

Long Term Survival of *Listeria monocytogenes* in Stress Conditions: High pH and Salt Concentrations

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ABSTRACT

Background: *Listeria monocytogenes* is a bacterium that is transmitted by food. This bacterium is the cause of Listeria disease, which causes severe symptoms such as meningitis, septicemia and abortion. Determination of the effect of different conditions such as pH, salt and temperature on survival of *L. monocytogenes* in food seems essential.

Methods: A standard strain of *L. monocytogenes* (ATCC: 19115) was used for this study. The bacteria was added in the concentration of 3×10^8 CFU/ml, to BHI broth medium at pH=4, 5, 6 and 7 and salt concentrations of 0, 7%, 14% and 21%. Temperature was 4°C and 20°C. Samples were collected every 24 hours from the cultured mediums in a special environment of PALCAM agar. This will be done until no colonies grow on the PALCAM agar environment.

Results: The effect of independent variable of storage temperature alone on the survival rate of *L. monocytogenes* in the level of 5% is not significant. Also, the increase in salt concentrations and pH together, increased on the survival of bacteria at 5% level. The minimum survival time was pH=4 and the salt concentration was 21% at 4°C for 19 days.

Conclusion: The results of this study showed that the effect of three factors (pH=4) and high salt concentration (21%) and temperature can reduce the bacterial survival rate in foods. This means that survival at low pH and salt concentration is strongly dependent on temperature.

Key words: *Listeria monocytogenes*, Salt concentration, Listeriosis, PALCAM agar

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INTRODUCTION

Listeria monocytogenes is the cause of food infections. This bacterium can be isolated from soil, animal food, water, faeces, meat and its products, milk and dairy products, and vegetables. It also has the ability to create biofilms on many surfaces in contact with food [1]. *L. monocytogenes* is the main cause of Listeriosis that causes primary meningitis, encephalitis, or septicemia in adult males and non-pregnant women [2]. The ability to tolerate adverse environmental conditions, including changes in pH, temperature and salt, leads to the spread of *Listeria* species in different food sources. *L. monocytogenes* is

capable of growing at 4°C due to having cold species, and on the other hand, some of its strains can survive under poorly pasteurized conditions [3]. Therefore, the risk of infection with *Listeria* is increased through the consumption of contaminated food. Finding a way to remove *L. monocytogenes* from food is of great importance. In order to increase the shelf-life of foods and prevent the growth of pathogens, especially the *Listeria* strains, salt is widely used in the food industry [4]. Salt prevents the growth of bacteria by reducing the aqueous activity. The use of salt in meat products reduces the number of aerobic serotypes of bacteria, *Enterobacteriaceae*, and lactic acid bacteria, thereby increasing the food shelf-life [5]. Salt is also used in traditional foods produced at home or in small workshops [6]. The main concern in this regard is the presence of salt-resistant pathogens in salt sources, as well as the frequent use of this salt in the production of

food, in particular the production of various types of cheese [7]. Edible acids are also used in a variety of foods such as fish, canned foods, flour products, fruit and vegetable extracts. Edible acids, like salt, control the growth of pathogens [8]. Edible estrogens have been very much considered due to their low-cost, safe and low levels of organoleptic properties of meat products [9]. The use of 2.5% sodium acetate, sodium lactate and sodium citrate, without undesirable effects on the organoleptic properties of salmon fish, increased the shelf-life of the product [10]. However, the key to using salt and edible acids in food production is to use their appropriate concentration to prevent the growth of pathogens, especially *L. monocytogenes*, because it has been shown that the use of less than lethal amounts of salt and acid in food causes resistance in microorganisms, including *L. monocytogenes* [10]. Because of the high level of *L. monocytogenes* in high risk groups, knowing how to behave in different conditions in terms of salt and pH can have an important effect on improving food safety [11]. The aim of the present study was to evaluate the survival of *L. monocytogenes* in different stress conditions, different salt concentration and pH at 2°C and 4°C.

MATERIALS AND METHODS

Microorganisms and culture media

In this study, standard strain of *Listeria monocytogenes* (ATCC: 19115) was prepared from the Iran Research Institute of Science and Technology. In order to activate the bacteria, lyophilized bacterium was cultured in BHI broth medium at 37°C for 24 hours. After activation, in order to obtain a single colony, the bacterium was cultured on a BHI Agar medium at 37°C for 24 hours. Single colony of bacteria was transferred to 10 ml of BHI broth medium and kept on incubator shaker at 37°C. The bacterial opacity was evaluated using a spectrophotometer at a wavelength of 600 nm. When absorption at 600 nm equalled 1.8, the turbidity of bacteria was twice as high as McFarland and equal to 6×10^8 CFU/ml.

Preparation of different concentrations of salt and pH

Using NaCl, different concentrations of 0%, 7%, 14% and 21% in pH 4, 5, 6, and 7 were stored in a broth medium of BHI according to standard method and stored in a refrigerator at 4°C.

Survival study at different salt and pH concentrations

In order to investigate the survival of various salt and pH concentrations, bacterial suspensions were added to the BHI medium with different concentrations of salt and pH to achieve a bacterial concentration equivalent to 3×10^8 CFU/ml. Samples were stored at 4°C and 20°C.

Samples were collected every 24 hours from cultured samples and cultured on a PALCAM agar medium containing polymixin B at a concentration of 5 mg and 2.5 mg acriflavin-HCL and maintained at 37°C for 48 hours. After this time, the plates were examined for bacterial growth and colony formation. This was done until no colonies grow up on the PALCAM agar medium. This procedure was repeated three times for each pH and salt concentration. In this study, *L. monocytogenes* in BHI broth was used as a positive control.

Detection of VBNC cells by culture method

To confirm formation of non-cultivable form of *Listeria*, one millimeter of each suspension from the conditions at which no colony was recovered transferred to 9 ml of BHI broth and kept it for 7 days at 37°C. Then, defined amounts of the culture was inoculated into PALCAM agar and appearance of the colonies was followed on the plates.

Statistical analysis

Data was analyzed using Design Expert software version 9. The response was evaluated by the survival of *L. monocytogenes* under the influence of three variables including temperature, salt concentrations, and pH.

RESULTS

L. monocytogenes survival data were collected under stress conditions with various salt and pH concentrations and listed in Table 1. Results showed that the minimum survival time of bacteria at pH 4 and 21% of salt concentration was 19 days and 22 days at 20°C and 4°C respectively.

It was determined that culture medium in temperature of 4°C, at a concentration of salt 0%, when pH elevates from 4 to 6, the amount of bacterial viability decreased, but at pH=7, bacterial viability was up to 88 days. By increasing the salt concentration to 14% and 21%, the survival rate was reduced to 35 days (Figure 1).

Table 1: *L. monocytogenes* survival data

Sample No.	pH	Salt concentration (%)	Lack of growth at 4°C after ... day	Lack of growth at 20°C after ... day
1	4	0	70	64
2	4	7	71	62
3	4	14	45	41
4	4	21	22	19

5	5	0	69	64
6	5	7	69	69
7	5	14	43	42
8	5	21	37	35
9	6	0	55	49
10	6	7	60	56
11	6	14	41	38
12	6	21	34	30
13	7	0	88	76
14	7	7	85	81
15	7	14	43	40
16	7	21	37	30

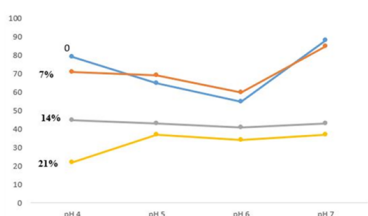


Figure 1: The survival rate of *L. monocytogenes* at different pH and different concentrations of salt at 4°C

At 0 and 7% salt concentrations, as pH increase from 4 to 6, decrease in survival of bacteria was seen, but at pH=7, the survival rate increased. At salt concentration of 14%, no significant decrease was observed in the amount bacterial survival, but in samples containing 21% salt concentration at pH 4, there was a significant reduction in bacterial survival.

With increasing pH, the bacterial survival rate increased slightly which indicated that reducing pH and increasing salt concentration together could be effective in reducing *L. monocytogenes* survival (Figure 2).

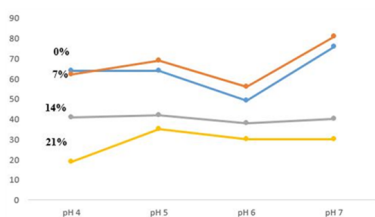


Figure 2: *L. monocytogenes* survival at different pH and salt concentrations at 20°C

Determination of the effect of salt concentration and Hydrogen ion concentration on the log time performed and results showed that when the temperature drop from 20°C to 4°C, growth of *L. monocytogenes* in colonies increased. The effect of increasing temperature with increasing salt concentration together is statistically

significant on the level of bacterial viability. Consequently, as the salt concentration increased at 20°C, the viability of the bacteria decreased in comparison with 20°C. Also, the reduction of salt concentration, especially at high levels of pH, in both temperatures (4°C and 20°C), increased the growth of *L. monocytogenes* (Figures 3 and 4).

ANOVA test analysis of bacterial survival in different stress conditions showed that the effect of salt concentration and the effect of temperature were statistically significant (Table 2). The results showed that the effect of independent variable of storage temperature alone on the survival rate of *L. monocytogenes* was not significant. Also, increasing salt concentrations and pH together, increased the survival of bacteria significantly.

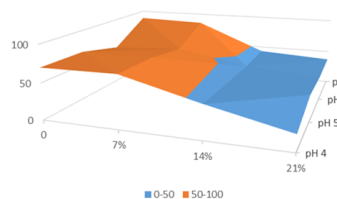


Figure 3: Effect of salt concentration and concentration of hydrogen ion on the logarithm of the time of significant growth of *L. monocytogenes* at 4°C

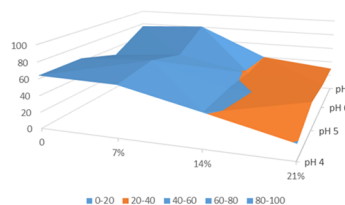


Figure 4: The effect of salt concentration and hydrogen ion concentration on the logarithm of the time of significant growth of *L. monocytogenes* at 20°C

Table 2: ANOVA test analysis of bacterial survival in different stress conditions

Variables	Sum of Squares	Coefficient	p-value
Temperature (4°C)	0.28	-0.17	0.3626
Salt concentration (4°C)	3.21	-0.57	0.0134
pH (4°C)	1.77	0.42	0.0452
Temperature (21°C)	0.18	-0.15	0.2931
Salt concentration (21°C)	2.81	-0.36	0.0122
pH (21°C)	2.1	0.34	0.0381

DISCUSSION

Listeriosis is known as a food related illness, due to its high resistance to environmental conditions, the presence of this bacterium is possible in raw or even processed food, and therefore it seems necessary from the beginning of the cycle to make food on the farm, take necessary measures and oversight [12]. The issue of the survival of food pathogenic bacteria in naturally acidic foods and therefore low in pH or foods used to maintain and improve their microbial quality have been used in organic acids in order to maintain a high level of consumer health is so important [13]. Generally, microorganisms experience different stresses relative to environmental stress and change their ideal conditions. Therefore, studying the survival rate of *L. monocytogenes* is very important for changing salt and pH conditions.

In this regard, Karabiyikli *et al.* showed that the antimicrobial effect of orange juice on *Salmonella typhimurium* and *L. monocytogenes* is due to its low pH and if neutralized, it has no antimicrobial effect [14]. Survival of *L. monocytogenes* in osmotic stress and low pH is largely influenced by temperature. The low temperature (4°C), in which the rate of growth and metabolism of the bacteria is reduced, allows the bacteria to survive more at lower pH and high salt concentration than the high temperature (20°C). This low salt concentration creates a protective state against low levels of pH in *L. monocytogenes*. These results are quite consistent with the results of this study because according to our results, in low salt concentration (7% and 14%) at 4°C, a slight decrease in the bacterial survival rate than the concentration of 21% salt was observed while at 20°C the highest survival rate of *L. monocytogenes* was observed at pH 4 and 21% salt concentration for 19 days. Faezi-Ghasemi *et al.* used antibiotics to prevent the presence of *L. monocytogenes* in foods, which creates drug resistance in bacteria as well as food consumers [15]. In the present study, the interaction effect of increasing temperature with a decrease in salt percent with bacterial viability was reported at a significant level of 5%. Also, the reduction of salt percent, especially at higher pH, increased the bacterial viability, while this increase was not statistically significant. Also, in this study, the significance of the effect of salt concentration on survival of *L. monocytogenes* in stored temperatures at the level of $p < 0.05$ was confirmed. In a study by Gallo *et al.* the effect of environmental conditions (pH and temperature) on increasing the

antibacterial effects of Nysin on *Listeria Innocua*. Their results showed that at pH=5, the effect of Nysin was greater than pH=7 [16]. Results of this study are consistent with the present study, because in this study, at low pH, the effect of salt concentration was higher than pH=7. In fact, *Listeria Innocua* is a non-pathogen strain, which is used as an indicator of *L. monocytogenes* due to their similarity of its physiochemical and biological properties. Razavilar *et al.* determined the pH, the growth and non-growth border and the survival and death rate of *Staphylococcus aureus* using organic and inorganic acids in different storage temperatures [17]. They compared the antimicrobial properties of the pH of three organic acids (acetic acid, citric acid, lactic acid) and an inorganic acid (hydrochloric acid) against *Staphylococcus aureus*. In this study, they examined the behaviour of *Staphylococcus aureus* in terms of growth at different pH levels (4 to 6). In this study, Razavilar *et al.* stated that the lowest pH as the border of the lack of growth was pH=5 [17].

In the present study, at salt concentrations of 14% and 21% in pH=5, a slight improvement in bacterial survival and significant reduction in pH=7 was observed. Therefore, the growth rate of bacteria in the presence of two factors of low pH and high salt concentration together, showed a decrease from 53% to 95% which is statistically significant.

CONCLUSION

The findings of this study indicated that environmental factors (temperature, pH and salt concentration) significantly decrease the survival rate of *Listeria monocytogenes*. It is recommended to study the effects of temperature, pH and salt concentration in laboratory conditions and dietary models such as meat and meat products, as well as dairy products.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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