OPEN ACCESS
Journal of Animal Health and Production

Research Article

Molecular Detection of *Listeria* Species Isolated From Raw Milk with Special Reference to Virulence Determinants and Antimicrobial Resistance in *Listeria monocytogenes*

Hams M.A. Mohamed[1*], Katreen K.G.[2], M.W. Abd Al-Azeem[1], Faysal A. Wasel[2], Ahmed M. Abd-Eldayem[3]

[1]Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt; [2]Department of Microbiology, Animal Health Research Institute, Sohag, Egypt; [3]Departement of Pharmacology, Faculty of Medicine, Assuit University, Assuit, Egypt.

Abstract | *Listeria monocytogenes* is classified as a major foodborne pathogen associated with high mortalities due to severe invasive listeriosis, meningitis and abortion in humans. With particular emphasis on *Listeria monocytogenes'* capacity to form biofilms, antimicrobial resistance profile and virulence determinants, the purpose of this study is to discuss the presence of *Listeria* species in raw milk. In total, 150 samples of raw milk from vendors and nearby dairy farms in Sohag, Egypt, were collected. The samples underwent a bacteriological investigation. The results showed that 22 out of 150 samples had *Listeria* spp. contamination. Polymerase chain reaction (PCR) using the *Listeria iap* gene identified 13 of the 22 isolates that were reported as biochemically positive *Listeria* spp. Eight isolates of *Listeria monocytogenes* were molecularly confirmed, while the remaining five were subjected to *16S rRNA* sequence analysis and identified as *L. innocua* (three isolates) and *L. welshimeri* (two isolates). The antibiotic susceptibility profiling revealed multidrug resistance of *L. monocytogenes* strains against several antimicrobials in addition, they harbored antibiotic resistance genes, including. *amp*C, *aad6*, *tet*M each present in 100% of our isolates and *mef*A (37.5%). Furthermore, different virulence genes, including the most often found virulence-associated genes *hly*A and *inl*B (100% for each), *inl*A (50%) were present in our isolates. Interestingly, all of the isolates demonstrated varying degrees of biofilm forming capability. The pathogenicity of *L. monocytogenes*, particularly virulent, drug-resistant and biofilm-forming strains, is highlighted in this study and can result in a public health danger when present in raw milk. Therefore, it is essential to keep an eye on bacterial resistance in the setting of food production.

Keywords | *Listeria* spp., *L. monocytogenes*, Virulence genes, Biofilm, antimicrobial resistance

Received | August 20, 2022; **Accepted** | September 20, 2022; **Published** | November 15, 2022
***Correspondence** | Hams M.A. Mohamed, Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt; **Email:** drhams85@yahoo.com; hams.mohamed@vet.svu.edu.eg
Citation | Mohamed HMA, Katreen KG, Abd Al-Azeem MW, Wasel FA, Abd-Eldayem AM (2022). Molecular detection of *listeria* species isolated from raw milk with special reference to virulence determinants and antimicrobial resistance in *listeria monocytogenes*. J. Anim. Health Prod. 10(4): 492-505.
DOI | http://dx.doi.org/10.17582/journal.jahp/2022/10.4.492.505
ISSN | 2308-2801

INTRODUCTION

Food contamination may be a vital issue for public health round the world. The foremost perishable and frequently consumed animal food by individuals is milk (Borena et al., 2022). Because of this, microbial contamination is considered to represent a heavy health risk to the public (Akrami-Mohajeri et al., 2018). The *Listeria* genus is cosmopolitan in nature, and it's frequent in farms (Tabit, 2018). *Listeria* spp. is spore-free, facultative anaerobic,

rod-shaped, and gram-positive (Odetokun and Adetunji, 2016). There are twenty-one species belongs to this genus, from which, *Listeria monocytogenes* (*L. monocytogenes*) and *Listeria ivanovii* are the 2 species that are recognized as being infective (Quereda et al., 2020).

L. monocytogenes is an important foodborne zoonotic pathogen, according to Reda et al. (2016). The contamination of milk and its products with *Listeria* spp., especially *L. monocytogenes*, could lead to severe infections in people. Human listeriosis is a serious or even deadly sickness for particular populations, including newborns, pregnant women, the elderly, and individuals with weakened immune systems, with fatality rates for listeriosis ranging from 20 to 30%. It causes sporadic and epidemic outbreaks of the disease (Phraephaisarn et al., 2017; Sarfraz et al., 2017; Şanlıbaba, 2018).

Typically, *Listeria* spp. are recognized using a range of diagnostic methods, such as growing on particular media. The required selective medium for the detection of *Listeria monocytogenes* is Agar *Listeria* according to Ottaviani and Agosti (ALOA), which has an advantage over other selective media for the isolation of *L. monocytogenes* and permits the distinction between pathogenic and non-pathogenic *Listeria* spp. because additional *Listeria* species frequently co-isolate from *L. monocytogenes*-positive food samples (Jamali et al., 2013). In addition, the likelihood of finding *L. monocytogenes* may be decreased if *L. innocua* is present in foods contaminated with the pathogen (Zitz et al., 2011).

Listeria is usually diagnosed through the time-consuming processes of isolation and organic chemistry identification. However, molecular techniques like polymerase chain reaction (PCR) ought to be employed in laboratories responsible for inspecting foods of animal origin as they permit a faster and correct identification of *L. monocytogenes* by targeting specific genes (El-Malek et al., 2010). The nucleotide sequence analysis of *L. monocytogenes* is a good trendy tool for genotyping and investigation of the relation of *Listeria* species to different native or international lineages.

Numerous molecular markers that are concerned within various stages of the infection, like the virulence genes responsible for the host cells invasion (*inl*A, *inl*B and *iap*), phagosomal escape (*hly*A, *plc*A and *plc*B) wherever the *hly*A gene mediates the discharge of the microorganism cells into the host's cytoplasm, and positive regulative factor A (*Prf*A), are usually accustomed assess the virulence potential of *L. monocytogenes* (Liu et al., 2007). All of those aid the bacterium's intracellular development and dissemination inside a mammalian host (Tirumalai et al., 2012). One of the intense risk factors to public health is antibiotic resistance because it makes infections tougher to treat since medication lose their effectiveness, prolongation of hospital stays, raising mortality rates and increasing medical expenses (WHO, 2018). Antibiotic-resistant microorganism has considerably accrued round the world, leading to harder to treat human and animal diseases (SCENIHR, 2009). The flexibility of *Listeria* to quickly develop antimicrobial agent resistance could be a recently discovered feature that represents a growing danger to each human and animal health.

Listeria monocytogenes has been concerned in many deadly ill health outbreaks. Future outbreaks could also be harder to manage due to the increase of antibiotic resistance among food *L. monocytogenes* isolates that has been exaggerated particularly for those antibiotics usually accustomed to treat listeriosis like tetracycline, penicillin, ampicillin, and gentamicin (Olaimat et al., 2018) with variable percentages. In 1990, acquired resistance to antibiotics was initial delineated in *L. monocytogenes* by Poyart-Salmeron et al. (1990). Since then, unpredictable animal and human listeriosis-causing multidrug-resistant (MDR) microorganisms are found in food samples (Haubert et al., 2016). However, early administration of the proper combination of antibiotics will cut back the severity of listeriosis, with a cure rate of 70% (Abdallahzadeh et al., 2016).

In dairy farms, biofilm development could be a common phenomenon and is assumed to be a possible infective agent transmission technique since the setting is contributing to microorganism survival. Biofilms from dairy farm niches are coupled to *L. monocytogenes* (Latorre et al., 2010). Biofilm formation starts within twenty minutes (Weiler et al., 2013) on completely different food contact surfaces, together with stainless steel and plastic, as *L. monocytogenes* multiplies quickly on improperly clean dairy farm appliances. This protects the organism from environmental stresses and will increase its resistance to cleaners and sanitizers utilized in the food industry. To lower the chance of tainted milk and human infections, it's crucial to inhibit biofilm creation on milking instrumentation (Latorre et al., 2010).

Hence, the objective of this study was to determine the incidence of *listeria* species in raw milk in addition to detection of the virulence, phenotypic and genotypic antibiotic resistance profiles as well as biofilm formation of *L. monocytogenes* strains.

MATERIALS AND METHODS

Collection of Samples

In Sohag governorate, Egypt, a total of 150 raw milk samples were collected from supermarkets, local vendors, and small-scale producers. In sterile plastic bags, the samples were taken and transferred to the laboratory in refrigera-

tor for bacteriologic investigation.

Isolation and Identification of different species of *Listeria*

Listeria spp. were isolated using *Listeria* selective enrichment broth (CM0862, Oxoid), that was supplemented with the *Listeria*-selective enrichment agent (SR0141, Oxoid) for twenty-four hours at 28 °C and plated onto ALOA (Merck, Germany), that was supplemented with the *Listeria*-selective supplement (SR0140, Oxoid) and incubated for twenty-four hours at 37 °C as described by Ottaviani and Agosti in ISO 11290–1 (1997). The potential colonies were transferred to Tryptic Soy Agar with 0.6% Yeast Extract (TSA-YE) (Sigma, Germany), wherever they were incubated for 24-48 hours at 37 °C. The biochemical tests comprised; colony morphology, gram staining reaction, catalase test, oxidase test and sugar fermentation test (xylose, rhamnose, mannitol) were carried out (MacFaddin, 2000). Furthermore, *Staphylococcus aureus* strain (((identified *Staph. aureus* strain was obtained from South valley university, Department of Microbiology, Qena, Egypt) was treated using Christie Atkins Munch Petersen's (CAMP) reaction, streaked on blood agar in a straight line across the plate center, then *L. monocytogenes* strain was streaked in a vertical direction to *S. aureus* then, incubated at 37 ℃ for 24 h and checked for β-hemolysis appearance as an arrowhead or circle shape in the positive reaction (CFSAN, 2001).

PCR-based confirmation of presumptive *Listeria* spp.

The QLA amp DNA mini kit (catalogue number 51304) was used for DNA extraction according to the manufacturer's instructions.

PCR was performed to confirm the *Listeria* colonies using the *Listeria iap* gene. 12.5 µl of Emerald Amp GT PCR Master combined (2x premix), 1 µl each of the forward and reverse primers, 5.5 µl of PCR grade water, and 5 µl of template DNA created up all the 25 µl total. Initial denaturation occurred at 5°C for five minutes then there have been thirty-five cycles of 94 °C for 30 seconds, 60 °C for one minute, and 72 °C for one minute, followed by a final extension lasting 12 minutes at 72 °C. PCR products were seen and captured using 1.5% agarose gel electrophoresis stained with ethidium bromide below ultraviolet illumination. The amplicon size and primer sequences are listed in Table (1).

Detection of *L. monocytogenes* using species specific primers

The PCR amplification was done in a thermal cycler (Applied Biosystem 2720) at 50 µl total volume included; 25 µl the EmeraldAmp GT PCR Master mix (2x premix), 1.5 µl of forward and reverse primers derived from *16SrRNA* gene (Table.1), 17 µl of PCR grade water, and 5 µl of template DNA created up the reaction mixture. The template DNA was initial denaturized at 95°C for four min to perform 35 cycles of PCR amplification. Then, the subsequent temperatures were used: DNA extension for one minute at 72°C, deoxyribonucleic acid denaturation for 45 s at 95°C, primer tempering for 45 s at 60°C, and a final extension for 5 minutes at 72°C.

Amplified *16SrRNA* sequencing and phylogenetic analysis

The distinct *Listeria* spp. were identified by the sequencing of amplified *16S rRNA* PCR product. Table (1) lists all-purpose primers. The PCR reaction was effectively carried out in 25 µl of the reaction mixture, which contained bacterial DNA (1 µl), each primer (0.5 µl), EmeraldAmp GT PCR Master Mix (12.5 µl), and nuclease-free water (10.5 µl). The PCR settings were first established by Rohwer et al. (2002). On 1.5% agarose gel, electrophoresis was used to analyses the outcome. The PCR products were subsequently cleaned using the QIA Rapid PCR Product Extraction Kit (Qiagen, Valencia, CA). On an automated DNA sequencer, purified PCR products were sequenced using the Big Dye terminator V3.1 kit (Bio system Cat. No.4336917, Applied Bio systems). Isolates were analyzed both forward and reverse for the *16S rRNA* gene sequence. MegAlign generated sequence alignment using multiple alignment techniques (DNASTAR, Window version 3.12e).

Phylogenetic analysis

MegAlign from the Lasergene program (version7) was used to create a phylogenetic tree based on the *16S rRNA* gene nucleotides sequence for our isolates in order to explore the identity of these isolates with each other and with reference strains registered with GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi? PROGRAM= blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch).

Evaluation of antimicrobial susceptibility of *L. monocytogenes*

The Kirby-Bauer disk diffusion assay was used with slight modification in consistent with CLSI (2011), to perform the antimicrobial sensitivity check for *L. monocytogenes* strains Briefly; 0.1ml of bacterial suspension ($1x10^8$ CFU/mL) corresponding to (0.5 McFarland) was plated on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. The plates were left for five minutes to dry then antimicrobial disks were deposited on the agar surface and incubated at 35°C±2 for 24-48hrs. Completely different antimicrobials were used like Penicillins: penicillin G (25 µg), amoxicillin/clavulanic acid (20/10 µg), oxacillin (1 µg), ampicillin (25 µg); Aminoglycosides: gentamicin

(10 µg), streptomycin (10 µg); Tetracyclines: tetracycline (30 µg), oxytetracycline (30 µg); Macrolides: erythromycin (15 µg), clarithromycin (15 µg) and vancomycin (30 µg); Lincosamides: lincomycin (2 µg); Fluoroquinolones: ciprofloxacin (5 µg), and levofloxacin (5 µg). Zone diameter was interpreted according CLSI (2011).

Biofilm formation

L. monocytogenes isolates were cultured for 24 hours at 37°C in 10 ml of brain heart infusion broth containing 1% glucose, in accordance with Stepanovic et al (2000)'s protocol. A sterile 96-well polystyrene microtiter plate with three wells was filled with 20 µL of each bacterial solution along with 180 µL of BHI with 1% glucose and 200 µL of uninoculated BHI as a negative control. The microtiter plate was incubated at 37°C for 24 hours. After that, the broth was taken out and the wells were cleaned three times with sterile phosphate-buffered saline. Biofilms were turned over and air-dried in a warm place for around 30 minutes after being fixed with methanol for 20 minutes. Biofilms were stained with crystal violet (2%) for 15 minutes. After two rinses with distilled water, the wells were dehydrated. The pigmented adherent cells were resolubilized in 150 µL of 33% acetic acid for 30 minutes at room temperature. Finally, a microtiter plate reader was used to measure the OD of each well at 595 nm (OD595). Based on the absorbance, the bacteria were categorized into four groups: no biofilm producer (OD≤ODc), weak biofilm producer (ODc<OD2X ≤ODc), moderate biofilm producer (2X <ODc ≤OD 4X ODc), and strong biofilm producer (OD>4X ODc). The optical density for the negative control was calculated as ODc (uninoculated broth).

Detection of antimicrobial resistance and virulence genes in *L. monocytogenes* by PCR

β-lactams antibiotics resistance gene *(amp*C*)* along with other drug-resistant genes to the following antibiotics: tetracycline *(tet*M*)*, aminoglycoside *(aad6)* and macrolides *(mef*A*)* were determined by PCR using the primers displayed in Table 1. Furthermore, *L. monocytogenes* isolates were screened for the presence of three virulence markers *hly*A, *inl*A and *inl*B (Table 1).

Uniplex PCR reaction was done in 25 µL PCR reaction mixture containing 1 µL of bacterial DNA, 0.5 µL of each primer, 12.5 µL of EmeraldAmp GT PCR Master Mix, and 10.5 µL nuclease-free water. The cycling conditions used for the uniplex PCR amplification of the *amp*C β-lactamase gene were: 5 minutes, 94°C; followed by 35 cycles (30 seconds, 94°C; 40seconds, 50°C; 45 seconds, 72°C); 10 minutes, 72°C. For *Aad6* gene: 5 minutes, 94°C; 35 (30 seconds, 94°C; 40 seconds, 55°C; 50 seconds, 72°C); 10 minutes, 72°C. For *tet*M tetracycline gene: 5 minutes, 94°C; followed by 35 cycles (30 seconds, 94°C; 40 seconds, 55°C; 40 seconds, 72°C); 10 minutes, 72°C and for *mef*A gene: 5 minutes, 94°C; 35 (30 seconds, 94°C; 40 seconds, 55°C; 40 seconds, 72°C); 10 minutes, 72°C. For *hly*A gene, the initial duration at 94°c for 5 min, followed by 35 cycles of (94°c for 30 s., 50°c for 30s.and 72°c for 30 s.) with a final extension at 72°c for 7min. For the *inl*A gene, the initial duration at 94°c for 5 min, followed by 35 cycles of (94°c for 30 s., 55°c for 45s.and 72°c for 45 s.) with a final extension at 72°c for 10min and for *inl*B gene, the initial duration at 94°c for 5 min, followed by 35 cycles of (94°c for 30 s., 55°c for 40s. and 72°c for 40 s.) with a final extension at 72°c for 10min. The PCR products were visualized using ethidium bromide-stained 1.5% agarose gel electrophoresis under UV light and photographed.

Statistical Analysis

The values were compared using SPSS (Version 28) data processing. The Scheffe and Duncan tests were conducted with a p value ≤ 0.05 indicating a significant difference after the one-way ANOVA analysis.

RESULTS

In our investigation, 150 samples of raw milk were analyzed, and 22 isolates (14.6%) were recognized as *Listeria* spp. by cultural characteristics on ALOA agar plates (bluish green color) and traditional biochemical identification of the suspected colonies. It was noted that the biochemical testing showed catalase positive, oxidase negative, CAMP test was positive for eight isolates and negative for other suspected *Listeria* isolates and variable results were showed in the fermentation tests particularly rhamnose and xylose sugars, these tests yielded an inconsistent and unreliable findings. Therefore, we employed PCR to validate the findings. PCR confirmed the presence of *Listeria* DNA using *iap* gene in 13 out of 22 *Listeria* isolates that were classified as positive biochemically (Fig.1a). Additionally, the use of species-specific *L. monocytogenes* primer derived from *16S rRNA* gene showed that 8 out of 13 *Listeria* spp. were *L. monocytogenes* (Fig.1b). The results of sequencing of the amplified universal *16S rRNA* gene showed that the remaining five isolates (5/13) were classified as; *L. innocua* (3 isolates) and *L. welshimeri* (2 isolates)

Our isolates were found to have the following relationships with reference strains that were registered in the gene bank (Table 2 and Fig. 2): *L. monocytogenes* grouped with *L. monocytogenes* (NR044823) with an identity percentage of 98.8%, *L. innocua* isolates grouped with *L. innocua* (FJ774201) with an identity percentage ranging from (99.1 to 99.5%), and *L. welshimeri* grouped with *L. welshimeri* (DQ065846) with identity percentage ranged from (99.9-100%) (Table 3).

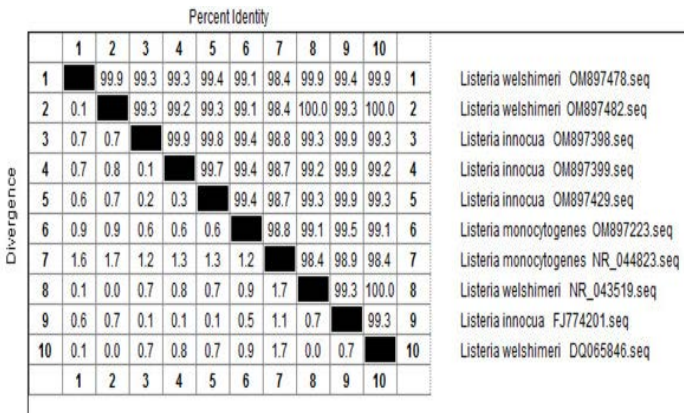
Table 1: Primers sequence

Gene	Primer Sequence 5'-3'	Amplified product (bp)	Reference
iap	ATGAATATGAAAAAAGCAAC	1450-1600 bp	Chen and Knabel, (2007)
	TTATACGCGACCGAAGCCAAC		
L. monocytogenes species specific *16S rRNA*	GGA CCGGGGCTA ATA CCG AAT GAT AA	1200 bp	Kumar et al., (2015)
	TTC ATG TAG GCG AGT TGC AGC CTA		
Universal *16S rRNA*	AGAGTTTGATC MTGGCTCAG	1,500-bp	Chèneby et al., (2000|)
	TACGGYTACC TTGTTACGACTT		
*inl*A	ACG AGT AAC GGG ACA AAT GC	800 bp	Liu et al., (2007)
	CCC GAC AGT GGT GCT AGA TT		
*inl*B	CTGGAAAGTTTGTATTTGGGAAA	343 bp	
	TTTCATAATCGCCATCATCACT		
*hly*A	GCA-TCT-GCA-TTC-AAT-AAA-GA	174 bp	Deneer and Boychuk, (1991)
	TGT-CAC-TGC-ATC-TCC-GTG-GT		
aad6	AGAAGATGTAATAATATAG	978 bp	Morvan et al., (2010)
	CTGTAATCACTGTTCCCGCCT		
*mef*A	AGTATCATTAATCACTAGTGC	345 bp	
	TTCTTCTGGTACTAAAAGTGG		
*tet*M	GTGGACAAAGGTACAACGAG	405 bp	
	CGGTAAAGTTCGTCACACAC		
*amp*C	TTCTATCAAMACTGGCARCC	550 bp	SRINIVASAN et al., (2005)
	CCYTTTTATGTACCCAYGA		

Table 2: Accession numbers of *Listeria* spp.

Accession Number	No. of isolates
OM897223	*L.monocytogens*(1)
OM897398	*L. innocua*(2)
OM897478	*L. welshimeri*(3)
OM897399	*L. innocua*(4)
OM897429	*L. innocua*(5)
OM897482	*L. welshimeri*(6)

Table 3: Identity percentages of our isolates with reference strains registered on gene bank



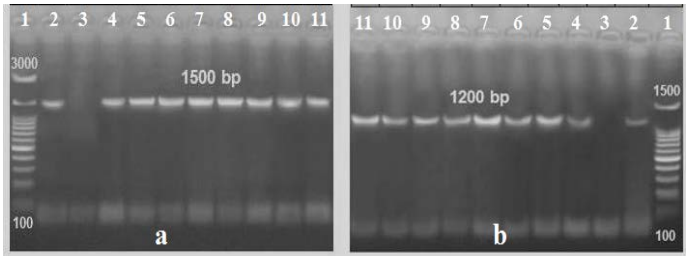


Figure 1: (a):Amplified profile of *Listeria* spp. DNA positive for *iap* gene at 1500 bp. , Lane(1):Gel Pilot 100 bp ladder , Lane2: positive control, Lane3:Negative control, Lane 4,5,6,7,8,9,10,11 positive isolates. (b): Amplified profile of *Listeria monocytogenes*. DNA positive for species specific *16S rRNA* gene at 1200 bp., Lane(1):Gel Pilot 100 bp ladder, Lane2: positive control, Lane3:Negative control, Lane 4,5,6,7,8,9,10,11positive isolates.

The Results in Table (4) demonstrated the antimicrobial resistance of eight isolates of *L. monocytogenes*. All of the isolates tested positive for the greatest levels of resistance to tetracycline (100%), lincomycin (100%) and gentamycin (100%), as well as penicillin, ampicillin, oxacillin, and amoxicillin/clavulanic acid (100%). Vancomycin (50%) and erythromycin (37.5%) both exhibited intermediate resistance, but all isolates shown great sensitivity to ciprofloxacin and levofloxacin (100%). All of the *L. monocytogens* milk isolates in this investigation (100%) showed multi-drug resistance.

Table 4: Antimicrobial sensitivity pattern of *L. monocytogenes* isolated from raw milk.

Type of Antibiotics	No. of *L. monocytogenes* isolates (n =8)					
	Sensitive (S)		Intermediate (I)		Resistant (R)	
	N.	%	N.	%	N.	%
Penicillin G (25 µg)	0	0%	0	0%	8	100%
Ampicillin (25 µg)	0	0%	0	0%	8	100%
Amoxicillin/clavulanic acid (20/10 µg)	0	0%	0	0%	8	100%
Oxacillin (1 µg)	0	0%	0	0%	8	100%
Gentamicin (10 µg)	0	0%	0	0%	8	100%
Streptomycin (10 µg)	0	0%	0	0%	8	100%
Tetracycline (30 µg)	0	0%	0	0%	8	100%
Oxytetracycline (30 µg)	0	0%	0	0%	8	100%
Erythromycin (15 µg)	2	25%	3	37.5%	3	37.5%
Clarithromycin (15 µg)	2	25%	2	25%	4	50%
Lincomycin (2 µg)	0	0%	0	0%	8	100%
Vancomycin (30 µg)	3	37.5%	1	12.5%	4	50%
Ciprofloxacin (5 µg)	8	100%	0	0%	0	0%
Levofloxacin (5 µg)	8	100%	0	0%	0	0%

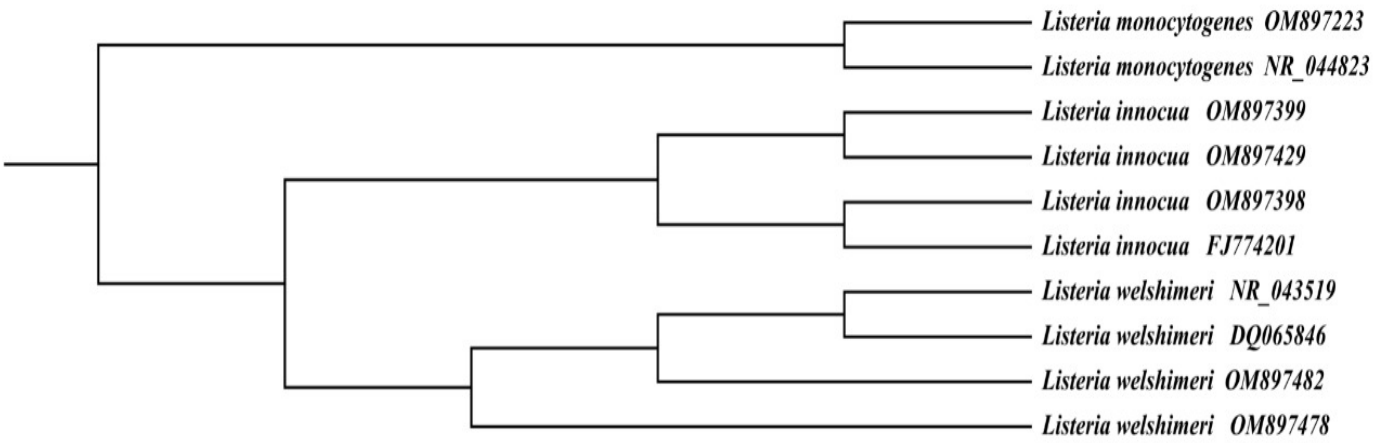


Figure 2: Phylogenetic Tree of the Entire Nucleotide Sequence of *16SrRNA* Gene of Our *Listeria spp.* Isolates Compared with Reference Strains Regained from Gene Bank

Data illustrated in Figure (3) revealed that all the isolates (100%) could form biofilms. After 24 hours incubation, five (62.5%) *L. monocytogenes* strains formed moderate biofilm while 3 (37.5%) *L. monocytogenes* isolates formed weak biofilm.

Statistical analysis showed a significant relationship between multidrug resistance and biofilm formation ($P < 0.05$) among *L. monocytogens* isolates (Figure 4).

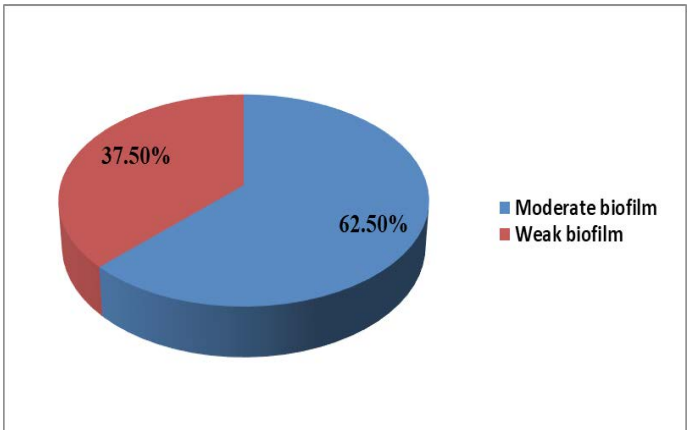


Figure 3: Biofilm formation by *L. monocytogenes* isolates

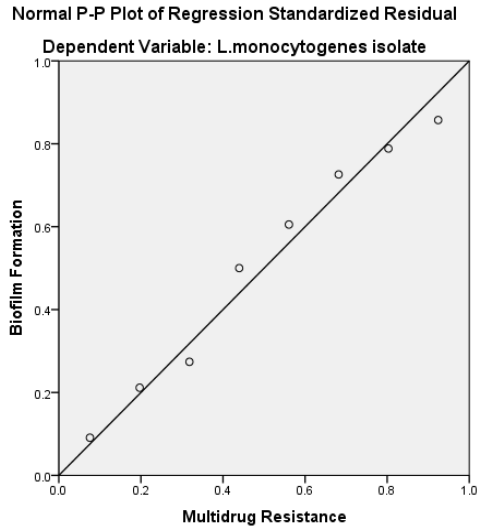


Figure 4: Relationship between Multidrug resistance and biofilm formation among ***L.monocytogenes*** isolates

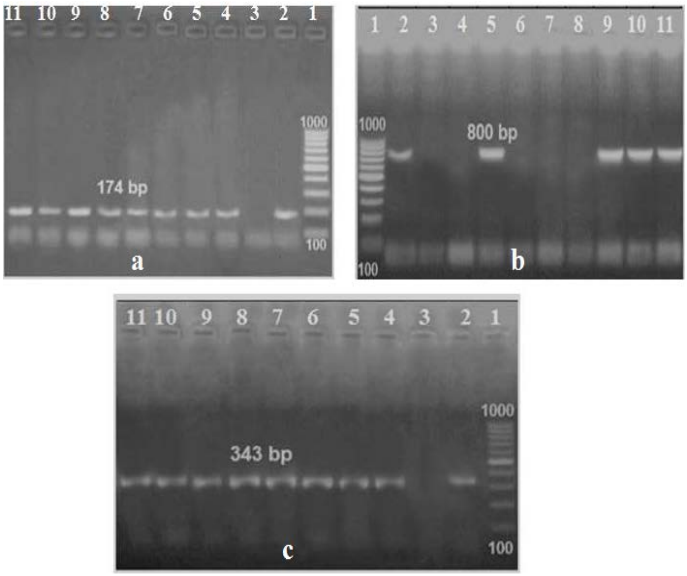


Figure 5: PCR products of the amplified hlyA, inlA and inB genes in *L.monocytogenes* were electrophoresed on an agarose gel.(a)amplified prodcut of *hlyA* gene at 174bp, lane1: Gel Pilot 100 bp ladder, lane2:postive control, laneL:3negative control, lane:4-11postive isolates. (b) amplified prodcut of *inlA* gene at 800bp,lane1: Gel Pilot 100 bp ladder, lane2: postive control, lane3:negative control, lane (5,9,10 and 11) postive isolates. (c) amplified prodcut of *inB* gene at 343 bp,lane1: Gel Pilot 100 bpladder, lane 2: postive control, lane 3: negative control, lane: 4-11postive isolates.

The inspection for three key virulence genes (*hly*A, *inl*A and *inl*B) in *L. monocytogens* isolates by conventional PCR revealed that *hly*A and *inl*B genes were detected in 100% of the isolates and *inl*A gene is detected in 50% of *L. monocytogenes* isolates of raw milk samples as shown in (Figure 5). The molecular detection of antimicrobial resistance genes in *L. monocytogenes* isolates revealed that the resistance to β-lactam antibiotics (penicillin, ampicillin, oxacillin and amoxicillin/clavulanic acid), tetracycline, gentamycin, and erythromycin was associated with the presence of *amp*C *β-lactamases* (100%), *tet*M (100%), *aad6* (100%) and *mef*A (37.5%) genes in the identified strains respectively (Figure 6).

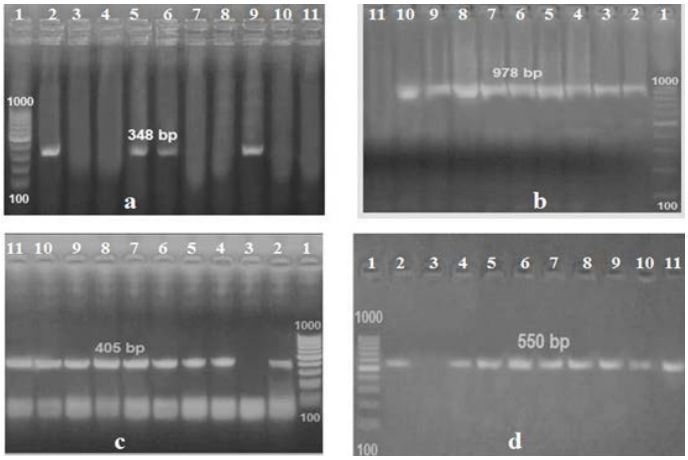


Figure 6: PCR products of the amplified *mef*A, *aad6*, *tet*M and *amp*C genes in *L.monocytogenes* were electrophoresed on an agarose gel.(a)amplified prodcut of *mef*A gene at 348bp, lane1: Gel Pilot 100 bp ladder, lane 2:postive control, lane3: negative control, lane:5,6 and9 postive isolates. (b) amplified prodcut of *aad6* gene at 978bp,lane1: Gel Pilot 100 bp ladder, lane2:postive control, lane(3-10)postive isolates, lane11:negative control. (c) amplified prodcut of *tet*M gene at 405bp, lane1: Gel Pilot 100 bp ladder, lane2: postive control, lane3: negative control, lane: 4-11postive isolates.(d) amplified prodcut of *amp*C gene at 550bp,lane1: Gel Pilot 100 bp ladder, lane2: postive control,lane3: negative control, lane:4-11postive isolates

DISCUSSION

In addition to being the third most frequent cause of food poisoning-related fatalities in humans, *Listeria monocytogenes* is one of the most significant foodborne bacteria that is linked to food-borne disease outbreaks and high hospitalization rates globally (Osman et al., 2020; Luque-Sastre et al., 2018). Because of its potential to cause severe and occasionally fatal listeriosis disease, its widespread distribution in the environment and food (Gasanov et al., 2005), its ability to survive in environmental niches by producing biofilms (Lee et al., 2017), and its capacity to grow and endure challenging conditions like low temperature and high salt concentrations (NicAogáin and O'Byrne, 2016), it poses serious health risks.

Culture techniques based on colony shape, sugar fermentation, and hemolytic characteristics were used in traditional identification. In our investigation, 14.6% of samples were considered to be contaminated with *Listeria* spp. based on

ALOA and traditional biochemical assays. These techniques constitute the gold standard (Gasanov et al., 2005). Despite the fact that ALOA is the first chromogenic media to be developed to yield presumptive identification of pathogenic *Listeria* species in a shorter amount of time after initial sample examination (Ottaviani et al., 1997) and enables more efficient isolation of *L. monocytogenes* from foods contaminated with *L. monocytogenes* in addition to other *Listeria* spp., they can occasionally result in false positive results (Nwaiwu, 2015). In order to assess *L. monocytogenes* contamination in food samples, we must instead employ more precise molecular techniques like PCR (Angelidis et al., 2012).

The *Listeria iap* gene, a highly specific housekeeping gene used for genus-level identification of *Listeria*, was used in the PCR findings to establish the presence of *Listeria* spp. DNA in (13 out of 22 isolates) (Atil et al., 2011). The most often recovered species (8 out of 13) was *L. monocytogenes*. Similar to this, *L. monocytogenes* was discovered in Egypt from several raw milk samples (Khedr et al., 2016). By employing the *16S rRNA* species-specific gene, some publications are successful in identifying *L. monocytogenes* (Haggag et al., 2019; El-Banna et al., 2016; Osman et al., 2016).

On the other hand, *16S rRNA* gene sequencing revealed the identities of the last 5 *Listeria* spp. as 3 isolates of *L. innocua* and 2 isolates of *L. welshimeri*, where its function is irreplaceable and may be regarded as the definitive method for species identification (Clarridge, 2004; Srinivasan et al., 2015).

Other incidences were recorded by Borena (2022) (7%) where *L. monocytogenes* was 1.82% and Atil et al. (2011) (1.19%) and 0.19% of *L. innocua* were detected. Another research, such as those by Aygun and Pehlivanlar S. (2006) and Kobayashi et al. (2017) did not find any *L. monocytogenes* strains. Contrarily, EL-Naenaeey et al. (2019) discovered a higher incidence of *Listeria* spp. (19%) in which both *L. monocytogenes*, *L. welshimeri*, and *L. innocua* were all identified from 4% and 2% of raw milk samples, respectively. Additionally, Albastami et al. (2020) found *Listeria* spp. in 25% of raw milk samples, with 2% and 5% of those samples isolating *L. monocytogenes* and *L. innocua*, respectively.

The variation in the prevalence of *L. monocytogenes* in milk observed in various reports could be caused by a number of factors, including geographic location, seasonal variations in milk sample collection, types of samples, isolation techniques, management system, time allocated for the study, and the hygienic status of the milk production and processing (Teshome et al., 2019). Conversely, listeriosis is largely a ruminant illness, thus it seems logical that bovine hosts may be important in maintaining the pathogen predominance in cattle ranches. Additionally, the incidence of *L. monocytogenes* may be significantly impacted by the use of untreated animal waste as fertilizer, polluted irrigation water, and inadequate hygiene measures during milking (Shiwakoti, 2015). All studies show that raw cow milk can be a source of *Listeria* infections in people, despite the widely known variance in *L. monocytogenes* prevalence in milk (Buchanan et al., 2017).

The identity and provenance of our isolates were validated by phylogenetic analysis, which revealed that they belonged to a group with reference strains of *Listeria* spp. that were recorded in the gene bank under the accession numbers (NR044823, FJ774201, and DQ065846) and came from food samples (Volokhov et al., 2006).

Raw milk contamination with antibiotic-resistant foodborne bacteria is a significant public health concern that might endanger human food safety. Recent investigations have demonstrated a decrease in the sensitivity of *L. monocytogenes* to a number of antibiotics (Korsak et al., 2012). All *L. monocytogenes* strains from raw milk used in this study had 100% resistance to gentamycin, tetracycline, ampicillin, ampicillin/clavulanic acid, penicillin and oxacillin. Our findings are important since penicillin or ampicillin and gentamicin make up the standard treatment for listeriosis and are often used to treat animal and human diseases (Aras & Ardiç, 2015). Our results supported prior research by Marian et al. (2012), AL-Ashmawy et al. (2014), Akrami-Mohajeri et al. (2018), Olaimat et al. (2018), and Borena et al., (2022). These findings demonstrate that penicillin, the first line antibiotic used to treat active listeriosis infections, may not be successful and that second line antibiotics may be necessary. Our strains' penicillin resistance may be a result of the suggested use of penicillin for treating infectious illnesses like mastitis in ruminants (EVIRA, 2018). However, the study's findings also demonstrated that all of the isolates were extremely susceptible to ciprofloxacin, which was supported by earlier data (Tahoun et al., 2017; Amajoud et al., 2018).

During the last few decades, multidrug resistance (MDR) among foodborne pathogens, including *L. monocytogenes*, has emerged (Zhang et al., 2007). MDR now poses a public health risk because it may result in unsuccessful treatment, which could increase hospitalizations costs, lengthen the time antibiotics must be administered, and increase the number of people who die from foodborne illness.

Egypt has reported the multi-drug resistance *L. monocytogenes* isolates from raw milk to certain conventional antibiotics (Aksoy et al., 2018). In our investigation, *L. monocytogenes* strains found in milk samples had a significant incidence of MDR phenotypes (100%) against the anti-

biotics examined. Our findings were consistent with those noted by Sharma et al. (2017). It was shown to be greater than the results that had previously been published by Jamali et al. (2013) (71.4%), Kevenk and Gulel (2016) (37%), and Kayode et al. (2022) (38.10%).

The predominance of MDR milk strains in our investigation may have come from dairy animals or acquired from repeated contact with antibiotics used in animal husbandry. However, given that biofilm-forming bacteria have been shown to have a higher tolerance to clinical antimicrobials and disinfectants due to regular exposure to sanitizers below the required doses, such resistance may be connected to the isolates' capacity for biofilm-formation (Doulgeraki et al., 2017).

The propensity of *L. monocytogenes* to infect and spread through food is associated with its capacity to create biofilms, which provide the microbe sticky and protective qualities (Oliveira et al., 2010). Because these biofilms are persistent for several months or even years, food can be contaminated repeatedly (Markkula et al., 2005). Our research confirms the findings of Skowron et al. (2019), who showed that *L. monocytogenes* of milk origin frequently exhibit biofilm activity and possess a number of genes linked with virulence and antibiotic resistance. Additionally, *L. monocytogenes* only moderately or weakly produced biofilm on different surfaces in earlier investigations by Djordjevic et al. (2002) and Harvey et al. (2007). Also, Conficoni et al. (2016) found that even under harsh settings including arid environments, high salt concentrations (10%, wt/vol), at refrigeration temperatures and a wide pH range (4.7-9.2), *L. monocytogenes* develops and lives in a variety of habitats. It is challenging to regulate the genus *Listeria* in food processing facilities due to its capacity to survive and grow in the food environment, even by forming biofilms.

Our findings showed a substantial connection between the development of biofilm and multidrug resistance in *Listeria monocytogenes* isolates ($P < 0.05$). The bacteria that form biofilms are naturally resistant to many antibiotics, increasing antibiotic resistance by up to 1000 times, and requiring high antimicrobial dosages to make these organisms inactive (Uruen et al., 2021; Thien-fah et al., 2001; Stewart et al., 2001). This is done so that biofilms, which are assemblages of surface-attached bacteria encapsulated in an extracellular matrix, may tolerate antimicrobial treatments far better than non-adherent, planktonic cells can. Therefore, biofilm-based illnesses are exceedingly challenging to treat (Clayton et al., 2017).

The discovery of several virulence markers, including internalins (*inl*A, *inl*B), listeriolysin O (*hly*A), and others that are crucial in *L. monocytogenes* infection and pathogenesis, provides the basis for the detection of pathogenic *L. monocytogenes* (Di Ciccio et al., 2012). The identification of pathogenic *L. monocytogenes* strains carrying these genes has also been done using PCR-based techniques (Swetha et al., 2012). The crucial and well-known *L. monocytogenes* gene *hly*A generates listeriolysin O (LLO), which facilitates bacterial entry into the host's cytoplasm and promotes pathogen proliferation both within and outside of host cells (Poimenidou et al., 2018). Both host cell adhesion and invasion as well as *L. monocytogenes* internalization inside host epithelial cells are regulated by the *inl*A and *inl*B genes.

The presence of the *hly*A gene in all strains examined for our study's virulotyping analysis was consistent with research by Osman et al. (2016), Şanlıbaba et al. (2018), Owusu-Kwarteng et al. (2018), El-Demerdash et al. (2019) and Skowron et al. (2019). Additionally, it was found that *L. monocytogenes'* ability to form biofilms is impacted by the presence of the *hly*A virulence gene (Price et al., 2018), as this organism is better able to withstand ineffective disinfection during cleaning procedures, which results in the continued presence of *L. monocytogenes* in the final product. According to Kayode et al. (2022), Indra-wattana et al. (2011), and Sant'Ana et al. (2012), practically all tested *L. monocytogenes* isolates from food samples included the internalin gene *inl*B. Additionally, the 50% of *L. monocytogenes* isolates that have the *inl*A gene support previous research by Coroneo et al (2016). The presence of these crucial virulence genes clearly suggested that *L. monocytogenes* from the examined raw milk may contribute to the pathogenesis of human listeriosis, according to Cotter et al (2008)'s explanation.

A clear proliferation of antibiotic determinants was seen by several researchers, particularly in food isolates where the incidence rate of MAR strains reached 30% or higher (Keet et al., 2021). It was discovered that all of the *L. monocytogenes* strains from milk samples utilized in this investigation contained the *amp*C B-lactamase gene. Consistent findings by Njagi et al. (2004) and Hassan et al. (2018) revealed *amp*C genes in 100% of the *L. monocytogenes* isolates recovered from milk and dairy products in Egypt. In contrast, a previous investigation discovered that none of the *L. monocytogenes* isolates recovered from milk samples have the *amp*C gene (Harshani et al., 2022). According to Meletis (2016), this may be related to mutations in «penicillin-binding proteins» (PBPs) that cause B-lactam resistance, contradicting other studies that claimed that the high percentage of ampicillin-resistant strains suggested amoxicillin/clavulanic acid as a better treatment option (Rezai et al., 2018; Fischer et al., 2020).

Beta-lactam antibiotics are the most frequently used in veterinary medicine because of their low toxicity and effec-

tiveness; however, the emergence of beta-lactam resistant pathogenic bacteria poses a serious threat to their widespread use (Ghazaei, 2019).

The most often reported resistance in *Listeria* species is to tetracyclines (Luque-Sastre et al., 2018). It has mostly been linked to the ribosomal protection proteins-granting *tet*M and *tet*S genes (Granier et al., 2011). Although greater than that was also noted by (Elsayed et al., 2022) (41.6%), the prevalence of *tet*M in our study was in agreement with that found by (Granier et al., 2011; Hassan et al., 2018). Additionally, among clinical isolates of *L. monocytogenes*, the *tet*M gene was reported by (Morvan et al., 2010).

In this investigation, 100% of the *L. monocytogenes* strains had high prevalence of the (*aad6*) gene. Additionally, 37.5% of the *L. monocytogenes* strains in our investigation had Macrolides, which are encoded by the (*mef*A) gene, amplified. In contrast, research by Hassan et al. (2018) found that 100% and 71.4% of *L. monocytogenes* strains isolated from milk and dairy products in Egypt, respectively, had the (*mef*A) and (*aad6*) genes. Efflux pumps like *mef*(A) identified in *Streptococcus pneumonia* can mediate the resistance to macrolides, notably erythromycin (Granier et al., 2011). This is a major discovery since, when combined with penicillin, gentamicin is a first-choice medication for treating listeriosis. Additionally, erythromycin is a second-choice antibiotic used to treat listeriosis in persons who are penicillin-sensitive or pregnant (Hof, 2004).

Antibiotic resistance in *L. monocytogenes* is brought on by a number of mechanisms, including the acquisition of mobile genetic elements, self-transferable, mobilizable plasmids, conjugative transposons, and target gene alterations, such as those in genes producing efflux pumps (Luque-Sastre et al., 2018). Additionally discovered to be a potential scenario was the transfer of erythromycin and tetracycline ARGs from lactic acid bacteria (LAB) to *L. monocytogenes* in fermenting milk (Toomey et al., 2009).

However, the differences in the susceptibility patterns of *L. monocytogenes* isolates may be due to the management of the farm and the broad, indiscriminate use of antibiotics to treat diseases and increase animal development (Lungu et al., 2011). Pre-exposure adaptation may be brought on by *L. monocytogenes*' ongoing exposure to low doses of antibiotics in the food manufacturing chain, allowing the organism to withstand larger antibiotic concentrations (Olaimat et al., 2018). Additionally, *L. monocytogenes* food isolates were exposed to various environmental variables, such as salt, heat, and cold, and they adapted to those settings, which increased their resistance to different antibiotics (Al-Nabulsi et al., 2015).

In addition, *L. monocytogenes* resistance to several antimicrobial medicines has evolved and altered during the past few decades (Olaimat et al., 2018). Tetracycline and ciprofloxacin resistance in *L. monocytogenes* isolates from humans, high prevalence of clindamycin and oxacillin resistance in food from meat and fish production chains, and significant percentages of resistance to ampicillin, penicillin G, and tetracycline in *L. monocytogenes* strains isolated from meat, fish, and dairy production chains were all reported in the environment (Caruso et al., 2019).

CONCLUSION

The capacity of these strains to resist various antibiotics as well as the finding that their ability to build biofilm was higher among multidrug resistant strains played a significant role in the identification of *Listeria* spp. and emphasized the pathogenicity function of *L. monocytogenes*. Therefore, to combat the issue of resistance and biofilm development, antibiotics must be administered in accordance with a certain regimen and at precise dosages.

ACKNOWLEDGMENTS

The authors are appreciative to all staff members in Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Egypt , Department of Microbiology, Animal Health Research Institute, Sohag, Egypt and Department of pharmacology, Faculty of Medicine, Assuit University, Assuit, Egypt.

CONFLICT OF INTEREST

The authors declared that no conflict of interest.

NOVELTY STATEMENT

This study focus on the role of *16SrRNA* in identification of *Listeria* species also directed the light on virulence determinants harbored by *L. monocytogenes specially multidrug resistance and its relationship with biofilm formation among these isolates*, which makes these isolates pose a threat on public health.

AUTHORS CONTRIBUTION

All authors contributed in this work equally.

REFERENCES

Abdollahzadeh E, Ojagh SM, Hosseini H, Ghaemi EA, Irajian G, Heidarlo MN (2016). Antimicrobial resistance of *Listeria monocytogenes* isolated from seafood and humans in Iran.

Microb. Pathogen. 100:70–74. https://doi.org/10.1016/j.micpath.2016.09.012 PMID: 276223 45

Akrami-Mohajeri F, Derakhshan Z, Ferrante M, Hamidiyan N, Soleymani M, ContiGO, Tafti R D (2018). The prevalence and antimicrobial resistance of *Listeria* spp. in raw milk and traditional dairy products delivered in Yazd, central Iran. Food Chem. Toxicol. 114:141–144.

Aksoy A, Sezer C, Vatansever L, Gulbaz G (2018). Presence and antibiotic resistance of *Listeria monocytogenes* in raw milk and dairy products. Kafkas Univ. Vet. Fak. Dergis. 24: 415–421

Al-Ashmawy MAM, Gwida MM, Abdelgalil KH (2014). Prevalence, Detection Methods and Antimicrobial Susceptibility of *Listeria monocytogenes* Isolated from Milk and Soft Cheeses and its Zoonotic Importance. World Appl. Sci. J. 29: 869-878.

Al-Nabulsi A, Osaili T, Shaker R, Olaimat A, Jaradat Z, Zain Elabedeen N, Holley R (2015). Effects of osmotic pressure, acid, or cold stresses on antibiotic susceptibility of *Listeria monocytogenes*. Food Microbiol. 46:154–160.

Albastami I, Wajiej A, Aburagaegah S (2020). Microbiological study on *Listeria* species isolated from some food products of animal origin. Damanhour J. Vet. Sci. 4 (1):15-19.

Amajoud N, Leclercq A, Soriano J M, Bracq-Dieye H, El Maadoudi M, Senhaji NS, Abrini J (2018). Prevalence of *Listeria* spp. and characterization of *Listeria monocytogenes* isolated from food products in Tetouan, Morocco. Food Control. 84: 436–441.

Angelidis AS, Georgiadou SS, Zafeiropoulou V, Velonakis EN, Papageorgiou DK, Vatopoulos A (2012). A survey of soft cheeses in Greek retail outlets highlights a low prevalence of *Listeria* spp. Dairy Sci. Technol. 92: 189–201.

Aras Z, Ardiç M (2015). Occurrence and Antibiotic Susceptibility of *Listeria* Species in Turkey Meats. Korean J. Food Sci. Anim. Resour. 35 (5): 669–673.

Atil E, Ertas HB, Ozbey G (2011). Isolation and molecular characterization of *Listeria* spp. from animals, food and environmental samples. Vet. Med. 56:386-94. https://doi.org/ 10.17221/1551-VETMED

Aygun O, Pehlivanlar S (2006). *Listeria* spp. in the raw milk and dairy products in Antakya, Turkey. Food Control. 17 (8): 676–679.

Borena BM, Dilgasa L, Gebremedhin EZ, Sarba EJ, Marami LM, Kelbesa KA, Tadese ND (2022). *Listeria* Species Occurrence and Associated Risk Factors and Antibiogram of *Listeria Monocytogenes* in Milk and Milk Products in Ambo, Holeta, and Bako Towns, Oromia Regional State, Ethiopia. Vet Med Int.5643478. doi: 10.1155/2022/5643478. PMCID: PMC9023178. PMID: 35465403.

Buchanan RL, Gorris LG, Hayman MM, Jackson TC, Whiting RC (2017). A review of *Listeria monocytogenes*: An update on outbreaks, virulence, dose-response, ecology and risk assessments. Food Control. 75:1–13.

Caruso M, Fraccalvieri R, Pasquali F, Santagada G, Latorre LM, Difato LM, Miccolupo A, Normanno G, Parisi A (2019). Antimicrobial susceptibility and multilocus sequence typing of *Listeria monocytogenes* isolated over 11 years from food, humans, and the environment in Italy. Foodborne Pathog. Dis. 17 (4): 284–294. https://doi.org/10.1089/fpd.2019.2723.

CFSAN (Center of Food safety and Applied Nutrition) (2001).Bacteriological Analytical Manual Online, *Listeria monocytogenes* 198:212 Food & drug Administration, Silver Spring, MA, USA..

Chastre J (2008). Evolving problems with resistant pathogens," Clin. Microbiol. Infect., 14: 3–14,.

Chen Y, Knabel SJ (2007). Multiplex PCR for Simultaneous Detection of Bacteria of Genus *Listeria*, *Listeria monocytogenes*, and Major Serotypes and Epidemic Clones of *L. monocytogenes*. Appl. Environ. Microbiol. 73(19): 6299–6304.

Chèneby D, Philippot L, Hartmann A, Hénalut C, Germon J C (2000). *16S rDNA* analysis for characterization of denitrifying bacteriaisolated from three agricultural soils. FEMS Microbiol. Ecol. 34: 121–128.

CLSI (2011). Clinical Laboratory Standards Institute, 2011. Performance standards for antimicrobial disk susceptibility tests; document M2-A9. 26:1. Available from: https://clsi.org/media/ 1631/m02a12_sample.pdf

Clarridge JE (2004). 3rd. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. 4:840-62. https://doi.org/10.1128/CMR.17.4.840-862.2004. PMID: 15489351; PMCID: PMC523561

Clayton W Hall, Thien-Fah Mah (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria, FEMS Microbiol. Rev. 41 (3) 276–301. https://doi.org/10.1093/ femsre/fux010

Conficoni D, Losasso C, Cortini E, Di Cesare A, Cibin V, Giaccone V, Corno G, Ricci A. (2016). Resistance to Biocides in *Listeria monocytogenes* Collected in Meat-Processing Environments. Front Microbiol. 19: 7-1627.

Coroneo V, Carraro V, Aissani N (2016). Detection of Virulence Genes and Growth Potential in *Listeria monocytogenes* Strains Isolated from Ricotta Salata Cheese. J. Food Sci. 81: M114 –M120. https://doi.org/10.1111/1750-3841.13173

Cotter PD, Draper LA, Lawton EM, Daly KM, Groeger DS, et al. (2008). Listeriolysin S, a novel peptide haemolysin associated with a subset of lineage I *Listeria monocytogenes*. PLoS Pathog. 4: e1000144. https://doi.org/10.1371/journal.ppat. 1000144 PMID: 18787690.

Deneer HG, Boychuk I (1991). Species-Specific Detection of *L. monocytogenes* by DNA Amplification. Appl. Environ. Microbiol. 606-609.

Di Ciccio P, Meloni D, Festino AR, Conter M, Zanardi E, Ghidini S, Vergara A, Mazzette R, Ianieri A (2012). Longitudinal study on the sources of *Listeria monocytogenes* contamination in cold-smoked salmon and its processing environment in Italy. International J. Food Microbiol. 158:79-84.

Djordjevic D, Wiedmann M, McLandsborough LA. **(**2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl. Environ. Microbiol. 68(6):2950–2958. https://doi.org/10.1128/AEM.68.6.2950-2958.2002

Doulgeraki AI, Di Ciccio P, Ianieri A, Nychas GJE (2017). Methicillin-resistant food-related *Staphylococcus aureus*: a review of current knowledge and biofilm formation for future studies and applications. Res. In Microbiol. 168(1): 1–15. https://doi.org/10.1016/j.resmic.2016. 08.001 PMID: 27542729

El-Banna TES, Sonbol FI, Zaki MES, El-Sayyad HHI (2016). Clinical and Environmental Prevalence and Antibiotic Susceptibility of *Listeria monocytogenes* in Dakahlea Governorate, Egypt. Clin. Microbiol. 5:249. https://doi.org/10.1016/10.4172/2327-5073.1000249

El-Demerdash AS, Raslan MT (2019). Molecular characterization of *Listeria monocytogenes* isolated from different animal-origin food items from urban and rural areas. Adv. Anim.

Vet. Sci. 7(s2): 51-56.
El-Malek AMA, Ali SFH, Hassanein R, Mohamed MA, Elsayh KI (2010). Occurrence of *Listeria* species in meat, chicken products and human stools in Assiut city, Egypt with PCR use for rapid identification of *Listeria monocytogenes*. Vet. World. 3:353–359.
EL-Naenaeey EYM, Abdel-Wahab AMO, Merwad AMA, Abdou HMA (2019). Prevalence of *Listeria* Species in Dairy Cows and Pregnant Women with Reference to Virulotyping of *Listeria monocytogenes* in Egypt; Zag. Vet J. 473): 248-258.
Elsayed MM, Elkenany RM, Zakaria AI, Badawy BM (2022). Epidemiological study on *Listeria monocytogenes* in Egyptian dairy cattle farms' insights into genetic diversity of multi-antibiotic-resistant strains by ERIC-PCR. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-022-19495-2.
EVIRA (2018). The Faculty of Veterinary Medicine at the University of Helsinki. Recommendations for the use of antimicrobials in the treatment of the most significant infectious and contagious diseases in animals. Helsinki (Finland): The Finnish Food Safety Authority.
Fischer M, Wamp S, Fruth A, Allerberger F, Flieger A, Halbedel S (2020). Population structure-guided profiling of antibiotic resistance patterns in clinical *Listeria monocytogenes* isolates from Germany identifies pbpB3 alleles associated with low levels of cephalosporin resistance. Emerg. Microbes Infect. 9: 1804–1813.
Gasanov U, Hughes D, Hansbro PM (2005). Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: A review. FEMS Microbiol. Rev. 29: 851–875.
Ghazaei C (2019). Phenotypic and molecular detection of Beta-Lactamase enzyme produced by *Bacillus cereus* isolated from pasteurized and raw milk. J. Med. Bacteriol. 8:1–7.
Granier SA, Moubareck C, Colaneri C, Lemire A, Roussel S, Dao TT, Brisabois A (2011). Antimicrobial resistance of *Listeria monocytogenes* isolates from food and the environment in France over a 10-year period. Appl. Environ. Microbiol. 77: 2788–2790.
Haggag YN, Nossair MA, Shehab SA (2019). Is Raw Milk Still Vehicle for Transmitting *Listeria* species To Pregnant Women? AJVS. 61(1): 67-73.
Harshani HBC, Ramesh R, Halmillawewa AP, Wijendra WAS (2022). Phenotypic and genotypic characterization of antibiotic resistance of *Listeria monocytogenes* isolated from raw milk samples collected from Polonnaruwa, Sri Lanka. Emirates J. Food Agric. 34(1): 16-25
Harvey J, Keenan KP, Gilmour A. (2007). Assessing biofilm formation by *Listeria monocytogenes* strains. Food Microbiol. 24(4):380–392. https://doi.org/10.1016/j.fm.2006.06.006
Hassan WMM, Fatma AAA, El-Hofy I, Zaki HT (2018). phenotypic and genotypic characterization of *Listeria* species isolated from chicken and milk products. Int.J.Adv. Res. 6(3):833-841.
Haubert L, Mendonça M, Lopes GV, de Itapema Cardoso MR, da Silva WP (2016). *Listeria monocytogenes* isolates from food and food environment harbouring *tetM* and *ermB* resistance genes. Lett. Appl. Microbiol. 62: 23-29.
Hof H (2004). An update on the medical management of listeriosis. Expert Opin. Pharmacotherap. 5 (8): 1727-1735.
Indrawattana N, Nidabbhasobon T, Sookrung N, Chongsa-Nguan M, Tungtrongchitr A, Makino S, Tungyong W, Chaicumpa W. (2011). Prevalence of *Listeria monocytogenes* in raw meats marketed in Bangkok and characterization of the isolates by phenotypic and molecular methods. J. Health Popul. Nutr. 29 (1): 26 –38. https://doi.org/10.3329/jhpn.v29i1.7565
Jamali H, Radmehr B. (2013). Frequency, virulence genes and antimicrobial resistance of *Listeria* spp. isolated from bovine clinical mastitis. Vet J. 198 (2):541–542. https://doi.org/10.1016/j.tvjl.2013.06.012.
Jamali H, Chai LC, Thong KL (2013). Detection and isolation of *Listeria* spp. and *Listeria monocytogenes* in ready-to-eat foods with various selective culture media. Food Control.32: 19–24.
Kayode AJ, Okoh AI (2022). Assessment of multidrug-resistant *Listeria monocytogenes* in milk and milk product and One Health perspective. PLOS ONE 17(7): e0270993. https://doi.org/10.1371/ journal.pone.0270993.
Keet R, Rip D (2021).*Listeria monocytogenes* isolates from Western Cape, South Africa exhibit resistance to multiple antibiotics and contradicts certain global resistance patterns. AIMS Microbiol. 7: 40–58.
Kevenk TO, Gulel GT (2016). Prevalence, antimicrobial resistance and serotype distribution of *Listeria monocytogenes* isolated from raw milk and dairy products. J. Food Safety. 36: 11–18.
Khedr A, Elmonir W, Sobeih A (2016). Public health risk of *Listeria monocytogenes* in raw milk in Egypt: virulence genes, antibiotic-resistance and high-risk consumption practices. Inter J. Appl. Nat. Sci. 5: 57-64.
Kobayashi PF, Carvalho AF, Fredrigo RC, Costa AM, Piatti RM, Pinheiro ES (2017). Detection of *Brucella* spp., *Campylobacter* spp. and *Listeria monocytogenes* in raw milk and cheese of uninspected production in the metropolitan area of São Paulo. Ciências Agrárias, Londrina. 38(4):1897-1904.
Korsak D, Borek A, Daniluk S, Grabowska A, Pappelbaum K. (2012). Antimicrobial susceptibilities of *Listeria monocytogenes* strains isolated from food and food processing environment in Poland. Int. J. Food Microbiol. 158: 203–208. https://doi.org/10.1016/j.ijfoodmicro.2012.07.016 PMID: 22874767.
Kumar A, Grover S, Batish VK (2015). Exploring specific primers targeted against different genes for a multiplex PCR for detection of *Listeria monocytogenes*.3 Biotech. 5: 261–269.
Latorre AA, Van Kessel JS, Karns JS, Zurakowski MJ, Pradhan AK, Boor KJ, Jayarao BM, Houser BA, Daugherty CS, Schukken YH (2010). Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes*. J. Dairy Sci. 93(6):2792–2802.
Lee S, Ward TJ, Jima DD, Parsons C, Kathariou S (2017). The arsenic resistance-associated *Listeria* genomic island LGI2 exhibits sequence and integration site diversity and a propensity for three *Listeria monocytogenes* clones with enhanced virulence. Appl. Environ. Microbiol. 83:e01189-17.
Liu D, Lawrence ML, Austin FW, Ainsworth AJ (2007). A multiplex PCR for species and virulence-specific determination of *Listeria monocytogenes*. J. Microbiol. Methods. 71: 133–140.
Lungu B, O'Bryan CA, Muthaiyan A, Milillo SR, Johnson, MG, Crandall PG , Ricke SC (2011). *Listeria monocytogenes:* Antibiotic resistance in food production. Foodborne Pathog. Dis. 8 (5): 569-578.
Luque-Sastre L, Arroyo C, Fox EM, McMahon BJ, Bai L, Li F, Fanning S. (2018). Antimicrobial resistance in *Listeria*

species. Microb. Spectrum. 6 (4): ARBA-0031-2017.
MacFaddin J F (2000). Biochemical Tests for Identification Medical Bacteri, Warery Press Inc, Baltimore, MD, USA.
Marian M (2012). MPN-PCR detection and antimicrobial resistance of *Listeria monocytogenes* isolated from raw and ready-to-eat foods in Malaysia. Food Control. 28: 309–314
Markkula A, Autio T, Lunden J, Korkeala H (2005). Raw and processed fish show identical *Listeria monocytogenes* genotypes with pulsed-field gel electrophoresis. J. Food Prot. 68: 1228-1231.
Meletis G (2016). Carbapenem resistance: Overview of the problem and future perspectives. Ther. Adv. Infect. Dis. 3: 15–21.
Morvan A, Moubareck C, Leclercq A, Hervé-Bazin M, Bremont S, Lecuit M, Courvalin P, Le Monnier A (2010). Antimicrobial Resistance of *Listeria monocytogens* Strains Isolated from Humans in France. Antimicrob. Agents Chemother. 54 (6):2728–2731.
NicAogáin K, O'Byrne CP (2016).The role of stress and stress adaptations in determining the fate of the bacterial pathogen. Front. Microbiol. 7:1865.
Njagi PG, Mbuthia LW, BeboraL C, Nyaga PN, Minga U, Olsen JE (2004).Carrier status for *Listeria monocytogenes* and other *Listeria* species in free range farm and market healthy indigenous chickens and ducks. East African Med. J. 81:529-533.
Nwaiwu O (2015). An overview of *Listeria* species in Nigeria. International Food Research Journal. 22:455-464.
Odetokun IA, Adetunji VO (2016). Prevalence and persistence of *Listeria monocytogenes* in dairy and other ready-to-eat food products in Africa. Microbes Food Health. 319: 349-361.
Olaimat AN, Al-Holy MA, Shahbaz HM, Al-Nabulsi AA, Abu Ghoush MH, Osaili TM, Ayyash MM, Holley RA (2018). Emergence of Antibiotic Resistance in *Listeria monocytogenes* Isolated from Food Products: Comprehensive Rev. Food Sci. Food Safety. Vol.17. doi: 10.1111/ 1541-4337.12387.
Oliveira MM, Brugnera DF, Alves E, Piccoli RP (2010). Biofilm formation by *Listeria monocytogenes* on stainless steel surface and biotransfer potential. Braz J. Microbiol. 4: 97-106.
Osman KM, Samir A, Abo-Shama UH, Mohamed EH, Orabi A, Zolnikov T (2016). Determination of virulence and antibiotic resistance pattern of biofilm producing *Listeria* species isolated from retail raw milk. BMC Microbiol. 16:263.
Osman KM, Kappell AD, Fox EM, Orabi A, Samir A (2020). 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. 9:5.
Ottaviani F, Ottaviani M, Agosti M (1997). Esperienza su un agar selettivo e differentiale per *Listeria monocytogenes*. Ind. Aliment. 36:1–3.
Owusu-Kwarteng J, Wuni A, Akabanda F, Jespersen L (2018). Prevalence and Characteristics of *Listeria monocytogenes* Isolates in Raw Milk, Heated Milk and Nunu, a Spontaneously Fermented Milk Beverage, in Ghana. Beverages. 4(2): 40.
Phraephaisarn C, Khumthong R, Takahashi H, Ohshima C, Kodama K, Techaruvichit P, Vvesaratchavest M, Taharnklaew R, Keeratipibul S (2017). A novel biomarker for detection of *Listeria* species in food processing factory. Food Control. 73: 1032-1038.
Poimenidou SV, Dalmasso M, Papadimitriou K, Fox EM, Skandamis PN, Jordan K (2018). Virulence Gene Sequencing Highlights Similarities and Differences in Sequences in *Listeria monocytogenes* Serotype 1/2a and 4b Strains of Clinical and Food Origin From 3 Different Geographic Locations. Front. Microbiol. 9:1103. https://doi.org/ 10.3389/fmicb.2018.01103 PMID: 29922249
Poyart-Salmeron C, Carlier C, Trieu-Cuot P, Courtieu AL, Courvalin P (1990). Transferable plasmid-mediated antibiotic resistance in *Listeria monocytogenes* Lancet (London, England). 335: 1422–6. http://www.ncbi. nlm. nih. gov/ pubmed/ 1972210.
Price R, Jayeola V, Niedermeyer J, Parsons C, Kathariou S (2018). The *Listeria monocytogenes* key virulence determinants *hly* and *prfa* are involved in biofilm formation and aggregation but not colonization of fresh produce. Pathogens. 7(1): 18. https://doi.org/10.3390/pathogens7010018 PMID: 29389865.
Quereda JJ, Leclercq A, Moura A, Vales G, Gomez-Martin A, Garcia-Munoz A, Thouvenot P, Tessaud-Rita N, Bracq-Dieye H, Lecuit M (2020). *Listeria* valentine sp. nov., isolated from a water trough and the faeces of healthy sheep. Int.J.Syst.Evol. Microbiol. 70: 5868-5879.
Reda WW, Abdel-Moein K, Hegazi A, Mohamed Y, Abdel-Razik K (2016). *Listeria monocytogenes*: An emerging food-borne pathogen and its public health implications. J. Infect. Dev. Ctries. 10(2):149-154. https://doi.org/10.3855/jidc.6616
Rezai R, Ahmadi E, Salimi B (2018). Prevalence and Antimicrobial Resistance Profile of *Listeria* Species Isolated from Farmed and On-Sale Rainbow Trout (Oncorhynchus mykiss) in Western Iran. J. Food Prot. 81: 886–891.
Rohwer F, Edwards R (2002).The phage proteomic tree: a genome – based taxonomy for phage. J. Bacteiol. 184:4529-4535.
SR EN ISO 11290–1(1997). Microbiology of food and animal feeding stuffs-Horizontal method for the detection of *Listeria monocytogenes* - Part 1: Detection method.
SCENIHR (2009). (Scientific Committee on Emerging and Newly Identified Health Risks). Assessment of the Antibiotic Resistance Effects of Biocides, European Commission Health & Consumer Protection DG Directorate C: Public Health and Risk Assessment Unit C7 - Risk Assessment Office: B232 B-1049 Brussels.
Şanlıbaba P, Uymaz Tezel B, Çakmak GA (2018). Detection of *Listeria* spp. in raw milk and dairy products retailed in Ankara. GIDA. 43(2):273-282. https://doi.org/10.15237/gida.GD17107.
Sant'Ana AS, Igarashi MC, Landgraf M, Destro MT, Franco BD (2012). Prevalence, populations and pheno and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil. Int. J. Food Microbiol. 155: 1–9.
Sarfaz M, Ashraf Y, Ashraf S (2017). A Review: Prevalence and antimicrobial susceptibility profile of *Listeria* species in milk. Matrix Sci. Med. 1(1): 03-09.
Sharma S, Sharma V, Dahiya DK, Khan A, Mathur M, Sharma A (2017). Prevalence, virulence potential, and antibiotic susceptibility profile of *Listeria monocytogenes* isolated from bovine raw milk samples obtained from Rajasthan, India. Foodborne Pathog. Dis. 14 (3): 132–40.
Shiwakoti S (2015). Prevalence of *Listeria monocytogenes* in the pre-harvest environment; a landscape epidemiology approach. Master's Thesis. The Graduate Faculty of the

North Dakota State University of Agric. Appl. Sci. USA.
Skowron K, Walecka-Zacharksa E, Grudlewska K, Wiktorczyk N, Kaczmarek A, Gryn G, Kwiecinska-Pirog J, Juszczuk K, Paluszak Z, Kosek-Paszkowska K, Gospodarek-Komkoweska E (2019). Characteristics of *Listeria monocytogenes* Strains Isolated from Milk and Humans and the Possibility of Milk-Borne Strains Transmission. Polish J. Microbiol. 68(3):353–369. https://doi.org/10.33073/ pjm-2019-038
Srinivasan V, Nam HM, Nguyen LT, Tamilselvam B, Murinda se Oliver SP (2005). Prevalence of Antimicrobial Resistance Genes in *Listeria monocytogenes* Isolated from Dairy Farms. Foodborne Pathog. Dis. 2(3).
Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S, Nadarajan R, Brodie EL, Lynch SV (2015). Use of *16S rRNA* gene for identification of a broad range of clinically relevant bacterial pathogens. PLoS One. 10 (2): e0117617. https://doi.org/10.1371/journal.pone.0117617. PMID: 25658760; PMCID: PMC 4319838.
Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microb. Methods. 40(2): 175–179.
Stewart PS, Costerton JW (2001). Antibiotic resistance of bacteria in biofilms. Lancet. 358:135–8.
Swetha CS, Rao TM, Krishnaiah N, Vijaya KA (2012). Detection of *Listeria monocytogenes* in fish samples by PCR assay. Ann. Biolog. Res. 3:1880-1884.
Tabit FT (2018). Contamination, prevention and control of *Listeria monocytogenes* in food processing and food service environments. In: Nyila MA editor. *Listeria Monocytogenes*. Intech Open. 71. https://doi.org/10.5772/intechopen.76132.
Tahoun AB, Abou Elez RM, Abdelfatah EN, Elsohaby I, El-Gedawy AA, Elmoslemany AM (2017). *Listeria monocytogenes* in raw milk, milking equipment and dairy workers: molecular characterization and antimicrobial resistance patterns. J. Global Antimicrob. Resist. 10:264–270. https://doi. org/10.1016/j. jgar. 2017.07. 008
Teshome Y, Giragn F, Gudeta D, Desa G, Bekele D (2019). Isolation and Prevalence of *Listeria* Species in Milk and Milk Product samples Collected from Bishoftu and Dukemtowns, Oromia, Ethiopia. World J. Dairy Food Sci. 14(2):196–201.
Thien-fah UK, George PA (2001). Mechanism of biofilm resistance to antimicrobial agents. Trends Microbiol. 9:34–9.
Tirumalai PS, Prakash S (2012). Expression of virulence genes by *Listeria monocytogenes*. J0161 in natural environment. Braz. J. Microbiol. 43(2):834–843. https://doi.org/10.1590/S1517-83822012000200050.
Toomey N, MonaghanA´, Fanning S, Bolton DJ (2009). Assessment of antimicrobial resistance transfer between lactic acid bacteria and potential foodborne pathogens using in vitro methods and mating in a food matrix. Foodborne Pathog. Dis. 6(8): 925–933. https://doi.org/10.1089/fpd.2009.0278 PMID:19799525.
Uruen C, Chopo-Escuin G.,Tommassen J, Mainar-Jaime RC, Arenas J (2021). Biofilms as Promoters of Bacterial Antibiotic Resistance and Tolerance. Antibiotics (Basel) 10(1):3. https://doi.org/10.3390/antibiotics10010003 PMID: 33374551 PMCID: PMC7822488.
Volokhov D, George J, Anderson C, Duvall RE, Hitchins AD (2006). Discovery of natural atypical non-hemolytic *Listeria seeligeri* isolates. Appl Environ Microbiol. 72(4):2439-48. https://doi.org/10.1128/AEM.72.4.2439-2448.2006. PMID: 16597942; PMCID: PMC1449060.
WHO (2018). World Health Organization, Antibiotic resistance. Retrieved from https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance.
Weiler C, Ifland A, Naumann A, Kleta S, Noll M (2013). Incorporation of *Listeria monocytogenes* strains in raw milk biofilms. Int J Food Microbiol. 161 (2): 61–68. https://doi.org/10.1016/j.ijfoodmicro.2012.11.027.
Zhang Y, Yeh E, Hall G, Cripe J, Bhagwat AA, Meng J (2007). Characterization of *Listeria monocytogenes* isolated from retail foods. Int. J. Food Microbiol. 113: 47–53.
Zitz U, Zunabovic M, Domig KJ, Wilrich PT, Kneifel W (2011). Reduced detectability of *Listeria monocytogenes* in the presence of *Listeria innocua*. J. Food Prot. 74:1282–1287.