Effects of osmotic stress on *Listeria monocytogenes* ATCC 7644: persistent cells and heat resistance

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Abstract

Persistent bacteria are a microbial subpopulation that, exposed to bactericidal treatment, is killed at a slower rate than the rest of the population they are part of. They can be triggered either following stress or stochastically without external signals. The hallmark of persistent bacteria is the biphasic killing curve, a sign that, within a microbial population, two subpopulations are inactivated at a different rate. Furthermore, when plated into a fresh medium and in the absence of stressors, persistent bacteria typically remain in the lag phase longer before resuming active replication. This study aims to evaluate *in vitro* whether the formation of persistent cells in a strain of *Listeria monocytogenes* can be triggered by exposure to osmotic stress and if this phenomenon can increase heat resistance in the bacterial population. In a first experiment, the lag time distribution of a *L. monocytogenes* strain grown in a 6% NaCl broth was evaluated using the software ScanLag. A stationary phase broth culture was inoculated on agar plates placed on an office scanner inside an incubator at 37°C. The plates were scanned every 20' for 4 days and the acquired images were automatically elaborated with the aid of MatLab software in order to evaluate the appearance times of every single colony. The experiment was also carried out on a control culture obtained by growing the strain in the broth without salt. In a second experiment, the same broth cultures, after proper dilutions to rebalance NaCl concentration, were subjected to a heat treatment at 51°C and the death curves obtained were parameterized using the GinaFit system. Results showed that the lag phase of 31.40% of the salt culture colonies was long enough to suppose the formation of persistent bacteria. Analyses of the thermal survival curves showed that the *shoulder* and *tail* model was the one that best represented the inactivation trend of the salt culture, unlike the control culture, whose trend was essentially linear. Results of the present study show how exposure to salt could induce the formation of persistent bacteria in a *L. monocytogenes* strain. The last raises concerns as persistent cells may not only be undetected with common analytical techniques but they even show a greater heat resistance.

Introduction

The defence strategies adopted by bacteria to survive lethal treatments are several and, over the years, increasingly sophisticated mechanisms have been discovered. Much of the knowledge in this field is due to studies on antibiotic *resistance* which have shed light on the different molecular processes triggered by bacteria when exposed to antibacterial treatments (Reygaert, 2018; Ed-Dra *et al.*, 2021; Mancuso *et al.*, 2021). In particular, the term *resistance* was coined to describe the heritable ability of bacteria to grow at higher antibiotic concentrations regardless of the time of exposure to the treatment (Blair *et al.*, 2015; Ed-Dra *et al.*, 2020; Kowalska-Krochmal and Dudek-Wicher, 2021). Other defence strategies, instead, do not depend on the concentration of the antibacterial but on the duration of the treatment. The terms *tolerance* and *persistence* refer to these different modes of survival which consist in the ability of bacteria to survive transient bactericidal treatments longer despite the minimum antibiotic concentration required to arrest their growth remains the same (Brauner *et al.*, 2016). The mechanisms behind *tolerance* and *persistence* are essentially the same, however, while *tolerance* is a phenomenon that concerns an entire microbial population, *persistence* only occurs in a subpopulation of bacterial cells (Liu *et al.*, 2022). We can define persistent bacteria as a genetically uniform microbial subpopulation that, exposed to a bactericidal treatment, is killed at a slower rate than the susceptible population it arose from (Balaban *et al.*, 2019). In this regard, the hallmark of persistent bacteria is the biphasic killing curve which assumes the presence of one initially major subpopulation, that is more sensitive to the treatment, and one minor subpopulation, that is more resistant to the treatment (Cerf, 1977). The ability of persistent bacteria to survive lethal treatment for longer is usually associated with the ability of these cells to enter a transient non-growing state (Levin-Reisman

and Balaban, 2016). Bacteria can enter a non-growing state according to two distinct mechanisms: either following a stressful stimulus (Type I persistence) or stochastically in absence of external signals (Type II persistence) (Levin-Reisman and Balaban, 2016). The distinctive feature of Type 1 persisters is that they remain in the lag phase longer than the rest of the population once the stressor has been removed and the culture resumes growth in a fresh medium (Balaban *et al.*, 2019). Even Type II persisters have a longer lag phase before growth resumes but they spontaneously generate without any trigger signal when the microbial population is in exponential growth. Therefore, exploring the lag time distribution at the single-cell level can allow determining the number of persisters in a bacterial population (Kaplan *et al.*, 2021). To date, most of the studies have investigated the formation of persisters by studying the typical biphasic killing curve of a culture exposed to a bactericidal treatment.

Through a more technological and automated approach, the open-source software *ScanLag* was implemented and successfully used to study the persistence fraction of different bacterial strains based on the colony appearance times on solid media (Levin-Reisman *et al.*, 2010). *ScanLag* uses office scanners to automatically record the growth of colonies on agar plates and evaluates the frequency distribution of their appearance times by automated image analysis. To date, the phenomenon of *persistence* has been extensively studied due to concerns about the ability of these bacteria to elude antibiotic treatments resulting in persistent infections that are difficult to eradicate (Balaban *et al.*, 2019). On this background, *persistence* also raises concerns about food safety since it cannot be ruled out that foodborne pathogens adopt this defence strategy to survive common manufacturing processes such as the use of bactericidal compounds or heat treatments (Doyle *et al.*, 2001; Wu *et al.*, 2017). For example, persistent bacteria could be involved in the listeriosis outbreak occurred in August 2019 in Andalusia, Spain, in which 222 cases of infection, three deaths and six miscarriages were associated with the consumption of cooked pork products (WHO, 2019). We could speculate that the most common preservation techniques (refrigeration, salting, drying, *etc.*) may act as stressors that trigger the formation of persistent bacteria able to survive subsequent bactericidal treatment and grow later in foods during storage. Therefore, this study aims to evaluate *in vitro* whether the formation of persistent cells in a strain of *L. monocytogenes* can be triggered by exposure to high salt concentration (osmotic stress) and if this phenomenon can increase the heat resistance of the bacterial population.

Materials and methods

Preparation of the bacterial strain

The strain used in this study was *L. monocytogenes* ATCC 7644 belonging to the collection of the *Food Microbiology Laboratory* of the Department of Veterinary Sciences, University of Messina (Messina, Italy). The stock culture was kept frozen at –80°C in 85% brain heart infusion broth (Biolife, Milan, Italy) and 15% glycerol (v/v) (Merck, Darmstadt, Germany). From the frozen stock, a 10 μL loopful was streaked onto a plate of tryptone soy yeast extract agar (TSYEA; Biolife, Milan, Italy) and incubated at 37°C for 24 hours. A single colony was inoculated in 10 ml of tryptone soy yeast extract broth (TSYEB; Biolife, Milan, Italy)

and incubated overnight at 37°C. The overnight culture was ten-folds diluted into saline peptone water (SPW; Merck, Darmstadt, Germany) and used to prepare two different broth cultures, both with a concentration of ~10^3 CFU/ml: one in TSYEB (control culture) and the other in TSYEB with the addition of 6% NaCl (Merck, Darmstadt, Germany) (salt culture). The salt concentration chosen allowed the growth of the microorganism and, based on previous studies, was high enough to induce relevant adaptive responses in *Listeria monocytogenes* (Al-Nabulsi *et al.*, 2015). The broth cultures thus obtained were incubated at 37°C until reaching the stationary phase with a concentration of ~10^8 CFU/mL and then used in the experiments described below. An optical density calibration curve was created in advance by a spectrophotometer (Biorad, Hercules, California, USA) at a wavelength of 600 nm in order to follow the microbial growth in each broth.

Experiment 1: evaluation of the lag phase duration at single-cell level

The distribution of the lag phase duration at single-cell level of the salt and control cultures was evaluated using the *ScanLag* software. The analytical protocol used for the present study was inspired by Levin-Reisman and Balaban (2016), suitably modified. In detail, control and salt cultures were ten-folds diluted in SPW to reach a final concentration of ~10^3 CFU/ml in TSYEB and 100 μL were plated onto plates of Agar Listeria according to Ottaviani and Agosti (ALOA; Biolife, Milan, Italy). The lid of each plate was covered with sterile white blotting paper to avoid condensation falling on the medium. The plates were placed in a homemade plate holder on the top of an office scanner (Epson-XP305, Suwa, Japan) placed inside an incubator (IC 150-R, Argo Lab, Carpi, Italy) set at 37°C. The scanner was configured using the application *ScanningManager* (v. 2016) so that the plates were scanned periodically every 20 minutes for 4 days. The images were acquired at a resolution of 600 dpi and were converted to grayscale using Photoshop (v. 2020; Adobe, California, USA). The images were analysed through specific functions implemented on *Matlab* (R2022a; Mathworks, Massachusetts, USA) by Levin-Reisman *et al.* (2010) that allow determining the appearance times (lag phase) of every single colony in the ALOA plates. Colonies were detected when their size exceeded a size threshold of ten pixels. To estimate the persistence level, the appearance times of the colonies were fit to a Gaussian distribution and the means and

standard deviations were calculated. The amount of persistent bacteria was established by summing the number of colonies whose appearance times were three standard deviations above the mean (cut-off value) (Levin-Reisman and Balaban, 2016). The persistence level was first calculated for each single culture then, to assess if the osmotic stress induces the formation of persistent bacteria, the colony appearance times of the salt cultures were compared with the cut-off value measured for the control cultures.

Experiment 2: heat treatment

The same control and salt cultures in the stationary phase used in experiment 1 were subjected to a heat treatment for 24 h using a water bath (WB22; Argo Lab, Carpi, Italy) set at 51°C. The heat treatment temperature was chosen as it allowed us to best follow the microbial inactivation based on the analytical protocol adopted in the present study and described below. Lower or higher temperatures would have inactivated the microorganism either too slowly or too quickly. Each broth culture, after appropriate dilution to rebalance the salt concentration, was inoculated into three sterile 20 ml tubes containing 9 ml of TSYEB preheated in the water bath so that the final volume in each tube was 10 ml at a concentration of ~10^7 CFU/ml. Another tube was prepared with uncontaminated broth and used to monitor the temperature with a probe thermometer (Checktemp Electronic Thermometer, Hanna Instruments, Woonsocket, Rhode Island, USA). The test began immediately after inoculating the control and salt cultures since the decrease in temperature observed in the uncontaminated tube following the inoculation could be considered negligible (±0.3°C). The broth cultures were sampled at preset time intervals (approximately every hour and a half), plated on TSYEA plates after proper ten-folds dilutions in SPW and incubated at 37°C for 48 hours before cell's enumeration. All the plates were inoculated with 100 μl of bacterial suspension except for the time points with expected low concentrations of viable bacteria that were plated with 1 ml. For each time point, ten randomly selected colonies were identified by MALDI-TOF technology to confirm their species and exclude contamination during analysis. In plates where the number of colonies grown was <10, all colonies were tested. MALDI-TOF analysis was performed according to Trabelsi *et al.* (2021) using a Vitek MS Axima Assurance mass spectrometer (bioMerieux, Firenze, Italy) set in positive linear mode, with a laser frequency of 50 Hz, an acceleration voltage of

20 kV, and an extraction delay time of 200 ns. The mass spectra (MS) range was set to detect from 2000 to 20,000 Da. MALDI-TOF generated a unique MS spectrum for each colony tested which was compared with a database of bacterial reference spectra and SuperSpectra using the SARAMIS software (Spectral ARchive and Microbial Identification System – Database version V4.12 – Software year 2013, bioMerieux, Firenze, Italy). Only the identifications with a 70% match were considered reliable.

Data analysis

The normal distribution of colony appearance times was tested by the D'Agostino-Pearson omnibus test and significant differences between the control and salt cultures were evaluated using the Mann-Whitney test using Graph Pad Prism 9.1.1 software (San Diego, California, USA). The statistical test was performed two-tailed and the critical significance level (*P*) was set at 5% (0.05). After the heat treatment, the counts of the surviving *L. monocytogenes* were converted to Log_{10} values and plotted against time to construct inactivation curves. Inactivation curves are triplicates of repeated measurements of the concentration (Log CFU/ml) of the heat-injured cells of the *L. monocytogenes* strain over time (hours). The curves of thermal inactivation of the control and salt cultures were fitted with different linear and non-linear microbial survival models and inactivation parameters were obtained using the freeware add-in for Microsoft Excel GInaFiT (v. 1.6) (Geeraerd *et al.*, 2005).

Results

Experiment 1

The normal distribution of colony appearance times of the control and salt cultures are shown in Figure 1A-B, respectively. As regards the control culture, the results refer to a total of 156 processed colonies, for which an average appearance time of 23.29±2.25 hours was calculated within a range of 20.70 and 31.70 hours. The cut-off value calculated for the control culture was 30.02 hours based on which the number of potential persistent colonies was 2 (1.28%). As regards the salt cultures, the results refer to a total of 293 processed colonies, for which an average appearance time of 29.22±4.37 hours was calculated within a range of 21.03 and 54.03 hours. The cut-off value calculated for the salt culture was 42.34 hours based on which the number of potential persistent colonies was 5 (1.71%). The results of the statistical analysis showed a significant difference between the appearance times of the two cultures stressing that the lag phase of the salt culture was significantly longer than that of the control culture ($P<0.0001$, u=3579).

By comparing the colony appearance times of the salt culture with the cut-off value of the control culture (Figure 1C), it was established that the number of persistent colonies following osmotic stress was 92 (31.40%) whose times of appearance ranged between 30.37 and 54.03 hours with an average value of 33.69±4.85 hours.

Experiment 2

The thermal inactivation curves of both control and salt cultures were fitted using different linear and non-linear microbial survival models and the results are reported in Tables 1 and 2, respectively. Overall, the best correlations were obtained with the log-linear model with *shoulder* and *tail* (Geeraerd *et al.*, 2000, Eq. 1) for salt culture (Figure 2A) while the log-linear model with *shoulder* (Geeraerd *et al.*, 2000, Eq. 2) was the one that best represented the inactivation curve of the control culture (Figure 2B). In detail, the equations and the relevant parameters of these models are as follows:

$$N = (N_0 - N_{res}) * exp(-k_{max} * t) * (exp(k_{max} * Sl))/ (1 + (exp(k_{max} * Sl) - 1) * exp(-k_{max} * t))) + N_{res} \quad [Eq.\ 1]$$

$$N = N_0 * exp(-k_{max} * t) * (exp(k_{max} * Sl))/ (1 + (exp(k_{max} * Sl) - 1) * exp(-k_{max} * t))) \quad [Eq.\ 2]$$

where *N* is the concentration (Log CFU/ml) of *L. monocytogenes* at time t (hour), N_0 is the initial inoculum concentration, Sl is the duration of the shoulder effect, K_{max} is the speed of decrease (Log CFU/ml/hour) of the population per time unit and N_{res} is the residual cell concentration after the stabilisation at the end of the decrease.

As regards the control culture, no outputs were obtained by fitting the microbial loads either with the log-linear models with *tail* or with the biphasic models, which, therefore, were not suitable to represent the shape of the thermal inactivation curve observed. As regards the salt culture, on one
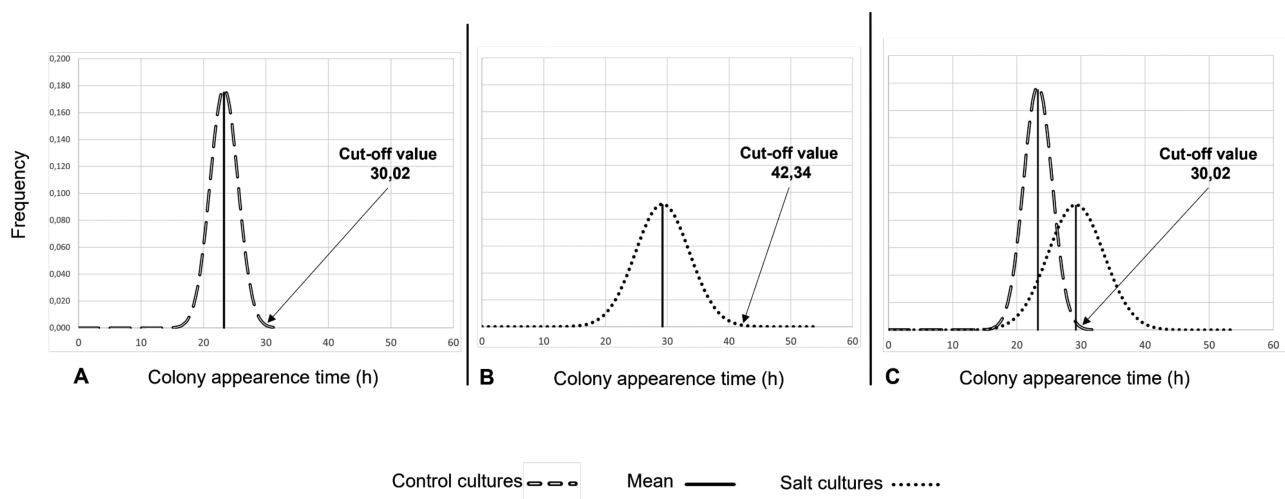
Figure 1. Lag time distribution measurements of the control (A) and salt (B) cultures determined by ScanLag. The effect of the osmotic stress on the formation of persistent bacteria was determined by comparing the lag time distribution of the salt culture with the cut-off value measured for the control cultures (C). The cut-off value was used to establish the persistence level and, according to Levin-Reisman and Balaban (2016), corresponds to the sum of the number of colonies whose appearance times were three standard deviations above the mean.

Table 1. Results of the inactivation parameters (expressed as value ± standard error) and statistical analysis obtained by fitting the *L. monocytogenes* counts of the salt culture after the thermal treatment with different linear and non-linear microbial survival models available on GInaFit (v. 1.6).

		Microbial survival model					
	Parameter	Log-Linear Regression				Biphasic	
		Simple	With shoulder	With tail	With shoulder and tail	Simple	With shoulder
Salt culture	$Log_{10}(N_0)$	5,66±0,63	No Fitting	7,17±0,38	6,61±0,49	7,02±0,46	6,57±0,54
	Shoulder lenght[1]	NA		NA	1,45±1,06	NA	1,45±1,21
	$K1_{max}$[2]	0,73±0,15		1,60±0,19	1,82±0,31	1,55±0,26	1,78±0,38
	$K2_{max}$[3]	NA		NA	NA	0,11±0,22	0,03±0,21
	$Log_{10}(N_{res})$	NA		0,65±0,39	0,67±0,36	NA	NA
	4D reduction[1]	±12,8		±5,80	±6,60	±6	±6.8
	RMSE	1,4319		0,6207	0,6018	0,7061	0,6533
	R^2	0,6928		0,9481	0,9566	0,9402	0,9552

[1]Time in hours; [2]$K1_{max}$: Speed of decrease per time unit of the major subpopulation more sensitive to the heat treatment expressed as CFU/mL/hour; [3]$K2_{max}$: Speed of decrease per time unit of the minor subpopulation more resistant to the heat treatment expressed as CFU/mL/hour; No Fitting, it is unlikely that the model reproduces the trend of the data examined; NA: not applicable.

Table 2. Results of the inactivation parameters (expressed as value ± standard error) and statistical analysis obtained by fitting the *L. monocytogenes* counts of the control culture after the thermal treatment with different linear and non-linear microbial survival models available on GInaFit (v. 1.6).

		Microbial survival model					
	Parameter	Log-Linear Regression				Biphasic	
		Simple	With shoulder	With tail	With shoulder and tail	Simple	With shoulder
Control culture	$Log_{10}(N_0)$	7,47±0,10	7,47±0,10	No Fitting	No Fitting	No Fitting	No Fitting
	Shoulder lenght[1]	NA	0,50±0,28				
	$K1_{max}$[2]	1,64±0,05	1,71±0,06				
	$K2_{max}$[3]	NA	NA				
	$Log_{10}(N_{res})$	NA	NA				
	4D reduction[1]	±5,68	±5,92				
	RMSE	0,1645	0,1441				
	R^2	0,9936	0,9959				

[1]Time in hours; [2]$K1_{max}$: Speed of decrease per time unit of the major subpopulation more sensitive to the heat treatment expressed as CFU/mL/hour; [3]$K2_{max}$: Speed of decrease per time unit of the minor subpopulation more resistant to the heat treatment expressed as CFU/mL/hour; No Fitting: It is unlikely that the model reproduces the trend of the data examined; NA: not applicable.
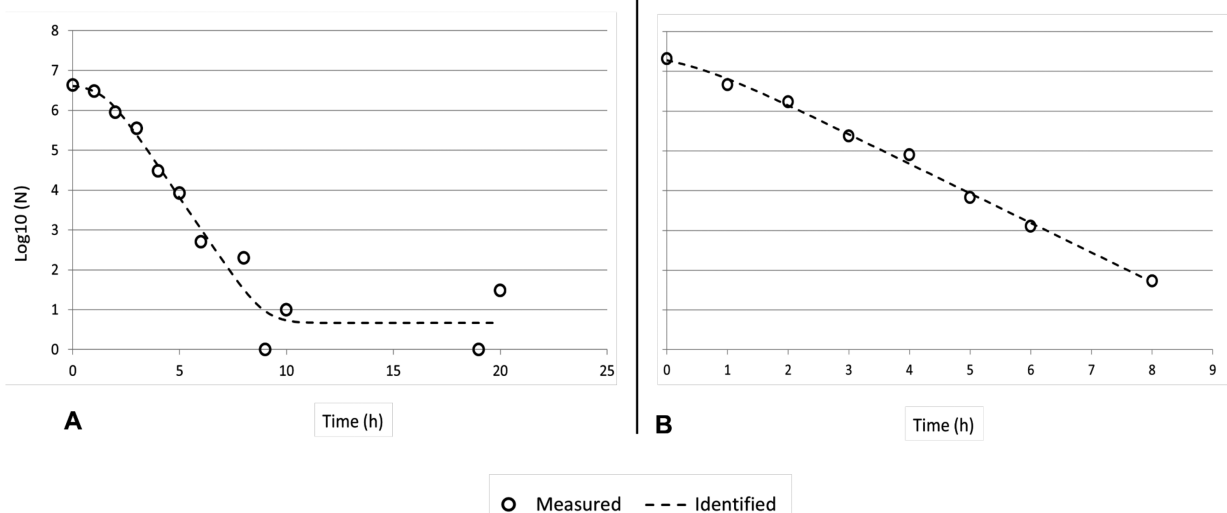

Figure 2. Thermal inactivation curves of the salt (A) and control (B) cultures. The graph shows the values of the loads measured during the heat treatment (51°C) and the trend line identified by fitting the load values of the salt cultures with a log-linear model with *shoulder* and *tail* and the load values of the control cultures with a log-linear model with *shoulder*. Fitting tests were performed on GInaFiT (v. 1.6). Both cultures were subjected to heat treatment for 24 hours. The graphs show the timepoints up to the moment in which microbial growth was observed.

hand, no outputs were obtained only with the log-linear model with shoulder and a low fitting was obtained with the simple log-linear model. On the other hand, the biphasic models, as well as the log-linear models with tail, reproduced the thermal death curve of the salt culture with good agreement.

Discussion

The results of the present study show how the exposure of a *L. monocytogenes* strain to a salt concentration that allows its growth could trigger the formation of persistent bacteria able to survive longer to mild heat treatment. The first observed hallmark of persistent bacteria was the extension of the lag phase that occurred once the stressor (NaCl) was removed and the culture resumed growth into a fresh medium. In the present study, the software ScanLag was used, to our knowledge for the first time, to assess the formation of persistent bacteria in a strain of *L. monocytogenes* following osmotic stress by evaluating the lag phase distribution at single-cell level. Overall, the distributions of the colony appearance times showed that the lag phase of the salt culture was ~6 hours longer than the control culture while the number of colonies present in the tails of the respective distributions was substantially the same. These results are similar to those obtained by Guiller *et al.* (2005) who observed an extension of 6,01 hours in the lag phase of a *L. monocytogenes* strain exposed for 25 h at room temperature to a concentration of 25% NaCl. As regards the persistence level, the lag phase of 92 colonies was long enough to assume the formation of persistent bacteria which, on average, appeared in the medium ~10 hours later than the colonies of the control culture. It is noteworthy that 3 of these persistent colonies started growing ~54 hours after plating which is well beyond the incubation time of 48 hours required by the ISO 11290-2:2017 for the enumeration of *L. monocytogenes* in foods. Since many factors influence the duration of the lag phase, it is difficult to explain the molecular mechanisms involved comprehensively. According to Robison *et al.* (1998), the duration of the lag phase depends on two main factors. The first factor is the amount of work required by the cells to recover a metabolic state that allows them to multiply. The second factor is the rate at which this process is carried out. Bacteria that experienced stress trigger several metabolic processes that involve the expression of specific genes and the activation of cellular structures to rebalance internal homeostasis and

prepare to grow again. The number and complexity of these processes affect the recovery times of the microorganisms and, therefore, the duration of the lag phase (Giarratana *et al.*, 2022). There is evidence that the adaptive responses following stress exposure not only affect the growth dynamics but would also induce cross-protection against different bactericidal treatments (Fang *et al.*, 2021). For example, Aryani *et al.* (2015) reported that cultures of *L. monocytogenes* grown in broth at pH 5 and 6 showed greater thermal resistance, while the growth at temperatures of 7°C and 15°C caused a significant decrease in the D_{60} value. When *L. monocytogenes* is exposed to salt concentration ≥6%, the overexpression of specific membrane pumps that regulate the ionic exchange between Na^+ and K^+ ensures adequate cellular turgor by modifying the internal osmotic pressure (Maria-Rosario *et al.*, 1995). Change in the osmotic pressure induces the cross-transcription of proteins, such as DnaK and GroE, typically expressed in response to sub-lethal thermal treatments which would explain the greater thermal resistance of bacteria exposed to salt (Kilstrup *et al.*, 1997). We could speculate that the salt acts as a stressor that induces the formation of persistent bacteria which have greater thermal resistance due to the cross-protection induced by the salt. The stress-induced responses may not be expressed by all cells in a population, and it is reasonable to expect some variability depending on their individual ability to stressors adaptation (Gefen and Balaban, 2009). This mechanism could underlie the increased thermal resistance of a microbial subpopulation in the salt culture which would explain its biphasic killing curve, the second hallmark of persistent bacteria observed in the present study. In this regard, the inactivation curves of the tested cultures were fitted with different predictive models of microbial survival. The best correlation was obtained with the *shoulder* and *tail* model as regards salt cultures and with the linear model with *shoulder* as regards control cultures. The biphasic trend of the killing curve of the salt culture is due to the presence of the *tail* which indicates the occurrence of a minor subpopulation more resistant to the heat treatment. The presence of a *shoulder* in the inactivation curves of both cultures would indicate an initial resistance of the microbial population to heat treatment during which the number of inactivated cells would be almost zero (Baranyi, 1996). The shoulder length in the salt cultures was greater than in the control culture, probably due to a greater general resistance

induced by the previous salt exposure. No significant difference was instead observed between the logarithmic inactivation rates of the two cultures as evident from the k_{max} values which were only slightly higher in the salt cultures. Similar results were obtained by Aryani et al. (2015) who observed that exposure to 2.5% and 5% of salt induces only a slight increase in the D_{60} value in several strains of *L. monocytogenes*. Other authors reported that the thermal resistance of *L. monocytogenes* exposed to salt depends on the physiological state of the cells at the time of treatment. Doyle *et al.* (2001) showed that *L. monocytogenes* cells in the stationary phase were the most resistant to thermal stress with a D_{56} value up to 8 times higher than those for cells in exponential growth. The stationary growth phase, in fact, is also a stressful condition due to the depletion of energy sources which may trigger an adaptive response in some cells capable of providing cross-protection to subsequent bactericidal treatment (Balaban *et al.*, 2019). In the present study, both cultures were in the stationary phase at the time of heat treatment, so the control cultures may also have developed higher thermal resistance which may explain why only a slight increase in thermal resistance was observed in salt cultures.

Conclusions

The results of the present study show that the *L. monocytogenes* ATCC 7644 strain grown in a salty broth was characterized by a longer lag phase and greater thermal resistance. The extension of the lag phase is of concern as the incubation time of 48 h required by the ISO 11290-2:2017 for the enumeration of *L. monocytogenes* in foods was not enough to detect the growth of some colonies that appeared in the medium ~54 h after plating. Additional concerns arise from the formation of heat-resistant subpopulations following sublethal stressors such as the salt concentration used in the present study. However, although the results obtained are supported by those of other Authors, the present study was conducted only on one *L. monocytogenes* reference strain and it cannot be excluded that different strains, both reference and wild, could show a different behaviour in response to stressors. Therefore, future studies should evaluate if other food preservation techniques may trigger the formation of persistent bacteria, considering, however, a greater number of strains to be tested, both reference and wild.

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