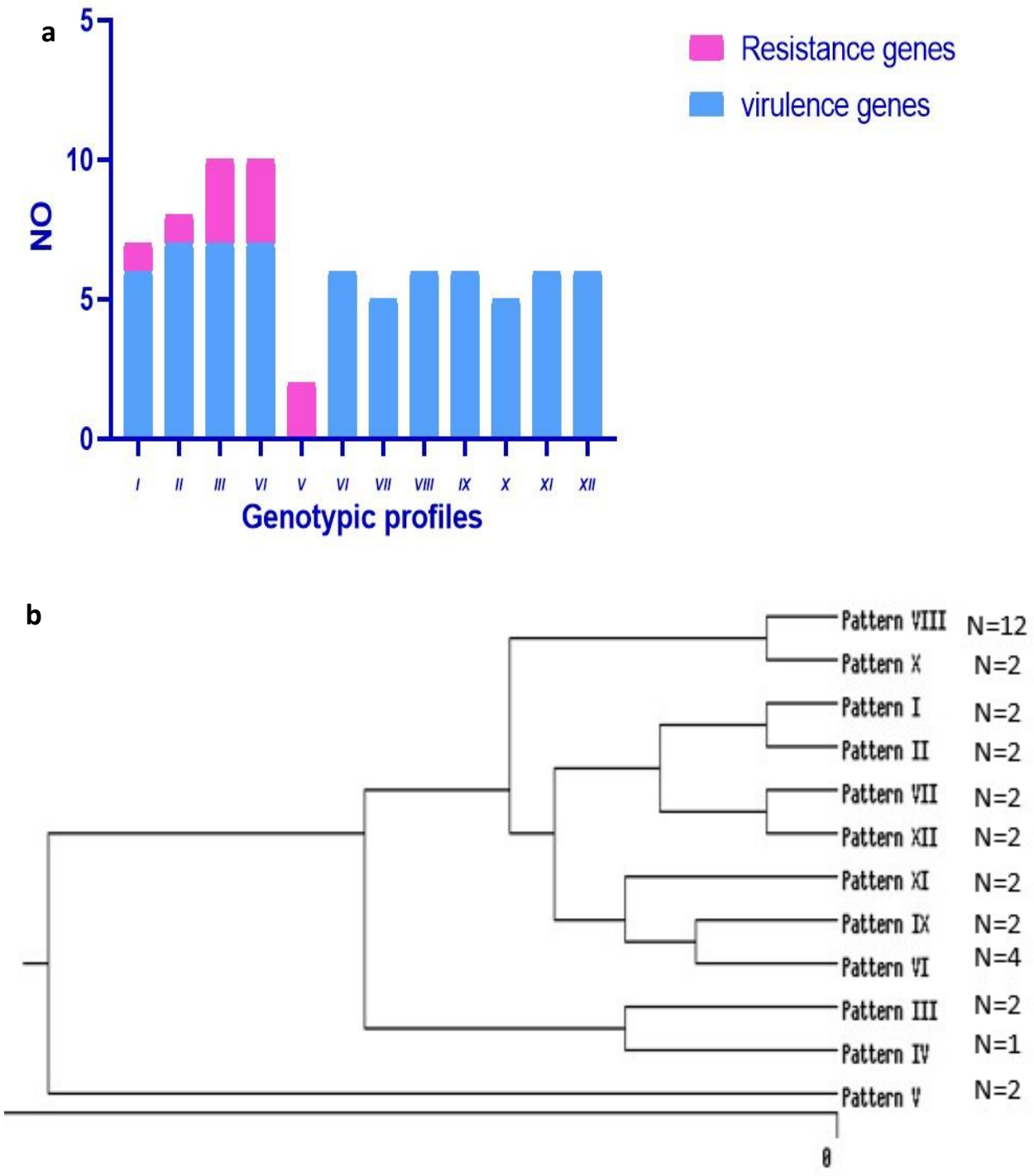


Figure 3. Distribution of the genetic profiles of antimicrobial resistance and virulence genes *among L. monocytogenes* isolates. a: Frequencies of antimicrobial resistance and virulence genes in each pattern, b: Dendrogram of antimicrobial resistance and virulence genes patterns of *L. monocytogenes* isolates. N is the number of isolates in each pattern. Cluster analysis was generated with the Dice similarity coefficient and the UPGMA clustering method. N.B. All Patterns mentioned in **table (5).**



Discussion

Listeria monocytogenes bacteria are commonly present in ready-to-eat and frozen foods by cross-contamination during food processing or in food-related environments [34]. Remarkably, previous studies have indicated the occurrence of *Listeria* in different Egyptian food products, which may pose a risk to public health [21,35,36]. The Egyptian code for food hygiene and regulations is overseen by the National Food Safety Authority (NFSA). The NFSA has modernized the regulatory framework to ensure food safety and quality. This includes requirements for specialty foods, and guidelines for food handling, processing, storage, and distribution to prevent contamination and ensure public health. The regulations align with international standards to facilitate trade and protect consumers [37].

In the present study, *L. monocytogenes* was identified in samples of various frozen food products. Out of 331 frozen food samples, it was found that 47 (14.2%) confirmed positive for *L. monocytogenes*. These findings were consistent with a study conducted in China by **Wu et al.** [18], which showed that frozen food products had the highest percentage of *L. monocytogenes* contamination. The authors reported that 44.9% of frozen foods was positive for *L. monocytogenes* [18]. In addition, **Soni et al.** (2014) from India detected that 6 (10 %) of the different vegetables tested were positive for *L. monocytogenes* [38]. **Abdeen et al.** study (2021) from Egypt reported that the overall prevalence of *L. monocytogenes* was 6.8% in dairy and meat products [39]. These findings agree with our results that frozen foods are a significant source of *L. monocytogenes.* The variation in the detection rate of *L. monocytogenes* throughout studies may be related to varied types of foods, sample size, geographical region and the degree of safety measures used during food processing and production [39]. Because *L. monocytogenes* may colonize food processing environments, there is a severe risk of end-product contamination and subsequent outbreaks of listeria food poisoning for consumers and food industry operators [40].

The first-line antimicrobials for listeriosis treatment include beta-lactams ampicillin and penicillin G, which are typically combined with gentamicin or another aminoglycoside. Antimicrobial susceptibility testing results in the current study revealed high resistance levels to ampicillin (91%), which agrees with previous reports [39,41]. Compared to this study, earlier studies revealed complete sensitivity or low resistance rate to ampicillin and penicillin among *Listeria* spp. isolates [38,39,41,42]. The resistance rates to cefotaxime and clindamycin were 87% and 66%, respectively. In a study from Egypt in 2019, **Osman et al.** found that all 27 *L. monocytogenes* isolates were resistant to cefotaxime or clindamycin. In the present study, an average resistance was found for gentamicin (32%), ciprofloxacin (17%), amoxicillin-clavulanic acid (17%) and tetracycline (15%). However, higher rates of resistance were reported in a previous study by **Soni et al.**, where 90% of *L. monocytogenes* were resistant to ciprofloxacin and 85 % were resistant to cefoxitine [38]. **Kwartenget et al.** (2018) revealed a high level of resistance to neomycin (61.3%) and tetracycline (24.2%) in *L. monocytogenes* isolates [42]. Similarly, an Egyptian study by **Abdeen et al.** [39] found that 76.4%, 29.5%, and 29.5% of *L. monocytogenes* were resistant to oxytetracycline, gentamicin and ciprofloxacin, respectively. In this study, *L. monocytogenes* isolates showed lower resistance to imipenem (4%) and ampicillin-sulbactam (6%) antibiotics. Consistent with these findings, **Şanlıbaba et al.** (2018) [43] in Turkey found high resistance to penicillin G and ampicillin and full susceptibility to imipenem. The presence of mobile genetic components from unrelated bacterial species shows that Gram-negative and Gram-positive bacteria may play a role in *L. monocytogenes* resistance gene acquisition [44].

In the current study, only 21.4% of *L. monocytogenes* isolates were strong biofilm producers, with the rest of the isolates forming weak (66.6%) or moderate (11.9%) biofilms. Comparable findings were reported by **Doijad et al.** (2015) [45], who identified 9.18% of their isolates as strong biofilm formers, 63.26% as weak and 27.55% as moderate biofilm formers. Previous studies have revealed that *L. monocytogenes* cells form weak to moderate biofilms [46,47]. The ability of microorganisms to form biofilms is an adapting and resisting mechanism that facilitates the transfer of genetic material, increases the availability of nutrients for growth and provides resistance to antimicrobial agents . Thus, it is easier for the microorganisms that make up biofilms to survive on equipment and surfaces that come into contact with food, establishing a continuous source of contamination [48]. Altogether, reducing or

eliminating the risk of recontamination due to the development of biofilms on industrial surfaces is the best strategy to control *L. monocytogenes* in food industry settings. As a result, every potential contamination source must be identified, and control strategies must be implemented to eliminate *L. monocytogenes* [49].

Listeria monocytogenes strains have been grouped into four lineages, with lineages I and II being the most common. Lineage I include serotypes 1/2b and 4b frequently associated with human listeriosis disease. Lineage II, consisting of serotypes 1/2a and 1/2c, is the most prevalent in foods and food processing facilities [50]. Although *L. monocytogenes* bacteria comprise 13 serotypes based on somatic and flagellar antigens, the serotypes 1/2b, 4b, 1/2a and 1/2c account for 90% of global listeriosis outbreaks [51]. Based on multiplex PCR-based serotyping in this study, the most predominant serotypes in *L. monocytogenes* isolates belonged to molecular serogroups 1/2a-3a. Serotypes 1/2a, 1/2b, 1/2c, and 4b have been linked to 98% of human listeriosis cases worldwide.

Various serotypes have been reported to develop different population patterns and may have varying abilities to oppose environmental stress [52]. Regarding prevalence, *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2b, and 4b, and, to a lesser extent, 1/2c, cause the vast majority of listeriosis cases. In accordance with these findings, isolates of serotype 1/2a are more common in food processing environments than isolates of serotype 4b [42,53]. The increased occurrence and higher capability of serotype 1/2a to persist in dietary environments may be due to the group's potential possession of more plasmids, which often frequently confer tolerance to hazardous chemicals [54].

Listeria monocytogenes pathogenicity depends on many virulence factors and antimicrobial resistance genes. In this study, some isolates carried genes encoding resistance to different antimicrobial classes, including β -lactams *ampC* (30%), tetracycline *tetA* (70%), aminoglycosides *strB* (70%), quinolone *parE* (30%). These results were in agreement with a study in Egypt by **El-Banna et al.** (2016) [55], where the incidence of antimicrobial resistance genes among *L. monocytogenes* isolates was *strA* (38%), *tetM* (20%) and *ampC* (18 %). Another study in South Africa by **Kayode et al.** (2022) demonstrated that the antimicrobial resistance genes among their isolates were as follows: β -lactams (bla_{TEM}, bla_{SHV} and bla_{TEM} variants (35.71%), *tetA* (33.33%) and aminoglycoside resistance gene aph(3)-IIa (aphA2) (11.11%) [56]. Additionally, a study from Sri Lanka by **Harshani et al.** (2022) detected *tetA* (14%) and aminoglycoside resistance gene (14%) while they reported the absence of the β -lactams resistance gene *ampC* [57].

Although β-lactams had the highest phenotypic resistance profiles in *L. monocytogenes* isolates in this search, the gene responsible for resistance to these antibiotics, *ampC*, was not highly found among isolates (30%). This agreed with previous studies [55-57], where the phenotypic resistance does not completely match the genotypic data. The inconsistent genotype-phenotype connection might be attributed to the presence of other resistance genes or factors that were not addressed in this study. In addition, antimicrobial resistance genes are not the leading cause of phenotypic antimicrobial resistance [57]. Several possible pathways involving efflux pumps that leak antimicrobial compounds outside the cell have also been implicated [58]. On the other hand, the incidence of *tetA* and *strB* were higher than their phenotypic expression in this study, and this might be because the favourable expression of the antimicrobial resistance gene is affected by multiple factors, such as adequate nutrition, temperature variation and antibiotic use [57].

Screening of the seven virulence genes *prs-prfA, plcB, plcA, iap, hlyA, prfA, flaA* showed that the *prfA, plcB* and *flaA* were the most detected virulence genes with prevalence rates of 97%, 90% and 90%, respectively, while the prevalence of *prs-prfA, iap, plcA* and *hlyA* were 87%, 87%, 83% and 30%, respectively. Of note, simultaneous detection of the seven virulence genes and three antimicrobial resistance genes was found in three *L. monocytogenes* isolates. Our findings support a recent study in Egypt that detected *hlyA* (70.6%), *iap* (70.6%) and *actA* (52.9%) as the most prevalent virulence genes [39]. Another study in Ghana showed that all *L. monocytogenes* tested isolates (100 %) harboured the virulence-associated genes *plcA, actA, hlyA, iap* and *prfA* [42]. In an Egyptian study, four virulence genes *prf A, plcA, plcB,* and *actA* were detected in all isolates; the potential virulence of their isolates was further confirmed by infection and mortality of mice, chick embryos and Vero cells [41]. Moreover, **Kaur and colleagues** in India found that all *L. monocytogenes* isolates

possess the *prfA, plcA, actA, hlyA* and *iap* virulence-related genes [59]. The carriage of *L. monocytogenes* isolates for the virulence genes *iap, inlA, actA, plcB, inlB* and *prfA* possibly enable them to cause listeriosis in humans [60]. In the current study, *hlyA* (30%) gene was carried by a small number of *L. monocytogenes* isolates. Many previous studies found a similar low prevalence of *hlyA* gene [41,61]. The lower percentage of the listerolysin O encoding gene *hlyA*, which is crucial for intracellular pathogenesis, could indicate a reduction in sequence homology, indicating the evolutionary adaptation of the *hlyA* gene.

Conclusion

The study highlights the significance of *L. monocytogenes* as a major contaminant of frozen vegetables and fruits and the necessity for greater awareness and safety measures across the food supply chain. Most *L. monocytogenes* isolates were resistant to many tested drugs, adding to existing serious concerns about global antibiotic resistance.

Limitation

Further research is needed to find genetic relatedness between *L. monocytogenes* isolates and worldwide clones to demonstrate the spread of these strains throughout the international food trade. The study did not correlate the phenotypic character of biofilm activity with the biofilm-associated genes.

Conflict of Interest

Authors declare no conflict of interest

Authors' contributions

Conceptualization, H.M.A.; Methodology, L.I.F., H.M.A; software, L.I.F.; formal analysis, L.I.F, H.M.A.; investigation, L.I.F., H.M.A.; data curation, L.I.F; writing—original draft preparation, L.I.F, H.M.A., writing—review and editing, H.M.A.; All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Acknowledgements

We thank Dr. Mohamed Abdalla Abdelmonem and Rasha I. Mohamed from the Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Foods (Microbiology Analysis Department) at Agricultural Research Center Dokki, Giza, Egypt, for help in samples collection and processing.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in creating this article.

References

1- **Mohan V, Cruz C D, van Vliet AH, Pitman AR, Visnovsky SB, Rivas L, et al.** Genomic diversity of Listeria monocytogenes isolates from seafood, horticulture and factory environments in New Zealand. International Journal of Food Microbiology (2021) ; 347: 109166.

2- **Sibanda T, Buys E M** .Listeria monocytogenes Pathogenesis: The Role of Stress Adaptation. Microorganisms (2021) ;10(8): 1522.

3- **Gandhi M, Chikindas ML.** Listeria: a foodborne pathogen that knows how to survive. International journal of food microbiology (2007),113(1):1-15.

4- **Liu D, Bai X, Helmick, HD, Samaddar M, Amalaradjou, MAR, Li X. et al.** Cell-surface anchoring of Listeria adhesion protein on L. monocytogenes is fastened by internalin B for pathogenesis. Cell Reports (2023),2(5).

5- **Nightingale K, Schukken Y, Nightingale C, Fortes E, Ho A, Her Z, et al.** Ecology and transmission of Listeria monocytogenes infecting ruminants and in the farm environment. Applied environmental microbiology (2004); 70(8): 4458-4467.

6- **Lekkas P.** The Microbial Ecology Of Listeria monocytogenes As impacted by three environments: A cheese microbial community; a farm environment; and a soil microbial community. Envirnomental Science, Biology and Agricultural and Food Sciences (2016).

7- **Buchanan RL, Gorris LG, Hayman MM, Jackson TC, Whiting RC.** A review of Listeria monocytogenes: An update on outbreaks, virulence, dose-response, ecology,

and risk assessments. Food control (2017); 75: 1-13.

8- **Todd E, Notermans S.** Surveillance of listeriosis and its causative pathogen, Listeria monocytogenes. Food control (2011); 22(9):1484-1490.

9- **Kang Q h, Guo Y, Hong S-q, Jiang L**. Listeria monocytogenes meningitis in infants: report of two cases. Journal of Contemporary Pediatrics (2013); 15(12) : 1150-1152.

10- **Mateus T , Silva J, Maia RL , Teixeira P.** Listeriosis during pregnancy: a public health concern. International Scholarly Research Notices (2013).

11- **Osek J, Wieczorek K.** Listeria monocytogenes—How this pathogen uses its virulence mechanisms to infect the hosts. Pathogens (2022);11(12):1491.

12- **Schuppler M, Loessner M J. The** opportunistic pathogen Listeria monocytogenes: pathogenicity and interaction with the mucosal immune system. International journal of inflammation (2010).

13- **Esbelin J, Santos T, Hébraud M.** Desiccation: an environmental and food industry stress that bacteria commonly face. Food microbiology (2018); 69:82-88.

14- **Lee BH, Cole S, Badel-Berchoux S, Guillier L, Felix B, Krezdorn N, et al.** Biofilm formation of Listeria monocytogenes strains under food processing environments and pan-genome-wide association study. Frontiers in Microbiology (2019); 10: 2698.

15- **Bai X, Liu D, Xu L, Tenguria S, Drolia R, Gallina N L, et al.** Biofilm-isolated Listeria monocytogenes exhibits reduced systemic dissemination at the early (12–24 h) stage of infection in a mouse model. Microbiomes (2021a); 7(1):18.

16- **Luque-Sastre L, Arroyo C, Fox EM, McMahon BJ, Bai L, Li F, et al.** Antimicrobial resistance in Listeria species. Microbiology spectrum (2018); 6(4):6.4. 19.

17- **Chen M, Wu Q, Zhang J, Wu S, Guo W.** Prevalence, enumeration, and pheno-and genotypic characteristics of Listeria monocytogenes isolated from raw foods in South China. Frontiers in Microbiology (2015); 6:1026.

18- **Wu S, Wu Q, Zhang J, Chen M , Yan, Z a, Hu H.** *Listeria monocytogenes* prevalence and characteristics in retail raw foods in China. PLoS One (2015); 10(8):e0136682.

19- **Matereke LT, Okoh AI.** Listeria monocytogenes virulence, antimicrobial resistance and environmental persistence: A review. Pathogens (2020); 9(7):528.

20- **ISO E.** 7937. Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of Listeria monocytogenes (2004).

21- **Mohamed RI, Abdelmonem, Abdelmonem A Mohamed AHM.** Virulence and antimicrobial susceptibility profile of Listeria monocytogenes isolated from frozen vegetables available in the Egyptian market. African journal of microbiology research (2018); 12(9):218-224.

22- **Clinical and Laboratory Standards Institute.** Performance standards for antimicrobial susceptibility testing, 30th ed. CLSI Supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA. 2020.

23- **Danese PN, Pratt LA, Dove SL, Kolter R.** The outer membrane protein, antigen 43, mediates cell-to-cell interactions within Escherichia coli biofilms. Molecular Microbiology (2000); 37(2): 424-432.

24- **Naves P, Del Prado G, Huelves L, Gracia M, Ruiz V, Blanco J , et al.** Measurement of biofilm formation by clinical isolates of Escherichia coli is method-dependent. Journal of applied microbiology (2008); 105(2): 585-590.

25- **Niu C, Gilbert E.** Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Applied environmental microbiology (2004); 70(12):6951-6956.

26- **Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P.** Differentiation of the major Listeria monocytogenes serovars by multiplex PCR. Journal of clinical microbiology 42(8): 3819-3822.microbe (2004); 23(4):470-484. e477.

27- **Mureddu A, Mazza R, Fois F, Meloni D, Bacciu R, Piras F, et al.** *Listeria monocytogenes* persistence in ready-to-eat sausages and in processing plants. Italian Journal of Food Safety (2014); 3(1).

28- **Johnson J, Jinneman K, Stelma G, Smith B, Lye D, Messer J, et al.** Natural atypical Listeria innocua strains with Listeria monocytogenes pathogenicity island 1 genes. Applied environmental microbiology (2004); 70(7):4256-4266.

29- **Jung H-J, Park S-H, Ha S-D, Lee K-H, Chung D H, Kim C-H, et al.** Species-specific detection of Listeria monocytogenes using polymerase chain reaction assays targeting the prfA virulence gene cluster. Bioscience, biotechnology, biochemistry (2009); 73(6):1412-1415.

30- **Zhang Y, Yeh E, Hal G, Cripe J, Bhagwat A A, Meng J.** Characterization of Listeria monocytogenes isolated from retail foods. International Journal of Food Microbiology (2007):113(1), 47-53.

31- **Guillaume G, Verbrugge D, Chasseur-Libotte M-L, Moens W, and Collard J-M.** PCR typing of tetracycline resistance determinants (Tet A–E) in Salmonella enterica serotype Hadar and in the microbial community of activated sludges from hospital and urban wastewater treatment facilities in Belgium. FEMS Microbiology Ecology (2000); 32(1):77-85.

32- **Poyart-Salmeron C, Carlier C, Trieu-Cuot P, Courvalin P, Courtieu A.** Transferable plasmid-mediated antibiotic resistance in Listeria monocytogenes. The Lancet (1990); 335(8703):1422-1426.

33- **Lanz R, Kuhnert P, Boerlin P.** Antimicrobial resistance and resistance gene determinants in clinical Escherichia coli from different animal species in Switzerland. Veterinary microbiology (2003); 91(1): 73-84.

34- **Ferreira V, Wiedmann M, Teixeira P, Stasiewicz M**. Listeria monocytogenes persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. Journal of food protection (2014); 77(1): 150-170.

35- **Elshinaway S, Meshref A, Zeinhom M, Hafez D.** Incidence of listeria species in some dairy products in beni-suef governorate. Assiut Veterinary Medical Journal (2016); 63(152):5-13.

36- **Ayaka N, HajimeT, Takashi K, Bon K.** Microbial safety and biodiversity of bacterial communities in traditional Egyptian cheese types. African Journal of Food Science (2022); 16(8):203-214.

37- **Ibrahim NA, Abdel-Haleem AMH.** Food Regulations and Enforcement in Egypt. Module in Food Sciences. Elsevier, (2017); 1–6.

38- **Soni DK , Singh M, Singh DV, Dubey SKJBm.** Virulence and genotypic characterization of Listeria monocytogenes isolated from vegetable and soil samples (2014); 14(1): 1-10.

39- **Abdeen EE, Mousa WS, Harb OH, Fath-Elbab GA, Nooruzzaman M, Gaber A, et al.** Prevalence, antibiogram and genetic characterization of Listeria monocytogenes from food products in Egypt. Foods (2021); 10(6):1381.

40- **Truchado P, Gil MI, Querido-Ferreira AP, Capón CL, Álvarez-Ordoñez A, Allende A.** Frozen Vegetable Processing Plants Can Harbour Diverse *Listeria monocytogenes* Populations: Identification of Critical Operations by WGS. Foods (2022); 11(11):1546.

41- **Osman KM, Kappell AD, Fox EM, Orabi A, Samir A.** Prevalence, pathogenicity, virulence, antibiotic resistance, and phylogenetic analysis of biofilm-producing Listeria monocytogenes isolated from different ecological niches in Egypt: Food, humans, animals, and environment. Pathogens (2019); 9(1): 5.

42- **Owusu-Kwarteng J, Wuni A, Akabanda F Jespersen L.** Prevalence and characteristics of Listeria monocytogenes isolates in raw milk, heated milk and nunu, a spontaneously fermented milk beverage, in Ghana. Beverages (2018); 4(2): 40.

43- **Şanlıbaba P, Tezel BU, Çakmak GA** . Prevalence and antibiotic resistance of Listeria monocytogenes isolated from ready-to-eat foods in Turkey. Journal of Food Quality (2018).

44- **Walsh D, Duffy G, Sheridan J, Blair I, McDowell D.** Antibiotic resistance among Listeria, including Listeria monocytogenes, in retail foods. Journal of Applied Microbiology (2001); 90(4): 517-522.

45- **Doijad SP, Barbuddhe SB, Garg S, Poharkar KV, Kalorey DR, Kurkure NV, et al.** Biofilm-forming abilities of Listeria monocytogenes serotypes isolated from different sources. PloS one (2015); 10(9): e0137046.

46- **Borucki MK, Peppin JD, White D, Loge F, Call DR**. Variation in biofilm formation among strains of Listeria monocytogenes. Applied environmental microbiology (2003); 69(12), 7336-7342.

47- **Harvey J, Keenan K, Gilmour A.** Assessing biofilm formation by Listeria monocytogenes strains. Food Microbiology (2007); 24(4):380-392.

48- **Yin W, Wang Y, Liu L, He J.** Biofilms: the microbial “protective clothing” in extreme environments. International journal of molecular sciences (2019); 20(14):3423.

49- **Mazaheri T, Cervantes-Huamán BR, Bermúdez-Capdevila M, Ripolles-Avila C, Rodríguez-Jerez JJ.** Listeria monocytogenes biofilms in the food industry: is the current hygiene program sufficient to combat the persistence of the pathogen? Microorganisms (2021); 9(1): 181.

50- **Alvarez-Molina A, Cobo-Díaz JF, López M, Prieto M, de Toro M, Alvarez-Ordóñez A.** Unraveling the emergence and population diversity of Listeria monocytogenes in a newly built meat facility through whole genome sequencing. International Journal of Food Microbiology (2021); 340: 109043.

51- **Zhang X, Niu Y, Liu Y, Lu Z, Wang D, Cui X, et al**. Isolation and characterization of clinical Listeria monocytogenes in Beijing, China, 2014–2016. Frontiers in microbiology (2019); 10: 981.

52- **Dunn KA, Bielawski JP, Ward TJ, Urquhart C, Gu H**. Reconciling ecological and genomic divergence among lineages of Listeria under an "extended mosaic genome concept". Molecular Biology and evolution (2009); 26(11):2605-2615.

53- **Ortiz S, López V, Villatoro D, López P, Dávila JC, Martinez-Suarez JV.** A 3-year surveillance of the genetic diversity and persistence of Listeria monocytogenes in an Iberian pig slaughterhouse and processing plant. Foodborne pathogens disease (2010); 7(10):1177-1184.

54- **Korsak D, Borek A, Daniluk S, Grabowska A, Pappelbaum K.** Antimicrobial susceptibilities of Listeria monocytogenes strains isolated from food and food processing environment in Poland. International Journal of Food Microbiology (2012); 158(3): 203-208.

55- **El-Banna T, Sonbol F, Zaki M, El-Sayyad H.** Clinical and environmental prevalence and antibiotic susceptibility of Listeria monocytogenes in Dakahlea Governorate, Egypt. Clin Microbiol (2016); 5(249): 2.

56- **Kayode AJ, Okoh AI** . Assessment of multidrug-resistant Listeria monocytogenes in milk and milk product and One Health perspective. Plos one (2022); 17(7): e0270993.

57- **Harshani HBC, Ramesh R, Halmillawewa AP, and Wijendra WA.** Phenotypic and genotypic characterization of antibiotic resistance of Listeria monocytogenes isolated from raw milk samples collected from Polonnaruwa, Sri Lanka. Emirates Journal of Food Agriculture (2022).

58- **Olaimat AN, Al-Holy MA, Shahbaz H M, Al-Nabulsi AA, Abu Ghoush MH, Osaili TM, et al.** Emergence of antibiotic resistance in Listeria monocytogenes isolated from food products: a comprehensive review. Comprehensive Reviews in Food Science Food Safety (2018); 17(5): 1277-1292.

59- **Kaur S, Singh R, Sran MK, Gill J.** Molecular characterization of Listeria monocytogenes in white meat samples from Punjab, India. Indian Journal of Animal Research (2018); 52(11): 1635-1641.

60- **Cao X, Wang Y, Wang Y, Ye C.** Isolation and characterization of Listeria monocytogenes from the black-headed gull feces in Kunming, China. Journal of Infection Public Health(2018); 11(1):59-63.environmental microbiology 68(2): 728-737.

61- **Al-Nabulsi AA, Osaili TM, Awad AA, Olaimat AN, Shaker RR, Holley RA .** Occurrence and antibiotic susceptibility of Listeria monocytogenes isolated from raw and processed meat products in Amman, Jordan. CYTA-J FOOD (2015) ; 13(3):346–352.