

Resuscitation and growth kinetics of sub-lethally injured *Listeria monocytogenes* strains following fluorescence activated cell sorting (FACS)

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Highlights

- Flow cytometry and cell sorting were used to study cell injury in *L. monocytogenes*.
- The degree of injury was influenced by stress treatments and strain variations.
- Stress injured cells were capable of repair and growth after cell sorting.
- Resuscitation kinetics were influenced by temperature and strain variations.
- Recovery ability of stress injured cells has implications in food safety.

Abstract

This study investigated the effect of acid (pH 4.2), osmotic (10% NaCl) and heat (55 °C for 30 min) stress induced injury on *Listeria monocytogenes* strains ATCC19115, 69, 159/10 and 243 using differential plating and flow cytometry coupled with membrane integrity indicators, thiazole orange (TO) and propidium iodide (PI) staining. Growth kinetics of injured cells sorted by fluorescence activated cell sorting (FACS) were studied at 4, 25 and 37 °C. The percentage of cell injury detectable by both flow cytometry and differential plating varied significantly among strains and stress treatments ($p < 0.0001$). Based on flow cytometry and TO/PI staining, acid stress caused the highest level of injury followed by heat and osmotic stress. Following cell sorting, acid and osmotic stress injured cells were capable of resuscitation and re-growth while heat injured cells (except for strain 69) were incapable of re-

growth despite having a high level of membrane intact cells. The lag phase duration (λ) of sorted stress injured cells resuscitated in brain heart infusion (BHI) broth was significantly influenced by strain variations ($p < 0.0001$), stress treatments ($p = 0.007$) and temperature of resuscitation ($p \leq 0.001$). Following repair, the maximum specific growth rate (μ_{\max}) of resuscitated cells was not different from untreated control cells regardless of strain differences and stress treatments. Only temperature had a significant effect ($p < 0.0001$) on growth rate. Sorted cells were also capable of growth at 4 °C, with the time to detectable growth ($\geq 1.40 \text{ Log}_{10} \text{ CFU ml}^{-1}$) ranging from 3 to 15 days. Overall, re-growth potential of sorted cells showed that while membrane integrity was a good indicator of cell injury and viability loss for acid and osmotic stress, it was not a sufficient indicator of heat stress injury. Once injured cells repair the cellular damage, their growth rate is not different from non-injured cells regardless of form of stress and strain differences. Thus highlighting the potential food safety risks of stress injured *L. monocytogenes* cells.

Keywords

Listeria monocytogenes, Stress, Injury, Flow Cytometry, Cell Sorting, Resuscitation.

1. Introduction

Listeria monocytogenes is a Gram-positive anaerobic facultative non-spore forming rod that is widely distributed in nature. The bacterium is the causative agent of the human disease listeriosis, an infection commonly associated with the consumption of contaminated minimally processed and processed ready to eat (RTE) foods (Aureli et al., 2000; Olsen et al., 2005). Owing to its ubiquity, the organism is easily associated with raw foods of both plant and animal origin (Jamali, Radmehr, & Thong, 2013; Wang et al., 2013). From raw food materials, *L. monocytogenes* readily colonizes and establishes persistence in food processing equipment, and associated environment (Muhterem-Uyar et al., 2015; Pagadala et al., 2012) forming a

major reservoir for the subsequent contamination of finished products (Bolocan et al., 2015; Strydom, Bester, Cameron, Franz, & Witthuhn, 2013).

The persistence of *L. monocytogenes* in food processing environments and its subsequent ability to overcome preservation hurdles associated with RTE foods is key to its pathogenesis (Jensen, Williams, Irvin, Gram, & Smith, 2008; Kastbjerg, Larsen, Gram, & Ingmer, 2010; Silva et al., 2015). Cell populations surviving in such environments are chronically exposed to sub-lethal physical and chemical stresses such as reduced a_w , pH, and temperature shifts as well as nutrient stress (Dalzini et al., 2015; Giaouris, Chorianopoulos, & Nychas, 2014; Sadeghi-Mehr, Lautenschlaeger, & Drusch, 2016). Due to individual cell heterogeneity, response to such chronic sub-lethal stress exposure varies among sensitive and resistant cell subpopulations resulting in a mixture of bacterial cells in various physiological states (Casadesús & Low, 2013; Ryall, Eydallin, & Ferenci, 2012). Stress sensitive cell populations exhibit a partial and reversible damage to the structural and functional cellular components and hence exist a sub-lethally injured state (Wesche, Gurtler, Marks, & Ryser, 2009; Wu, 2008).

The occurrence of sub-lethally injured cells of pathogenic bacteria has huge implications in food safety since such cells require a period of repair in non-selective media before the pathogen can be detected by conventional selective techniques (Wu, 2008). Detection therefore is influenced by the extent of injury and rate of repair. Following a sub-lethal stress exposure, the occurrence of injured cells in mixed populations with live undamaged cells makes it difficult to study their resuscitation and growth behavior as their response is often masked by the latter. This problem is made worse by the occurrence of such cells in low numbers and often in complex microbial communities (Waage, Vardund, Lund, & Kapperud, 1999).

Flow cytometry and Fluorescence Activated Cell Sorting (FACS) allows for the quantitative measurement of optical characteristics of individual cells passing through a

focused light beam (Veal, Deere, Ferrari, Piper, & Attfield, 2000). When coupled with fluorescent labeling, the technique allows for the differentiation of cell subpopulations in various physiological states (Kennedy, Cronin, & Wilkinson, 2011). As an indicator of cell viability, injury or death, bacterial cells are often subjected to a double staining protocol using nucleic acid binding dyes excitable with laser but with different emission spectra (Joux & Lebaron, 2000; Veal et al., 2000). A combination of membrane permeant dyes such as arcidine orange, thiazole orange, ethidium bromide, 4',6-diamidino-2-phenylindole (DAPI) and SYBR green (Martin, Leonhardt, & Cardoso, 2005; Perfetto et al., 2006; Shapiro, 2001) with impermanent dyes such as propidium iodide, TO-PRO-3, SYTOX green (Mortimer, Mason, & Gant, 2000; Novo, Perlmutter, Hunt, & Shapiro, 2000) allows for the differentiation of bacterial cell physiological states on the basis of membrane integrity (da Silva, Piekova, Mileu, & Roseiro, 2009; Díaz, Herrero, García, & Quirós, 2010). Because of their compromised membranes, injured cells are distinguished by their ability to take up both stains and therefore show strong fluorescence of both permeant and impermeant dyes. The addition of FACS allows the differently stained cells to be recovered as separate subpopulations. This is a microfluidics based cell separation technique used to separate cells with rare phenotypes from complex ecological communities and heterogeneous populations (Bhagat et al., 2010; Gossett et al., 2010) allowing for subsequent study of such unique features in isolation.

While the stress response of *L. monocytogenes* has been extensively studied, models for the behavior of stressed cells in food systems are often based on heterogeneous mixtures of live and injured cells with the response taken to represent the average for the whole population. Given the heterogeneity of such populations, the response of sub-lethally injured cells is often underestimated thus impacting on the safety of RTE foods where *L. monocytogenes* has a potential of resuscitation and growth. In this study, the effect of sub-lethal acid, heat and NaCl stress response of food-borne *L. monocytogenes* strains was investigated using flow cytometry. Sub-lethally injured subpopulations based on a double

fluorescent staining were recovered by FACS and their resuscitation and growth kinetics in broth were studied under different temperatures.

2. Materials and methods

2.1 Bacterial strains

The strains of *L. monocytogenes* used in this study included three isolates (69, 159/10 and 243) obtained from the Department of Food Science and Biotechnology, University of Free State, Bloemfontein, South Africa, having been isolated from an avocado processing plant (Strydom et al., 2013) as well as strain ATCC19115. The strains were positively confirmed as *L. monocytogenes* by matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry (Bruker, Germany) with Spectral Archiving and Microbial Identification System (SARAMIS™) (Anagnostec, Potsdam, Germany) database. Stock cultures of the strains were maintained in cryovials of 25% glycerol at -80°C. The cultures were activated by inoculating 100 µl of stock culture into 10 ml of brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) and incubation at 37°C for 24 hours.

2.2 Stress treatments and induction of injury

Cultures of test organisms were subjected to acid, salt and heat stress in order to induce cell injury. Working cultures were prepared by transfer of 100 µl of activated culture into 10 ml of BHI broth and incubation at 37°C for 18 hours which was repeated twice before exposure to stress. For stress exposure, cultures were centrifuged at 5000 g for 10 minutes in 2 ml microcentrifuge tubes (Eppendorf, Hauppauge, USA). Harvested cells were washed twice with sterile phosphate buffered saline (PBS, pH 7.3) (Oxoid) and re-suspended at a cell density of approximately 10^8 CFUml⁻¹ (McFarland 0.5) in the following solutions in 10 ml volumes; (i) sterile acidified saline solution (pH 4.2) adjusted with 0.1 M lactic acid (Sigma-Aldrich, Steinheim, Germany) for acid stress; (ii) sterile 10% NaCl (Merck, Darmstadt,

Germany) solution (pH 7.0) for osmotic stress; (iii) sterile PBS pre-warmed to 55°C for heat stress. Treatment conditions were chosen to represent the potential sub-lethal stresses encountered by *L. monocytogenes* in foods and food processing environments. Acid and osmotic stress cell suspensions were incubated in a static incubator (Labcon, Mogale City, South Africa) at 25°C for 24 hours while heat stress suspensions were immediately heated to 55°C in a water bath (Labotec, Midrand, South Africa) for 30 minutes. Untreated overnight cultures of each test organism were used as positive controls.

2.3 Determination of sub-lethal injury by plate counts

The percentage injury after each stress treatment was determined by comparison of growth of treated cells on selective and non-selective agar. Each sample of stressed cells was serially diluted in sterile 0.1% peptone water and plated on BHI agar (Oxoid) as non-selective media and PALCAM agar (Oxoid) as selective media. As positive controls, untreated overnight cultures of each test organism were similarly plated on both selective and non-selective media. Plates were incubated at 37°C for 48 hours and viable cell counts were used to calculate percentage injury using the following equation (Busch & Donnelly, 1992).

$$\% \text{ Injury} = \left[1 - \left(\frac{\text{Counts on Selective Agar}}{\text{Counts on Non-selective Agar}} \right) \right] \times 100 \quad (1)$$

2.4 Preparation of cell suspensions and staining

After each stress treatment, cell suspensions were centrifuged at 5000 g for 10 minutes and washed twice with sterile PBS. The cell pellets were re-suspended in PBS and diluted to a cell density of 10^6 to 10^7 cells/ml. A 0.5 ml of diluted cell suspension in sterile flow cytometry tubes was then stained with 5 μ l of 0.42 μ M thiazole orange (TO) (BD Biosciences, San Jose, USA) solution in dimethyl sulfoxide (DMSO) and 5 μ l of 4.3 mM propidium iodide (PI) (BD Biosciences) solution in water. Stained cells were incubated at room temperature in the dark

for 10 min. Positive controls consisted of untreated overnight cultures of each test organism. The cultures were centrifuged at 5000 g for 10 minutes, washed twice with sterile PBS and stained with TO/PI. As negative controls, cells from fresh overnight cultures were suspended in 70% ethanol for 30 minutes at room temperature. The ethanol killed cells were re-centrifuged at 5000 g for 10 minutes, washed twice and re-suspended in sterile PBS followed by staining with TO/PI.

2.5 Flow cytometry analysis

Flow cytometry analysis was performed using a flow cytometer (BD FACSAria™, BD Biosciences) equipped with a 20 mW argon laser emitting at 488 nm. For each cell crossing the focus point of the laser, two light-scattering signals, forward scatter (FSC) and side scatter (SSC) and two fluorescence signals for red and green fluorescence were recorded. The fluorescence of thiazole orange was collected in the FL1 photomultiplier with a band pass filter of 525 nm. The fluorescence of propidium iodide was recorded in the FL3 photomultiplier with a short pass filter of 620 nm. For each sample, 10,000 events were measured at a flow rate of approximately 800 events per second (eps). The recorded light-scattering and fluorescence signals were collected as logarithmic signals and the obtained data was analyzed using a BD FACSDiva v8.0.1 software. Data was presented as density dot plots of Red vs Green Fluorescence. Gates representing, live intact (TO-positive), and dead (PI-positive) cells were drawn using control samples consisting of live and dead ethanol treated TO/PI stained cells. The injured cell subpopulation was defined as cells with a combined high fluorescence of both stains (TO-positive, PI-positive) (Arku, Fanning, & Jordan, 2011). To avoid overlap in the emission spectra of the two fluorochromes, controls consisting of live unstained, live TO stained and dead ethanol treated PI stained cells were used for fluorescence compensation. In order to exclude cell doublets and clumps, a plot of FSC-H vs FSC-A was done for every analysis (Wersto et al., 2001). Cells that were not along the diagonal cluster of

the FSC-H vs FSC-A plot were gated out and excluded in the analysis. The percentage of cells for each subpopulation was determined as the number of events in each gate as a proportion of the total number of events.

2.6 Sorting of subpopulations

The cell sorting process was done using a BD FACSAria™ cell sorter (BD Biosciences), following the procedure described by Le Roux et al. (2015) with conditions and parameters as previously described by Kennedy et al. (2011). Before sorting, the cell sorter was calibrated using the standard protocols of BD BioSciences. The stream was calibrated using the Accudrop delay function. This was followed by checking the sort accuracy and purity using the purity function. Accuracy and purity were tested by using internally labeled fluorescent beads (cytometer setup and tracking (CST) beads, BD BioSciences) excitable with 488 nm laser. After sorting approximately 40,000 beads for each subpopulation, the sorted beads were re-analyzed to determine the sort efficiency which was confirmed to be > 99% at all times. Sorting of membrane damaged cells was based on drawing defined gates of the three main subpopulations (Intact, TO-positive/PI-negative; membrane damaged, TO-positive/PI-positive; dead, TO-negative/PI-positive). The population of interest was defined as cells with fluorescence signal of both TO and PI. Due to partial cell membrane damage, such cells exhibit an incomplete fluorescence resonance energy transfer (FRET) between thiazole orange and propidium iodide resulting in the cells emitting high fluorescence of both red and green fluorescence in both FL1 and in FL3 channels (Tamburini et al., 2013). Cells were sorted using a two way sort into separate sterile 5 ml flow cytometry tubes with filter-sterilized PBS as sheath fluid, a 70 µm nozzle, a drop delay of 52 - 56 and a sort rate of 500 eps. Sorting was stopped after achieving a target of 10,000 events for the membrane damaged cell subpopulation. A sort of untreated intact cells was used as positive control.

2.7 Resuscitation of injured cells and growth kinetics

After sorting 10,000 events (10^4 cells) in about 200 – 500 μl of sterile sheath fluid, the volume was adjusted to 1 ml using sterile PBS (pH 7.3) to give a suspension with a cell density of 10^4 cells/ml. An aliquot of 100 μl was used to inoculate 10 ml of BHI broth which was subsequently incubated at 37°C, 25°C and 4°C. Resuscitation and growth of injured cells was monitored by viable counts at intervals of 1, 2, 4, 8, 12, 18, 24, 30 hours for tubes incubated at 37°C and 25°C. Recovery at 4°C was monitored at intervals of 3, 5, 10 and 15 days. At each sampling time, dilutions were made in maximum recovery diluent (0.1% peptone (Biolab, South Africa) and 0.85% NaCl (Merck) in distilled water), plated on BHI agar and incubated at 37°C for 48 hours. The Log_{10} transformed growth data were fitted to the growth model of Baranyi and Roberts (1994) using DMFit version 3.5 Excel add-in software to determine specific growth rate (μ_{max}) and lag phase duration (λ). The limit of detection was $1.40 \text{ Log}_{10} \text{ CFUml}^{-1}$. Positive controls of live intact

2.8 Recovery of sorted cells by plate counts

In order to check the re-growth ability of sorted cells, 100 μl of the sorted cell suspension was diluted with 900 μl of sterile maximum recovery diluent and inoculated onto BHI agar immediately after sorting. Inoculated plates were incubated at 37°C for 48 hours.

2.9 Data analysis

All experiments were repeated three times. Analysis of variance (ANOVA) ($\alpha = 0.05$) was used to determine the effect of strain, stress treatment, and temperature factors on percentage injury, lag phase duration and growth rates of *L. monocytogenes*. For multiple comparisons, ANOVA with Tukey's HSD test for correction in GraphPad Prism 6.0 was used to test for any significant differences between strains and stress treatments. A significance level of 0.05 with adjusted *P* values was used in each case. A heat map exploring the relationship between

percentage injury, lag phase duration, and growth rate was constructed using Xlstat 2016 statistical package version (Addinsoft, New York, USA) after normalization of the data.

3 Results

3.1 Stress induced injury by differential plating

The percentage of *L. monocytogenes* injury after exposure to osmotic, acid and heat stress based on differential plating on BHI agar and PALCAM agar is shown in Fig 1. Stress treatments had a significant effect ($p < 0.0001$) on percentage injury of *L. monocytogenes* strains (Table 1). The degree of injury to acid, acid and heat stress ranged from 21.29 - 100%, 50.76 - 100% and 44.05 - 100% respectively (Fig. 1). With the exception of strain 69, which showed a consistent lack of ability to grow on PALCAM agar after exposure to all forms of stress and therefore had an injury percentage of 100%, significant differences ($p < 0.0001$) in the degree of injury were observed among *L. monocytogenes* strains in response to the three forms of stress for strains ATCC19115, 159/10 and 243 (Table 1).

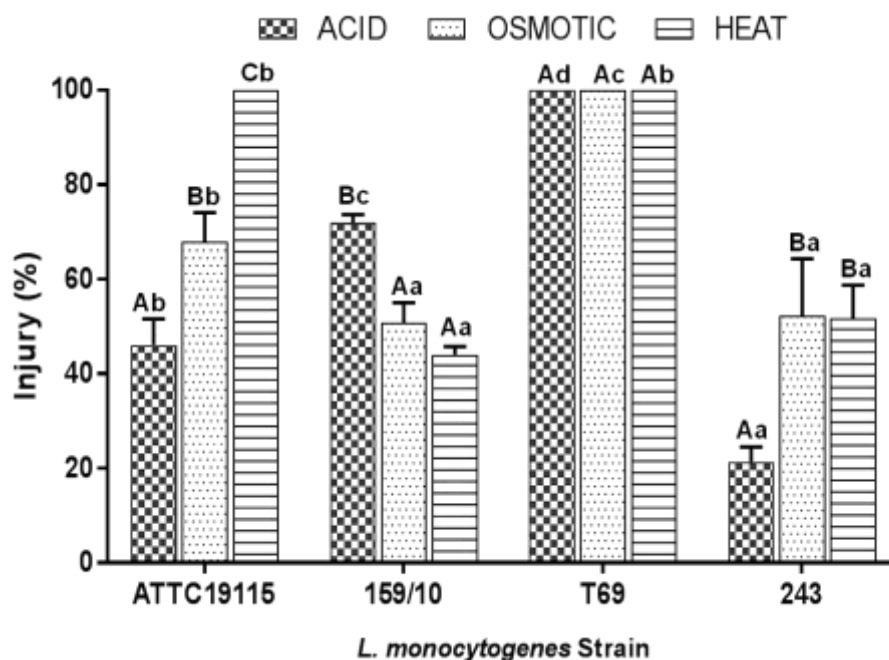


Fig. 1. Percentage of sub-lethal injury of *L. monocytogenes* after osmotic, acid and heat stress exposure based on differential plating. Bars with different uppercase letters for each strain indicate significant differences ($p \leq 0.05$). For each stress treatment, bars with different lowercase letters indicate significant differences ($p \leq 0.05$) (Tukey's HSD).

Table 1: Analysis of variance showing the effect of strain and stress factors on percentage injury of *L. monocytogenes* based on differential plating and flow cytometry

Factor	DF	P value	
		Differential Plating	Flow Cytometry
Strain (ATCC19115, 159/10, 69, 243)	3	< 0.0001	< 0.0001
Stress (Acid, Osmotic, Heat)	2	< 0.0001	< 0.0001
Strain*Stress	6	< 0.0001	< 0.0001

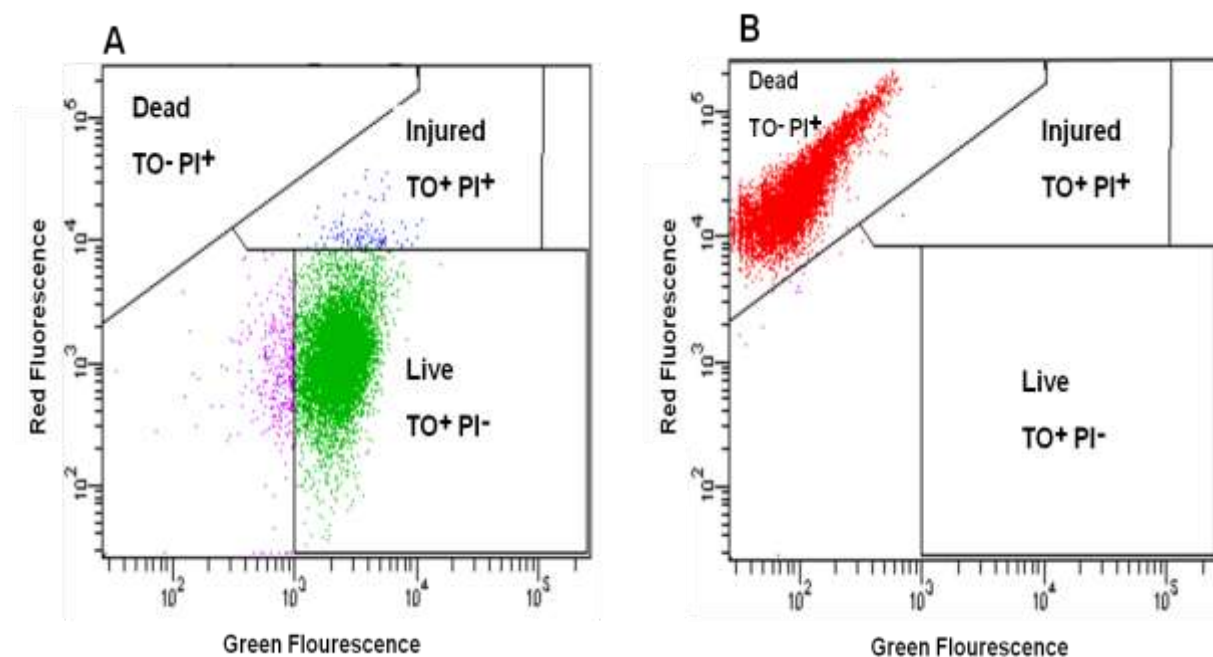


Fig. 2. Representative fluorescence density dot plots of live *L. monocytogenes* cells stained with TO/PI (A) and ethanol treated cells stained with TO/PI (B) showing gates of live, injured and dead cells.

3.2 Stress induced injury by flow cytometry and differential staining

Fig. 2 shows representative fluorescence density dot plots indicating the gates for the three subpopulations of live intact, injured and dead *L. monocytogenes* cells. Responses to each stress treatment are shown as fluorescence density dot plots in Fig. 3 (A – C). In general, exposure to the three forms of stress increased the intensity of the red fluorescence resulting from PI. The proportion of injured cells showing the fluorescence signal of both stains was highest for acid treated cells (Fig. 3A), followed by heat (Fig. 3C) and osmotic stress (Fig. 3B). The summary of the percentage proportion of injured cells (TO positive, PI positive) and intact cells (TO positive) for each of the stress treatments is shown in Fig. 4. As observed with

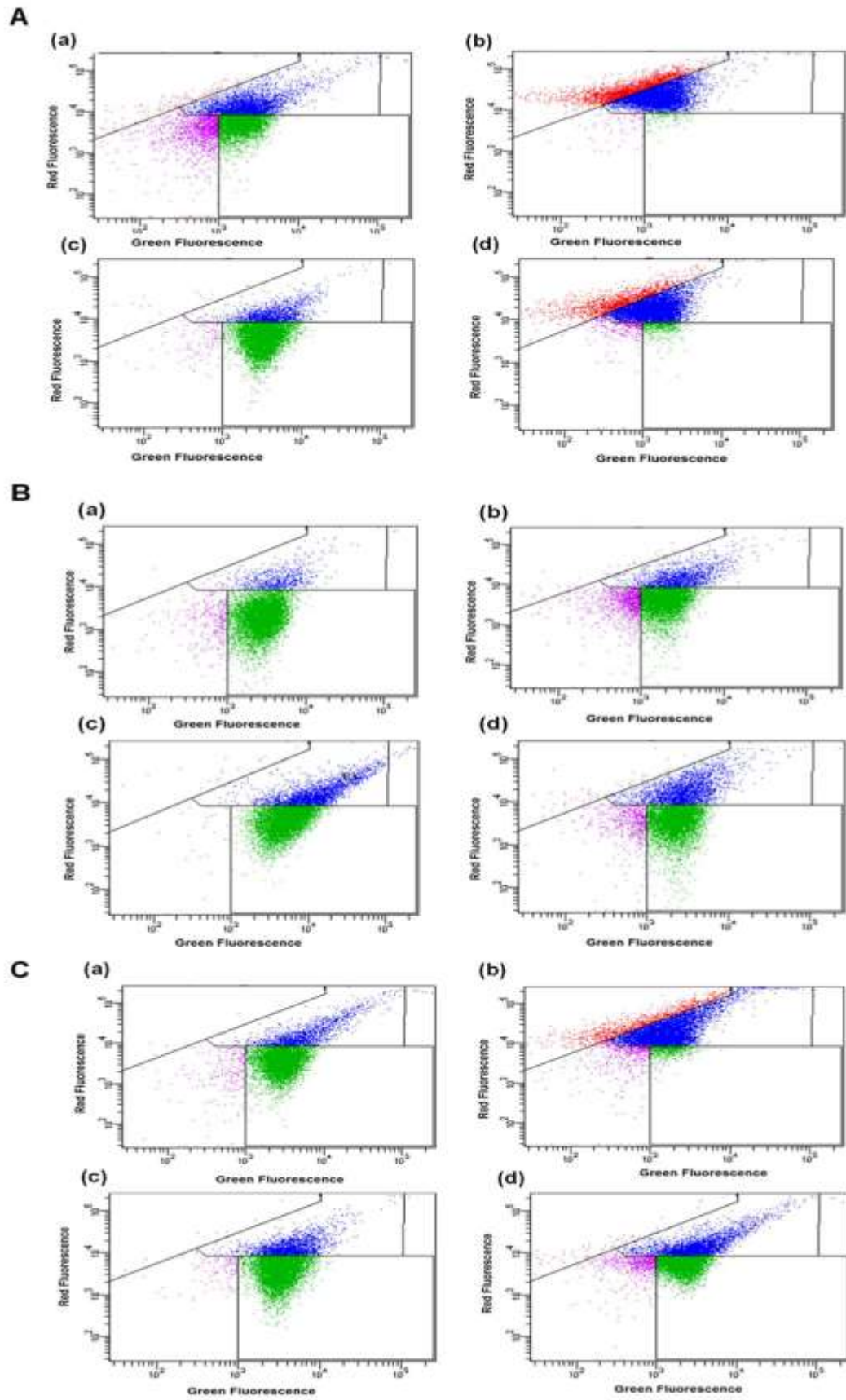


Fig. 3. Fluorescence density dot plots of acid (A), osmotic (B) and heat (C) stress treated *L. monocytogenes* cells stained with TO/PI. (a) - 159/10, (b) - ATCC19115, (c) - 69, (d) - 243.

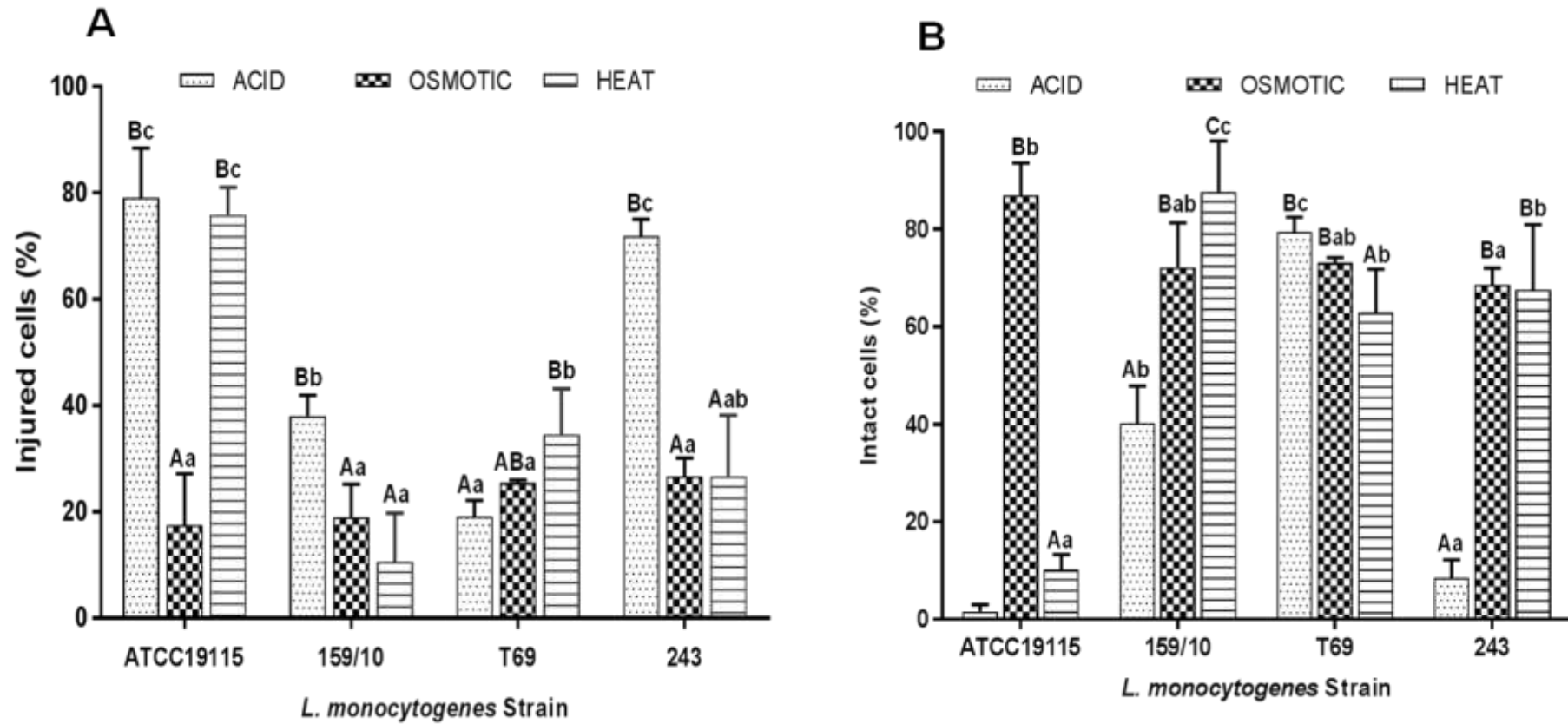


Fig. 4. Percentage of sub-lethally injured (**A**) and intact (**B**) *L. monocytogenes* cells after exposure to osmotic, acid and heat stress based flow cytometry and TO/PI differential staining. For each strain, bars with different uppercase letters indicate significant differences ($p \leq 0.05$). For each stress treatment, bars with different lowercase letters indicate significant differences ($p \leq 0.05$) (Tukey's HSD).

differential plating, the degree of cell injury by flow cytometry was significantly influenced ($p < 0.0001$) by stress treatment and strain of *L. monocytogenes* (Table 1). Osmotic stress resulted in the lowest degree of cell injury for all strains (17.5 – 26.6%) (Fig. 4) with most of the surviving cells in the intact cell gate (Fig. 3B). Strains ATCC19115 and 243 displayed a high susceptibility to acid stress with 79.1% and 71.6% injury respectively (Fig. 4) which included some of the cells in the dead cell gate (Fig. 3A).

Contrary to the high degree of injury observed for strain 69 with differential plating, flow cytometry showed that the same strain had consistently the highest proportion of membrane intact cells and low percentage injury (Fig.3 and Fig. 4).

3.3 Re-growth potential of sorted cells on BHI agar

After cell sorting, the growth potential of injured cells was assessed by their ability to form colonies on BHI agar (Table 2). Sorted acid and NaCl treated cells were all capable of re-growth although the proportion of cells capable of re-growth as a percentage of sorted cells was significantly lower ($p \leq 0.05$) for strains ATCC19115 and 243. Among heat injured, only strain 69 cells were capable of re-growth.

3.4 Lag phase duration (λ) of resuscitating injured cells

The lag phase duration for resuscitating injured cells was significantly influenced by variations among *L. monocytogenes* strains ($p < 0.0001$), from stress treatment ($p \leq 0.007$), and resuscitation temperature ($p \leq 0.001$) (Table 5). At 25°C, λ values were 4.51 – 9.14 and 3.26 – 9.22 days for acid and osmotic stress injured cells respectively (Table 3). When resuscitated at 37°C, the same cells had λ values of 1.96 – 7.15 and 0.50 – 7.14 days respectively. When individual strains were compared, *L. monocytogenes* strain 69 had the shortest lag phase duration ($p < 0.05$) at 37°C for its acid and osmotic stressed cells (Table 4) while strains with a high susceptibility to stress (ATCC19115 and 243) had longer periods of

Table 2. Re-growth potential of FACS sorted injured *L. monocytogenes* cell subpopulations re-grown on BHI agar at 37°C for 48 hours (sorted cells = 10,000 cells per ml)

<i>L. monocytogenes</i> Strain	Acid		Osmotic		Heat	
	Log ₁₀ cells/ml	% Re-growth	Log ₁₀ cells/ml	">% Re-growth	Log ₁₀ cells/ml	">% Re-growth
ATTC19115	2.81 ^a ±0.09	6.6±1.4	3.04 ^a ±0.06	10.9±1.5	ng	ng
159/10	3.98 ^c ±0.03	96.29±5.3	3.86 ^c ±0.08	70.0±11.3	ng	ng
69	3.96 ^c ±0.34	92.9±11.3	3.91 ^c ±0.03	81.5±4.9	3.46±0.02	29.0±1.4
243	3.58 ^b ±0.05	44.0±15.2	3.46 ^b ±0.08	29.2±5.3	ng	ng

Values are means ± standard deviations of three replicate experiments. Means with different letters in the same column indicate significant differences ($p \leq 0.05$). ng – no detectable growth after sorting. *% Re-growth as a proportion of sorted cells.

Table 3. Lag phase duration (λ) and growth rate (μ_{\max}) of FACS sorted osmotic, acid and heat injured *L. monocytogenes* cells resuscitated in BHI broth at 25°C

<i>L. monocytogenes</i> Strain	Treatment	λ (h)	μ_{\max} (Log ₁₀ CFUml ⁻¹ h ⁻¹)	R ²
ATCC19115	Acid	6.48 ^{Aa} ±0.74	0.40 ^{Aa} ±0.08	0.98
159/10		9.14 ^{Ba} ±1.46	0.46 ^{Aab} ±0.08	0.94
69		4.98 ^{Aa} ±0.60	0.49 ^{Ab} ±0.09	0.99
243		4.51 ^{Aa} ±0.80	0.38 ^{Ab} ±0.01	0.99
ATCC19115	Osmotic	7.65 ^{Ba} ±0.77	0.47 ^{Ba} ±0.02	0.99
159/10		7.61 ^{Ba} ±0.23	0.54 ^{Bb} ±0.01	0.97
69		3.26 ^{Aa} ±0.05	0.42 ^{Ab} ±0.03	0.97
243		9.22 ^{Bb} ±0.10	0.50 ^{Bc} ±0.03	0.99
ATCC19115	Heat	ng	ng	
159/10		ng	ng	
69		5.98 ^{ab} ±0.22	0.47 ^b ±0.01	0.99
243		ng	ng	
ATCC19115	Control	nd	0.50 ^{Ba} ±0.01	0.97
159/10		nd	0.38 ^{BAa} ±0.01	0.99
69		nd	0.25 ^{Aa} ±0.04	0.99
243		nd	0.27 ^{Aa} ±0.01	0.98

λ and μ_{\max} values are means \pm standard deviations of three replicate experiments. Means with different uppercase letters in the same column for each treatment indicate significant differences ($p \leq 0.05$). For each strain, means with different lowercase letters indicate significant differences ($p \leq 0.05$). ng – no growth after sorting. nd – lag phase duration not detectable. R² – Goodness of fit for the Baranyi and Roberts (1994) model (Coefficient of determination).

Table 4. Lag phase duration (λ) and growth rate (μ_{\max}) of FACS sorted osmotic, acid and heat injured *L. monocytogenes* cells resuscitated in BHI broth at 37°C

<i>L. monocytogenes</i> Strain	Treatment	λ (h)	μ_{\max} (Log ₁₀ CFUml ⁻¹ h ⁻¹)	R ²
ATCC19115	Acid	7.15 ^{Ca} ±0.07	0.69 ^{BCb} ±0.01	0.99
159/10		4.14 ^{Ba} ±0.01	0.47 ^{Aa} ±0.03	0.98
69		1.96 ^{Aa} ±0.19	0.65 ^{ABb} ±0.04	0.97
243		5.12 ^{Ba} ±0.29	0.54 ^{ABb} ±0.02	0.99
ATCC19115	Osmotic	5.39 ^{Ba} ±0.05	0.62 ^{Cb} ±0.04	0.99
159/10		6.52 ^{Bb} ±0.09	0.69 ^{Cc} ±0.01	0.97
69		0.50 ^{Aa} ±0.16	0.52 ^{Ba} ±0.01	0.97
243		7.14 ^{Ba} ±0.59	0.49 ^{Ab} ±0.06	0.99
ATCC19115	Heat	ng	ng	
159/10		ng	ng	
69		5.31 ^b ±0.01	0.69 ^b ±0.00	0.97
243		ng	ng	
ATCC19115	Control	nd	0.42 ^{Aa} ±0.05	0.95
159/10		nd	0.58 ^{Bb} ±0.01	0.99
69		nd	0.66 ^{Bb} ±0.03	0.99
243		nd	0.44 ^{Aa} ±0.04	0.98

λ and μ_{\max} values are means \pm standard deviations of three replicate experiments. Means with different uppercase letters in the same column for each treatment indicate significant differences ($p \leq 0.05$). For each strain, means with different lowercase letters indicate significant differences ($p \leq 0.05$). ng – no growth after sorting. nd – lag phase duration not detectable. R² – Goodness of fit for the Baranyi and Roberts (1994) model (Coefficient of determination).

injury repair. λ values for unstressed control cells could not be determined by the DMfit software owing to their quick commencement of growth (Tables 3 and 4).

3.5 Maximum specific growth rate (μ_{max}) of resuscitated cells

The temperature of incubation had a significant effect ($p < 0.0001$) on the maximum specific growth rate of resuscitated *L. monocytogenes* cells (Table 5). At 37°C, μ_{max} values were in the range of 0.47 – 0.69 Log₁₀ CFUml⁻¹h⁻¹ (Table 3) compared to 0.38 – 0.54 Log₁₀ CFUml⁻¹h⁻¹ at 25°C (Table 3). *L. monocytogenes* strain variations ($p \leq 0.330$) and stress treatments ($p \leq 0.407$) had no significant effect on growth rate (Table 5). μ_{max} values were 0.38 - 0.69 and 0.42 – 0.69 Log₁₀ CFUml⁻¹h⁻¹ for acid and NaCl injured cells respectively (Tables 3 and 4). However, when compared to their respective untreated controls, μ_{max} values for injured cells at 25°C were significantly higher ($p < 0.05$) for strains 69, 159/10 and 243 (Table 3).

Table 5. Analysis of variance showing the effect of strain, stress and temperature factors on lag phase duration, maximum specific growth rate of *L. monocytogenes*

Factor	DF	P value	
		λ	μ_{max}
Strain (ATCC19115, 159/10, 69, 243)	3	< 0.0001	0.330
Stress (Acid, Osmotic, Heat)	2	0.007	0.407
Temperature (25°C, 37°C)	1	0.001	< 0.0001

3.6 Recovery of injured cells at 4°C

Table 6 shows the growth potential of injured *L. monocytogenes* resuscitated in BHI broth at 4°C. No growth was detectable in the first 3 days of incubation with a detection limit of 1.40 Log₁₀ CFUml⁻¹, for all strains except for *L. monocytogenes* 69 whose population had risen to greater than 3.0 Log₁₀ CFUml⁻¹ in the same period. The population of acid and osmotic stress injured strain 159/10 reached levels of 2.72 Log₁₀ CFUml⁻¹ and 2.92 Log₁₀ CFUml⁻¹ respectively after 10 days. Acid and osmotic stress injured *L. monocytogenes* strains

ATCC19115 and 243 took 15 days to reach levels $\geq 2.30 \text{ Log}_{10} \text{ CFUml}^{-1}$. With the exception of strain 69, heat injured cells could not recover after 15 days of incubation.

Table 6. Recovery of injured *L. monocytogenes* cells in BHI broth at 4°C and time to detection of $\geq 1.40 \text{ Log}_{10} \text{ CFUml}^{-1}$.

<i>L. monocytogenes</i> Strain	Osmotic		Acid		Heat	
	Time (d)	$\text{Log}_{10} \text{ CFUml}^{-1}$	Time (d)	$\text{Log}_{10} \text{ CFUml}^{-1}$	Time (d)	$\text{Log}_{10} \text{ CFUml}^{-1}$
ATCC19115	15	2.30 ± 0.06	15	3.16 ± 0.16	nd	
159/10	10	2.92 ± 0.44	10	2.72 ± 0.33	nd	
69	3	3.06 ± 0.08	5	6.19 ± 0.02	3	2.30 ± 0.06
243	15	3.44 ± 0.46	15	3.25 ± 0.07	nd	

nd – Not detectable

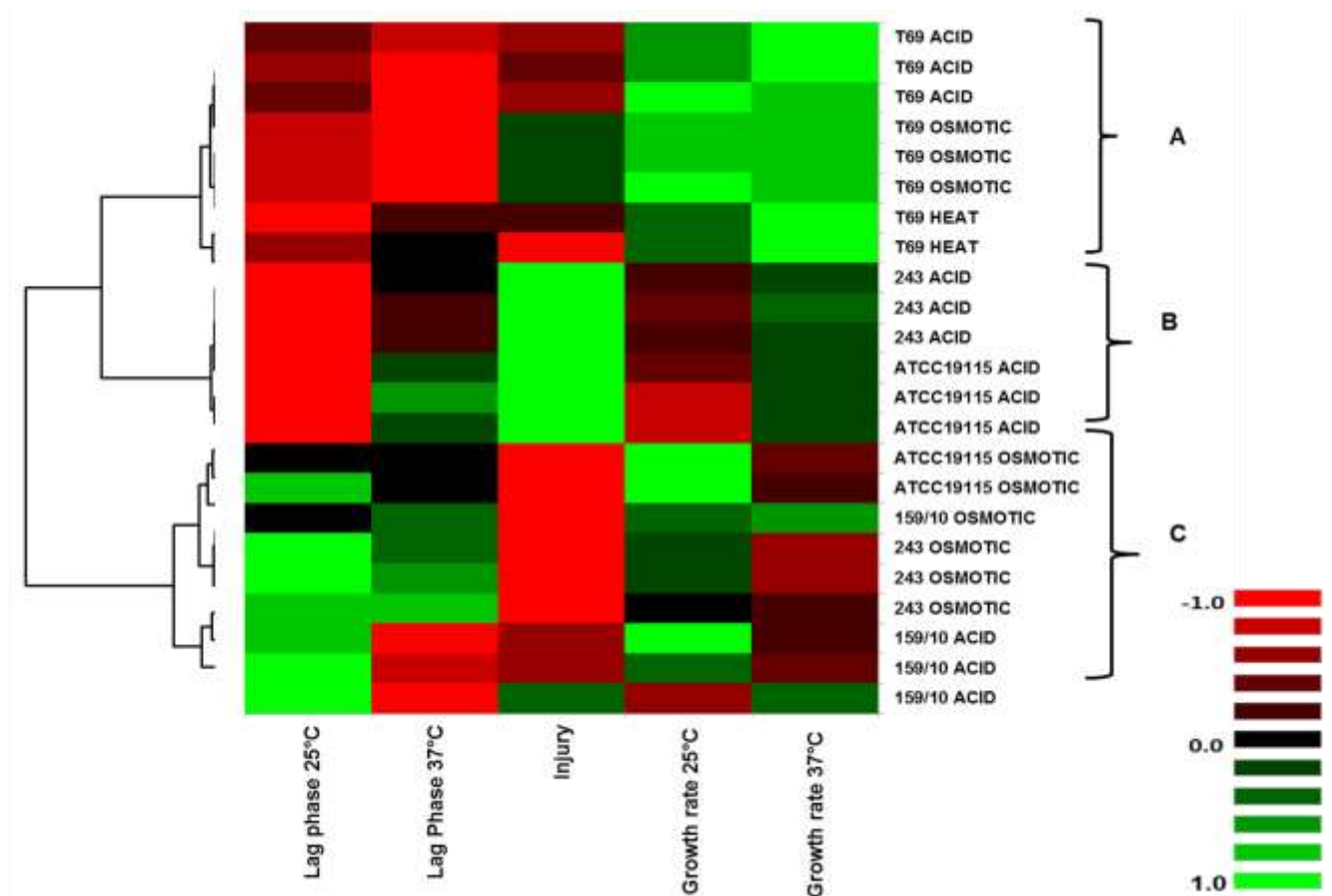


Fig. 5. Heat map of the relationship between cell injury and growth parameters of FACS sorted osmotic, acid and heat injured strains of *L. monocytogenes* resuscitated in BHI broth. Green (+1.0) represents high values of cell injury and growth parameters, red (-1.0) - low values and black (0.0) - median values. Response clustered into three main groups. A - Strain 69 with short lag phase, B - High acid injury with long lag phase, C - Low osmotic and acid injury with short lag phase.

3.7 Heat map clustering of cell injury, lag phase duration and growth rate

Heat map clustering of cell injury, lag phase duration and growth rate (Fig. 5) showed that the response clustered into three main groups indicating an association between percentage injury of individual strains and lag phase duration. Strain 69 which had a low percentage cell injury tended to have a short lag phase duration at both 25°C and 37°C. In addition, strains with a high degree of cell injury particularly related to acid stress tended to have with long lag phase durations.

4 Discussion

The objective of this study was to examine the effect of stress induced cell injury on the resuscitation and growth behavior of resuscitated *L. monocytogenes* cells following cell sorting. The initial step in the study was to assess the degree of stress induced injury based on differential plating and flow cytometry. This was followed by recovery of injured cells by FACS sorting and their re-growth in BHI broth.

On the analysis of stress imposed injury, both methods showed significant differences in response among *L. monocytogenes* strains and also among stress treatments. No clear patterns of response were observed with the plate count method with the most conspicuous observation being the high percentage of injury (100%) recorded for strain 69 (for all stress treatments) and heat stressed ATCC19115, which manifested as a complete lack of growth on selective media after stress exposure. In contrast to flow cytometry and differential staining, a clear pattern of response was observed in which acid stress caused the highest degree of cell membrane damage with osmotic stress causing the least, regardless of strain. The complete cell injury of strain 69 (based on plate counts) was unexpected given that $\leq 40\%$ of the cells (for all forms of stress) were identified as membrane damaged by flow cytometry. Possible reasons for this observation could be that stressed non-injured cells may be susceptible to selective agents incorporated in selective media. The presence of acriflavine, nalidixic acid

and lithium chloride in PALCAM agar has been shown to be inhibitory to some stressed *L. monocytogenes* strains (Liamkaew, Thipayarat, & Saranak, 2014). This result emphasizes the drawbacks of culture based methods for detection of sub-lethal injury as it implies a potential for over-estimation of injury and under-estimation of live cells (Van Nevel et al., 2017). However, in some cases, the two methods showed a good agreement in detection of injury. For instance, the 100% injury for strain ATCC19115 (lack of growth on selective media) was strongly supported by cytometric data which revealed that 73% of the cells were injured with about 12% of the population dead. Positive correlation between the two methods has been reported in previous studies (Massicotte et al., 2017).

Differences in percentage injury among the three stress treatments based on flow cytometry and differential staining are likely an indication of differences in mechanism of antimicrobial action and extent to which the stress exposures affect cell membrane integrity. The high degree of injury arising from acid stress is likely related to the antimicrobial mechanism of lactic acid. While acid stress is not associated with direct degradation of cell membrane structural components (Wood, 1999), the increased influx of protons (associated with acid exposure) disrupts the trans-membrane potential thereby affecting the proton motive force and membrane permeability (Alakomi et al., 2000). This explanation has been supported by previous studies using flow cytometry and membrane permeability markers which showed that lactic acid can increase membrane permeability in Gram-negative bacteria (Alakomi et al., 2000). The resultant effect on membrane permeability could be the cause of the increased uptake of PI by acid stressed *L. monocytogenes* cells.

The low percentage of cell injury resulting from NaCl stress is a reflection of the intrinsic salt resistance of *L. monocytogenes*. Previous studies have reported the ability of *L. monocytogenes* strains to survive and grow in high NaCl containing media (Bergholz, de Bakker, Fortes, Boor, & Wiedmann, 2010). The effect of osmotic stress on bacterial cells arises from changes in hydrostatic pressure that results in cell membrane tension and bilayer

deformations (Wood, 1999). The low level of injury implies that the effect of the osmotic stress was not sufficient to cause any significant influence on membrane permeability resulting in a low level of PI uptake by stressed cells.

Except for *L. monocytogenes* ATCC19115, the level of heat induced injury was $\leq 40\%$. Heat stress response of *L. monocytogenes* has been shown to vary considerably among strains from different serotypes (Aryani, de Besten, Hazeleger, & Zwietering, 2015). Apart from the cell membrane, heat stress affects several other cellular targets such as enzymes, RNA and DNA (Kramer & Thielmann, 2016). Using a multi-method approach, Kramer and Thielmann, (2016) concluded that several cellular targets such as the respiratory pathway are affected by heat stress before loss of membrane integrity. This highlights the fact that membrane integrity indicators like PI may not be good indicators of cell injury and viability loss following heat exposure.

Following cell sorting, the percentage of re-growth of injured cells showed a link to the extent of cell damage recorded by PI uptake of stressed cells. While PI staining has been reported to be a sensitive marker of cell damage, it is a poor indicator of cell viability loss (Novo et al., 2000) thus some TO positive/PI positive cells may not be viable. It is likely that a high degree of cell damage for stress sensitive strains, ATCC19115 and 243 could have resulted in loss of viability for some sorted injured cells resulting in low percentage of re-growth. Amor et al. (2002) observed a similar reduction in the re-growth potential of sorted injured *Bifidobacterium* cells following bile salt stress.

The lag phase duration of FACS sorted injured cells, resuscitated in BHI broth varied significantly with temperature, stress treatment and strains of *L. monocytogenes*. In bacterial growth kinetics, the lag phase duration is a measure of the time a bacterial population takes to commence exponential growth following a transition to a new environment (Swinnen, Bernaerts, Dens, Geeraerd, & Van Impe, 2004). The duration of this period is influenced by the physiological state of the cell population prior to environmental change (Augustin, Rosso,

& Carrier, 2000). The shorter lag phase at 37 °C could be a result of a quicker rate of repair which is associated with the rate of initiation of protein synthesis, in particular the enzymes necessary for the synthesis of membrane lipids needed for repair of damaged cell membranes (D. García, Mañas, Gómez, Raso, & Pagán, 2006). With the exception of strain 69, the lag phase duration of resuscitating acid and osmotic stress injured *L. monocytogenes* was at least 4.5 h at 25 °C and 4.1 h at 37 °C which was expectedly higher than non-injured control cells. The extended length of the lag phase for injured cells results from the need to resolve cell damage before cells can commence division. In addition to cell membrane damage, which was the basis of cell sorting, stress induced cell injury can result in damage to other functional components of the cell such as DNA, RNA and membrane potential (Chilton, Isaacs, Manas, & Mackey, 2001). Resuscitation of such injured cells has been shown to depend on the degree of damage which is influenced by the magnitude of stress (Mellefont & Ross, 2003). The short lag phase of strain 69 was particularly unique from the other three strains. The quick recovery of this strain appeared to be consistent with its low percentage of membrane damage after stress exposure.

Unlike acid and osmotic stress, heat injured cells of strains ATCC19115, 159/10 and 243 were unable to re-grow under any conditions when sorted into BHI broth. This indicates that in addition to membrane damage, heat stress results in damage to other cellular targets such as proteins, DNA, rRNA, ribosomes, respiratory processes and electron transport chain (Kramer & Thielmann, 2016). The fact that membrane intact cells were detectable after heat exposure is an indication that heat induced disruption of cellular processes occurs before membrane damage (Kramer & Thielmann, 2016). This signifies that membrane integrity alone is not a sufficient indicator of injury and viability of heat stressed cells.

The long lag phases of resuscitating *L. monocytogenes* cells have huge implications for the detection of injured cells in food systems. For effective detection, bacterial concentration must reach about 10^2 to 10^4 CFU ml⁻¹ in the enrichment broth (Dupont & Augustin, 2009). In this

study, the inoculation level of injured cells was meant to achieve a cell density of 10^2 cells/ml in the growth medium. However, in the case of acid injured cells, the proportion of cells capable of growth dropped below the detection limit of $1.40 \text{ Log}_{10} \text{ CFU ml}^{-1}$ for the first 4 h. This initial response is perhaps due to the fact that individual cell variation influences the probability of growth post stress exposure. Dupont and Augustin (2009), observed that stress exposure reduces the growth probability of individual cells of *L. monocytogenes* in half-fraser broth. This highlights the importance of including a step of injured cell repair in a non-selective enrichment broth before selective enrichment in pathogen detection methods.

The growth rate of resuscitated *L. monocytogenes* showed no variation among strains and was expectedly lower at $25 \text{ }^\circ\text{C}$ than at $37 \text{ }^\circ\text{C}$. Growth rate differences between $25 \text{ }^\circ\text{C}$ and $37 \text{ }^\circ\text{C}$, reflect temperature dependence of growth processes in exponential phase. Within the growth permitting temperature range, bacterial growth rate is influenced by the rate of biological reactions which follow first order kinetics, a situation that has been observed for *L. monocytogenes* growth in foods (Lee et al., 2014). In addition, at temperatures below optimum, reduction in cell membrane fluidity lowers nutrient affinity which subsequently reduces growth rate (Nedwell, 1999).

The importance of *L. monocytogenes* as a pathogen in RTE foods arises from the fact that refrigeration is invariably used as a terminal hurdle in preservation of such foods and yet the organism is a psychrotroph capable of growth at refrigeration temperatures (Schmid et al., 2009). After inoculating 100 cells/ml of injured cells into BHI broth at $4 \text{ }^\circ\text{C}$, the number of cells capable of growth dropped below the detection limit of $1.40 \text{ Log}_{10} \text{ CFU ml}^{-1}$ in the first 5 days except for strain 69. Considering that the stress treatments and subsequent sorting and inoculation of cells was done at room temperature, the transfer of injured cells to a $4 \text{ }^\circ\text{C}$ environment could have induced cold shock to which some of the injured cells were susceptible. Differences in the cold growth behavior of strains indicated a link to the percentage of cell damage observed in flow cytometry analysis with strain 69 showing the

fastest growth while strains ATCC19115 and 243 had extended growth periods. Depending on the physicochemical properties of the food, the control of *L. monocytogenes* in RTE foods is largely based on limiting its cold growth by extension of the lag phase period (Angelidis, Papageorgiou, Tyrovouzis, & Stoforos, 2013). The findings of this study show that cold stress may be insufficient to prevent recovery of injured *L. monocytogenes* cells. However, resuscitation of these cells was done in a broth model without any physicochemical hurdles to limit growth. It is likely that in a food matrix with additional hurdles to prevent growth, response of such injured cells could be different. García, Hassani, Mañas, Condón, & Pagán (2005) observed that sub-lethally injured *E. coli* cells after pulsed electric fields treatment were sensitive to refrigerated storage in apple juice, thus stressing the need to study the resuscitation of sorted cells in different food matrices.

5 Conclusions

This study underscored the importance of flow cytometry in the study of stress imposed cell injury and the potential of FACS cell sorting in the study of injured cells. Generally, flow cytometry and fluorescent staining with membrane integrity indicator probes showed a clear pattern of response with acid stress causing the highest level of injury followed by heat and osmotic stress. Lack of re-growth of FACS sorted heat injured cells showed that while membrane integrity was a good indicator of cell injury and viability loss for acid and osmotic stress, it was not a sufficient indicator of heat stress injury. The length of the lag phase duration (indicating repair period) is the major difference in the resuscitation behavior of *L. monocytogenes* strains. Once injured cells repair the cellular damage, their growth rate is not different from non-injured cells regardless of form of stress and strain differences. Thus highlighting the potential food safety risks of stress injured *L. monocytogenes* cells.

Acknowledgements

The authors would like to acknowledge financial support from the South African National Research Foundation (NRF) Centre of Excellence in Food Security (Project 140203: Wastage and Loss). We also wish to acknowledge Amy Strydom and Prof. R. C. Witthuhn, Department of Food Science and Biotechnology, University of Free State for providing us with the strains used in this study.

References

- Alakomi, H. L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. *Applied and Environmental Microbiology*, *66*, 2001–2005.
- Amor, K. Ben, Breeuwer, P., Verbaarschot, P., Rombouts, F. M., Akkermans, A. D. L., De Vos, W. M., & Abee, T. (2002). Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead *Bifidobacterium* cells during bile salt stress. *Applied and Environmental Microbiology*, *68*, 5209–5216.
- Angelidis, A. S., Papageorgiou, D. K., Tyrovouzis, N. A., & Stoforos, N. G. (2013). Kinetics of *Listeria monocytogenes* cell reduction in processed cheese during storage. *Food Control*, *29*, 18–21.
- Arku, B., Fanning, S., & Jordan, K. (2011). Flow cytometry to assess biochemical pathways in heat-stressed *Cronobacter* spp. (formerly *Enterobacter sakazakii*). *Journal of Applied Microbiology*, *111*, 616–624.
- Aryani, D. C., de Besten, H. M. W., Hazeleger, W. C., & Zwietering, M. H. (2015). Quantifying variability on thermal resistance of *Listeria monocytogenes*. *International Journal of Food Microbiology*, *193*, 130–138.
- Augustin, J. C., Rosso, L., & Carrier, V. (2000). A model describing the effect of temperature history on lag time for *Listeria monocytogenes*. *International Journal of Food*

- Microbiology*, 57, 169–181.
- Aureli, P., Fiorucci, G. C., Caroli, D., Marchiaro, D., Marchiabo, G., Novara, O., ... Salmaso, S. (2000). An outbreak of febrile gastroenteritis associated with corn contaminated with *Listeria monocytogenes*. *The New England Journal of Medicine*, 342(17), 1236–1241.
- Baranyi, J., & Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology*, 23, 277–294.
- Bergholz, T. M., de Bakker, H. C., Fortes, E. D., Boor, K. J., & Wiedmann, M. (2010). Salt stress phenotypes in *Listeria monocytogenes* vary by genetic lineage and temperature. *Foodborne Pathogens and Disease*, 7, 1537–1549.
- Bhagat, A. A. S., Bow, H., Hou, H. W., Tan, S. J., Han, J., & Lim, C. T. (2010). Microfluidics for cell separation. *Medical and Biological Engineering and Computing*, 48, 999–1014.
- Bolocan, A. S., Oniciuc, E. A., Alvarez-Ordóñez, A., Wagner, M., Rychli, K., Jordan, K., & Nicolau, A. I. (2015). Putative cross-contamination routes of *Listeria monocytogenes* in a meat processing facility in Romania. *Journal of Food Protection*, 78, 1664–1674.
- Busch, S. V., & Donnelly, C. W. (1992). Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and *Listeria innocua*. *Applied and Environmental Microbiology*, 58, 14–20.
- Casadesús, J., & Low, D. A. (2013). Programmed heterogeneity: Epigenetic mechanisms in bacteria. *The Journal of Biological Chemistry*, 288, 13929–13935.
- Chilton, P., Isaacs, N. S., Manas, P., & Mackey, B. M. (2001). Biosynthetic requirements for the repair of membrane damage in pressure-treated *Escherichia coli*. *International Journal of Food Microbiology*, 71, 101–104.
- da Silva, T. L., Piekova, L., Mileu, J., & Roseiro, J. C. (2009). A comparative study using the dual staining flow cytometric protocol applied to *Lactobacillus rhamnosus* and *Bacillus licheniformis* batch cultures. *Enzyme and Microbial Technology*, 45, 134–138.
- Dalzini, E., Cosciani-Cunico, E., Bernini, V., Bertasi, B., Losio, M.-N., Daminelli, P., &

- Varisco, G. (2015). Behaviour of *Escherichia coli* O157 (VTEC), *Salmonella* Typhimurium and *Listeria monocytogenes* during the manufacture, ripening and shelf life of low fat salami. *Food Control*, 47, 306–311.
- Díaz, M., Herrero, M., García, L. A., & Quirós, C. (2010). Application of flow cytometry to industrial microbial bioprocesses. *Biochemical Engineering Journal*, 48, 385–407.
- Dupont, C., & Augustin, J.-C. (2009). Influence of stress on single-cell lag time and growth probability for *Listeria monocytogenes* in half fraser broth. *Applied and Environmental Microbiology*, 75, 3069–3076.
- García, D., Hassani, M., Mañas, P., Condón, S., & Pagán, R. (2005). Inactivation of *Escherichia coli* O157:H7 during the storage under refrigeration of apple juice treated by pulsed electric fields. *Journal of Food Safety*, 25, 30–42.
- García, D., Mañas, P., Gómez, N., Raso, J., & Pagán, R. (2006). Biosynthetic requirements for the repair of sublethal membrane damage in *Escherichia coli* cells after pulsed electric fields. *Journal of Applied Microbiology*, 100, 428–435.
- Giaouris, E., Chorianopoulos, N., & Nychas, G. J. (2014). Acquired acid adaptation of *Listeria monocytogenes* during its planktonic growth enhances subsequent survival of its sessile population to disinfection with natural organic compounds. *Food Research International*, 64, 896–900.
- Gossett, D. R., Weaver, W. M., Mach, A. J., Hur, S. C., Tse, H. T. K., Lee, W., ... Di Carlo, D. (2010). Label-free cell separation and sorting in microfluidic systems. *Analytical and Bioanalytical Chemistry*, 397, 3249–3267.
- Jamali, H., Radmehr, B., & Thong, K. L. (2013). Prevalence, characterisation, and antimicrobial resistance of *Listeria* species and *Listeria monocytogenes* isolates from raw milk in farm bulk tanks. *Food Control*, 34, 121–125.
- Jensen, A., Williams, D., Irvin, E. A., Gram, L., & Smith, M. A. (2008). A processing plant persistent strain of *Listeria monocytogenes* crosses the fetoplacental barrier in a pregnant

- guinea pig model. *Journal of Food Protection*, 71(5), 1028–1034.
- Joux, F., & Lebaron, P. (2000). Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes and Infection*, 2(12), 1523–1535.
- Kastbjerg, V. G., Larsen, M. H., Gram, L., & Ingmer, H. (2010). Influence of sublethal concentrations of common disinfectants on expression of virulence genes in *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 76(1), 303–309.
- Kennedy, D., Cronin, U. P., & Wilkinson, M. G. (2011). Responses of *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus* to simulated food processing treatments, determined using fluorescence-activated cell sorting and plate counting. *Applied and Environmental Microbiology*, 77, 4657–4668.
- Kramer, B., & Thielmann, J. (2016). Monitoring the live to dead transition of bacteria during thermal stress by a multi-method approach. *Journal of Microbiological Methods*, 123, 24–30.
- Le Roux, K., Prinsloo, L., & Meyer, D. (2015). Fourier Transform Infrared spectroscopy discloses different types of cell death in flow cytometrically sorted cells. *Toxicology In Vitro*, 29, 1932–1940.
- Lee, J. Y., Jung, B. S., Yoon, H. J., Kim, K. T., Paik, H. D., & Lee, J. Y. (2014). Predictive model for the growth kinetics of *Listeria monocytogenes* in raw pork meat as a function of temperature. *Food Control*, 44, 16–21.
- Liamkaew, R., Thipayarat, A., & Saranak, J. (2014). *Listeria* detection in microscale solid state inoculation with minimal selective agents. *Food Control*, 43, 183–192.
- Martin, R. M., Leonhardt, H., & Cardoso, M. C. (2005). DNA labeling in living cells. *Cytometry Part A*, 67(1), 45–52.
- Massicotte, R., Mafu, A. A., Ahmad, D., Deshaies, F., Pichette, G., & Belhumeur, P. (2017). Comparison between flow cytometry and traditional culture methods for efficacy assessment of six disinfectant agents against nosocomial bacterial species. *Frontiers in*

Microbiology, 8, 112.

- Mellefont, L. A., & Ross, T. (2003). The effect of abrupt shifts in temperature on the lag phase duration of *Escherichia coli* and *Klebsiella oxytoca*. *International Journal of Food Microbiology*, 83, 295–305.
- Mortimer, F. C., Mason, D. J., & Gant, V. A. (2000). Flow cytometric monitoring of antibiotic-induced injury in *Escherichia coli* using cell-impermeant fluorescent probes. *Antimicrobial Agents and Chemotherapy*, 44(3), 676–681.
- Muhterem-Uyar, M., Dalmasso, M., Bolocan, A. S., Hernandez, M., Kapetanakou, A. E., Kuchta, T., ... Wagner, M. (2015). Environmental sampling for *Listeria monocytogenes* control in food processing facilities reveals three contamination scenarios. *Food Control*, 51, 94–107.
- Nedwell, D. B. (1999). Effect of low temperature on microbial growth : lowered affinity for substrates limits growth at low temperature. *FEMS Microbiology Ecology*, 30, 101–111.
- Novo, D. J., Perlmutter, N. G., Hunt, R. H., & Shapiro, H. M. (2000). Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. *Antimicrobial Agents and Chemotherapy*, 44, 827–834.
- Olsen, S. J., Patrick, M., Hunter, S. B., Reddy, V., Kornstein, L., MacKenzie, W. R., ... Mead, P. (2005). Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. *Clinical Infectious Diseases*, 40, 962–967.
- Pagadala, S., Parveen, S., Rippen, T., Luchansky, J. B., Call, J. E., Tamplin, M. L., & Porto-Fett, A. C. S. (2012). Prevalence, characterization and sources of *Listeria monocytogenes* in blue crab (*Callinectes sapidus*) meat and blue crab processing plants. *Food Microbiology*, 31, 263–270.
- Perfetto, S. P., Chattopadhyay, P. K., Lamoreaux, L., Nguyen, R., Ambrozak, D., Koup, R. A., & Roederer, M. (2006). Amine reactive dyes: An effective tool to discriminate live and

- dead cells in polychromatic flow cytometry. *Journal of Immunological Methods*, 313, 199–208.
- Ryall, B., Eydallin, G., & Ferenci, T. (2012). Culture history and population heterogeneity as determinants of bacterial adaptation: the adaptomics of a single environmental transition. *Microbiology and Molecular Biology Reviews*, 76(3), 597–625.
- Sadeghi-Mehr, A., Lautenschlaeger, R., & Drusch, S. (2016). Behavior of *Salmonella* spp. and *Listeria monocytogenes* throughout the manufacture and shelf-life of dry-cured formed ham. *Food Control*, 64, 22–28.
- Schmid, B., Klumpp, J., Raimann, E., Loessner, M. J., Stephan, R., & Tasara, T. (2009). Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions. *Applied and Environmental Microbiology*, 75, 1621–1627.
- Shapiro, H. M. (2001). Multiparameter flow cytometry of bacteria: Implications for diagnostics and therapeutics. *Cytometry*, 43, 223–226.
- Silva, A., Genovés, S., Martorell, P., Zanini, S. F., Rodrigo, D., & Martinez, A. (2015). Sublethal injury and virulence changes in *Listeria monocytogenes* and *Listeria innocua* treated with antimicrobials carvacrol and citral. *Food Microbiology*, 50, 5–11.
- Strydom, A., Bester, I. M., Cameron, M., Franz, C. M. A. P., & Witthuhn, C. C. (2013). Subtyping of *Listeria monocytogenes* isolated from a South African avocado processing facility using PCR-RFLP and PFGE. *Food Control*, 31, 274–279.
- Swinnen, I. A. M., Bernaerts, K., Dens, E. J. J., Geeraerd, A. H., & Van Impe, J. F. (2004). Predictive modelling of the microbial lag phase: a review. *International Journal of Food Microbiology*, 94, 137–159.
- Tamburini, S., Ballarini, A., Ferrentino, G., Moro, A., Foladori, P., Spilimbergo, S., & Jousson, O. (2013). Comparison of quantitative PCR and flow cytometry as cellular viability methods to study bacterial membrane permeabilization following supercritical CO₂ treatment. *Microbiology*, 159, 1056–1066.

- Van Nevel, S., Koetzsch, S., Proctor, C. R., Besmer, M. D., Prest, E. I., Vrouwenvelder, J. S., ... Hammes, F. (2017). Flow cytometric bacterial cell counts challenge conventional heterotrophic plate counts for routine microbiological drinking water monitoring. *Water Research, 113*, 191–206.
- Veal, D. A., Deere, D., Ferrari, B., Piper, J., & Attfield, P. V. (2000). Fluorescence staining and flow cytometry for monitoring microbial cells. *Journal of Immunological Methods, 243*, 191–210.
- Waage, A. S., Vardund, T., Lund, V., & Kapperud, G. (1999). Detection of small numbers of *Campylobacter jejuni* and *Campylobacter coli* cells in environmental water, sewage, and food samples by a seminested PCR assay. *Applied and Environmental Microbiology, 65*(4), 1636–1643.
- Wang, X. M., Lü, X. F., Yin, L., Liu, H. F., Zhang, W. J., Si, W., ... Liu, S. G. (2013). Occurrence and antimicrobial susceptibility of *Listeria monocytogenes* isolates from retail raw foods. *Food Control, 32*, 153–158.
- Wersto, R. P., Chrest, F. J., Leary, J. F., Morris, C., Stetler-stevenson, M., & Gabrielson, E. (2001). Doublet Discrimination in DNA Cell-Cycle Analysis. *Cytometry (Communications in Clinical Cytometry), 46*, 296–306.
- Wesche, A. M., Gurtler, J. B., Marks, B. P., & Ryser, E. T. (2009). Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. *Journal of Food Protection, 72*, 1121–1138.
- Wood, J. M. (1999). Osmosensing by bacteria: signals and membrane-based sensors. *Microbiology and Molecular Biology Reviews, 63*, 230–262.
- Wu, V. C. H. (2008). A review of microbial injury and recovery methods in food. *Food Microbiology, 25*, 735–744.