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Effect of Sub-Lethal Environmental Stresses on the Cell Survival and Antibacterial Susceptibility of Listeria monocytogenes PTCC1297

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Abstract

***Background*:** *Listeria monocytogenes* is slowly becoming drug resistant, study on resistance of this pathogen is important to ensure effective treatment of human listeriosis. The aim of this work was evaluating the changes in susceptibility to antibiotics and cell survival of *L. monocytogenes* PTCC1297 (serotype 4a) after exposure to some stresses.

***Materials and Methods*:** In this descriptive-analytic study *L. monocytogenes* PTCC1297 subjected to sub-lethal environmental stresses including ethanol (5% v/v), sodium chloride (7% w/v), acid (HCl, pH=5.0), hydrogen peroxide (600 ppm) and heat (45ºC). After the stress treatments, antibacterial susceptibility and cell survival were determined.

***Results*:** Exposing to hydrogen peroxide (600 ppm) and heat (45ºC) significantly (p<0.05) increased resistance to all selective antibiotics. But treating to stresses such as hydrochloric acid (pH=5.0), sodium chloride (7% w/v) and ethanol (5% v/v) decreased resistance (p<0.05) to antibiotics. *L. monocytogenes* PTCC1297 cell survival was decreased at 60ºC which is considered as lethal condition. Exposing to sub-lethal acid stress increased survival to high acidic conditions (pH=3.0). But upon increasing hydrogen peroxide the viability of cell decreased. Treating the cells with ethanol (14% v/v) and NaCl (20% w/v) increased cell survival.

***Conclusion*:** Adaptation to some stresses including hydrogen peroxide and heat increase resistance to antibiotics. Stresses such as ethanol, hydrochloric acid and sodium chloride act in adverse. Exposing to some sub-lethal stresses increased cell survival when lethal doses of the same stress such as acid, ethanol and sodium chloride were used. But, when we treating the cells to sublethal doses of H_2O_2 and heat the cell survival decreased.

Introduction

Listeria monocytogenes is an important foodborne pathogen. It plays a significant role in food safety control due to its wide distribution in nature.

The genus listeria contains 6 species of low G+C content gram-positive bacteria closely related to the genus bacillus [1]. The species are *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. grayi*. Studies show that *L. monocytogenes* strains from food and food-environments are susceptible to the antibiotics commonly used in veterinary and human listeriosis treatment.

The treatment of choice for listeriosis remains the administration of ampicillin or penicillin G combined with an aminoglycoside, classically gentamicin. The association of trimethoprim with a sulfonamide, such as sulfamethoxazole in co-trimoxazole, is considered to be a second-choice therapy. Considering that *L. monocytogenes* is slowly becoming drug resistant, a continued surveillance of emerging drug resistance of this pathogen is important to ensure effective treatment of human listeriosis.

Antibiotics to which some *L. monocytogenes* are resistant include tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulphonamide, trimethoprim-sulfamethoxazole (co-trimoxazole) and rifampicin [2-4]. Studies about stress responses are very important in biological and medicine [5]. Microbial population encounter different environmental stresses and usually adaptation to these stresses causes extended tolerance to multiple other lethal stressors. This phenomenon is defined as stress hardening, which refers to the increased resistance to lethal factors after adaptation. A study by Lou and Yousef showed that treating *L. monocytogenes* with sub-lethal stresses increased resistance to lethal doses of the same stresses [6]. Mauro et al. reported that *L. monocytogenes* serotype 4b and 1/2c are become more resistant to sulphafurazole [4].

Although considerable progress has been made in understanding the *L. monocytogenes* stress response, but there are little reports concerning the effect of environmental stresses on increasing or decreasing antimicrobial susceptibility of *L. monocytogenes* to variety of antibiotics. After exposing to sublethal stresses, evaluating the increasing or decreasing cell survival of *L. monocytogenes* cells is significant in the biology of this pathogen. The aim of this work was evaluating the changes in drug resistance and cell survival of

L. monocytogenes PTCC1297 (serotype 4a) to antibiotics after exposure to environmental stresses including acid, osmotic pressure, ethanol, oxidative and heat.

Materials and Methods

In this descriptive-analytic all media and materials used in this study were obtained from (Merck Co. Darmstadt, Germany). Antibiotics were obtained from (Mast Group Ltd., UK). The strain of *L. monocytogenes* PTCC1297 (serotype 4a) was obtained from Persian Type Culture Collection (PTCC, Tehran, Iran). Originally, this strain isolated from mammal, brain sheep circling disease. Lyophilized vial of *L. monocytogenes* PTCC1297 was cultured in Listeria CHROMagar (LCA). For preparation of pre-culture, individual colonies from streaked plates of LCA medium were selected and grown at 30ºC for 24 h in Listeria Enrichment Broth (LEB). LEB medium was used to induce sub-lethal environmental stresses. The following stress factors were used: ethanol, sodium chloride, hydrochloric acid, hydrogen peroxide and heat.

Conditions used for different stresses: For heat stress a portion (5 mL) of the *L. monocytogenes* PTCC1297 culture in LEB tubes was inoculated into 100 mL of the same medium in a 250 mL flask. The inoculated medium was incubated at 30ºC with shaking at 150 rpm in a shaker-incubator (JEIO TECH, Model SI-600R, and South Korea). When the optical density of the cells at 600 nm reached 0.4, the cells were harvested by centrifugation at 3,000 g at 4ºC. The harvested cells were washed once in 5 mL of LEB and suspended in 10 mL of the same medium. The cell suspension was heat shocked by keeping it at 45ºC for 2 h in a thermostatically controlled circulating water bath. The cell suspension was then cooled in a bath-water mixture and washed twice with 10 mL of pre-chilled (4ºC) 0.1 M phosphate buffer (PB) and kept in refrigerator for evaluating cell survival and susceptibility to antibiotics.

In acidic stress *L. monocytogenes* PTCC1297 was grown in LEB to an optical density at 600 nM of 0.1-0.2. Then the cells were harvested by centrifugation at 3,000 g and transferred into the media with the pH value of 5.0. Incubation was performed at 30ºC with shaking at 150 rpm for 1 h. The cell suspension was then cooled in a bath of ice-water mixture and washed twice with 10 mL of pre-chilled (4ºC) 0.1 M phosphate buffer (PB) and kept in refrigerator for evaluating cell survival and susceptibility to antibiotics.

For evaluating the oxidative, ethanol and osmotic stresses, sub-lethal dose of these stresses including hydrogen peroxide (H_2O_2) at concentration of 600 (ppm), ethanol at concentrations of 5% (v/v) and sodium chloride at concentration of 7% (w/v) were used, respectively. *L. monocytogenes* PTCC1297 cultures were incubated for 1 h in the presence of the stress factors, and the cell suspensions were centrifuged washed and suspended in 0.1 M phosphate buffer (PB) and kept in refrigerator for evaluating cell survival and susceptibility to antibiotics.

Antimicrobial susceptibility tests:

a) Disk diffusion method: A suspension of *L. monocytogenes* PTCC1297 with optical density of 0.5 McFarland Turbidity Standard (1.5×10^8) was made using the stressed cells kept in refrigerator. Antimicrobial susceptibility tests were done by Kirby-Bauer disk diffusion method. A 0.1 mL portion of suspension was cultured on Muller-Hinton Agar (MHA) and disks containing antibiotic were placed onto the surface of the medium. After incubation, the zones of inhibition surrounding the disks were measured and compared with the standard for each antibiotic according to guidelines of Clinical and Laboratory Standard Institute (CLSI-M45-P-2006) for *L. monocytogenes*. All incubations were performed at 35ºC for an overnight. The following antibiotics were used (µg/disk): tetracycline (30 µg), rifampicin (5 µg), gentamycin (10 µg), penicillin (10 µg), ampicillin (10 µg), trimethoprim-sulfamethoxazole (5 µg) and chloramphenicol (30 µg).

b) Minimum inhibitory concentration (MIC): For the broth micro dilution method, stressed cells of *L. monocytogenes* PTCC1297 were grown for 24 h at 30ºC. One drop of 24 h growth was added to 8 mL of LEB and incubated at 30ºC for 4 to 6 h. The turbidity was adjusted spectrophotometrically to a density equal to McFarland 0.5 standard, and the growth was then diluted 1:100 in LEB. Serial two fold dilutions of antibiotics were made in tubes with PBS. The inoculum containing approximately 10^6 colony forming units (CFU)/ml was added to wells in microtiter plates with 0.05 mL dropping pipettes. Equal volumes (0.05 mL) of antibiotic dilutions were added to the microtiter plates. Plates were covered with parafilm and incubated for 18 h at 30ºC. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic that prevented macroscopically visible growth after 18 h of incubation.

Viability estimation: For determining the viability of *L. monocytogenes* PTCC1297 (serotype 4a) stressed cells were cultured in different environmental conditions as follows: temperatures of 30ºC, 40ºC, 45ºC, 50ºC, 55ºC, and 60ºC. The pH values of 2.0, 3.0, 4.0, 5.0, 6.0, hydrogen peroxide (H_2O_2) at concentrations of 0%, 0.06%, 0.3%, 0.6%, 2.0%, 6.0%, ethanol at concentrations of 0%, 5%, 10%, 15%, 20%, 25% (v/v), and sodium chloride at final concentrations of 0%, 2%, 7%, 14%, 20%, 25% (w/v). The colony count method was used for identifying the sub-lethal dose of each stress. The inoculum containing approximately 10^6 colony forming units (CFU)/mL was added into 250 mL-Erlenmeyer flasks containing 50 mL of LEB and incubated on a rotary shaker 150 rpm at 30ºC for 24 h. Survivor plots (log 10 CFU/mL) were determined for each ranges of environmental conditions on Listeria Selective Oxford Agar and compared to the cells without stress.

Results

In this study exponential phase of *L. monocytogenes* PTCC1297 (serotype 4a) cells was subjected to some sub-lethal environmental stresses such as ethanol (5% v/v), sodium chloride (7% w/v), acid (HCl, pH=5.0), hydrogen peroxide (600 ppm) and heat (45ºC) in order to evaluate changes in cell survival and susceptibilities to different

antibiotics. As shown in the table 1, the most potent selected antibiotic is trimethoprim-sulfamethoxazole and the lowest activity was observed upon using rifampicin. Inductions of oxidative (600 ppm) and heat stresses at 45ºC lead to complete resistance to all antibiotics, but exposure to hydrochloric acid (pH=5.0), sodium chloride (7% w/v) and ethanol (5% v/v) stresses increased sensitivity to the antibiotics (p<0.05).

The minimum inhibitory concentration ranges (µg/mL) of *L. monocytogenes* PTCC1297 to selected antibiotics was shown in table 2. According to the obtained results, sensitivity to all antibiotics was in the approximate MIC breakpoints suggested by (CLSI) except the heat (45ºC) stress. Breakpoints for the used antibiotics are: penicillin (≤1.5), ampicillin (<1.5), tetracycline (≤4.0), chloramphenicol (≤12.5), gentamycin (≤6.0), rifampicin (≤0.5), trimethoprim-sulfamethoxazole (≤0.5/9.5).

MBCs for penicillin, ampicillin, tetracycline, chloramphenicol, gentamycin, rifampicin and trimethoprim-sulfamethoxazole were determined. The MBCs end points for penicillin, ampicillin and tetracycline were about three-fold dilution of MIC. But the MBCs for chloramphenicol, gentamycin, rifampicin and trimethoprim-sulfamethoxazole were about two-fold dilution above the MICs.

Figure 1 shows viability of *L. monocytogenes* PTCC1297 (serotype 4a) stressed cells exposed to different conditions and results are as follows:

Response to different temperature: *L. monocytogenes* stressed cells were cultured separately and exposed to 6 temperature conditions, (I) 30ºC, (II) 40ºC, (III) 45ºC, (IV) 50ºC, (V) 55ºC and (VI) 60ºC. The results showed that upon increasing the temperature viable cell decreased especially at 60ºC which is considered as lethal condition (Fig. 1A).

Response to ethanol and NaCl: The viability and resistance of stressed *L. monocytogenes* cells were increased to lethal doses of ethanol (14% v/v) and NaCl (20% w/v) concentrations as shown in (Fig. 1B, 1C) (p<0.05).

Response to hydrogen peroxide: Stressed *L. monocytogenes* cells treated with hydrogen peroxide (H_2O_2) at concentration of (600 ppm) showed increased resistance to antibiotics (p<0.05), but the viability decreased upon increasing hydrogen peroxide (H_2O_2) as shown (Fig. 1D) no growth occurred at 2.0%, 6.0% concentrations of hydrogen peroxide.

Response to acid conditions: Stressed *L. monocytogenes* cells were separately subjected to 5 acid conditions, (I) pH=2.0, (II) pH=3.0, (III) pH=4.0, (IV) pH=5.0, (V) pH=6.0. These treatments show that upon exposing to sub-lethal stress resistance of *L. monocytogenes* increased to high acid conditions (pH=3.0). *L. monocytogenes* growth was stopped in pH=2.0 (Fig. 1E).

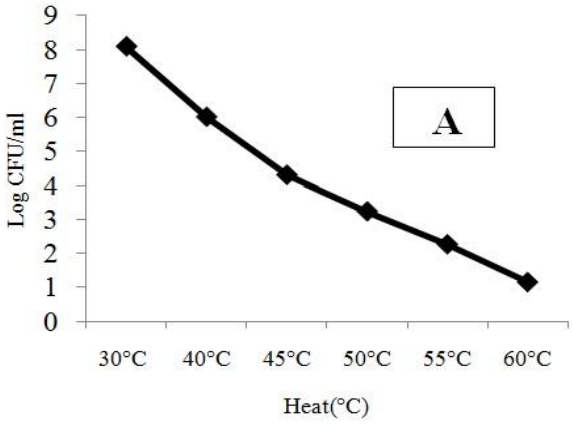
Table 1. Susceptibility of L. monocytogenes PTCC1297 to selected antibiotics before and after induction of stresses

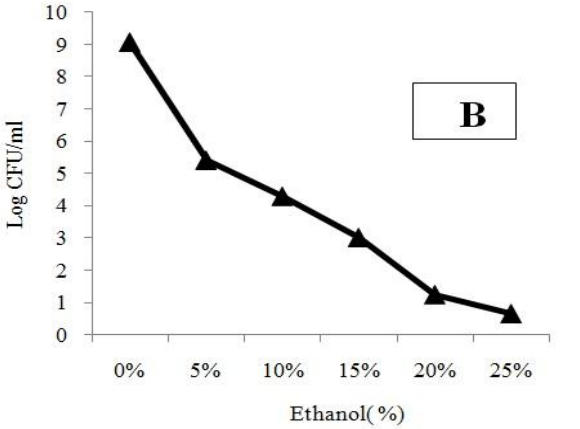
Sub-lethal stresses / Antibiotics	Listeria monocytogenes PTCC 1297 (control) (mean±SD)	Hydrochloric acid (pH=5) (mean±SD)	Sodium chloride (7% w/v) (mean±SD)	Ethanol (5% v/v) (mean±SD)
Trimethoprim-sulfamethoxazole	32-35[a]±0.6	35-39±2.1	35-38±1.6	33-36±0.9
Tetracycline	21-26±1.3	28-32±1.3	34-36±2.4	27-30±1.3
Chloramphenicol	22-25±0.9	17-30±1.5	28-33±2.1	26-29±2.4
Penicillin	20-23±1.1	25-28±0.9	33-37±1.9	29-34±0.8
Ampicillin	16-18±1.6	19-23±1.2	25-28±2.7	23-26±2.2
Gentamycin	14-17±0.6	16-20±1.8	21-23±1.4	18-22±0.9
Rifampicin	9-11±0.5	11-14±0.9	10-13±1.2	7-11±1.9

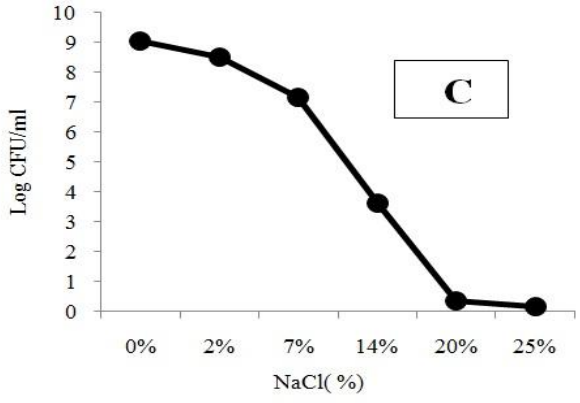
[a] Diameter of zone of inhibition (mm)

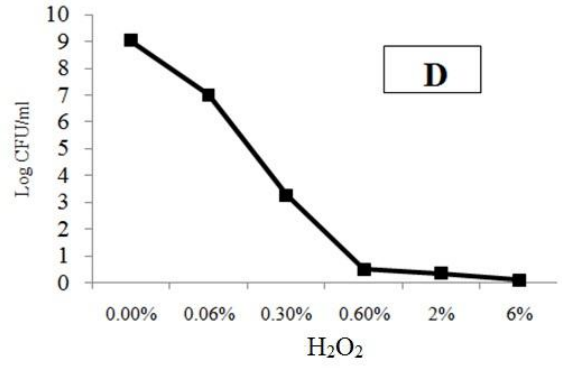
Table 2. Minimum inhibitory concentration ranges (µg/mL) of L. monocytogenes PTCC1297 to selected antibiotics after induction of stresses

Sub-lethal stresses / Antibiotics	Listeria monocytogenes PTCC 1297 (control) (mean±SD)	Hydrochloric acid (pH=5) (mean±SD)	Sodium chloride (7% w/v) (mean±SD)	Ethanol (5% v/v) (mean±SD)	Hydrogen peroxide (600 ppm) (mean±SD)	Heat (45°C) (mean±SD)
Trimethoprim-sulfamethoxazole	0.016-0.03±0.02	0.016-0.03±0.03	0.016-0.02±0.04	0.06-0.12±0.05	0.12-0.25±0.05	0.12-0.25±0.08
Tetracycline	0.12-0.5±0.03	0.12-0.25±0.06	0.06-0.25±0.05	0.12-0.25±0.03	0.25-0.5±0.05	0.5-1±0.03
Chloramphenicol	0.12-0.5±0.06	0.12-0.25±0.08	0.06-0.25±0.02	0.12-0.25±0.06	0.25-0.5±0.09	0.5-1±0.06
Penicillin	0.25-1.0±0.07	0.5-1.0±0.12	0.125-0.5±0.07	0.25-.05±0.05	0.5-1±0.03	1.0-2.0±0.07
Ampicillin	1.0-2.0±0.12	1.0-2.0±0.03	0.5-1.0±0.09	0.5-1.0±0.04	1.25-2±0.07	2.0-4.0±1.3
Gentamycin	1.0-2.0±0.16	0.5-1.25±0.16	0.5-1.25±0.02	0.5-1.25±0.07	1.25-2±0.1	2.0-4.0±1.2
Rifampicin	2.0-4.0±0.15	1.0-2.0±0.08	1.0-2.0±0.06	2.0-4.0±0.08	4.0-6.0±1.2	4.0-8.0±1.5









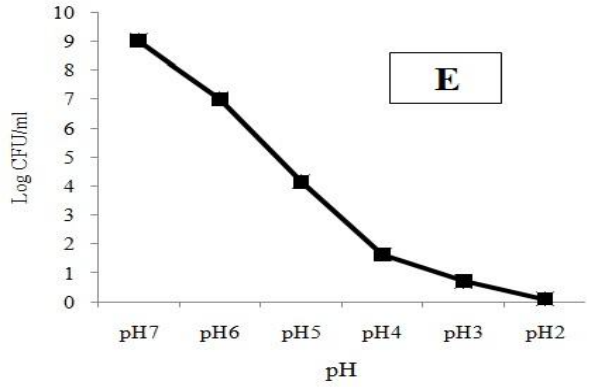


Figure 1. Viability curves of L. monocytogenes PTCC1297 exposed to different degrees of stresses (A) Heat, (B) Ethanol, (C) NaCl, (D) H_2O_2, (E) HCl incubation was performed in 250 mL-Erlenmeyer flasks containing 50 mL of listeria enrichment broth on a rotary shaker 150 rpm at 30ºC for 24. Survivor plots (log 10 CFU/mL) were determined for each range of environmental conditions on listeria selective oxford agar

Discussion

In this study, exposure to hydrochloric acid (pH=5.0), sodium chloride (7% w/v) and ethanol (5% v/v) stresses increased sensitivity of *L. monocytogenes* to all selected antibiotics. It seems that acidification leads to proton and anion influx into the cytoplasm and disrupts metabolic functions as well as inducing damage to proteins, nucleic acids, and cell membranes, molecular processes and cellular components such as membranes, proteins, nucleic acids, and enzymes. The main reason for increasing the sensitivity to antibiotics is cellular damages caused by sub-lethal factors. Upon treating the cells to heat and H_2O_2 cell survival and resistance to antibiotics increased.

L. monocytogenes is an important public health problem. Also, as a foodborne pathogen, *L. monocytogenes* primarily targets immune compromised individuals, including the elderly, pregnant women and newborns, leading to listeriosis, a disease that, although less frequent in occurrence (2.4 cases/million), is associated with relatively high (averaging 20-30%) mortality rates [7].

L. monocytogenes is very important in food safety control due to its wide distribution in nature and its capacity to survive and grow on the food products in spite of exposure to stressful conditions associated with food processing and preparation. However, a phenomenon called stress hardening, which refers to the increased resistance to lethal doses of stress after exposing to sub-lethal environmental stresses may interfere with food preservation and safety. It is presumed that *L. monocytogenes* cells depend on various stress-sensing mechanisms [6].

Also, from the point of clinical view, for treatment of *L. monocytogenes* infections, ampicillin or penicillin is the drug of choice and synergistic combination of *β*-lactam with gentamicin is sometimes used in immune compromised and neutropenic patients. Microbiological studies have shown that these drugs are not bactericidal

against *L. monocytogenes* [8]. Charpentier and Courvalin reported resistance of *L. monocytogenes* to different antibiotics such as tetracycline, gentamicin, penicillin, ampicillin, streptomycin, erythromycin, kanamycin, sulphonamide, trimethoprim, and rifampicin [3]. So, finding new ways to reduce resistance to different antibiotics is important in food industry and clinical therapy. Oxidative stress is deleterious to various. The responses of *L. monocytogenes* to sub-lethal dose of ethanol (5% v/v), acid (HCl, pH=4.5-5.0), H_2O_2 (500 ppm) were studied by Lou and Yousef [6]. They found that treating *L. monocytogenes* with sub-lethal doses of environmental stresses increases resistance of this strain to lethal effects of the stresses. Pre-incubation of *L. monocytogenes* at 3 acid conditions (I) pH=5.0, (II) pH=4.5, and (III) pH=5.0 significantly increased resistance of the pathogen to the lethal acid condition at pH=3.5. Also, adaptation to 5% ethanol or 500 ppm of H_2O_2 provided the greatest protection against H_2O_2. Protection against lethal levels of hydrogen peroxide may be partly explained by the induction of a sigma factor (σS, or KatF), which accounts for the general resistance to environmental stresses in microbial cells [9-11]. Oxidative stresses are under control of PerR, OhrR and σ^Bfactors [12, 13].

The presence PerR and Fur regulons during oxidative stress indicated a response to damage and/or repair proteins and DNA [14]. Our results showed that using H_2O_2 at 600 ppm leads to complete resistance to all selected antibiotics which may correlate to PerR and Fur regulons function, in contrast cell survival decreased in higher concentrations of H_2O_2.

We heated shocked *L. monocytogenes* cells by keeping it at 45ºC for 2 h and the cell survival and resistance to antibiotics increased to lethal doses of ethanol (14% v/v) and NaCl (20% w/v) concentrations, which is in consistent to the results obtained by Mead et al. [7]. They reported heat shocking significantly increased the resistance of *L. monocytogenes* to (25% w/v) NaCl. There are reports implying that exposing to ethanol stresses induce stress proteins with a profile similar to that of stress proteins induced by heat shock [15, 16]. So, we can conclude that stress responses to ethanol and heat shock act in similar ways in *L. monocytogenes.* Bergholz et al. [17] assessed genome-wide changes in the *L. monocytogenes* H7858 transcriptome during short-term and long-term adaptation to salt stress at 7ºC and 37ºC to understand the impact of temperature on the responses of *L. monocytogenes* which uses to adapt to osmotic stress. They found that at both temperatures, the short-term response to salt stress increased transcript levels of sigB and sigB-regulated genes, as well as mrpABCDEFG, encoding a sodium/proton antiporter. This antiporter was found to play a role in adaptation to salt stress at both temperatures.

A study by Morvan et al. [18] showed that the prevalence of drug resistance strains of *L. monocytogenes* was estimated at 1.27% amongst 4816 isolates from human samples. An explanation for acid resistance is adaptive response of *L. monocytogenes* to weak or strong acid food preservatives includes an increase in the total lipid Tm (decreased membrane fluidity), decreasing the ability of the weak acid preservatives to pass through the membrane and to act into the microbial cell, and thus conferring protection. Furthermore, decreased membrane fluidity acts as strong defense mechanism in some conditions (in the cases of hydrochloric or acetic acid) or as mild defense mechanism (in the cases of benzoic or lactic acid) [19].

Altuntas et al. [20] reported that some *L. monocytogenes* strains were resistant to streptomycin and fosfomycin antibiotics. Also some studies showed that treating *L. monocytogenes* to sublethal concentrations of disinfectant such as benzalkonium chloride and triclosan reduced susceptibility to ciprofloxacin, gentamicin [21, 22].

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Authors' Contributions

All authors had equal role in design, work, and performing the lab works.

Conflict of Interest

The authors declare no conflict of interest.

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