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The effect of oxidative stress on proteomics of *Listeria monocytogenes* PTCC 1297

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Abstract

Listeria monocytogenes is a ubiquitous Gram-positive food-borne human bacterial pathogen that can cause listeriosis. This disease is a fatal with a high rate of hospitalization (>90%). The aim of this study is to determine the effect of hydrogen peroxide (H2O2) on L. monocytogenes PTCC 1297 proteomics. Bacterial cells exposed to gradually increasing sub-lethal concentrations of oxidative stress: 0.06, 0.3, 0.6, and 1.5 % of H2O2. Changes in protein profile of cells exposed to H2O2 and control (non-adapted cells) were determined by isoelectric focusing (IEF) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Protein spots visualized by staining with colloidal Coomassie Brilliant Blue. Based on the obtained results, one thousand four hundred spots identified on the gels. Out of these points, 85 spots were reproducibly detected with the help of software and eye confirmation. After analyzing these points, 9 spots showed the most significant changes. These points had appropriate density and indicate the response of bacteria to stress conditions. It can be concluded that the structure of proteome of L. monocytogenes PTCC 1297 changes when faced with oxidative stress. Nine protein spots on the gel were found to have substantial variations after protein spot analysis. These sites show how bacteria react under stress. Some of these spots' expressions had increased, while others had decreased.

Key words: Listeria monocytogenes PTCC 1297, proteomics, Oxidative Stress; Proteomics; IEF; SDS-PAGE

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1. Introduction

Listeria monocytogenes is a food-borne pathogen, Gram-positive and non-spore-forming bacterium which can grow under anaerobic and aerobic conditions cause serious infection of listeriosis. The disease primarily affects people with compromised immune systems (elderly adults, neonates, etc.) (Välimaa et al., 2015) (Vázquez-Boland et al., 2001). While the number of cases is low (0.1 to 11.3 per million capita), the fatality rate is very high (20 to 30%) (Swaminathan & Gerner-Smidt, 2007). Although listeriosis only affects 3% of healthy people on average, it is the most common foodborne illness and kills 250 people annually in the US (Bansal et al., 2018). Most microorganisms spend a long period of their lives facing environmental stress. Therefore, more information and findings about stress and stress responses are necessary for better understanding about physiology of them, which can lead to the development of new vaccines, new treatments for diseases, new methods of food hygiene, identification of new antimicrobial etc. Hence, studies on stress responses will continue to be an existing discussion of biological, medical and applied research (Albert G. Moat et al., 2002). In our previous studies, we determined the growth, cell morphology and biochemical characteristics of L. monocytogenes PTCC 1297 (Serotype 4a) under different doses of environmental stresses such as acid stress (HCl, pH 2.0-6.0), alkaline stress (NaOH, pH 8.0-12.0), ethanol stress (5.0%-25.0% V/V), oxidative stress (H2O2, 0.06%-6.0% V/V), osmotic stress (NaCl and sucrose, 2.0%-30.0% W/V), heat stress (40-60°C), and heavy metals including mercury (II) bromide (HgBr2), lead (II) oxide (PbO), and cadmium sulfate (CdSO4) (0.1% - 0.5% W/V). According to the results, the bacteria at pH≤4 and pH≥10 achieved by HCl and, NaOH, respectively, died. Also, concentrations of ethanol at ≥15 % V/V, H2O2 ≥ 0.3% V/V, NaCl ≥14% W/V, HgBr2 (0.1% W/V), CdSO4 (0.2% W/V) and heat ≥50°C were lethal for the bacteria. Unlike other stresses, sucrose did not kill bacteria but decreased their growth. In addition, different concentrations of PbO could not kill bacteria yet decreased their growth. Their phenotypical and biochemical characteristics of them changed when exposed to each stress. In addition, scanning electron microscope (SEM) micrographs showed very wide changes due to severe stresses induced on bacteria (Faezi-Ghasemi & Kazemi, 2015) (Kazemi & Faezi-Ghasemi, 2015b) (Kazemi & Faezi-Ghasemi, 2015a). Contaminated, often ready-to-eat (RTE) foods are the main transmission vehicles for human L. monocytogenes infections (Halbedel et al., 2020) (Halbedel et al., 2020) (Self et al., 2019). In food-processing environments, L. monocytogenes is commonly exposed to oxidative stress produced by sanitizers and disinfectants or antimicrobial rinses (Gao & Liu, 2014) (Michael J et al., 2013). Prototype change has a genotypic origin, which also is one of the ways of survival of microorganisms against some severe environmental stresses. Therefore, the purpose of the present study was to investigate the proteomic analysis of L. monocytogenes PTCC 1297 cells to treatments with sub-lethal doses of hydrogen peroxide (H2O2).

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

L.monocytogenes PTCC1297 was obtained from the Iranian Research Organization for Science and Technology (IROST). A lyophilized vial of L.monocytogenes PTCC 1297 was cultured in a Listeria CHROM agar (LCA) medium. 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 oxidative stress. All media and materials used in this research were purchased from Merck Co. Darmstadt, Germany.

2.2. Induction of Oxidative Stress

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. For oxidative stress, dilutions of 0.06%, 0.3%, 0.6%, 1.5%, and 6% (V/V) hydrogen peroxide were added to LEB. Then, a single colony from

the pre-culture was aseptically inoculated into the hydrogen peroxide-containing medium. The flasks were transferred to a shaking incubator (150 rpm), and incubated at 30°C until cultures reached the exponential phase that was defined by optical density (OD) of ≈1-2 at 600 nm for 24 h.

2.3. Sample Collection for Proteomics Analysis

According to our previous study on the impact of oxidative stress induced by H2O2 in L. monocytogenes (Kazemi & Faezi-Ghasemi, 2015b), the concentration of 0.24 % of H2O2 was accepted as the most effective stress concentration. To collect samples for proteomics analysis, 200 µL of bacteria, with a population density equivalent to 0.5 McFarland standards solution, were inoculated to 75 milliliters of nutrient broth medium and were inoculated at 37°C for 8-10 h to reach mid-log phase. Then, 5ml was poured into sterile tubes and centrifuged at 7000 rpm at 4°C for 30 min. The supernatant was discarded and the precipitate was washed with 2 ml of phosphate-buffered saline (PBS) and centrifuged again at 7000 rpm at 4°C s for 30 min. After washing 3 times, the supernatant solution was discarded and the sediment was transferred to a microtube under sterile conditions and kept at -80°C. Seventy-five ml of nutrient broth medium with a concentration of 0.24 % of H2O2 was prepared and bacterial sediment was transferred to it and placed in an incubator in dark conditions for 26-28 h. Then the pellet of the bacteria that was under stress conditions was obtained by the method described above and kept at -80°C.

2.4. Protein Extraction from Bacterial Cells

The bacterial sediment was centrifuged at 10,000 g at 4°C for 10 min. Then the supernatant solution was discarded and the sediment was washed with 500 µL distilled water. It was centrifuged again with the same conditions, washing with water twice. The 10 µL of phenylmethyl sulfonyl fluoride (PMSF) and 100 µL of 1% solution of protease inhibitor were added to 1 ml of lysis buffer. Then 1 ml of this buffer was added to 0.007 g of dithiothreitol (DTT). 250 µL of this buffer was added to the bacterial pellet. Then, using a sonicator, every 20 seconds was hit with 20 kHz and rested for 40 seconds. This process was repeated for 8 min. Then, it was centrifuged at 10000 g at 4 °C for 30 min. The supernatant solution, which is protein, was kept and the precipitate was discarded. The lysis buffer were including Urea (4.2g), Thio Urea (1.5g), CHAPS (0.4g), Tris stock solution (0.4ml), 100x Biolyte 3/10 (0.1ml), Ultra-pure water (Up to 10 ml). The Tris stock solution were including Tris base (6.05g), Ultra-pure water (35ml), HCl (Adjust to pH 7), and Double distilled water (To 50 ml). TCA solution was made from 10% protein solution and placed in ice for 1 h. Centrifugation was performed with 8000 g at 4°C for 45 min. Then, 800 µL of acetone -20°C was added to the solution. Incubation was done at -20 °C for 24 h. It was centrifuged for 10 min, washed with acetone, and incubated at -20°C for 30 min. The centrifuge was performed under the same conditions and the microtube was left open for acetone to escape. Then 100 µL of lysis buffer was added to the sediment.

2.5. Bradford Colorimetric Assay for Determining Protein Concentration

To prepare the standard curve, two volumes of concentrations of 10, 20, 40, 60, 80, and 100 µl from the standard solution of 1 mg/ml albumin were poured into the tube. In another tube, 100 µL of Bradford's reagent and water were added as a blank. The 5 µL of protein reagent was added to each tube. Then, the absorbance of the sample and standard was measured at 595 nm. The color cleated completely after 5 min, but after 10-15 min started to sediment. Especially in high protein concentrations, that is due to the tendency of proteins to sediment. Then, 2 µL of the unknown samples was mixed with 18 µL of water and 1 ml of the reagent, and the absorbance of each sample was read (Kielkopf et al., 2020).

2.6. Isoelectric focusing (IEF) for Protein Separation

To perform IEF, a 17 cm gel with a range of pH (3 to 10) was used. Protein was mixed with rehydration buffer. The total volume of this solution should be 125 µL (Table 1).

The rehydration buffer was including; Tris base (60 mg), bromophenol blue (100 mg), and ultra-pure water (Up to 10 ml). The IEF gels were placed in the prepared samples for 16 h, then placed in the protein IEF Cell to separate proteins based on isoelectronic pH. In this step, 300 µg pro-

teins are needed. The IEF gel was including Urea (9.61 g), 100x Biolyte 3/10 (200 µL), CHAPS (0.8 g), bromphenol blue stock solution (4 µL), ultra-pure water (Up to 20 ml) (Cornell, 2009).

2.7. SDS PAGE- (2-D) Protein Separation

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used to obtain high-resolution separation of complex mixtures of proteins. The SDS-PAGE gel was prepared according to the conditions which showed in Table 2.

Then equilibration buffer solution was added to the IEF gels. After 20 min, 150 mg iodoacetamide was added and allowed to stand for 20 min. Then, the IEF gel was placed on the SDS gel. For the first stage gel, 16 mA/gel is done for 30 min, and for the second stage gel, 24 mA/gel is done for 5 h. The equilibration based buffer was including Urea (36 g), SDS (2 g), 1.5M Tris/HCl pH 8.8 (5 ml), glycerol (17.4 ml), ultra-pure water (Adjust to 100 ml).

The SDS gel contained ultra-pure water (13.6 ml), acryl amid (16ml), resolving (10.4 ml), SDS 10% (200 µL), APS 10% (200 µL), TEMED (20µL). The resolving buffer was including Tris base (181.7g), ultra-pure water (750 ml), HCl (Adjust to pH 8.8), and ultra-pure water (To 1000 ml) (Berkelman, 2008).

2.8. Silver Nitrate Staining

After the second dimension of electrophoresis, staining was done. First, the gel was placed in the fixation solution overnight. Washing was done three times for 20 min with washing buffer and then it was washed three times with sensitizing buffer for 5 min. The dye solution was discarded. After the spots appeared on the gel, a stopping solution was added.

The staining solution was including silver nitrate (0.4 g), formaldehyde (152 µL), and ultra-pure water (200 ml). The Fixation solution was including; Ethanol (80 ml), Acetic acid (20 ml), and Ultra-pure water (100 ml). The Washing solution was including; Ethanol 96% (73 ml), ultra-pure water (127 ml). The sensitizing solution was including thiosulphate sodium (0.062 g), ultra-pure water (200 ml).The stopping solution was including methanol (100 ml), acetic acid (24 ml), ultra-pure water (76 ml).

2.9. 2DE Proteome Profiles

After electrophoresis, the stained gels were scanned using a densitometer GS-800 (Bio -Rad) with a resolution of 600 dots per inch. The relevant images were analyzed using ImageMaster 2D Platinum 6.0 software (Figure 1).

Table 1. The compounds of Isoelectric focusing (IEF) solutions

Sample no.	H_2O_2 concentration in sample (%V/Vl)	Sample (µL)	Rehydration buffer (µL)
1	Before the stress (0)	48.7	76.3
2	Before the stress (0)	44.1	80.9
3	Before the stress (0)	34.7	90.3
4	Oxidative stress (0.24)	23	102
5	Oxidative stress (0.24)	20.8	104.2
6	Oxidative stress (0.24)	16	109

Table 2. SDS-PAGE steps conditions

Step	Ramping Mode	Maximum Voltage	Time	Volt-Hour
1	Linear	250v	20 min	
2	Linear	10000v	2.5 h	
3	Rapid	10000v	4.3 h	40000-500000v-hr

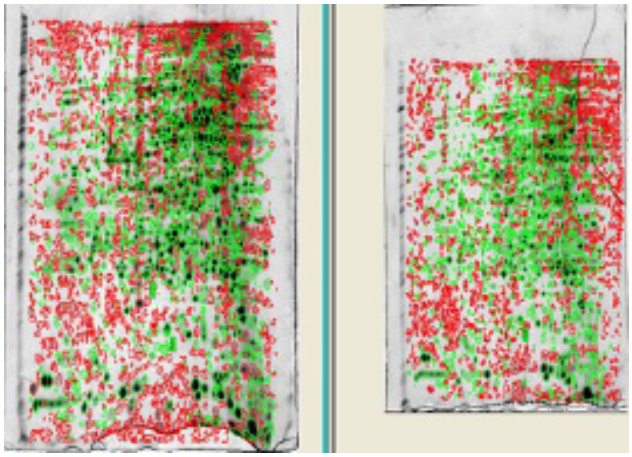

Figure 1. Spots determined on the 2DE gels using the ImageMaster 2D Platinum 6.0 software

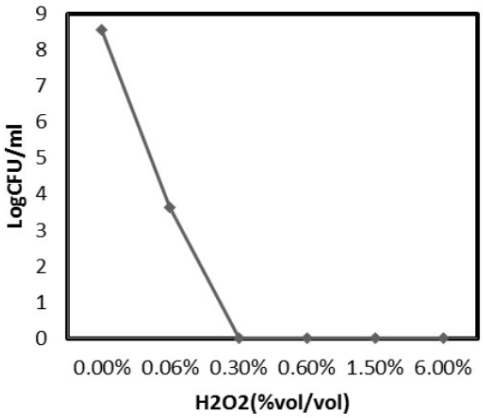

Figure 2. The viability of L. monocytogenes PTCC 1297 after 24 h starvation in different degrees of H2O2 at 35°C

3. Results

3.1. Bacteria growth

The viability of L. monocytogenes PTCC 1297 after 24 h starvation in different degrees of H2O2 at 35°C is shown in Figure 2.

3.2. Determination of protein concentration with the Bradford method

After bacterial cell lysis and protein extraction, the protein concentration was checked by the Bradford method. The results were as follows (Table 3):

Table 3. Protein concentration by Bradford method

Sample no.	H_2O_2 concentration in sample (%vol/vol)	Concentration (mg/ml)
1	Before the stress (0)	7.6
2	Before the stress (0)	6.8
3	Before the stress (0)	8.64
4	Oxidative stress (0.24)	13.1
5	Oxidative stress (0.24)	14.4
6	Oxidative stress (0.24)	18.6

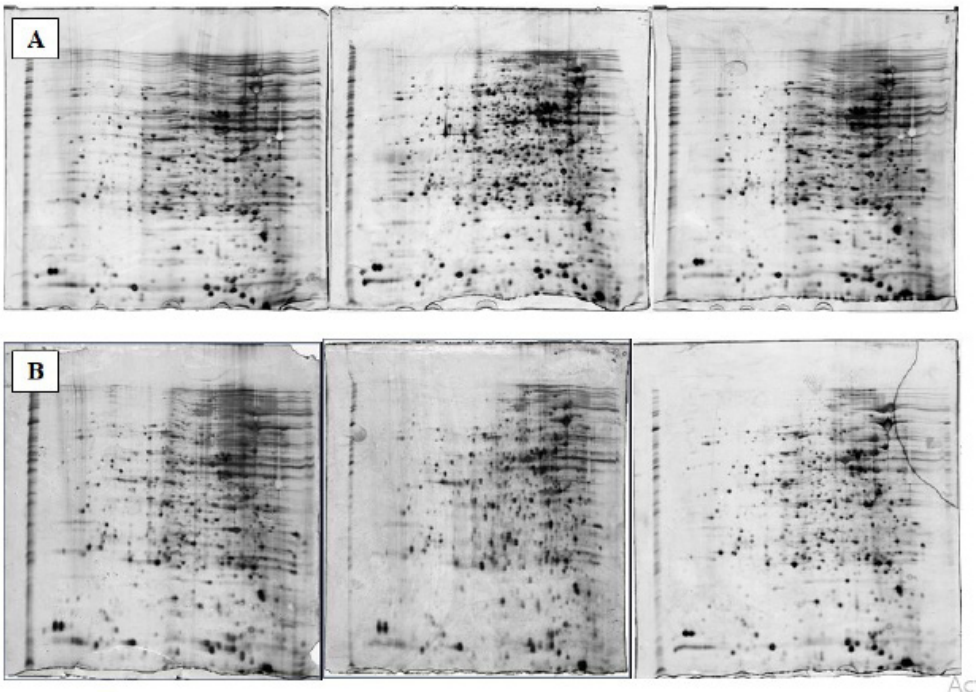

Figure 3. 2DE gels maps of three strains of Listeria monocytogenes PTCC 1297 (A) before stress, (B) after oxidative stress

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3.3. Proteomics analysis

During the analysis, first, an intra-group comparison was done and the common points were checked. Then, a comparison was made between the control group (before stress) and the sample group (after oxidative stress). In this sample group, the points that had significant differences were reported as follows. After taking the images of the gels with a densitometer and entering them into the Image Master 2D Platinum 6.0 software, at least 1400 spots were identified on the gels. Out of these points, 85 spots were reproducibly detected with the help of software and eye confirmation. After analyzing these points, 9 spots showed the most significant changes. The selected spots were shown in Figure 4. Statistical analyzes such as T tests were performed on them using the mentioned software (Table 4).

Selected spots had CVs less than 40. CV indicates the amount of intra-group difference, the lower the value, the better. Fold indicates protein density, the higher the value, the better.

4. Discussion

The objective of this study was to evaluate the protein changes against H2O2 stress in L. monocytogenes in laboratory conditions. Since oxidative stress is one of the many challenges infectious agents must face to persist within the host environment, it is vital to attempt to understand the mechanisms utilized to withstand

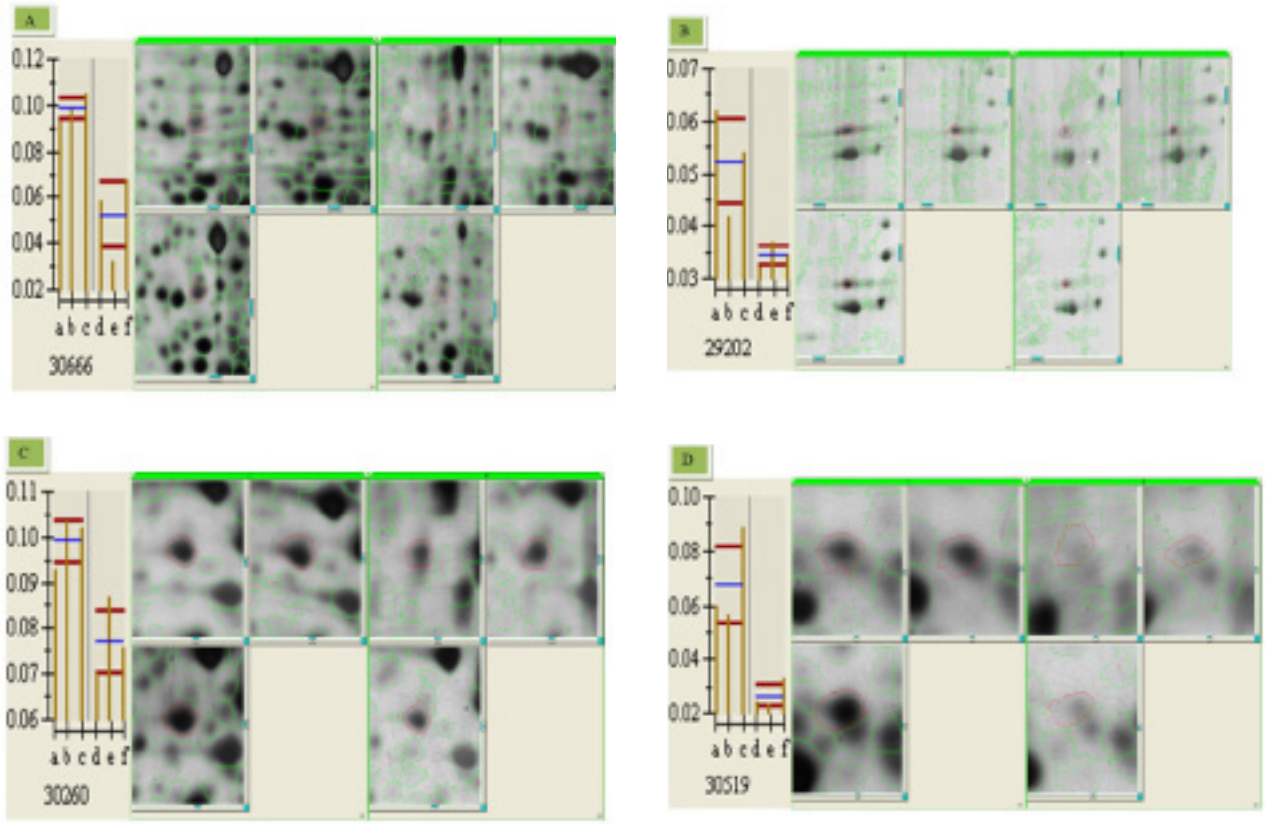

Figure 4. Selected Spots (A) 30666, (B) 29202, (C) 30260, and (D) 30519 using Image Master 2D Platinum 6.0 software

Table 4. Comparative expression of identified proteins in *L.monocytogenes* PTCC 1297

Spot no.	Student-t	C v C	C v M	Fold change C/M
30666	5	6.37254	34.64708	1.9
29202	2.9	19.38896	6.854321	1.5
30260	3.6	5.829068	11.1999	1.3
30519	4.6	18.22563	7.3156	1.7

these stressors. Adaptation to changes in growth conditions, which is necessary for cell survival, occurs at different levels including regulation of transcription, after transcription, and translation. Proteomics analysis using two-dimensional gel-based allows the following changes in the rate of protein synthesis. Faced with new conditions, the cell uses high translation capacity to synthesize sufficient amounts of essential proteins.

Huang et al. (2014) presented a preliminary view of the protein expression in two strains of L. monocytogenes species in New Zealand. According to this study, two local strains, SB92/844 and SB92/870 displayed identical growth profiles in comparison to the overseas strain under the same growth conditions. From 1D SDS PAGE, it was apparent that the protein banding patterns of three strains of L. monocytogenes were very similar with no significant differences in the intensities in bands in the gel. In contrast, 2DE gel maps and comparative spot analysis revealed that strains exhibited differences in the relative levels of some proteins when their respective strains were grown under similar physiological conditions. Furthermore, this study also highlighted the possibility that the stress response proteins of L. monocytogenes may have a role in the pathogenesis and clinical behavior of specific strains (Huang et al., 2014).

In a different investigation, representative isolates under stressful conditions were examined for the expression of two important genes for virulence (hly) and stress (clpC). The hly and clpC genes were unregulated during oxidative stress at low temperatures. Their findings suggest that L. monocytogenes may be able to grow under circumstances common in the food industry. Activating molecular mechanisms based on cross-protection that can improve virulence is one of the survival strategies that L. monocytogenes has developed. This might potentially increase the likelihood of virulent strains remaining in food processing facilities. They showed the effect of two different oxidizing agents at two temperatures (optimal growth temperature and the refrigeration temperature in food industries) at the same time on different genotypes of L. monocytogenes. The oxidative effect is temperature dependent, being lower at 10 °C than at 37 °C. The virulence LIPI-1 genes were more strongly expressed when oxidative agents were applied at refrigeration temperatures (Manso et al., 2020).

Welch (2021) investigated if oxidative stress might function as a signal from the environment that affects the level of cyclic-di-GMP inside the cell. Hydrogen peroxide was added to aerobic cultures to test this, and the levels of cyclic-di-GMP were compared to the controls. Results showed that treated cultures had much higher levels of cyclic-dimeric-GMP than untreated controls. The findings of this study imply that anaerobic circumstances and reactive oxygen species probably play a significant role in the control of cyclic-di-GMP expression within the cell (Welch, 2021).

Conclusion

Nine protein spots on the gel were found to have substantial variations after protein spot analysis. These sites have the right amount of density and show how bacteria react under stress. Some of these spots' expressions had increased, while others had decreased. This study highlights the need for more research to determine whether the virulence and pathogenicity of L. monocytogenes under diverse growth circumstances may be correlated with the variable expression of oxidative stress proteins. Identifying the proteins that are specifically created when H2O2 stress is induced and investigating their function, and further molecular studies to understand cellular mechanisms of L. monocytogenes stress adaptation response against H2O2.

CONFLICT OF INTEREST

No conflict of interest declared.

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