# Modelling the survival of *Listeria monocytogenes* strains in soft lactic cheese following acid and salt stress exposures

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Short title: Modelling *L. monocytogenes* survival

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## Significance and impact of the study

The ability to predict the growth and survival of *L. monocytogenes* in contaminated RTE foods is essential for listeriosis risk assessment. The results of this study provided valuable information on the kinetic parameters of survival of some *L. monocytogenes* strains found within the South African food environment. In addition to showing differences in the survival responses among strains, the study also showed the importance of the pre-contamination state of the cells in influencing survival kinetics.

## Abstract

This study evaluated the survival responses of *L. monocytogenes* strains (individually and mixed) in a soft lactic cheese following acid and salt stress exposures. The Weibull and log-linear with tail models were used to predict the survival responses of the pathogen in the cheese stored at 4°C for 15 d. Both models showed a good prediction accuracy for stressed *L. monocytogenes* cells ($Af \approx 1.00$) and higher prediction errors ($Af$ =1.12 -1.14) for non-stressed cells. The

inactivation rates ($\delta$ (d) and $k_{max}$ ($d^{-1}$)) were significantly lower ($p < 0.05$) for cells subjected to stress exposure indicating the enhanced tolerance to food stress. However, while enhanced tolerance appeared to be the main effect of stress pre-exposure, in one susceptible strain (69), stress exposure led to higher rates of inactivation. When introduced into the food as mixed strains, one strain out-lived others and remained as the sole survivor. Such strains that perhaps have a predilection for the food environment can provide more cautious estimates of kinetic parameters for predicting *L. monocytogenes* responses in foods especially if their stress-hardened tolerant cells are used.

**Keywords**: *Listeria monocytogenes*, modelling, survival, stress, soft lactic cheese.

## Introduction

*Listeria monocytogenes* is a foodborne pathogen that is the etiologic agent of the human disease listeriosis. Based on the Codex Allimentarius Commission (CAC) listeriosis risk profiling of ready to eat (RTE) foods, soft lactic cheeses fall in the category of low-risk ($pH \leq 4.4$) foods incapable of supporting *L. monocytogenes* growth (CAC, 2009). Hence most of the emphasis with respect to listeriosis risk in RTE foods is placed on the high-risk foods capable of supporting the growth of the pathogen. However, some RTE foods historically considered low-risk have recently been implicated in outbreaks as well. Notable examples include stone fruits in America as well as acid curd cheese in Germany (Fretz *et al*. 2010; Kase *et al*. 2017).

Accurate and reliable kinetic data obtained from models reflecting the behavior of the pathogen in foods are crucial in listeriosis risk assessment (Drosinos *et al*. 2006). However, pathogen

responses are influenced by exposure to environmental stresses in the food processing environment which determines the state of the cells at the point of product contamination (Giaouris *et al*. 2014). In addition, *L. monocytogenes* contamination of RTE foods can involve multiple strains as has previously been observed with contaminated cheese and cantaloupe (Laksanalamai *et al*. 2012; Rychli *et al*. 2014). The occurrence of multiple strains in broth co-cultures has been shown to affect the growth ability and detectability of other strains (Zilelidou *et al*. 2016), yet less is known about the survival of such co-contaminant strains in foods. The aim of the study was to model survival responses of *L. monocytogenes* strains individually and as mixed strains in a soft lactic cheese after exposure to acid and salt stress and to identify the surviving populations in cheese samples co-inoculated with mixed strains.

## Results and discussion

### Statistical evaluation of inactivation models

The LoF analysis of the four models is shown in Table 1. The Weibull model was accepted in 66.7% of the cases followed by the log-linear with tail model (58.3%). Consequently, the two models were used to obtain kinetic parameters. Differences were observed in the accuracy of prediction between stressed and non-stressed cells (Table 2). Both models showed good agreement between experimental data and model predictions (*Af* and *Bf* values close to 1.00) for stressed cells of strains ATCC19115, 159/10 and mixed strains while giving a slight over-prediction for non-stressed cells of the same strains. Previous studies have found the Weibull model to be satisfactory in describing the non-linear inactivation of *L. monocytogenes* in RTE foods (Angelidis *et al*. 2013; Mataragas *et al*. 2015). The log-linear with tail model has also been used to obtain kinetic parameters for some linear inactivation responses in which there is a

remaining resistant cell fraction (Mataragas *et al*. 2015). In the case of strain 69, prediction errors for both models were high (56 - 79%) once the organism was subjected to stress.

**Table 1:** Lack of fit (LoF) analysis of models used to describe the inactivation of *L. monocytogenes* in soft lactic cheese

| **Strain** | **Treatment** | **Model** | | | |
|---|---|---|---|---|---|
| | | **Biphasic** | **Weibull** | **Log-linear with tail** | **Weibull with tail** |
| ATCC19115 | NS† | Yes | Yes | Yes | Yes |
| | Acid | Yes | Yes | Yes | Yes |
| | Salt | Yes | Yes | Yes | Yes |
| 159/10 | NS | No | No | No | No |
| | Acid | Yes | Yes | Yes | No |
| | Salt | No | No | No | No |
| 69 | NS | No | Yes | Yes | No |
| | Acid | Yes | Yes | Yes | Yes |
| | Salt | No | No | No | No |
| Mixed strains | NS | No | Yes | No | No |
| | Acid | Yes | Yes | Yes | Yes |
| | Salt | No | No | No | No |
| | **Percentage** | **50.0** | **66.7** | **58.3** | **41.7** |

Yes - model passed the LoF test. No - model failed the LoF test. †NS – non-stressed

**Table 2:** Bias factor (*Bf*) and Accuracy factor (*Af*) analysis of the log-linear with tail and Weibull models

| **Strain** | **Treatment** | **Log-linear with tail** | | **Weibull** | |
|---|---|---|---|---|---|
| | | ***Bf*** | ***Af*** | ***Bf*** | ***Af*** |
| ATCC19115 | NS† | 1.01 | 1.14 | 0.98 | 1.20 |
| | Acid | 1.00 | 1.02 | 1.00 | 1.02 |
| | Salt | 1.00 | 1.04 | 1.00 | 1.03 |
| 159/10 | NS | 1.01 | 1.12 | 1.01 | 1.11 |
| | Acid | 1.00 | 1.01 | 1.00 | 1.01 |
| | Salt | 1.00 | 1.03 | 1.00 | 1.03 |
| 69 | NS | 1.01 | 1.08 | 1.00 | 1.08 |
| | Acid | 1.24 | 1.79 | 1.42 | 1.62 |
| | Salt | 1.15 | 1.73 | 1.42 | 1.56 |
| Mixed strains | NS | 1.01 | 1.12 | 1.01 | 1.13 |
| | Acid | 1.00 | 1.03 | 1.00 | 1.03 |
| | Salt | 1.00 | 1.02 | 1.00 | 1.02 |

†NS – non-stressed

**Inactivation curves and kinetic parameters**

A sharp decrease in the population of survivors (Figure 1) during the first few days of cheese storage was generally observed for non-stressed cells of both individual and mixed strains an indication that non-stressed cells comprise of both sensitive and resistant cell subpopulations. This upward concavity of the curves was a reflection of the quick inactivation of the sensitive subpopulation (Peleg 2003). For cells subjected to acid and salt stress prior to cheese inoculation, the lack of a sharp decline is an indication that stress exposures result in the development of adaptive stress tolerance responses and cell populations with reduced susceptibility to the food stress (NicAogáin, and O'Byrne, 2016).

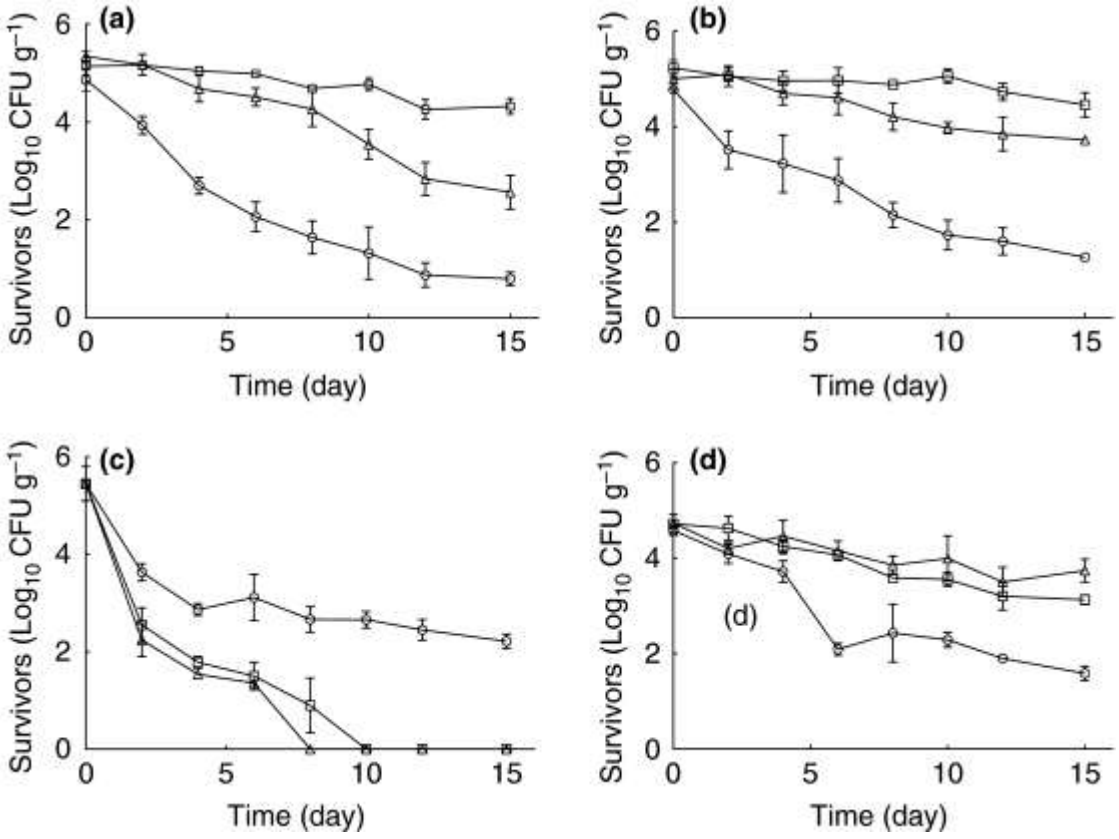


**Figure 1** Survival curves of non-stressed (○), acid-stressed (□) and salt-stressed (Δ) *L. monocytogenes* strains in lactic soft cheese stored at 4°C for 15 days. Strain: (a) – ATCC19115, (b) – 159/10, (c) – 69 and (d) – mixed strains. Results are expressed as means and standard deviations of three independent replicates.

Interestingly, while exposure to stress led to an enhanced survival for strains ATCC19115, 159/10 and mixed strains (Figure 1 (a), (b) and (d)), the opposite was observed for strain 69 (Figure 1 (c)). Such differences in stress susceptibility among *L. monocytogenes* strains have been reported previously (Komora *et al*. 2017) and are likely related to genetic differences, especially differences that have to do with evolutionary lineages (Horlbog *et al*. 2018). Although the genetic lineages of strains 159/10 and 69 which were recently isolated from the food processing environment are not known, evidence from many studies shows that some strains are inherently poorly adapted to the food environment (Horlbog *et al*. 2018; Lee *et al*. 2018). Another aspect that influences survival in foods is the extent of cell injury after stress exposure. A high level of stress-induced injury would lead to increased sensitization of the injured cell fraction to the subsequent food stress. Such stress-induced cell damage has previously been shown to result in sensitization of injured cells to subsequent acid and osmotic stresses (Barker and Park, 2001).

Table 3 shows the inactivation kinetic parameters obtained from the Weibull and log-linear with tail models. Overall, differences in strains ($p < 0.0001$) and stress exposure ($p < 0.05$) had significant effects on the kinetics of inactivation. With the exception of strain 69, the rate of cell reduction, was significantly lower ($p < 0.05$) for cells subjected to stress exposure an indication that in the majority of cases, pre-exposure to stress resulted in enhanced tolerance to the food stress. The ability of stressed cells to survive a second stress exposure has been seen to be influenced by the type of the first stress exposure (Skandamis *et al*. 2008; Tiganitas *et al*. 2009). *L. monocytogenes* cells surviving acid stress exposure develop an acid tolerance response (Davis *et al*. 1996) which protects such cells against subsequent lethal acid stress exposure thus

**Table 1:** Weibull and log-linear with tail model kinetic parameters of inactivation of non-stressed, and stressed strains of *L. monocytogenes* in soft lactic cheese

| Strain | Treatment | Model/Parameters | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Log-Linear with tail | | | | | Weibull | | | | |
| | | $N_o$ ($Log_{10}$ CFU $g^{-1}$) | $N_{res}$ ($Log_{10}$ CFU $g^{-1}$) | $k_{max}$ ($Log_{10}$ CFU$g^{-1}$ $d^{-1}$) | $R^2$ | RMSE | $N_o$ ($Log_{10}$ CFU $g^{-1}$) | $\delta$ (d) | $p$ | $R^2$ | RMSE |
| ATCC19115 | NS† | 4.22 (0.16) | 0.76 (0.17) | $0.77^{c}$ (0.07) | 0.95 | 0.28 | 4.22 (0.25) | $2.34^{a}$ (0.83) | 0.72 (0.12) | 0.92 | 0.36 |
| | Acid | 5.28 (0.07) | ND‡ | $0.16^{a}$ (0.05) | 0.87 | 0.13 | 5.16 (0.55) | $14.91^{c}$ (0.70) | 1.65 (0.29) | 0.92 | 0.10 |
| | Salt | 5.46 (0.10) | ND | $0.46^{b}$ (0.04) | 0.96 | 0.21 | 5.23 (0.09) | $6.84^{b}$ (0.54) | 1.38 (0.13) | 0.98 | 0.15 |
| 159/10 | NS | 3.95 (0.21) | 1.29 (0.32) | $0.53^{f}$ (0.09) | 0.84 | 0.39 | 4.14 (0.26) | $2.92^{d}$ (1.32) | 0.64 (0.15) | 0.85 | 0.38 |
| | Acid | 5.11 (0.05) | ND | $0.05^{d}$ (0.08) | 0.62 | 0.09 | 5.03 (0.02) | $19.81^{f}$ (1.68) | 3.46 (0.94) | 0.82 | 0.06 |
| | Salt | 5.17 (0.09) | 3.45 (0.30) | $0.29^{e}$ (0.05) | 0.91 | 0.16 | 5.14 (0.11) | $9.39^{e}$ (1.41) | 0.90 (0.18) | 0.89 | 0.17 |
| 69 | NS | 5.44 (0.24) | 2.65 (0.10) | $2.22^{g}$ (0.41) | 0.89 | 0.34 | 4.84 (0.25) | $0.23^{h}$ (0.33) | 0.27 (0.09) | 0.82 | 0.44 |
| | Acid | 4.60 (0.41) | 0.14 (0.26) | $1.45^{g}$ (0.27) | 0.85 | 0.69 | 5.45 (0.27) | $0.05^{g}$ (0.04) | 0.31 (0.04) | 0.96 | 0.38 |
| | Salt | 4.97 (0.39) | 0.23 (0.20) | $2.16^{g}$ (0.36) | 0.89 | 0.61 | 4.81 (0.36) | $0.03^{h}$ (0.02) | 0.27 (0.04) | 0.92 | 0.52 |
| Mixed Strains | NS | 4.41 (0.22) | 1.78 (0.19) | $0.71^{i}$ (0.13) | 0.86 | 0.38 | 4.30 (0.30) | $3.38^{i}$ (1.58) | 0.72 (0.20) | 0.81 | 0.44 |
| | Acid | 4.66 (0.05) | 2.91 (0.16) | $0.30^{h}$ (0.03) | 0.94 | 0.13 | 4.67 (0.07) | $8.53^{j}$ (0.80) | 0.83 (0.09) | 0.93 | 0.14 |
| | Salt | 4.64 (0.05) | 3.43 (0.14) | $0.25^{h}$ (0.04) | 0.87 | 0.13 | 4.65 (0.07) | $12.79^{k}$ (1.27) | 0.76 (0.12) | 0.87 | 0.14 |

Values for model parameters $N_o$, $N_{res}$, $k_{max}$, $\delta$ and $p$ are means with standard error in parenthesis of three replicate experiments. For each strain, $k_{max}$ and $\delta$ values with the same letters are not statistically different from each other ($p < 0.05$). NS† - non-stressed ND‡ - Not detected.

accounting for the reduced rate of inactivation for acid-stressed cells. Similarly, pre-exposure to a hyper-osmotic environment results in the development of osmotolerance which arises from the adaptive intracellular accumulation of compatible solutes (Duché *et al*. 2002). While both stress exposures resulted in the development of stress tolerance responses, salt-stressed cells had significantly higher ($p < 0.05$) rates of inactivation than their acid-stressed counterparts. Given that low pH is the major hurdle in soft lactic cheese, the acid tolerance response provides protection for such acid-adapted *L. monocytogenes* in acidic foods. In contrast, salt-adapted cells require an extra energy expenditure to counter the additional acid exposure in the soft cheese as this imposes a further homeostatic burden on the already stressed cells (Skandamis *et al*. 2008; Tiganitas *et al*. 2009).

**Identification of *L. monocytogenes* survivors from mixed strain challenge tests**

The majority of the surviving population from mixed strain challenge tests showed similar fingerprint patterns identical to parental strain 159/10 (Figure 2). The lack of strain 69 in the mixed strain challenge was probably related to its individual response which showed an inability to survive in the soft cheese. Although strain ATCC19115 showed high survival ability when inoculated in soft cheese alone, its absence in the cocktail challenge indicates a potential interaction among the strains. *L. monocytogenes* strains have been shown to exhibit competition in co-cultures (Zilelidou *et al*. 2016) resulting in reduction or total lack of growth for outcompeted strains. In addition, serotype and genetic lineage differences have been shown to influence stress survival and ecological niche preferences among *L. monocytogenes* strains (Horlbog *et al*. 2018). Serotype 4b (lineage I) strains like ATCC19115 have a predilection for the human host but lack the ability to compete well in the food environment where lineage II strains

dominate (Orsi *et al*. 2011). Over and above the genetic lineage differences influencing stress adaptation, recent evidence shows that *L. monocytogenes* stress resistance heterogeneity exists among sublineages and clonal complexes (Maury et al., 2019).

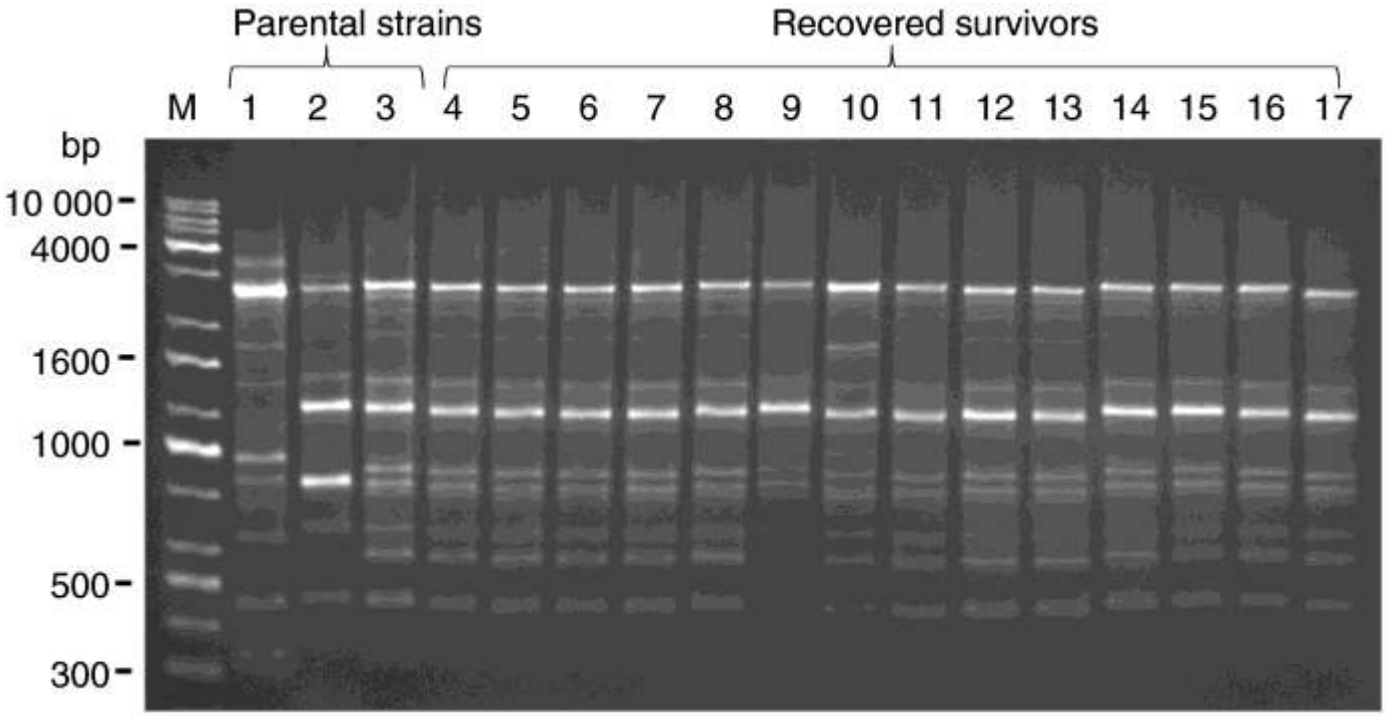


**Figure 2** Representative agarose gel image of $GTG_5$-REP-PCR fingerprint patterns of acid-stressed *L. monocytogenes* survivors and parental strains. M – Molecular weight marker. Lane 1- ATCC19115; Lane 2 - 69; Lane 3 - 159/10; Lanes 4 – 17 represent survivor isolates.

The results of this study indicate that the survival responses of *L. monocytogenes* in foods vary among strains and that this response is influenced by the state of the cells prior to food contamination. The ability to predict such responses requires kinetic data that takes into consideration the variations in strains and the development of stress tolerance responses that enhance survival of the pathogen. The implications in predictive microbiology are that strains that survive better and persist in contaminated foods (like 159/10) can provide more cautious

estimates of the pathogen's response in foods giving more representative kinetic parameters for risk assessment.

## Materials and methods

### Bacterial strains

The strains of *L. monocytogenes* used in this study included two isolates (69, and 159/10) obtained from the Department of Food Science and Biotechnology, University of Free State, Bloemfontein, South Africa (Strydom *et al*. 2013) as well as strain ATCC19115.

### Stress treatments

Fresh (< 18 h) cultures in BHI broth (Oxoid, Hampshire, UK) were centrifuged at 5000×*g* for 5 min. The cell pellets were washed twice with sterile phosphate buffered saline (PBS), pH7.3 (Oxoid) and re-suspended in acidified normal saline solution (pH 4.2) adjusted with 0.1 mol $l^{-1}$ lactic acid (Sigma-Aldrich, Steinheim, Germany) solution and sterile 10% NaCl (Merck, Darmstadt, Germany) solution (pH 7.0) for acid and salt stress treatments respectively. The suspensions were incubated at 25°C for 24 h. Fresh (< 18 h) cultures were used as non-stressed controls.

### Cheese preparation and inoculation

Commercial fat-free pasteurized milk was fermented with a mixture of *Lactococcus lactis* subspp. *lactis* and *Lactococcus lactis* subspp. *cremoris* (Cape Food Ingredients, South Africa) for 16 h at 25°C to pH of 4.6. After cutting, the coagulum was heated slowly to a temperature of 45°C over a period of 2 h and held at the same temperature for 10 min. After cooling to 25°C,

and draining, the curds were washed with cold and ice water left to drain the excess whey on a cheesecloth for 8 h. One hundred gram samples of the cheese (mean pH = 4.17; titratable acidity = 0.63%) were packaged in sterile 100 ml polypropylene tubs and inoculated with 100 µl of standardized bacterial suspensions to a final density of approximately $10^5$ CFU $g^{-1}$. Inoculated samples were stored at 4°C for 15 d.

**Enumeration of survivors**

Survivors (detection limit = 5 CFU $g^{-1}$) were was monitored by plating on PALCAM agar (Oxoid) at 0, 2, 4, 6, 8, 10, 12 and 15 d. Plates were incubated at 37°C for 48 h.

**Survival models**

The inactivation data ($Log_{10}$ CFU $g^{-1}$ survivors vs time) were fitted to four mathematical models (Equations 1 - 4) by non-linear regression using GInaFiT version 1.7 (Geeraerd *et al*. 2005).

Log-linear with tail model (Geeraerd *et al*. 2000).

$$Log_{10}N(t) = Log_{10}\left(\left(10^{Log_{10}(N_0)} - 10^{Log_{10}(N_{res})}\right) \times \exp(-K_{max} \times t) + 10^{Log_{10}(N_{res}}\right) \quad (1)$$

Weibull model (Mafart *et al*. 2002).

$$Log_{10}N(t) = Log_{10}N_0 - \left(\frac{t}{\delta}\right)^p \quad (2)$$

Weibull with tail model (Albert and Mafart, 2005).

$$Log_{10}\, N(t) = Log_{10}\left(\left(10^{Log_{10}(N_0)} - 10^{Log_{10}(N_{res})}\right) \times 10^{\left(\left(\frac{t}{\delta}\right)^{p}\right)} + 10^{Log_{10}(N_{res}}\right)$$

(3)

Biphasic model (Cerf, 1977).

$$\mathrm{Log}_{10}\, N(t) = \log_{10}(N_0) + \log_{10}(f \times exp(-k_{sens} \times t) + (1-f) \times \exp(-k_{res} \times t))$$

(4)

where $N_0$ and $N(t)$ are the initial and cell densities (CFU $g^{-1}$) at time $t$ (d) respectively, $k_{max}$ is the maximum inactivation rate ($d^{-1}$). $N_{res}$ is the resistant cell fraction (CFU $g^{-1}$), $\delta$ is the time to the first-decimal reduction (d) and $p$ is a shape parameter. $f$ is the fraction of sensitive subpopulation, ($1-f$) is the fraction of the resistant subpopulation, $k_{sens}$ and $k_{res}$ ($d^{-1}$) are the inactivation rates of the sensitive and resistant subpopulations respectively.

**Statistical evaluation of models**

Survival models were analyzed for their adequacy in describing the experimental data using the lack of fit (LoF) statistic (Zwietering *et al*. 1990), the root mean square error (RMSE) (Equation 6), accuracy factor (*Af*) (Equation 7), bias factor (*Bf*) (Equation 8) (Drosinos *et al*. 2006; Ross 1996).

$$RMSE = \sqrt{\frac{\sum(Observed - Predicted)^2}{n}} \quad (6)$$

$$Accuracy\ Factor = 10^{\left(\sum Log\left|\frac{Predicted}{Observed}\right|/n\right)} \quad (7)$$

$$Bias\ Factor = 10^{\left(\sum Log\left(\frac{Predicted}{Observed}\right)/n\right)} \quad (8)$$

where *n* is the number of data points. *Observed* and *Predicted* values represent the experimental data and the values predicted by the model at each respective data point.

**Identification of *L. monocytogenes* survivors from mixed strain challenge tests**

A total of 80 colonies representing both acid and salt stress-treated survivors from mixed strain challenge tests after 15 d were subjected to repetitive PCR (REP-PCR) fingerprinting to determine their similarity to any of the three mixed strains. Genomic DNA was extracted from cultures of survivor isolates grown in BHI broth using the ZR Fungal/Bacterial DNA MiniPrep$^{TM}$ kit (Zymo Research, Irvine, USA). The PCR reaction (20 µl) consisted of a 2 × Mastermix (Kapa Biosystems, Massachusetts, USA) containing 0.4 U Taq DNA polymerase; 0.2 mmol $l^{-1}$ deoxyribonucleotide phosphate; 1.5 mmol $l^{-1}$ $MgCl_2$; 35 pmol of primer ($GTG_5$; 5´-GTGGTGGTGGTGGTG-3´) (WhiteSci, Cape Town, South Africa); and 5 ng template DNA. The reaction conditions comprised of; initial denaturation at 95ºC for 7 min; 34 cycles of denaturation at 92°C for 30 s, annealing at 38°C for 1 min and extension at 65°C for 8 min; final extension at 65°C for 16 min. PCR products were separated by electrophoresis in 1.5% agarose gel and band patterns of each survivor were compared to the three strains by visual analysis.

**Data analysis**

Analysis of variance (ANOVA) with the Tukey's HSD test for multiple comparisons was used to compare the inactivation kinetic parameters among the strains and the stress treatments. The

analysis was performed using GraphPad Prism (GraphPad Software, Inc., USA). All experiments were done in duplicates and each experiment was repeated three times.

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**Conflict of interest**

The authors have no conflict of interest to declare.

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