

Effect of Heavy Metals Stresses on Growth, Surface Structure and Biochemical Features of *Listeria monocytogenes* PTCC 1297: An in Vitro Study

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Abstract

Background: Although many food-borne pathogens may generally cause illness, *Listeria monocytogenes* is an opportunistic organism that causes harm to individuals who are particularly vulnerable to disease. Unlike organic pollutants, heavy metals do not decay and thus pose a different kind of challenge for remediation. Microbial bioremediation is an efficient strategy due to its low cost and efficient targeting of heavy metals.

Objectives: The aim of this work was evaluate the growth, surface structure and biochemical features of *L. monocytogenes* PTCC 1297 endured to toxic heavy metals for humans.

Materials and Methods: The effects of various concentrations of mercury(II) bromide (HgBr₂), lead (II) oxide (PbO), and cadmium sulfate (CdSO₄) (0.1% - 0.5% wt/vol) were evaluated. All stresses were applied to exponential phase cells whereas non-stressed exponential phase cells served as a control and the cells were allowed to grow for 24 hours. For evaluating the growth of *L. monocytogenes* PTCC 1297 after the inoculation procedure and exposure of cells to selected stresses, the colony count method was used. Scanning Electron Microscopy (SEM) was used to visualize the surface structure of bacteria.

Results: The amount of HgBr₂ (0.1% wt/vol) and CdSO₄ (0.2% wt/vol) were considered as lethal doses for *L. monocytogenes* PTCC 1297 (Serotype 4a). Different concentrations of PbO could not kill bacteria yet decreased their growth. The bacteria showed different morphologic and biochemical characteristics under each stressor.

Conclusions: It can be concluded that *L. monocytogenes* PTCC 1297 can be resistant to lead.

Keywords: Biodegradation, Environmental, Metals, Heavy, *Listeria monocytogenes*

1. Background

Listeria monocytogenes is a human food-borne pathogen that can cause listeriosis. These bacteria have a worrying high fatality rate, especially in immunocompromised patients and pregnant women (1, 2). *Listeria monocytogenes* can remain viable in undesirable environmental conditions both in natural environments such as soil and streams, and within food-processing environments such as on food processing equipment (3).

In general, the microorganism encounters stress whenever an environment deviates from the ideal growth conditions. In addition, stress is said to exist whenever microorganisms express deviations in optimal growth patterns, sub-lethal injury, or any alteration to optimal functioning of metabolic reactions in the cell (4). According to the amount of the involved stress, it can be categorized as either "sub-lethal", "lethal", or "severe". When microorganisms are exposed to sub-lethal stress, this exposure can usually induce adaptation to subsequent

lethal levels of the same type of stress. This microbial adaptation is considered as "stress adaptation" (5). Lethal or severe stress can lead to immutable damage to the microbial cells (3). Stressed microbial populations may or may not possess the ability to recover from injury inflicted by food processing methods (4). Yousef et al. defined microbial stress as any injurious physical, chemical, or biological parameters that have a negative affect on microbial growth or survival (6). Reduced growth rate or induced entry into stationary phase is emblematic of general stress response (7).

2. Objectives

The overall focus of our work was to evaluate the growth, surface structure and biochemical characteristics of *L. monocytogenes* PTCC 1297 (Serotype 4a) when exposed to heavy metals, to better understand its stress adaptive response.

3. Materials and Methods

3.1. Microorganism and Media

Listeria monocytogenes PTCC 1297 was obtained from the Iranian Research Organization for Science and Technology (IROST) and was used throughout the experiments. A lyophilized vial of *L. monocytogenes* PTCC 1297 was cultured in Listeria Chromagar (LCA) medium. The plates were grown at 35°C for 24 hours and stored at 4°C for future studies. For preparation of pre-culture, individual colonies from streaked plates of LCA medium were selected and grown at 35°C for 24 hours in 10-mL of Listeria Enrichment Broth (LEB) before use to induce environmental stresses.

The effect of different stresses was studied by the addition of various degrees of the following compounds: HgBr₂, PbO, and CdSO₄.

All media and materials used in this research were obtained from Merck Co. (Darmstadt, Germany).

3.2. Inoculation Procedure and Exposure of Cells to Selected Stresses

HgBr₂, PbO, and CdSO₄ along *L. monocytogenes* PTCC 1297 were added to Listeria Enrichment Broth (LEB) at a final concentration of 0% (control), 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% (wt/vol), separately.

Furthermore, 25 mL of each concentration was transferred to different 50 mL Erlenmeyer flasks. All Erlenmeyer flasks were placed on an auto-clove. Next, one colony of culture was aseptically inoculated into 25 mL of LEB in a 50 mL flasks and on Listeria chromagar, separately. All flasks were exposed to shaking (150 rpm) inside a shaking incubator at 30°C and kept until cultures reached an exponential phase, based on an optical density (OD) of approximately 1-2 at 600 nm for 24 hours (8).

3.3. Evaluation of Growth

For evaluating the viability of *L. monocytogenes* PTCC 1297, the colony-count method was used, before (as control) and after induction to stresses. A portion (1 mL) of cell suspensions containing stressed cells, was added to 9 mL of 0.9% (wt/vol) NaCl, and was serially diluted in 0.9% (wt/vol) NaCl. One milliliter of each dilution was plated on Listeria selective oxford agar. Inoculated agar plates were incubated at 35°C and bacterial colonies were counted after 24 hours. Survivor plots (log₁₀ CFU/mL) were determined for each range of environmental conditions on Listeria Selective oxford agar and compared to the cells without stress (8).

3.4. Scanning Electron Microscopy (SEM)

Physical changes in the samples before (as control) and after induction of stresses were observed by SEM. The SEM micrograph for the last sub-lethal dose (before lethal dose) of each heavy metal was performed by the McMullan method (9).

3.5. Biochemical Characterization

The harvested cells and non-harvested cells (as control), were characterized according to the criteria described by Bailey and Scott's Diagnostic Microbiology (10). The following characteristics were studied: 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.

4. Results

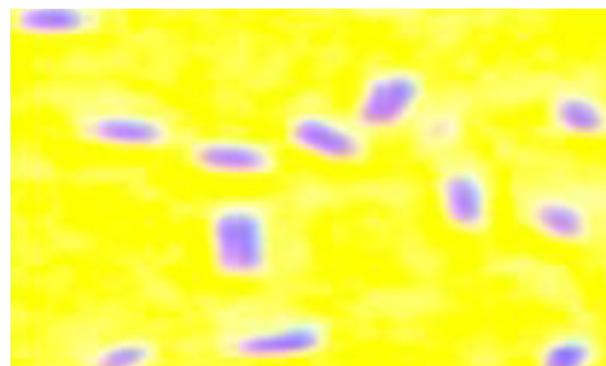
According to our results, all of the stress treatments reduced the initial population of *L. monocytogenes* PTCC 1297 compared to the control. The growth pattern of *L. monocytogenes* PTCC 1297 was evaluated, when the pathogen was exposed to environmental stress conditions (Figure 1). Non-harvested cells showed 8.56 Log CFU/mL. Harvested cells showed decreased Log CFU/mL after 24 hours and the presence of PbO had the lowest effect on the growth of bacteria. Also some concentrations of stresses were lethal for *L. monocytogenes* PTCC 1297 including HgBr₂ (0.1, 0.2, 0.3, 0.4, 0.5% wt/wt) and CdSO₄ (0.2, 0.3, 0.4, 0.5%wt/wt). These comparisons are seen in Figure 1.

Figure 2 shows the Gram staining results of *Listeria monocytogenes* PTCC 1297.

As shown in Figure 3, significant reductions were seen for all shock treatments of *L. monocytogenes* and resulted in morphology changes when monitored after the application of Scanning Electron Microscopy (SEM). Application of sub-lethal concentrations of stresses led to changes in the morphological characterization. Non-treated cells which observed, had ranging about 2 μm (Figure 3 A). Scanning Electron Microscopy figures indicated that cells appear small and rounded in high-pressure treatments. Additionally, the numbers of bacteria were reduced after induction of stresses. Biochemical characteristics of *L. monocytogenes* PTCC 1297 are seen in Figure 3.

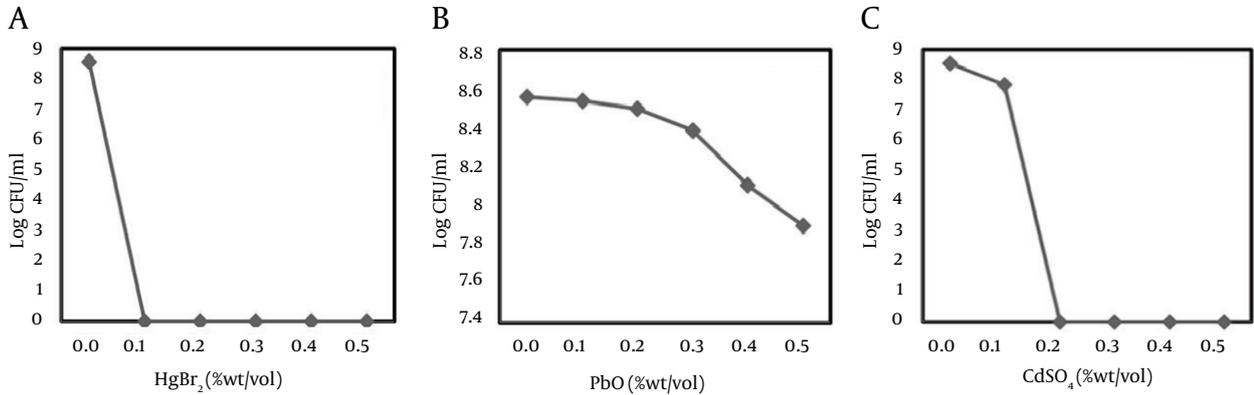
The stress response resulted in biochemical changes to selected sub-lethal stress conditions that microorganisms might encounter in food processing environments or nature (Table 1 and Figure 4 A - G).

Figure 2. Gram Staining of *Listeria monocytogenes* PTCC 1297



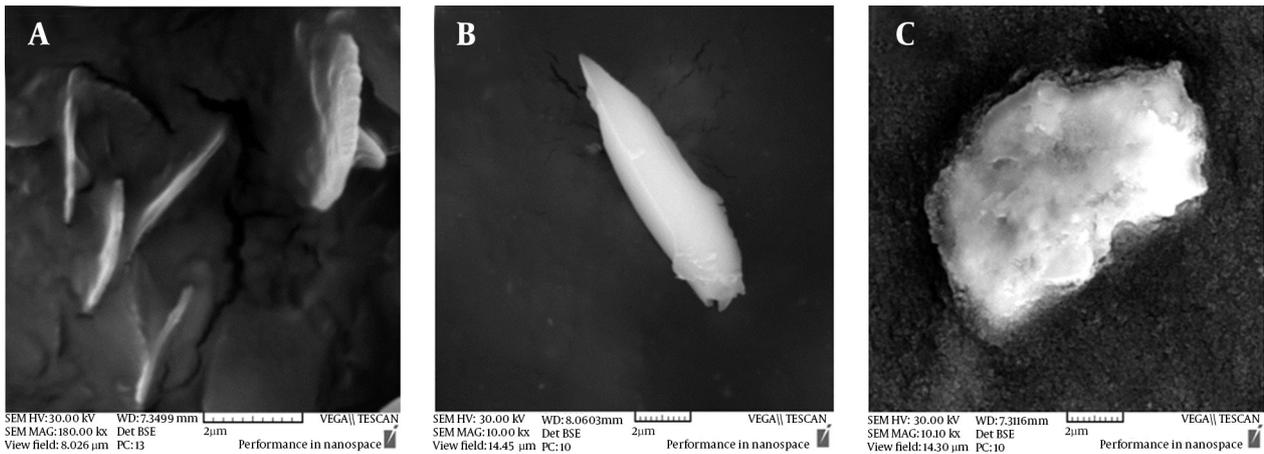
The bacteria were cultured on Listeria chromagar and stained by crystal violet and safranin, 100×.

Figure 1. Viability of *Listeria monocytogenes* PTCC 1297 After a 24-Hour Starvation at Different Degrees of Stresses at 35°C



A, HgBr₂; B, PbO; C, CdSO₄.

Figure 3. Scanning Electron Microscopy Images of *Listeria monocytogenes* PTCC 1297

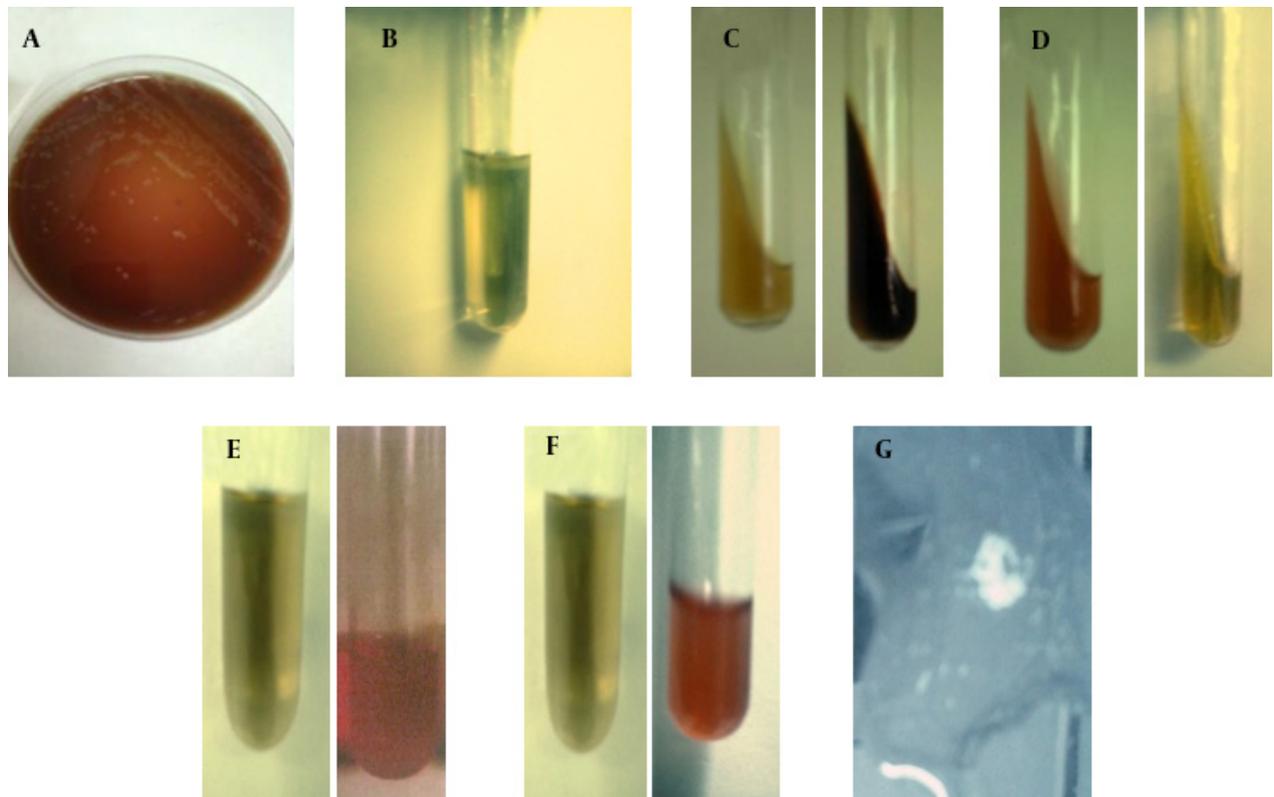


(A) Before induction of stress (as control), and after induction of sub-lethal stresses (B) PbO (0.5%wt/vol), (C) CdSO₄ (0.1%wt/vol).

Table 1. Biochemical Characteristics of *Listeria monocytogenes* PTCC 1297 After 24 Hours of Starvation at Sub-Lethal Levels of PbO and CdSO₄ (%wt/vol) at 35°C

| Biochemical Test | Sub-Lethal Stresses | | | | | | |
|-------------------------|---------------------|------------|------------|------------|------------|------------|--------------------------|
| | Control (0.00%) | PbO (0.1%) | PbO (0.2%) | PbO (0.3%) | PbO (0.4%) | PbO (0.5%) | CdSO ₄ (0.1%) |
| Hemolysis | + | + | + | + | + | + | - |
| Motility | + | + | + | + | + | + | + |
| Hydrolysis of esculin | + | + | + | + | + | + | - |
| Fermentation of glucose | A/A | A/A | A/A | A/A | A/A | A/A | Alk/Alk |
| Production of gas | - | - | - | - | - | - | - |
| MR | + | - | - | - | - | - | - |
| VP | + | - | - | - | - | - | - |
| Catalase | + | + | + | + | + | + | - |
| Oxidase | - | - | - | - | - | - | - |

Abbreviations: MR, Methyl Red; VP, Voges-Proskauer; +, positive; -, negative.

Figure 4. Biochemical Characteristics of *Listeria monocytogenes* PTCC 1297 After 24 Hours of Incubation

(A) Diffused narrow zone of β -hemolysis surrounding the colonies on sheep blood agar, (B) Characteristic umbrella-shaped motility pattern on SIM medium, (C) Hydrolysis of esculin on bile esculin medium (left to right: control, positive), (D) Fermentation of glucose without production of gas on TSI medium (left to right: control, positive), (E) Methyl-Red test (left to right: control, positive), (F) Voges-Proskauer test (left to right: control, positive), (G) Production of catalase.

5. Discussion

In the present study the effect of heavy metal stresses on growth, surface structure and biochemical features of *L. monocytogenes* PTCC 1297 was investigated in vitro. Food processing environments offer many opportunities for the induction of stress response in microorganisms. Stresses may enhance and cross protect against other stresses (11). On Gram staining, *L. monocytogenes* appears as a pleomorphic, non-spore-forming, Gram-positive bacillus. It is somewhat smaller than most Gram-positive bacilli, with cells ranging from 0.4 to 0.5 $\mu\text{m} \times 1$ to 2 μm . The cells may be coccobacillary and occur in pairs and short chains (12). Morphological and physiological changes occur during starvation of cells. Bacterial cells become smaller and more rounded during starvation (11). Wen et al. reported that during long term survival, *L. monocytogenes* cells appear small and rounded as well (13). This rounding and shrinking may increase the ability of the cell to absorb nutrients (14). The results obtained in this study confirm earlier reports.

A recent study by Faezi-Ghasemi and Kazemi (8) showed the effect of sub-lethal environmental stresses on the cell

survival and antibacterial susceptibility of *L. monocytogenes* PTCC 1297. They reported that adaptation to some stresses including hydrogen peroxide and heat increase resistance to antibiotics, but, ethanol, hydrochloric acid, and sodium chloride decrease resistance to antibiotics. Heavy metals are members of a loosely defined subset of elements that exhibit metallic properties. Living organisms require varying amounts of "heavy metals". The pollutants of heavy metals can localize via ion exchange into soil and mud or precipitation of their compounds, which can have intense effects on the environment. Unlike organic pollutants, heavy metals do not rot and thus pose a different kind of challenge for degradation. Plants, mushrooms, or microorganisms are sometimes successfully used to remove some heavy metals. Many heavy metals are toxic for humans and mercury (Hg), lead (Pb), cadmium (Cd), and arsenic (As) are the most hazardous, due to their toxicity and widespread use (15). The presence of heavy metals lead to resistance in the environment. (1). Microorganisms have developed mechanisms of resistance that lead to the selection of resistant varieties

with the ability to tolerate metals (10). Malekzadeh et al. (16) and Verma et al. (10) reported on strains which did not show growth inhibition in the presence of 1 mM/L of cadmium (Cd), lead (Pb), zinc (Zn), and copper (Cu). Our results indicated that unlike different concentrations of cadmium or mercury, which could be lethal for bacteria, different levels of lead did not generally have an effect on the growth of *L. monocytogenes* PTCC 1297.

The development of more effective food preservation methods depend on an improved understanding of fundamental changes that are instituted at the gene expression level in cells of these bacteria when challenged with adverse environmental stress conditions. Proteins encoded and governed by global general stress response regulator σ factors play vital roles in protecting *L. monocytogenes* cells against various stress stimuli (17).

In conclusion, we demonstrated that different doses of stresses have lethal or sub-lethal roles in the survival of these bacteria and lead to different changes in its characteristics. The results of this study showed that *L. monocytogenes* PTCC 1297 could be resistant to lead. By learning more about tolerant bacteria to heavy metals, researches may find better ways to use them for bioremediation of water and waste-water treatments and reduction of Chemical Oxygen Demand (COD). Microbial bioremediation is an efficient strategy due to its low cost and efficient alternative to target heavy metals.

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Footnotes

Authors' Contribution: All authors had equal roles in the design and experimental work of this study.

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