Hindawi
Journal of Food Quality
Volume 2022, Article ID 9696927, 12 pages
https://doi.org/10.1155/2022/9696927

Research Article

Prevalence of Multidrug-Resistant *Listeria monocytogenes* in Dairy Products with Reduction Trials Using Rosmarinic Acid, Ascorbic Acid, Clove, and Thyme Essential Oils

Mahmoud Elafify,[1] Mohamed Tharwat Elabbasy,[2,3] Rasha Samir Mohamed,[4] Esraa A. Mohamed,[5] Walaa Fathy Saad Eldin,[6] Wageh Sobhy Darwish,[3] Essam Hassan Eldrehmy,[7,8] and Radwa Reda Shata[1]

[1]*Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt*
[2]*Public Health Department, College of Public Health and Health Informatics, Ha'il University, Ha'il, Saudi Arabia*
[3]*Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt*
[4]*Department of Agricultural Microbiology, Faculty of Agriculture, Cairo University, Cairo 12613, Egypt*
[5]*Department of Medical Microbiology and Immunology, Faculty of Medicine, Tanta University, Tanta, Egypt*
[6]*Educational Veterinary Hospital, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt*
[7]*Department of Biology, Turabah University College, Taif University, Taif, Saudi Arabia*
[8]*Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt*

Correspondence should be addressed to Mohamed Tharwat Elabbasy; tharwat330@gmail.com

Received 10 December 2021; Accepted 16 February 2022; Published 12 March 2022

Academic Editor: Ahmed A. Tayel

Continuous monitoring of *Listeria* spp., particularly *Listeria monocytogenes,* in foods is a mandatory task for food safety and microbiology sectors. This study aimed to determine the prevalence and antimicrobial resistance patterns of *L. monocytogenes* in milk and dairy products retailed in Egypt. Furthermore, an experimental trial was conducted to investigate the antilisterial effects of some phytochemicals. A total of 200 samples (market raw milk, Kareish cheese, Damietta cheese, and plain yoghurt, 50 each) were collected and examined for detection of *Listeria* spp. The results revealed that 8, 12, 1, and 0 samples of market raw milk, Damietta cheese, Kareish cheese, and plain yoghurt were contaminated with *Listeria* spp., respectively. Antimicrobial sensitivity testing revealed that all *L. monocytogenes* isolates (15) were resistant to streptomycin and erythromycin. Molecular analysis revealed that 86.67% of *L. monocytogenes* harbored *hylA* virulent gene. Use of rosmarinic acid, ascorbic acid, thyme, and clove essential oils significantly ($P < 0.05$) reduced *L. monocytogenes* growth in soft cheese—artificially contaminated with *L. monocytogenes* throughout a 4-week incubation period. In conclusion, strict hygienic conditions should be adopted during the preparation and distribution of dairy products. In addition, rosmarinic acid, ascorbic acid, clove, and thyme essential oils are good candidates as food preservatives with antilisterial activities.

1. Introduction

Contamination of milk and dairy products during processing and storage by food poisoning organisms is still a significant concern in dairy plants [1, 2]. More than 250 various illnesses have been reported to be transmitted via the consumption of contaminated foods [3]. Among the causative agents of such food-borne illnesses, *Listeria monocytogenes (L. monocytogenes)* has been allocated. The pathogenicity of *L. monocytogenes* is attributed to its resistance to a wide range of environmental stressors. It can withstand chilling, thermal, and osmotic stressors [4].

Until now, *L. monocytogenes* is isolated from ready-to-eat (RTE) foods such as milk, cheese, seafood, and

vegetables. For instance, *L. monocytogenes* was isolated from raw milk, cheese, and butter at 29.2%, 14.1%, and 4%, respectively, in Yazd, central Iran [5]. Also, in Faisalabad, *L. monocytogenes* was detected in unheated dairy products such as raw milk at 17.78% and cheese at 5.55% [6].

The common preservation methods as chilling and osmotic stressors are used to minimize and reduce the growth of *L. monocytogenes.* Still, these methods are not adequate enough for preventing the growth of the pathogen. Furthermore, *L. monocytogenes* had acquired genetic changes and virulence factors to withstand such preservation methods [4].

Although the proper pasteurization of milk and dairy products might be a protective method for inhibiting *L. monocytogenes* growth, postpasteurization contamination is still a possible way to transmit food-borne pathogens [7, 8]. As reported before, pasteurized milk distributed to various markets was shown to be contaminated with *L. monocytogenes* due to the postpasteurization contamination [9, 10]. Furthermore, overheat treatment has a negative effect on the quality of milk and dairy products because of protein denaturation, loss of vitamins, and undesirable changes in the flavor [11].

The food industry sector is seeking for safe and friendly candidates to act as food preservatives. In this direction, several natural substances had been established to suppress the growth of pathogens in milk and dairy products. D-tryptophan is a natural amino acid that revealed inhibitory activities against *Salmonella* spp. in contaminated cheese [12]. Also, olive oil extract showed an inhibitory effect against *Bacillus cereus*' vegetative cells in contaminated milk [13]. Rosemary is one of the most popular phytochemicals cultivated worldwide and is used as a flavoring agent and possesses antioxidant and antimicrobial properties [14]. Ascorbic acid has also several beneficial functions in maintaining the level of collagen, wound healing, as an antioxidant, and with antimicrobial activities against several pathogens like *Staphylococcus aureus, Campylobacter* spp., and *Mycobacterium tuberculosis* [15]. Clove is one of the phytochemicals with several medical applications as an antiseptic, analgesic, and antimicrobial, and the Food and Drug Administration recognized it as a safe food additive [16]. Thyme is another phytochemical that has antispasmodic and antimicrobial activities and is also a safe food preservative [17]. However, the antilisterial activities of such phytochemicals have received less attention. Thus, this study first aimed at the investigation of the prevalence of *Listeria* spp., particularly *L. monocytogenes,* in various cow's dairy products retailed in Egypt. Second, the antimicrobial resistance profiling of the recovered *L. monocytogenes* isolates was further screened using the disk diffusion method. In an experimental trial, the antilisterial activities of rosmarinic acid, ascorbic acid, clove, and thyme essential oils were examined using soft cheese as a food matrix.

2. Materials and Methods

2.1. Collection and Preparation of Samples. A total of 200 samples of dairy products (market raw milk, Kareish cheese, Damietta cheese, and plain yoghurt, 50 each) were collected from healthy animals at dairy farms, various shops, and supermarkets in Mansoura, Tanta, and Zagazig cities, Egypt. All collected samples were transferred and cooled in an insulated box and were microbiologically examined on the same day at either the Food Control Department, Faculty of Veterinary Medicine, Mansoura University, or the Department of Medical Microbiology and Immunology, Faulty of Medicine, Tanta University, Egypt.

About 25 g of all examined dairy product samples, except raw milk and yoghurt, was aseptically mixed well with 225 ml of 0.1% bacteriological peptone and homogenized well via a blender, and this homogenate was incubated at 37°C/24 h, while raw milk and yoghurt were mixed well via a sterile spatula, and 25 ml of them was added to 225 ml of 0.1% bacteriological peptone and also incubated in the same way.

2.2. Isolation and Identification of Listeria spp. Isolation of *Listeria* spp. was performed according to Roberts et al. [18]. In a brief, 10 ml of each incubated homogenate was separately added to 90 ml of *Listeria* enrichment broth base (CM862, Oxoid) with *Listeria* selective enrichment supplement (Nalidixic acid, cycloheximide, and acriflavine hydrochloride) (SR141, Oxoid) and incubated at 37°C/48 h. Subsequently, a loopful was taken from the enriched broth and streaked onto Oxford agar plates as a selective media (CM856, Oxoid) supplemented with *Listeria* selective supplement (SR140, Oxoid) and incubated at 37°C/48h. At least five olive green colonies surrounded by black zones were picked up for further purification on the same media and stored in glycerol 15% at −80°C for further examination.

The representative stored colonies were refreshed onto tryptone soy agar (CM0131, Oxoid) supplemented with 0.6% yeast extract and incubated at 37°C/48 h. The refreshed isolates were microscopically examined (*Listeria* spp. appeared as coccobacilli and nonsporulating bacteria), and then, the isolates were exposed for biochemical examination (catalase test, oxidase, and sugar fermentation tests including D-glucose, L-rhamnose, xylose, and mannitol) and typical umbrella motility at 25°C (Table 1) [19]. The isolates exhibited that the positive results were exposed to hemolysin production via using blood agar media (CM854, Oxoid) supplemented with 5% sheep blood. Furthermore, the CAMP test was applied against *Staphylococcus aureus* [22]. Finally, the expected isolates were subjected to serological identification using a *Listeria* latex agglutination kit (Oxoid, Basingstoke, Hampshire, England) [23]. All procedures were carried out according to the manufacturer's instructions.

2.3. Antimicrobial Susceptibility Testing. The positive fifteen isolates of *L. monocytogenes* that revealed typical characteristics for the used biochemical tests were exposed to antimicrobial susceptibility testing using agar disk diffusion methods on Mueller-Hinton agar [24]. The tested antimicrobials were amikacin (AM) (30 μg); cefotaxime (CF) (30 μg); cephalothin (CN) (30 μg); chloramphenicol (C) (30 μg); ciprofloxacin (CP) (5 μg); erythromycin (E) (15 μg);

Table 1: Biochemical tests used to differentiate between Listeria spp.

Species	Hemolysis	Catalase test	Oxidase test	D-mannitol fermentation	Rhamnose fermentation	D-xylose fermentation	CAMP (*S. aureus*)	*Listeria* latex agglutination	Permissible counts*	Reference
L. monocytogenes	+ (β)	+	−	−	+	−	+	+++	ND	MacFaddin [19]
L. innocua	−	+	−	−	V	−	−	+	ND	
L. ivanovii	+	+	−	−	−	+	−	+	ND	
L. seeligeri	(+)	+	−	−	−	+	(+)	+	ND	
L. welshimeri	−	+	−	−	V	+	−	+	ND	
L. grayi	−	+	−	+	−	−	−	+	ND	

+: Positive test; -: negative test; (+): weak reaction; V: variable results; and +++: strong positive result. *The permissible count for L. *monocytogenes* in milk and dairy products is zero (ND: not detected) [20,21].

gentamicin (G) (10 μg); kanamycin (K) (30 μg); nalidixic acid (NA) (30 μg); neomycin (N) (30 μg); streptomycin (S) (10 μg); ampicillin (AM) (10 μg) oxytetracycline (T) (30 μg); and trimethoprim-sulfamethoxazole (SXT) (25 μg). The interpretation of the results was performed according to CLSI [25]. Multiple antibiotic resistance index was calculated according to the equation established by Singh et al. [26]:

MAR index = Number of resistance (isolates classified as intermediate were calculated as sensitive for MAR index) antibiotics/total number of antibiotics tested.

2.4. DNA Extraction. Bacterial DNA from three to five juvenile refreshed colonies of *L. monocytogenes* isolates was extracted using GeneJET Genomic DNA Purification Kit (Fermentas) according to the manufacturer's instruction.

2.5. Detection of hlyA Gene as a Virulent Gene of L. monocytogenes. L. monocytogenes isolates were screened for detection of listeriolysin O *(hlyA)* gene as a virulent gene presents only in the pathogenic types using PCR. The amplification was performed on a thermal cycler (Mastercycler, Eppendorf, Hamburg, Germany). The primer sequences were *hylA* (F) 5′ GCAGTTGCAAGCGCTTGGAGTGAA ′3 and *hylA* (R) 5′ GCAACGTATCCTCCAGAGTGATCG ′3 that used to produce 456 pb PCR products under the following protocol: an initial denaturation at 95°C for 2 min followed by 35 cycles each of 15-sec denaturation at 95°C and 30-sec annealing at 60°C and 1 min extension at 72°C, followed by a final extension of 10 min at 72°C and held at 4°C [27]. Amplified DNA fragments were run on 1.5% agarose gel electrophoresis (AppliChem, GmbH, Germany) in 1x TBE buffer stained with ethidium bromide and captured and visualized on a UV transilluminator.

2.6. Antilisterial Effects of Some Phytochemicals

2.6.1. Bacterial Preparations. Four strains of *L. monocytogenes* (ATCC7644, 19118, 15313, and 13932) were obtained from the American Type Culture Collection (Manassas, VA, USA) and were used in our experiment. All these isolates were stored at −80°C in tryptic soy broth (TSB; Merck) containing 15% glycerol. For refreshment of these isolates, a loopful was individually taken from each strain and cultured onto sterile TSB and incubated at 37°C/24 h and subsequently streaked onto tryptic soy agar (TSA), and all plates were incubated at 37°C/48 h. A single pure colony from each strain was taken via a sterile platinum loop and inoculated into an independent sterile of 10 mL TSB. Cocktail pathogen protocol was used in this experiment. Thus, equal volumes (1 mL) from each strain in inoculated TSB were collected and combined in a sterile plastic tube (10 mL) and mixed well through a vortex [9].

2.6.2. Preparation of the Tested Phytochemicals and Media. Rosmarinic and L-ascorbic acids were purchased from Sigma-Aldrich, CO, USA. Clove and thyme essential oils (100%) were locally purchased from the Animal Health Research Institute, Giza, Egypt. All used media as TSA and TSB were prepared according to the manufacturer's instructions.

2.6.3. Screening of the Antilisterial Activities of the Tested Phytochemicals Using Agar Well Diffusion Method. The antimicrobial activity of phytochemical extracts was performed according to Mayo [28]. Briefly, *L. monocytogenes* strains were refreshed on Müeller-Hinton agar (Oxoid) at 37°C/24 h. A single pure colony from each strain was picked up, cultured in TSB, and incubated at 37°C/24 h. Then, 1 mL of each strain was combined into a sterile plastic tube. The infective dose was adjusted at 10^5–10^6 CFU/mL. Wells with 9 mm diameters had been performed in Müeller-Hinton agar, and then, 50 μL of each tested phytochemical at a concentration range between 0.5% and 3.0% (0.5% interval) was placed in the wells of Müeller-Hinton that previously streaked with *L. monocytogenes*. The incubated plates were kept at 4°C to allow the diffusion of the extracts onto the medium, and then, the incubation was followed at 37°C/24 h. Subsequently, the inhibition zones at the lowest concentrations were recorded and used in the experimental trials.

2.6.4. Preparation and Inoculation of Soft Cheese. Fresh soft cheese was prepared according to Youssef et al. [29], and cow's raw milk was collected from dairy farms in Dakahlia province, Egypt, and pasteurized at 63°C/30 min in a water bath and kept warm at 40°C. Sodium chloride (2% and 3%), calcium chloride (0.02 g/L), and the cocktail pathogens of *L. monocytogenes* were added to milk. Subsequently, the warmed milk was distributed onto clean and sterile vats. Then, the rennet (0.2 g/L) and one of the tested phytochemicals either rosmarinic acid (1%), ascorbic acid (3%), clove essential oil (2%), or thyme essential oil (2%) were separately added to the inoculated milk and incubated at 37°C/45–60 min. These concentrations were chosen based on the results of the antimicrobial activity of the pre-experiment. In parallel, control cheese samples were also manufactured in the same manner without any inhibitory phytochemicals. Once the coagulation was performed, the curd was separated from whey via a clean, sterilized drainage cloth. Then, the obtained cheese was kept in a sterile container at 4°C and 10°C for four weeks. Samples were collected each week and compared with the control to determine *L. monocytogenes* counts. Three independent trials, with three samples for each time point, were carried out at 4°C and 10°C.

2.6.5. L. monocytogenes Counts in Cheese Samples. Under a complete septic condition, a tenfold serial dilution was performed for each collected sample via 0.1% sterile peptone water. The surface spreading method was used by culturing 100 μl aliquots of a suitable inoculated diluent onto sterile TSA followed by incubation at 37°C/18–24 h. All colonies were counted using a colony counter and compared with the control samples. The obtained results were

converted into log10 CFU/g. Each experiment in each condition was carried out in triplicate (three independent trials).

2.7. Statistical Analysis. To examine the significant differences between the treated and control samples, one-way ANOVA was conducted to compare results at various treatments and concentrations. Statistical analyses were carried out using R statistical software (v. 3.5.2, R Foundation for Statistical Computing, Vienna) with a p value of 0.05 that is considered to be significant.

3. Results

3.1. Prevalence of Listeria spp. including L. monocytogenes in Milk and Dairy Products. In this study, the achieved results illustrated that 16%, 24%, and 2% of market raw milk, Damietta cheese, and Kariesh cheese were positive for *Listeria* spp., respectively, while *Listeria* spp. could not be recovered from yoghurt. Various types of *Listeria* spp. were biochemically identified from examined milk and dairy products *as L. monocytogenes, L. innocua, L. ivanovii, L. grayi, L. seeligeri, and L. welshimeri* (Table 1). Fifteen out of twenty-five isolates were confirmed as *L. monocytogenes* as 6, and 9 isolates were recovered from raw milk and Damietta cheese, respectively.

3.2. Antimicrobial Resistance and Virulence Characterization of L. monocytogenes. According to the biochemical tests and serological identification using *Listeria* latex agglutination kit (Oxoid, Basingstoke, Hampshire, England), fifteen isolates exhibited positive results for *L. monocytogenes* and these isolates were screened for their antimicrobial resistance and for detection of one of the most virulent genes (*hylA*) that is necessary for *L. monocytogenes* virulence (Tables 2 and 3). The results revealed that 100% of *L. monocytogenes* isolates were resistant to streptomycin and erythromycin. Also, *L. monocytogenes* were phenotypically resistant to oxytetracycline (86.67%), (nalidixic acid, sulfamethoxazole, cephalothin, and amikacin, 73.33% each) and cefotaxim (60%). Lower than 50% of *L. monocytogenes* isolates were resistant to chloramphenicol (46.67%), kanamycin (33.33%), neomycin (33.33%), gentamicin (13.33%), ampicillin (13.33%), and ciprofloxacin (6.67%). The average of MAR indices for the isolates recovered from milk and cheese was 0.607 and 0.539, respectively, with no significant difference ($p = 0.4$).

All L. *monocytogenes* isolates were genotypically examined for detection of *hylA* gene, and the results revealed that 13 (86.67%) out of 15 isolates harbored *hylA* gene.

3.3. Antilisterial Activities of Some Phytochemicals in Soft Cheese with 2% NaCl. The inhibitory effects of rosmarinic and ascorbic acids, and the essential oils of clove and thyme against *L. monocytogenes* were investigated. The survival counts of *L. monocytogenes* in soft cheese with 2% NaCl are shown in Figure 1. The results showed that rosmarinic acid 1% had a bacteriostatic effect against *L. monocytogenes* at 4°C, and *L. monocytogenes* counts were reduced by 4.6 log CFU/g during a 4-week treatment with a reduction percentage of 58.95%. Furthermore, essential oils of clove at 2%, thyme at 2%, and ascorbic acid at 3% showed a significant ($P < 0.05$) reduction in *L. monocytogenes* counts by 5.0, 6.2, and 6.4 log CFU/g, respectively, during a nearly one-month storage period. Such reduction counts were 3.7, 5.0, 4.6, and 5.4 log CFU/g, after treatment with rosmarinic acid 1%, ascorbic acid 3%, and essential oils of both clove 2% and thyme 2%, respectively, in soft cheese artificially contaminated with *L. monocytogenes* and kept at 10°C (Figure 1, Table 4). It is noteworthy to confirm that storage of cheese at 4°C revealed a significant reduction than storage at 10°C in a combination with all tested additives.

3.4. Antilisterial Activities of Some Phytochemicals in Soft Cheese with 3% NaCl. *L. monocytogenes* counts in artificially contaminated soft cheese (3% NaCl) stored at 4°C and treated with rosmarinic acid (1%), and essential oils of clove (2%) and thyme (2%) exhibited a significant ($P < 0.05$) reduction in *L. monocytogenes* count by 4.4, 5.0, and 6.2 log CFU/g, respectively, through the 4-week storage period. *L. monocytogenes* could not be detected in soft cheese treated with ascorbic acid 3% at a 4-week storage period. Increasing the refrigeration temperature up to 10°C exhibited a significant ($P < 0.05$) reduction in *L. monocytogenes* count. Cotreatment of cheese with rosmarinic acid (1%), essential oils of clove (2%) and thyme (2%), and ascorbic acid (3%) had 4.1, 4.8, 5.6, and 5.9 log CFU/g reduction, respectively, in *L. monocytogenes* during the end of storage period (4 weeks) achieving a significant ($P < 0.05$) reduction in *L. monocytogenes* count by 51.54%, 60.51%, 71.15%, and 75.38%, respectively (Figure 2, Table 5). Comparison of the results of Tables 4 and 5 showed that cotreatment of contaminated cheese containing a high salt content (3% NaCl) with the used antilisterial additives exhibited slightly higher reduction percentages in *L. monocytogenes* growth than low salt content (2% NaCl).

4. Discussion

L. monocytogenes represents a great challenge to the food safety sector because of its apparent resistance to a wide range of environmental stressors [4]. The achieved results indicated that 16% of market raw milk, 24% of Damietta cheese, and 2% of Kariesh cheese were contaminated with *Listeria* spp. particularly *L. monocytogenes*. Thus, these contaminated samples did not meet the national and international standards [20, 21], which confirm that dairy products must be free from *L. monocytogenes*. The results of this study go in agreement with a study conducted in Ethiopia, which revealed that 13% of raw milk was contaminated with *Listeria* spp. [30]. Similarly, in the USA, 12.6% of milk filters were contaminated with *L. monocytogenes* [31]. Besides, in Iran, 21.7% of raw milk was contaminated with *Listeria* spp. [32]. Additionally, *Listeria* spp. was isolated from raw milk in Iran at 29.2%,

Table 2: Antimicrobial susceptibility of *L. monocytogenes* isolated from milk and soft cheeses ($n = 15$ isolates).

Antimicrobial agents	Antimicrobial group	Sensitive (S)		Intermediate (I)		Resistant (R)	
		No.	%	No.	%	No.	%
Streptomycin (S)	Aminoglycosides	—	—	—	—	15	100
Erythromycin (E)	Macrolide	—	—	—	—	15	100
Oxytetracycline (T)	Tetracycline	—	—	2	13.33	13	86.67
Nalidixic acid (NA)	Quinolone	2	13.33	2	13.33	11	73.33
Sulfamethoxazole (SXT)	Sulfonamide	4	26.67	—	—	11	73.33
Cephalothin (CN)	Cephalosporin	1	6.67	3	20	11	73.33
Amikacin (AK)	Aminoglycosides	3	20	1	6.67	11	73.33
Cefotaxim (CF)	Cephalosporin	6	40	—		9	60
Chloramphenicol (C)	Phenicols	4	26.67	4	26.67	7	46.67
Kanamycin (K)	Aminoglycosides	7	46.67	3	20	5	33.33
Neomycin (N)	Aminoglycosides	9	60	1	6.67	5	33.33
Gentamicin (G)	Aminoglycoside	12	80	1	6.67	2	13.33
Ampicillin (AM)	β-lactam	13	86.67	—		2	13.33
Ciprofloxacin (CP)	Fluoroquinolone	11	73.33	3	20	1	6.67

Table 3: Antimicrobial resistance profiling of L. *monocytogenes* isolated from milk and soft cheeses ($n = 15$).

No.	ID of the isolates	Sources	Antimicrobial resistance profile	MAR index	Virulent gene (*hylA*)
1	9	Raw milk	S, E, T, NA, STX, CN, AK, CF, C, K, N, AM	0.857	+
2	18	Raw milk	S, E, NA, STX, CN, AK, CF, K, N	0.643	+
3	24	Raw milk	S, E, T, NA, STX, CN, AK, K, N	0.643	+
4	31	Raw milk	S, E, NA, STX, CF, G, CP	0.500	+
5	41	Raw milk	S, E, T, CN, AK, CF, C	0.500	+
6	57	Raw milk	S, E, T, NA, STX, CN, AK	0.500	+
7	62	Damietta cheese	S, E, T, NA, STX, CF, C	0.500	+
8	69	Damietta cheese	S, E, T, NA, STX, CN, AK, CF, C, K, N	0.786	+
9	78	Damietta cheese	S, E, T, CN, AK, CF, C, G	0.571	+
10	79	Damietta cheese	S, E, T, NA, STX, CN, AK, CF, C, K, N	0.786	+
11	84	Damietta cheese	S, E,T, NA, STX, CN, AK, CF	0.571	+
12	88	Damietta cheese	S, E,T, NA, STX, CN, AK, C	0.571	+
13	96	Damietta cheese	S, E,T, CN, AK, AM	0.428	−
14	112	Damietta cheese	S, E,T, NA, STX	0.357	+
15	136	Damietta cheese	S, E,T, N	0.285	−

S: Streptomycin; E: erythromycin; NA: nalidixic acid; T: oxytetracycline; SXT: sulfamethoxazole; CN: cephalothin; AK: amikacin; CF: cefotaxim; C: chloramphenicol; K: kanamycin; N: neomycin; G: gentamicin; AM: ampicillin; CP: ciprofloxacin.

L. monocytogenes at 7.8%, and *L. innocua* at 15% [33]. In Egypt, *L. monocytogenes* was the prevalent *Listeria* spp., isolated from milk and dairy products in Assiut, Egypt [34]. Besides, seven raw milk samples were contaminated with *L. monocytogenes*, and 3 samples were contaminated with *L. innocua* in a recent study in Egypt [35].

Damietta cheese had the highest prevalence of *L. monocytogenes*. In general, the survival and growth of *L. monocytogenes* depend on several factors such as comparing microflora, pasteurization process, and pH [36]. A study reported that the suitable pH for the growth of *L. monocytogenes* is < 5.6 as in the case of soft cheese [37]. Damietta cheese is a common type of soft cheese consumed in Egypt. Its manufacture mainly depends on milk coagulation via rennet. Once the crude has been performed, it is cut into small blocks, followed by salting. This type of soft cheese is either consumed fresh (a few weeks to one month postsalting process) or as a ripened cheese. Therefore, the manufacture processes of Damietta cheese serve as ideal conditions for the growth of *L. monocytogenes* [36]. High isolation of *L. monocytogenes* in soft cheese was also reported before as Al-Ashmawy et al. [36] stated that 24% of Damietta cheese was contaminated with *L. monocytogenes.* Kariesh cheese came second in the prevalence of *Listeria* contamination. Kariesh cheese is another type of soft cheese that is commonly manufactured from unpasteurized milk in Egyptian villages. In agreement with the prevalence of *L. monocytogenes* in Kariesh cheese, *L. monocytogenes* was recovered from 3 Kariesh cheese samples in Egypt [38]. Furthermore, 4% of Kariesh cheeses were contaminated with *L. monocytogenes* [34]. However, another study failed to isolate *L. monocytogenes* from Kariesh cheese [39].

Yoghurt is one of the main fermented dairy products in Egypt. In this study, none of the examined yoghurt samples were contaminated with *L. monocytogenes*. This result agrees with other studies that failed to isolate *L. monocytogenes* from yoghurt [39, 40]. The absence of *L. monocytogenes* in the examined yoghurt samples might be attributed to the antimicrobial activities of some compounds secreted by lactic acid bacteria present in yoghurt [34].

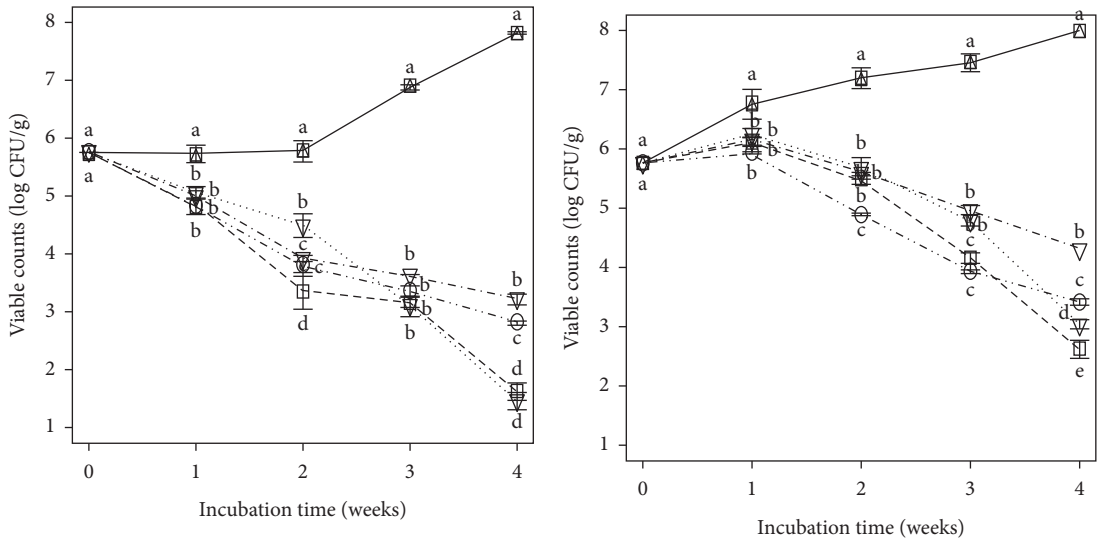

Figure 1: Inhibitory effect of some phytochemicals, solid line (0% control), dotted line (3% ascorbic acid), dot-dashed line (1% rosmarinic acid), long-dashed line (2% clove oil), and dashed line (2% thyme oil), on the survival and growth of *L. monocytogenes* in experimentally contaminated soft cheese with 2% NaCl at (a) 4°C and (b) 10°C. Results are the mean ± SD of three independent trials at each sampling point. Value with different letters at the same incubation time means a significant difference ($P < 0.05$).

Table 4: Mean ± standard deviation (SD) and reduction (%) in L. monocytogenes growth in soft cheese (2% NaCl) at 4 and 10°C.

Temperatures	The incubation period (weeks)	Control mean ± SD	Rosmarinic acid mean ± SD (reduction %)	Ascorbic acid mean ± SD (reduction %)	Clove oil mean ± SD (reduction %)	Thyme oil mean ± SD (reduction %)
4 C	0	5.75 ± 0.01^{a}	5.75 ± 0.01^{a}	5.75 ± 0.01^{a}	5.75 ± 0.01^{a}	5.75 ± 0.01^{a}
	1st	$5.73 \pm 0.15^{a*}$	$5.00 \pm 0.01^{b*}$ (12.74)	$5.06 \pm 0.09^{b*}$ (11.69)	$4.81 \pm 0.13^{b*}$ (16.05)	$4.83 \pm 0.13^{b*}$ (15.71)
	2nd	$5.78 \pm 0.18^{a*}$	$3.92 \pm 0.06^{c*}$ (32.18)	$4.49 \pm 0.19^{b*}$ (22.32)	$3.80 \pm 0.18^{c*}$ (34.26)	$3.36 \pm 0.32^{d*}$ (41.87)
	3rd	$6.89 \pm 0.04^{a*}$	$3.64 \pm 0.02^{b*}$ (47.17)	$3.10 \pm 0.17^{b*}$ (55.01)	$3.35 \pm 0.09^{b*}$ (51.38)	$3.16 \pm 0.09^{b*}$ (54.14)
	4th	$7.82 \pm 0.02^{a*}$	$3.21 \pm 0.09^{b*}$ (58.95)	$1.46 \pm 0.15^{d*}$ (81.33)	$2.81 \pm 0.03^{c*}$ (64.10)	$1.62 \pm 0.15^{d*}$ (79.28)
10 C	0	5.75 ± 0.01^{a}	5.75 ± 0.01^{a}	5.75 ± 0.01^{a}	5.75 ± 0.01^{a}	5.75 ± 0.01^{a}
	1st	6.76 ± 0.24^{a}	6.16 ± 0.09^{b} (8.87)	6.27 ± 0.07^{b} (7.25)	5.94 ± 0.02^{b} (12.13)	6.12 ± 0.07^{b} (9.47)
	2nd	7.20 ± 0.17^{a}	5.59 ± 0.03^{b} (22.36)	5.67 ± 0.18^{b} (21.25)	4.89 ± 0.02^{c} (32.08)	5.47 ± 0.07^{b} (24.03)
	3rd	7.46 ± 0.15^{a}	4.97 ± 0.07^{b} (33.38)	4.79 ± 0.10^{b} (35.79)	3.93 ± 0.03^{c} (47.32)	4.16 ± 0.09^{c} (44.23)
	4th	8.00 ± 0.01^{a}	4.30 ± 0.01^{b} (46.25)	3.04 ± 0.08^{c} (62.00)	3.41 ± 0.05^{d} (57.37)	2.62 ± 0.15^{e} (67.25)

Different letters in the same raw indicate significant differences at $P < 0.05$. Values carrying star mark (*) indicate significant difference at $P < 0.05$ between the corresponding values at 4°C and 10°C.

The abuse of antimicrobials during animal farming and livestock production might lead to the development of antimicrobial resistance [12, 41]. The achieved results in this study revealed that 100% of the recovered *L. monocytogenes* isolates showed resistance against streptomycin and erythromycin, while more than 70% of the isolates showed resistance against oxytetracycline, nalidixic acid, sulfamethoxazole, cephalothin, and amikacin. These results are in agreement with a previous study that recorded 100% of *L. monocytogenes* isolated from cheese had resistance to tetracycline, streptomycin, and sulfamethoxazole [36]. However, more than 70% of the recovered isolates were susceptible to gentamicin, ampicillin, and ciprofloxacin. This agrees with a previous study that reported the susceptibility of *L. monocytogenes* isolated from cheese to gentamicin [42].

There are many important virulence genes for *Listeria* spp. such as as *prfA, inlA, inlB, and irpA,* and others, but the most virulence factor associated with *L. monocytogenes* is listeriolysin O (LLO) that is produced by the microorganism and encoded by *hlyA* gene, and the LLO-encoding gene (*hlyA*) presents only in the virulent strains of *Listeria* spp. and is urgently required for their virulence [35]. Molecular

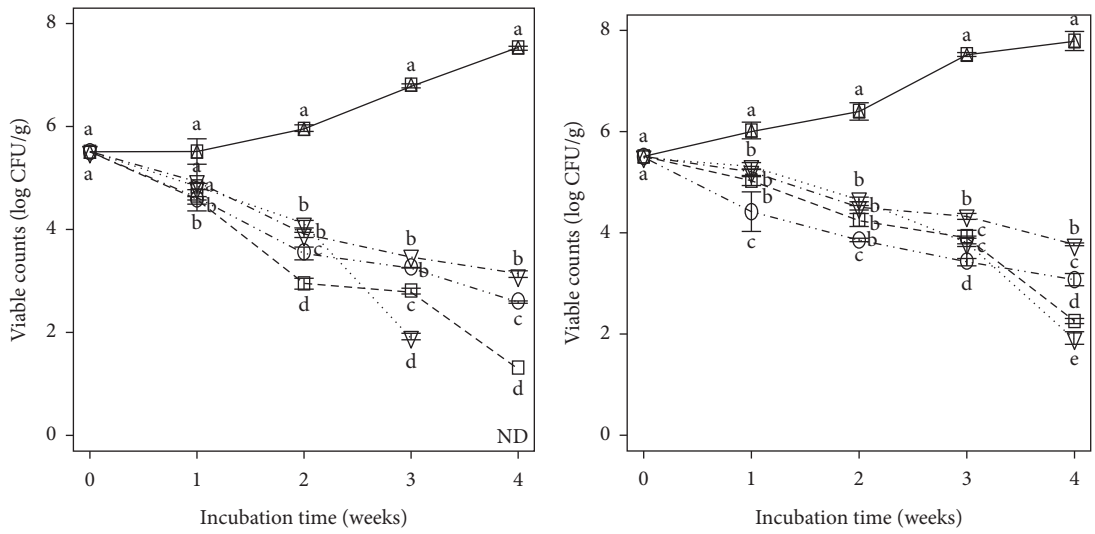

Figure 2: Inhibitory effect of some phytochemicals, solid line (0% control), dotted line (3% ascorbic acid), dot-dashed line (1% rosmarinic acid), long-dashed line (2% clove oil), and dashed line (2% thyme oil), on the survival and growth of *L. monocytogenes* in experimentally contaminated soft cheese with 3% NaCl at (a) 4°C and (b) 10°C. Results are the mean ± SD of three independent trials at each sampling point. Value with different letters at the same incubation time means a significant difference ($P < 0.05$). ND: not detected.

Table 5: Mean ± standard deviation (SD) and reduction (%) in *L. monocytogenes* growth in soft cheese (3% NaCl) at 4 and 10°C.

Temperatures (°C)	The incubation period (weeks)	Control mean ± SD	Rosmarinic acid mean ± SD (reduction %)	Ascorbic acid mean ± SD (reduction %)	Clove oil mean ± SD (reduction %)	Thyme oil mean ± SD (reduction %)
4	0	5.52 ± 0.01^{a}	5.52 ± 0.01^{a}	5.52 ± 0.01^{a}	5.52 ± 0.01^{a}	5.52 ± 0.01^{a}
	1st	5.52 ± 0.24^{a}	4.94 ± 0.56^{a} (10.51)	4.84 ± 0.06^{a} (12.32)	4.61 ± 0.03^{b} (16.48)	4.64 ± 0.13^{b} (15.94)
	2nd	5.97 ± 0.07^{a}	$3.90 \pm 0.05^{b*}$ (34.67)	$4.10 \pm 0.07^{b*}$ (31.32)	3.54 ± 0.13^{c} (40.70)	$2.95 \pm 0.09^{d*}$ (50.59)
	3rd	$6.80 \pm 0.04^{a*}$	$3.48 \pm 0.01^{b*}$ (48.82)	$1.92 \pm 0.06^{c*}$ (71.76)	3.26 ± 0.01^{b} (52.06)	$2.81 \pm 0.06^{d*}$ (58.68)
	4th	$7.52 \pm 0.03^{a*}$	$3.13 \pm 0.06^{b*}$ (58.38)	$0.00 \pm 0.00^{c*}$ (100.00)	2.59 ± 0.03^{d} (65.56)	$1.30 \pm 0.00^{e*}$ (82.71)
10	0	5.52 ± 0.01^{a}	5.52 ± 0.01^{a}	5.52 ± 0.01^{a}	5.52 ± 0.01^{a}	5.52 ± 0.01^{a}
	1st	6.02 ± 0.17^{a}	5.20 ± 0.07^{b} (13.62)	5.32 ± 0.06^{b} (11.63)	4.42 ± 0.39^{c} (26.58)	5.04 ± 0.08^{b} (16.28)
	2nd	6.40 ± 0.17^{a}	4.50 ± 0.05^{b} (29.69)	4.67 ± 0.05^{b} (27.03)	3.87 ± 0.03^{c} (39.53)	4.26 ± 0.13^{b} (33.44)
	3rd	7.53 ± 0.03^{a}	4.33 ± 0.05^{b} (42.50)	3.81 ± 0.09^{d} (49.40)	3.43 ± 0.08^{c} (54.45)	3.92 ± 0.13^{d} (47.94)
	4th	7.80 ± 0.18^{a}	3.78 ± 0.02^{b} (51.54)	1.92 ± 0.13^{c} (75.38)	3.08 ± 0.12^{d} (60.51)	2.25 ± 0.05^{e} (71.15)

Values carrying star mark (*) indicate significant difference at $P < 0.05$ between the corresponding values at 4°C and 10°C.

analysis of *L. monocytogenes* revealed the detection of *hlyA* gene as one of the most coding genes for its pathogenicity. Similarly, several previous studies detected *hlyA* in *L. monocytogenes* isolated from food samples [34, 36].

Several outbreaks of listeriosis have been linked with the consumption of improper heated dairy products such as soft and semisoft cheese. Proper pasteurization of milk should eliminate *L. monocytogenes* from milk. Still, the presence of *L. monocytogenes* in pasteurized milk or dairy products indicates postpasteurization contamination [43]. Therefore, this study was extended to investigate the antilisterial activities of some phytochemicals.

The tested chemicals at the used concentrations did not show deviations in the sensory characteristics of the soft cheese (data are not shown). Interestingly, rosmarinic acid showed significant antilisterial effects in soft cheese contaminated with *L. monocytogenes*. Rosmarinic acid is rich in phenols that have a vital role in disrupting the bacterial cell

wall [43]. The rosemary's antimicrobial effect was in agreement with Han et al. [43], who recorded a 2.5 log CFU/g reduction in *Listeria* counts within nine days of treatment at contaminated cheese. Besides, rosemary used for the preservation of flavored cheese made from cream cheese showed a significant ($P < 0.05$) reduction in the total bacterial counts of the cheese [44].

Ascorbic acid is one of the tested chemicals in this study and exhibited significant antilisterial activities. Several studies investigated the antimicrobial properties of ascorbic acid. For instance, ascorbic acid at a concentration of 0.31 mg/mL had an in *vitro* bactericidal effect against *Pseudomonas aeruginosa* [45]. Furthermore, ascorbic acid had a significant role in the inhibition of biofilm formation of methicillin-resistant *Staphylococcus aureus* at 8–16 mg/mL [46]. Ascorbic acid also showed antisalmonella activity in the digestive tract of chickens [47]. In addition, Verghese et al. [15] stated that ascorbic acid at a concentration of 20 mg/mL had clear inhibitory effects against *E. coli* and *Klebsiella pneumoniae* in broth culture. Regarding the use of ascorbic acid in dairy products, Shivaprasad et al. [48] recorded a significant reduction in *E. coli* EMC17 counts (1.5 log CFU/g) in treated cottage cheese with ascorbic acid. The same authors added that the antimicrobial effects of ascorbic acid are attributed to two main factors: the first is its prooxidant action on ROS generation (hydroxyl radicals, hydrogen peroxide, and superoxide). The high dose of ascorbic acid leads to the reduction of Fe+3 to Fe+2, leading to an intracellular pressure of ferrous ions. A load of ferrous ions leads to ROS generation, which has a vital role in bacterial cell death. The second factor is the leakage of protein and sugar due to ROS generation, which leads to bacterial cell death.

Clove oil is another natural product rich in phytochemicals that has several uses as an antiseptic, analgesic, and antimicrobial. In this study, clove oil had significant antilisterial activities in soft cheese contaminated with *L. monocytogenes*. Likely, Menon and Garg [49] reported the antilisterial effect of clove (1%) on contaminated cheese, and the results showed a 3 log CFU/g reduction in the viability counts of *Listeria* spp., compared to the control sample. However, Leuschner and Ielsch [50] recorded the bactericidal effect of clove oil against *L. monocytogenes* on the broth culture only without a significant effect of clove powder on the contaminated cheese. The antimicrobial effect of clove is due to its content of eugenol that might denature bacterial proteins and alter bacterial cell membrane [51].

Thyme oil also exhibited significant antilisterial activities in soft cheese contaminated with *L. monocytogenes*. Similarly, Bagamboula et al. [52] recorded bactericidal effects of thyme oil against *Enterobacteriaceae* at low concentration (0.1%) in naturally contaminated lettuce. Besides, Oliveira et al. [53] used thyme and rosemary oils to control *L. monocytogenes* in raw beef. The results showed a significant suppression in the pathogen growth by 1.25 log CFU/g within 48 h of using the inhibitory oils. Furthermore, Abdollahzadeh et al. [54] examined the inhibitory effect of thyme oils against *L. monocytogenes* in minced beef. The results stated that using thyme oil at a concentration of 0.8 to 1.2% reduced the viable count of *L. monocytogenes* in minced beef to less than 2 log CFU/g within six days of storage. They explained the antimicrobial activity of thyme due to its high contents of thymol and carvacrol that have vital roles in the disturbance of bacterial cell membrane permeability.

The combined effects of the used additives together with osmotic stress (NaCl 2%, and NaCl 3%) on L. monocytogenes viability were investigated. The obtained results indicated that the used antimicrobial additives exerted the greatest overall suppression of L. monocytogenes when combined with NaCl 3%. Similarly, Chen et al. [55] found that using D-tryptophan as an antimicrobial amino acid for suppression of the growth of *V. vulnificus* and *V. parahaemolyticus* at a high salt content (5% NaCl) exerted more bacteriostatic effect than low salt content (3.5% NaCl). Also, the same authors found that the total bacterial population in freshly shucked oysters in artificial seawater treated with 40 mM D-tryptophan with a high salt content revealed a significant decrease in the bacterial counts than that recorded at low salt content. The high NaCl content led to an increase in the withdrawal of water from the bacterial cells, followed by a more gradual reduction in bacterial populations [55].

Treatment of L. *monocytogenes* with natural additives at 4°C led to a significant reduction in the pathogen growth than at 10°C. This result agrees with other studies that used lactic acid and hot water for decontamination of *L. monocytogenes* on the surface of fresh beef stored at 4°C and 10°C as *L. monocytogenes* was not detected at 4°C, while it was still grown at 10°C [56]. Besides, Han et al. [43] examined the inhibitory effects of thyme and rosemary oils onto artificially contaminated cheese with *L. monocytogenes* at 4°C and 10°C, and the results revealed that thyme oil or rosemary oils (1%, each) had more potent inhibitory effects on *L. monocytogenes* at 4°C than 10°C. In general, the growth inhibition of pathogens at 4°C than 10°C might be via the extension of the lag phase produced by the inhibitory agents [43].

5. Conclusions

The obtained results of this study revealed isolation of multidrug-resistant *L. monocytogenes* from dairies retailed in Egypt. Rosmarinic acid, ascorbic acid, clove, and thyme essential oils showed significant antilisterial activities in soft cheese contaminated with *L. monocytogenes* compared with the control samples. Furthermore, cotreatment of contaminated cheese containing a high salt content (3% NaCl) with the used food additives exhibited slightly higher reduction percentages in *L. monocytogenes* growth than low salt content (2% NaCl). These results strongly recommend the use of rosmarinic acid, ascorbic acid, clove, and thyme essential oils as food preservatives with significant antilisterial activities besides the traditional food preservation conditions such as low chilling temperature and osmotic stressors. In addition, the adoption of strict hygienic measures during the manufacture and processing of dairy products is mandatory in food processing plants.

Data Availability

The data are available from the corresponding author upon request.

Ethical Approval

This study was approved by the research ethics committee (R/103) of the Faculty of Veterinary Medicine, Mansoura University, Egypt.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Acknowledgments

This research has been funded by the Scientific Research Deanship at the University of Ha'il, Saudi Arabia, through project number RG-20-211.

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