Original Article

An In Vitro Study on Impact of Environmental Stresses on Growth, Morphological and Biochemical Features of *Listeria monocytogenes* PTCC 1297

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Introduction: *Listeria monocytogenes* is a serious concern for the food industry due to its high case fatality rate, widespread distribution, ability to survive a wide variety of food processing conditions, and the severity of the illness associated with this pathogen infection. The objective of this study was to determine the growth, cell morphology and biochemical characteristics of *L. monocytogenes* PTCC 1297 (Serotype 4a) under selected environmental stresses. **Method:** The environmental stresses were acid stress (HCl, pH 2.0-6.0), alkaline stress (NaOH, pH 8.0-12.0), ethanol stress (5.0%-25.0% vol/vol), oxidative stress (H_2O_2, 0.06%-6.0% vol/vol), osmotic stress (NaCl and sucrose, 2.0%-30.0% wt/vol) and heat stress (40-60°C). All stresses were applied to the exponential phase bacteria whereas non-stressed exponential phase cells served as a control and the cells were allowed to grow for 24 h. For evaluating the growth of *L. monocytogenes* PTCC 1297 after inoculation procedure and exposure of cells to selected stresses we used colony count method. Scanning electron microscopy (SEM) was implemented to visualize the external appearance of the bacteria. **Results:** According to the results, the bacteria at pH≤4 and pH≥10 achieved by HCl and, NaOH, respectively, died. Also, concentrations ethanol at ≥15% vol/vol, H_2O_2 ≥0.3% vol/vol, NaCl ≥14% wt/vol, and heat ≥50°C were lethal for the bacteria. Unlike other stresses, sucrose did not kill bacteria but decreased their growth. The phenotypical and biochemical characteristics of them changed when exposed to each stress. **Conclusion:** Different doses of various stresses were either lethal or sub-lethal for this bacterium and lead to various changes in its characteristics. *J Med Microbiol Infec Dis, 2015, 3 (1-2): 11-17.*

Keywords: *Listeria monocytogenes,* Cell Biology, Environment, Stress Physiological.

INTRODUCTION

Listeria monocytogenes is a facultative intracellular bacterial pathogen with a variety of genetically encoded survival mechanisms to withstand environmental stresses such as heat, cold, salt, and acidic conditions. Given its unique adaptability to survive in adverse environmental conditions compared to other non-spore forming bacteria, this pathogen is a serious concern especially within the Ready-To-Eat (RTE) food industry. *L. monocytogenes* is considered an adulterant agent by the U.S. Food and Drug Administration, and hence from a regulatory perspective, there is a zero tolerance for this organism in foods, while this organism is ubiquitous and is found in soil, water, food processing equipment, and other environments [1].

L. monocytogenes is an opportunistic microorganism and causes infection in individuals who are particularly vulnerable to diseases such as immunocompromised individuals, elderly people, pregnant women, infants, and hospitalized patients [2]. In the United State, listeriosis is the second most costly foodborne illness after salmonellosis [3].

L. monocytogenes is associated with significant food safety control problems due to its wide distribution in nature and its capacity to survive and grow on the food products despite frequent exposure to harsh environmental conditions associated with food processing and preservation measures. It has been shown through several studies that the exposure of *L. monocytogenes* to sub-lethal stress induces the development of stress-conditioned organisms, which are physiologically more tolerant to increased levels of the same or different stresses [4]. The greatest relative risk for contaminating food in factories is where *Listeria* grow to high numbers in some niches. These growth niches must be actively sought and eliminated as soon as possible. Many factors affect microbial growth in niches, including moisture, nutrients, pH, oxidation-reduction potential, temperature, presence or absence of inhibitors, and interactions between cells in a population [5].

Stresses that *L. monocytogenes* cells endure during infection of humans and animals, storage of foods and survival in adverse environmental conditions may increase its virulence and resistance to subsequent food processing interventions.

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Food processing procedures offer many opportunities for the induction of stress response in microorganisms [2]. The present study is aimed to indicate which environmental stresses, pH (acid and alkaline), oxidative (H_2O_2), ethanol, osmotic pressure (NaCl and Sucrose) and heat on *L. monocytogenes* PTCC 1297 affect its survival, morphological features or biochemical characterization of the pathogen.

MATERIAL AND METHODS

Microorganism and media. *L. monocytogenes* PTCC 1297 was obtained from Iranian Research Organization for Science and Technology (IROST). A lyophilized vial of *L. monocytogenes* PTCC 1297 was cultured in Listeria CHROMagar (LCA) medium (Figure 1). The plates were grown at 35°C for 24 h and then stored at 4°C until used. For the preparation of pre-culture, individual colonies from streaked plates of LCA medium were grown at 35°C for 24 h in fresh LCA medium before induction of environmental stresses.

The effect of different stresses was studied by the addition of various degrees of following factors: hydrochloric acid, sodium hydroxide, ethanol, hydrogen peroxide, sodium chloride, sucrose, and heat. All media and materials used in this research were purchased from Merck Co. Darmstadt, Germany.

Inoculation procedure and exposure of cells to selected stresses. Amounts of 25 mL of *Listeria* Enrichment Broth (LEB) were transferred to 50 mL Erlenmeyer flasks. All flasks were placed on an auto-clove after induction stress to their medium. Next, a single colony from the pre-culture was aseptically inoculated into the stressed medium. The flasks were transferred to a shaking incubator (150 rpm), at 30°C and incubated until cultures reached the exponential phase that was defined by optical density (OD) of ≈1-2 at 600 nm for 24 h [6]. In the following, preparation of each stressed medium is explained.

Heat Stress. The cell suspension was heat stressed by inoculation into a fresh medium at temperatures 35°C, 40°C, 45°C, 50°C, 55°C, and 60°C for 24 h in a thermostatically controlled circulating water bath.

Acid and Alkaline Stress. For acidic and alkaline stresses, Hydrochloric acid (HCl, 0.1M) and Sodium Hydroxide (NaOH, 0.1M) were used for adjusting the desired pH. Exponentially growing *L. monocytogenes* PTCC 1297 was inoculated into the LEB media with the pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 9.0, 10.0, 11.0, 12.0 and 7.0 (control), respectively. Inoculations were performed at 35°C with shaking at 150 rpm in a shaker-incubator for 24 h.

Oxidative and Ethanol Stress. For evaluating the oxidative and ethanol concentrations stresses, hydrogen peroxide (H_2O_2) at concentrations of 0% (control), 0.06%, 0.3%, 0.6%, 1.5%, 6% (vol/vol) and ethanol at concentrations of 0% (control), 5%,10%, 15%, 20%, 25% (vol/vol) were used, respectively. The same procedure of preparing the cells for evaluating the acidic and alkaline stresses was used for studying oxidative and ethanol stresses.

Osmotic Stress. For evaluating osmotic pressure, sodium chloride and sucrose were added to medium cultures at final concentration of 0% (control), 2%, 7%, 14%, 20%, 25%, and 30% (wt/vol).

Evaluation of the bacteria growth. For assessing the viability of *L. monocytogenes* PTCC 1297, we used colony count method, after and before (as control) induction of stresses. One ml of suspensions of the stressed cells was added to 9 ml of 0.9 % (wt/vol) NaCl and was serially diluted in 0.9% (wt/vol) NaCl. Amounts of 1ml of each dilution were plated in duplicate on Listeria selective Oxford agar (Figure 2). Inoculated agar plates were incubated at 35°C, and bacterial colonies were counted after 24 hours. For heat stress, inoculated agar plates were incubated at different temperatures.

Evaluation of the surface structure of bacteria. Physical changes in the samples after induction of stresses and before them (as control) were examined by Scanning Electron Microscopy (SEM) Analysis. The SEM micrograph for the last sub-lethal dose (before a lethal dose) of each selected stresses was performed by the McMullan method [7].

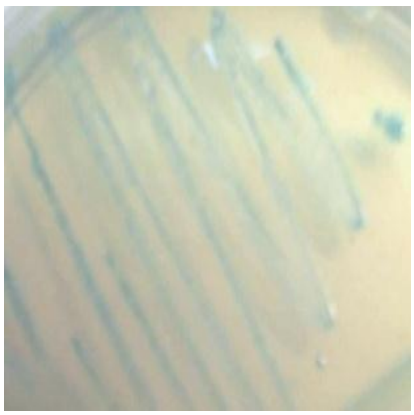


Fig. 1. Blue-green colonies with around zone of Listeria monocytogenes PTCC 1297 on Listeria chrom agar after 24-h incubation

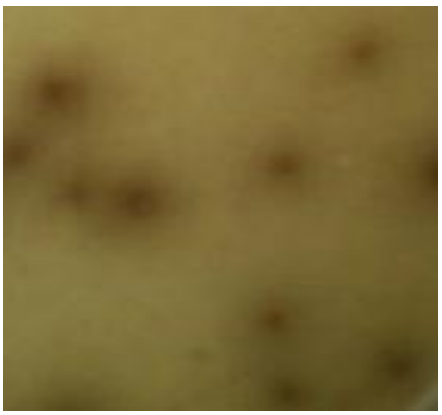


Fig. 2. Black colonies of Listeria monocytogenes PTCC 1297 on Listeria selective oxford agar after 24-h incubation, showing the diffuse dark zone surrounding the colonies

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Evaluation of the Biochemical characterization. The harvested cells and non-harvested cells (as control), were characterized according to the criteria described by others [8]. We studied the following characteristics: Hemolysis on blood agar, motility, hydrolysis of esculin, fermentation of glucose, production of gas, Methyl-Red test, Voges-Proskauer test and production of catalase and oxidase enzymes.

RESULTS

Bacteria growth. The growth curves of *L. monocytogenes* PTCC 1297 in the presence of various concentrations of each stress is reflected in figure 3a-3g. As shown, all the stress treatments (acid, alkaline, ethanol, oxidative, osmotic pressure and heat) reduced the initial population of bacteria compared to the control. The bacteria viability was assessed following its exposure to environmental stress conditions (Figure 3). Non-adapted cells exhibited 8.56 Log CFU/ml. Adapted cells had a decreased Log CFU/ml after 24 h, and the presence of sucrose had the lowest effect on the growth of bacteria. Also, The variables were lethal to the cells at some levels, including acid stress (pH 4.0, 3.0, 2.0), alkaline stress (pH 10.0, 11.0, 12.0), ethanol stress (15.0, 20.0, 25.0% vol/vol), oxidative stress (0.3, 0.6, 1.5, 3.0, 6.0% vol/vol) saline stress (14.0, 20.0, 25.0, 30.0% wt/vol), and heat stress (50, 55, 60°C) (Figure 3).

Morphology of *L. monocytogenes* PTCC 1297 stressed cells. Significant reductions were seen within all stress treatments of *L. monocytogenes* and resulted in morphology changes when monitored after the application of SEM (Figure 4). Application of sub-lethal levels of stresses led to morphological changes. Non-treated cells size was about 2 µm (Figure 4a). The SEM micrographs indicated that cells became smaller and more rounded in high-pressure treatments. Additionally, the numbers of bacteria were reduced after stress induction.

The biochemical characteristics of *L. monocytogenes* PTCC 1297 stressed cells. The Bacteria showed on biochemical changes to the above stress conditions (Tables 1-4).

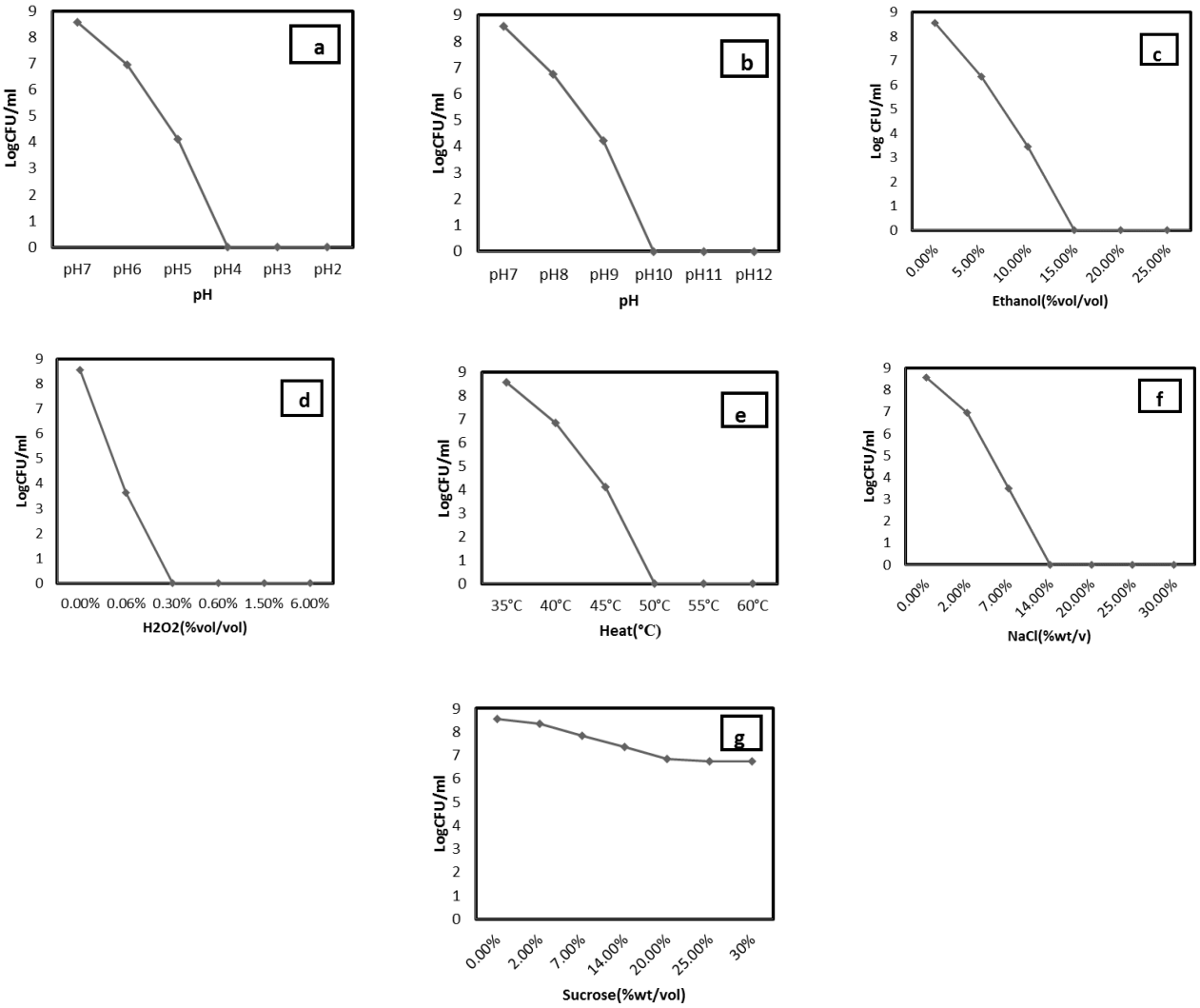


Fig. 3. Viability of *L. monocytogenes* PTCC 1297 after 24 h starvation in different degrees of stresses at 35°C (for heat stress a different temperature was used) (a) HCl, (b) NaOH, (c) Ethanol, (d) H_2O_2, (e) Heat, (f) NaCl, (g) Sucrose.

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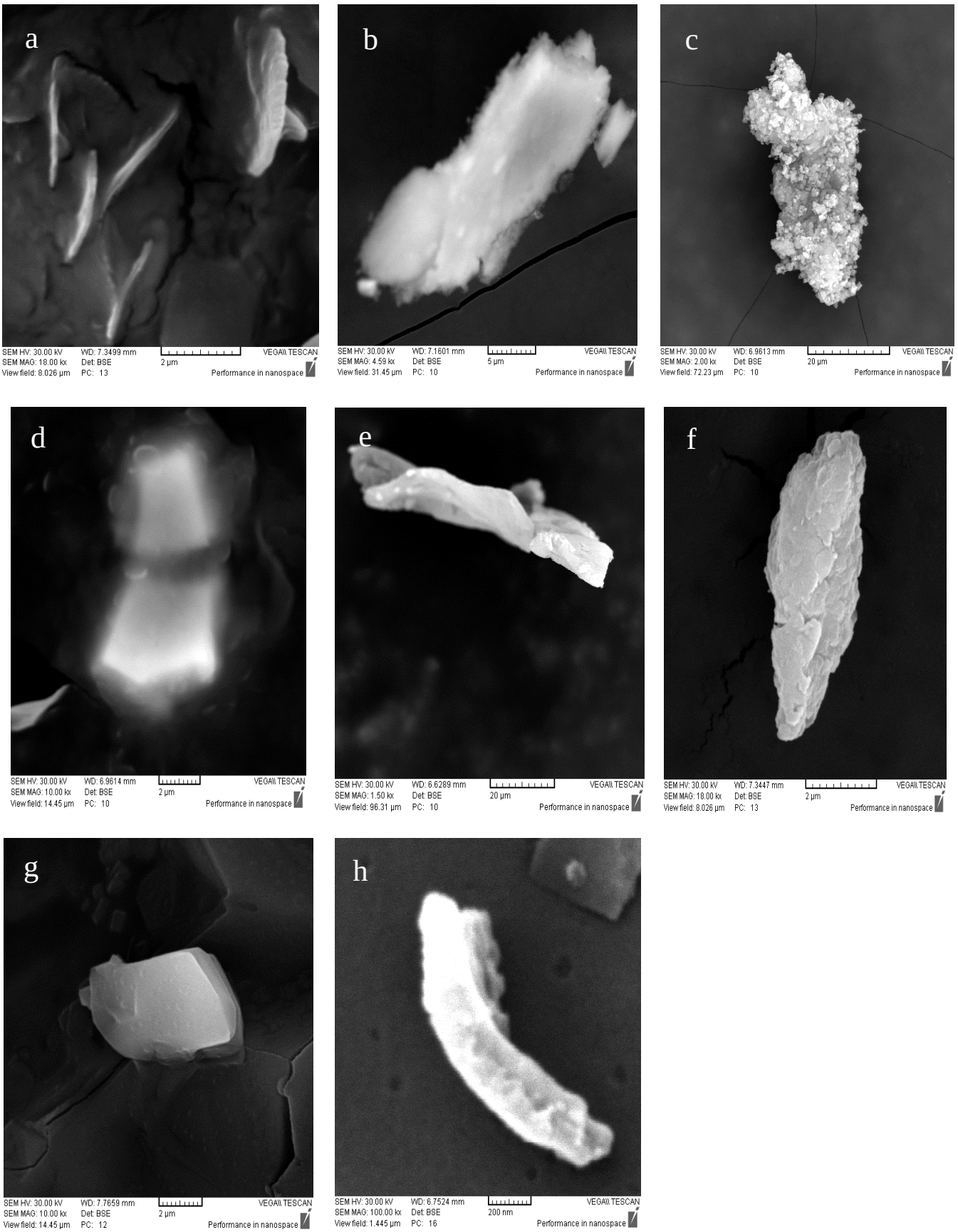


Fig. 4. SEM images of *L. monocytogenes* PTCC 1297 (a) before induction of stress (as control), and after induction of sub-lethal stresses (b) HCl (pH5), (c) NaOH (pH9), (d) Ethanol (10% vol/vol), (e) H_2O_2 (0.06% vol/vol), (f) Heat (45°C), (g) NaCl, (h) Sucrose.

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Table 1. Biochemical characteristics of *L. monocytogenes* PTCC 1297 after 24 h starvation in sub-lethal levels of HCl and NaOH at 35°C.

Biochemical test	**Sub-lethal stresses**				
	Control (pH 7)	pH 6	pH 5	pH 8	pH 9
Hemolysis	+	+	+	+	+
Motility	+	+	-	+	+
Hydrolysis of esculin	+	+	-	+	+
Fermentation of glucose	A/A	A/A	Alk/Alk	A/A	A/A
Production of gas	-	-	-	-	-
MR	+	+	+	+	+
VP	+	-	-	-	-
Catalase	+	+	+	+	-
Oxidase	-	-	-	-	-

Table 2. Biochemical characteristics of *L. monocytogenes* PTCC 1297 after 24 h starvation in sub-lethal levels of Ethanol and H_2O_2 (%vol/vol) at 35°C.

Biochemical test	**Sub-lethal stresses**			
	Control (0.00%)	Ethanol (5%)	Ethanol (10%)	H_2O_2 (0.06%)
Hemolysis	+	+	+	+
Motility	+	+	+	+
Hydrolysis of esculin	+	+	+	-
Fermentation of glucose	A/A	A/A	A/A	A/A
Production of gas	-	-	-	-
MR	+	-	-	-
VP	+	-	-	-
Catalase	+	+	+	-
Oxidase	-	-	-	-

Table 3. Biochemical characteristics of *L. monocytogenes* PTCC 1297 after 24 h starvation in sub-lethal levels of Heat (°C)

Biochemical test	**Sub-lethal stresses**		
	35 (°C)	40 (°C)	45 (°C)
Hemolysis	+	+	+
Motility	+	-	-
Hydrolysis of esculin	+	+	+
Fermentation of glucose	A/A	A/A	A/A
Production of gas	-	-	-
MR	+	-	-
VP	+	-	-
Catalase	+	-	-
Oxidase	-	-	-

Table 4. Biochemical characteristics of *L. monocytogenes* PTCC 1297 after 24 h starvation in sub-lethal levels of Sucrose and NaCl (%wt/vol) at 35°C.

Biochemical test	**Sub-lethal stresses**								
	Control (0.00%)	Sucrose (2%)	Sucrose (7%)	Sucrose (14%)	Sucrose (20%)	Sucrose (25%)	Sucrose (30%)	NaCl (2%)	NaCl (7%)
Hemolysis	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Hydrolysis of esculin	+	+	+	+	+	+	+	+	+
Fermentation of glucose	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A	A/A
Production of gas	-	-	-	-	-	-	-	-	-
MR	+	-	-	-	-	-	-	-	-
VP	+	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-

DISCUSSION

In general, microorganisms face various stresses whenever environmental conditions change from their ideal status. Also, stress may occur while microorganisms represent deviations in optimal growth patterns, sub-lethal injury, or any alteration to optimal functioning of metabolic reactions in the cell. Stress conditions constantly occur in microorganisms in the food processing environment and may include physical, chemical and nutritional stresses. Foods themselves may impose stress on microorganisms [9-11].

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Morphological and physiological changes occur during starvation of cells. Bacterial cells become smaller and more rounded during starvation [12]. Wen *et al.* (2009) reported that long-term survival of *L. monocytogenes* led to small and rounded cells [13], which is similar to our results as shown in our micrographs. This rounding and shrinking may increase the ability of the cell to absorb nutrients [14].

Since *L. monocytogenes* is a foodborne pathogen, it likely exists in the sour foods, for example, sour juices. Therefore, more findings of acidic stress in *L. monocytogenes* can be useful for discovering new ways to protect against *L. monocytogenes.* For example, with respect to resistance to heating in juice (watermelon, pH~5.3), Sharma *et al.* (2005), showed that acid-adapted cells were less resistant to heating at 56°C; whereas no difference in heat resistance of *L. monocytogenes* was seen in cantaloupe juice (pH 6.3) [15]. Despite these findings, the thermal resistance of acid-adapted *L. monocytogenes* was reported in various other juices (orange, white grape, and apple) [16]. Acid stress may induce cross protection to both chemical and physical food processing stresses. Acid stress at various pH conditions (4.0-6.0) was tested for stress hardening in further acid challenges [17].

The presence of alkaline in the food processing conditions may occur in various environments. Alkaline cleaning solutions can be used to clean processing equipment. Alkaline (NaOH and KOH) based detergents are presently utilized. Research has suggested that survival in cleaning detergents is possible [18]. Sodium hydroxide, sodium carbonate and sodium metasilicate are used in the formulation of alkali food processing detergents [19]. High pH resulted in some morphological changes in *L. monocytogenes* [20]. Alkaline treatment resulted in cells that were longer, larger ended and had an increased volume. Alkaline treatment in a buffered media increased *L. monocytogenes* length with a longer exposure time [21].

L. monocytogenes can be exposed to oxidative stress upon invasion of a human host. The immune response of cells can produce oxidizing molecules to delete pathogens and from this way can be the case of neutrophilic attacks on *L. monocytogenes* [22]. Additional of H_2O_2 in raw milk and liquid eggs is permitted in some countries. The activity of hydrogen peroxide in the raw milk lactoperoxidase system has also been affirmed [23]. The impact of oxidative stress may be seen in the demolition of enzymes, proteins, DNA and cellular membranes [4, 13]. Oxidative stress (H_2O_2) resulted in stress hardening and increased the resistance of the pathogen to lethal amounts of hydrogen peroxide [24].

Alcohol or ethanol-based sanitizers are recommended in areas that must be kept dry. The use of alcohol as a disinfectant can be found in food processing environments. A study examining 117 food industry sites noted that 26% of facilities used alcohol based products. This was the third most commonly used disinfectant reported in the survey [25]. The impact of ethanol stress in *L. monocytogenes* has relevance to the food processing industry and has significance in cross protection as well.

The disease of an animal or lack of refrigeration leading to elevated temperatures in milk may render *L. monocytogenes* more resistant to High-Pressure Processing (HPP). This is important when examining procedures to ensure the microbial safety of milk treated with this emerging technology. Reka (2009) reported that heat injury does occur when *L. monocytogenes* 4ab is exposed to 50°C [26]. Our experiments indicated the same result with Reka, too.

L. monocytogenes is a foodborne pathogen, and the possibility of its existence in the salty and sweet foods is more. In the United States and Canada, sporadic cases and some outbreaks caused by *L. monocytogenes* have been strongly associated with cheese, particularly soft and soft-ripened cheese. Food Directorate have conducted an appraisement of the risk posed by *L. monocytogenes* in soft-ripened cheese, according to reports from the U.S. Department of Health and Human Services, Food and Drug Administration (FDA) and Health Canada-Santé Canada (HC-SC) concern the safety in these products [27]. On March 13, 2015, CDC and FDA collaborated to investigate an outbreak of *Listeria*. The joint investigation found that certain Blue Bell brand ice cream products are the presumable source for some or all of these diseases [28]. Hence, more findings of osmotic pressure stress in *L. monocytogenes* can be useful for discovering new ways to protect against *L. monocytogenes.* In our study, the effect of osmotic pressure was investigated by the addition of various concentrations of NaCl and sucrose. It may be assumed that the presence of sucrose had little effect on the growth of *L. monocytogenes* PTCC 1297, therefore it is not suitable for food storage. Additionally, it can be concluded that the presence of *L. monocytogenes* in sweet food such as an ice-cream is more. In contrast, 14% NaCl was lethal for the bacterium. Although, our results were contradicted to the Farber *et al.* (1992) results that demonstrated *L. monocytogenes* has been shown to resist against high NaCl concentrations (up to 14% NaCl) [29].

Exposure to sub-lethal stresses can even affect to antibiotic resistance pattern on *L. monocytogenes*. Al-Nabulsi AA *et al.* (2015) reported that the use of sub-lethal stresses in food preservation systems might stimulate antibiotic resistance responses in *L. monocytogenes* strains. [30]. Faezi-Ghasemi and Kazemi (2015) reported that exposing to hydrogen peroxide (600 ppm) and heat (45ºC) significantly (p<0.05) increased *L. monocytogenes* cells resistance to selected antibiotics including Tetracycline, Gentamycin, Penicillin, Ampicillin, Trimethoprim-Sulfamethoxazole, Rifampicin, and Choloramphenicol. Also, they indicated that exposing to hydrochloric acid (pH=5.0), sodium chloride (7% wt/vol) and ethanol (5% vol/vol) decreased resistance (p<0.05) to antibiotics [31].

Currently, *L. monocytogenes* is known as a significant foodborne pathogen with a high annual fatality rate in humans. We found that different doses of various stresses were either lethal or sub-lethal for this bacterium and lead to various changes in its characteristics. Also, it is assumed that *L. monocytogenes* cells can enter into a viable but non-culturable (VBNC) state when exposed to some of the sub-lethal stresses and some of the sub-lethal stresses might lead to the emersion of MDR strains. Therefore, our results

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can be a basis for further research to discover new ways to protect against *L. monocytogenes.*

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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