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Deciphering the induction of Listeria monocytogenes into sublethal injury using fluorescence microscopy and RT-qPCR

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ABSTRACT

The adaptive response of bacterial cells to changing environmental conditions depends on the behavior of single cells within the population. Exposure of *Listeria monocytogenes* to sublethal acidic conditions in foods or in the gastrointestinal track of the host may induce injuries relevant to difficult physiological states within the dormancy continuum. In this study, exposure to acidic conditions (acetic-AA and hydrochloric acid-HCl adjusted to pH 3.0, 2.7, 2.5 at 20 °C for 5 h) was used to evaluate injury of *L. monocytogenes*, Scott A strain. To differentiate the resistant sub-population from the total, Tryptic Soy Agar with 0.6 % Yeast Extract (TSAYE) supplemented or not with 5 % NaCl were comparatively used. Sublethally injured cells were detected by comparing plate counts with fluorescence microscopy, using combinations of CFDA (viability) and Propidium-Iodide (death). Effect of acid stress on the relative transcription of *clpP*, *mazE*, *mazF*, *relA*, *gadC*, *gadD*, *gadB*, *sigB*, *inlA* and *prfA* upon transition of total population into different physiological stages was evaluated through RT-qPCR. AA treated cells showed measurable logarithmic reduction at pH 2.7 and 2.5, while there was a significant percentage of $CFDA^{-}/PI^{+}$ cells. Evaluation of the potentially culturable population on TSAYE, from the percentage of CFDA/PI-stained cells, revealed that unstained cells represented a non-culturable sub-population. Exposure to Ringer's solution pH 2.7, adjusted with AA, resulted in higher percentages of non-esterase active with membrane integrity cells ($CFDA^{-}/PI^{-}$) compared to the percentages of the enumerated culturable cells on TSAYE after 4 and 5 h. Under the same conditions, after 1 h of exposure macroscopic observation revealed size colony variations (SCVs) of the total population (CFU on TSAYE). *L. monocytogenes* retained its culturability after hydrochloric acid exposure, while cells remained metabolically active ($CFDA^{+}$). However, a stochastic change in cell's shape, was detected after exposure to pH 3.0 and 2.5, adjusted with HCl, for 2 h at 20 °C. A pattern of gene up-regulation was observed during treatment with AA pH 2.7 and HCl pH 3.0 at the 3rd h of exposure. Deciphering *L. monocytogenes* sublethal injury sheds light into the physiological and molecular characteristics of this state and provides the food science community with quantitative data to improve risk assessment.

1. Introduction

Listeria monocytogenes is a robust foodborne bacterial pathogen that can persist in a wide range of environmental and food-related niches due to its ability to sense and respond to physicochemical stresses (Gandhi and Chikindas, 2007; O'Byrne and Karatzas, 2008). Foodborne infections caused by *L. monocytogenes* are rather rare; however, they are associated with high mortality rates, reaching 20 to 30 %, making this pathogen a significant food safety threat (Swaminathan and Gerner-Smidt, 2007).

L. monocytogenes can encounter acidic conditions in foods or in the gastrointestinal track of the host, which may arise from weak organic acids, like lactate, benzoate, acetate or sorbate or by strong inorganic acids, such as hydrochloric acid (NicAogáin and O'Byrne, 2016). According to Federal Regulations, naturally acid foods are defined as those which have a naturally occurring pH of less than or equal to 4.6, while acidified foods (AF) are low-acid foods to which acid(s) or acid food(s) are added and which has a finished equilibrium pH of 4.6 or below. The

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use of weak-acid preservatives to inhibit growth of microorganisms in foods and beverages extends back many centuries. Inhibition by weak acids is more effective at low pH values where solutions contain increased concentrations of undissociated acids (Lambert and Stratford, 1999). Survival of foodborne pathogens under organic acid-induced stress is critical for their successful transmission from food to humans (Arcari et al., 2020). Depending on the intensity and the duration of exposure to stress, sublethal injury may be temporary and subsequently repaired from the cellular machinery or permanent, leading to cellular death (Hurst, 1984; Kell et al., 1998). Sublethally injured cells lose their ability to form colonies in media containing selective agents (e.g., sodium chloride), where non-injured cells are normally capable of multiplication (Yousef and Courtney, 2002) (Fig. 2).

Another physiological state, where bacterial cells have undergone loss of culturability in selective and non-selective, nutrient rich media, is the Viable But Non Culturable (VBNC) state (Fig. 2). VBNC state is one of the stages within the dormancy continuum (Ayrapetyan et al., 2015), closely related to persistence (Arvaniti and Skandamis, 2022). Dormant cells present low metabolic activity compared to actively growing or typical stationary phase cells (Balaban et al., 2019). Persister cells are antibiotic tolerant, as a consequence of a transient phenotypic switch (Abee et al., 2016), and genetically identical to non-persister cells (Ayrapetyan et al., 2015; Ayrapetyan and Oliver, 2016). There is evidence that persisters are nongrowing during antibiotic treatment, but regain their growth capacity after the removal of the antibiotic (Balaban et al., 2004). A difference between persisters and VBNC cells is their time needed for resuscitation (Ayrapetyan et al., 2015). Phenotypic switching refers to a switch between two phenotypic states, that normally occurs in a small fraction of the total population (Sousa et al., 2012). It may be a random event (stochastic switching), or it can be triggered by an external factor (Rossignol et al., 2009; van der Woude, 2006). Small colony variants (SCVs) are associated with increased bacterial pathogenicity and persistence (Sousa et al., 2012). They represent a reservoir of persistent and potential recurrent bacterial infections (Proctor et al., 2006). SCVs constitutes a slow growing sub-population with atypical colony morphology, nearly one-tenth the size of the colonies associated with the wild-type (Proctor et al., 2006), and results from specific defects in the electron transport chain (Curtis et al., 2016). They form pinpoint colonies have a variety of phenotypic characteristics, such as altered carbon metabolism, decreased toxin and lytic enzyme production, aminoglycoside resistance, and increased intracellular persistence (Curtis et al., 2016).

Persistence and VBNC states share common part of a dormancy continuum, sharing common molecular mechanisms (Ayrapetyan et al., 2018, 2015). As reported by Keren et al., type II toxin-antitoxin (TA) systems MazEF were found to be important in persister cell dynamics (Keren et al., 2004). Like all Type II TA systems, *mazEF* genes are co-transcribed into two proteins, MazE, that is an unstable antioxin that binds and inhibits the activity of MazF toxin. MazF is an endoribonuclease that cleaves mRNA at specific sites (Curtis et al., 2017). In silico predictions revealed two TA systems in *L. monocytogenes* EGDe, lmo0113-0114 and lmo0887-0888 (Xie et al., 2018). *relA* is also related to persistence and VBNC induction. The stringent response protein RelA synthesizes the alarmone guanosine pentaphosphate, (p)ppGpp, during amino acid starvation (Harshman and Yamazaki, 1971). (p)ppGpp leads to the accumulation of polyphosphates, which activates a Lon-mediated proteolytic process, involving the chaperone protein CplP, encoded by *clpP*, which begins to degrade antitoxin proteins (Germain et al., 2015). Free toxins inhibit translation and cause persistence induction (Maisonneuve and Gerdes, 2014). Persister formation and VBNC induction molecular mechanism has not been fully characterized in *L. monocytogenes*.

The glutamate decarboxylase (GAD) acid resistance system is important in survival and adaptation of *L. monocytogenes* in acidic conditions (Cotter et al., 2001). σ^B, encoded by *sigB*, regulates the transcription of genes contributing to acid resistance, including genes encoding components of the glutamate decarboxylase (GAD) system. In general, the GAD system consists of a glutamate decarboxylase enzyme and a glutamate-γ-aminobutyrate (GABA) antiporter, that acts in concert to reduce acidification within the cytoplasm of the cell (Chaturongakul et al., 2008). Furthermore, the alternative sigma B factor contributes to invasion and virulence of *L. monocytogenes* (Garner et al., 2006; Nadon et al., 2002). The sigma B regulon contains stress response as well as virulence genes and gene regulators, such as *gadB*, *inlA* and *prfA* (Kazmierczak et al., 2003; Nadon et al., 2002; Patrick et al., 2007). Transcriptional activator PrfA is the master regulator of virulence gene expression in *L. monocytogenes*, while *inlA* gene encodes a surface protein, an invasin, that mediates internalization of *L. monocytogenes* in epithelial cells (Dussurget, 2008).

The dynamic adaptive response and survival capacity of bacteria to changing environmental conditions depends on the behavior of single cells within the population (Abee et al., 2016). In our previous study, we have evaluated *L. monocytogenes* sublethal injury induced by acidic conditions at a population level, using plate counting. The results raised questions about the extent of sublethal injury that occurs in single cells, as well as the occurrence of any other physiological states in the total population after acidic treatment. Also, we sought to evaluate genes' transcription related with persister formation mechanism, VBNC induction, acid stress response, virulence and pathogenicity. As a result, gene transcription of *clpP*, *mazE*, *mazF*, *relA*, *gadC*, *gadD*, *gadB*, *sigB*, *inlA* and *prfA* was examined. The objectives of the present study were (i) to investigate, at population and single cell level, sublethal injury in *L. monocytogenes*, (ii) to describe the proportion of metabolically active, sublethally injured, VBNC and dead cells during exposure to acid stress using fluorescence microscopy and CFDA/PI staining, (iii) to identify size colony variations (SCVs) associated with persistence and (iv) to monitor the activation of the dormancy continuum associated molecular mechanism with RT-qPCR.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Listeria monocytogenes, Scott A strain (serotype 4b), was used in the present study, as one of the first available listeriosis outbreak isolates (Briers et al., 2011). Stock cultures were stored at −20 °C in Tryptic Soy Broth supplemented with 0.6 % Yeast Extract (TSBYE; Oxoid) and 20 % glycerol. Starting from them, the resuscitated cultures were maintained on plates of Tryptic Soy Agar with 0.6 % Yeast Extract (TSAYE; Oxoid) at 4 °C.

A single colony from TSAYE stock culture was inoculated into 10 ml TSBYE and incubated for 24 h at 30 °C. Subsequently, 0.1 ml of the 24-h culture was transferred in 10 ml TSBYE and incubated for 18 h at 30 °C. Cells were washed once in 1/4-strength Ringer's solution (Oxoid), to maintain osmotic balance of bacteria, and harvested by centrifugation (2, 434 ×*g* for 4 min at 4 °C). The activated 18 h cultures of *L. monocytogenes* were enumerated at approximately 10^9 CFU/ml.

2.2. Preparation of acid stress solutions

Acetic and hydrochloric acid in Ringer's solution, adjusted to pH 3.0, 2.7, 2.5 at 20 °C for 5 h, were used to evaluate sublethal cell injury of *L. monocytogenes* at single cell level (Fig. 1). Stock solutions of acetic acid 1 M and hydrochloric acid 3 N were gradually added to 50 ml sterile Ringer's solution, under aseptic conditions, in order to adjust pH up to the desired value (pH 3.0, 2.7 and 2.5). Exposure temperature was selected to simulate ambient conditions in food-processing environments. The treatment conditions were chosen to represent potential sublethal stress of *L. monocytogenes* in foods and food-processing environments and to comparatively assess the effect of weak organic versus strong inorganic acids. Stress solutions were prepared first and equilibrated for at least 20 min at 20 °C.

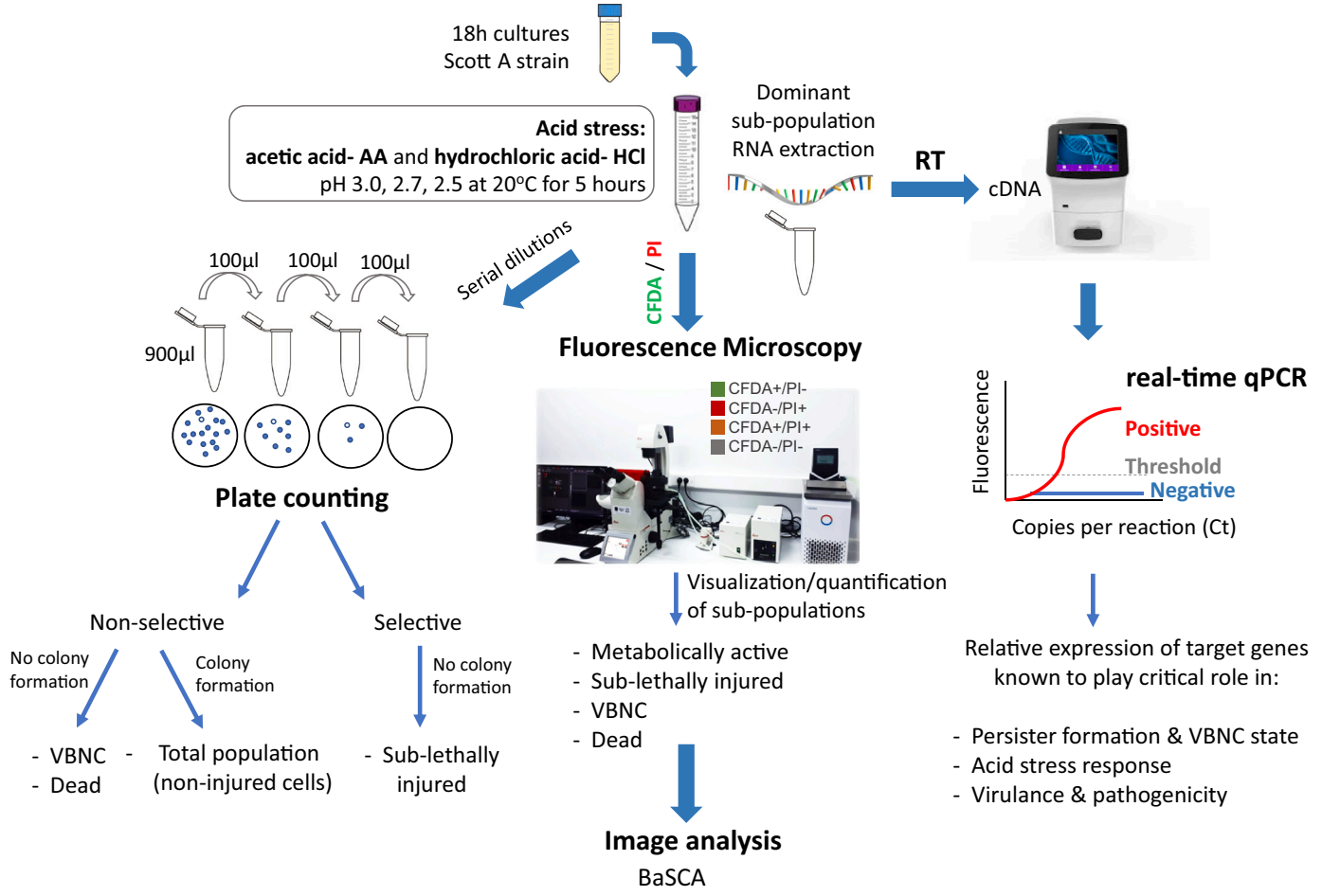

Fig. 1. Experimental design.

2.3. Exposure to stress

The activated 18 h cultures (10^9 CFU/ml), after being centrifuged once (2434 ×*g* for 10 min at 4 °C, to avoid any additional injury as a result of temperature increase during centrifugation), were resuspended: (i) in 4 ml of solution ($10^{9.5}$ CFU/ml) containing the stress agent, to assess sublethal injury with the parallel use of fluorescence microscopy and plate counting, and (ii) in 6 ml of stress solution ($10^{9.3}$ CFU/ml), to evaluate the effect of acid stress on the relative gene transcription of target genes.

pH values for acetic and hydrochloric acid solutions were measured after the resuspension of cells during their exposure to stress for 5 h at 20 °C (Tables 1 and 2).

2.4. Quantification of culturable cells

Percentage of culturable cells was calculated by the following method:

$$\%\text{of culturable cells} = \frac{100^{*}\ \text{CFU on TSAYE}}{\text{CFU initial population}} \quad (1)$$

Table 1
pH values after resuspension of cells in Ringer's solution, adjusted with AA, pH 3.0, 2.7 and 2.5 for 5 h at 20 °C.

AA 20 °C	Exposure time (h)		0	1	2	3	4	5
		Initial pH	pH after the resuspension of cells					
		3.01	3.37	3.42	3.42	3.4	3.4	3.4
		2.71	2.93	2.92	2.93	2.93	2.94	2.94
		2.52	2.72	2.70	2.70	2.71	2.71	2.70

Table 2
pH values after resuspension of cells in Ringer's solution, adjusted with HCl, pH 3.0, 2.7 and 2.5 for 5 h at 20 °C.

HCl 20 °C	Exposure time (h)		0	1	2	3	4	5
		Initial pH	pH after the resuspension of cells					
		3.01	3.50	3.51	3.51	3.51	3.51	3.51
		2.71	3.23	3.23	3.25	3.25	3.25	3.25
		2.52	2.96	2.96	2.98	2.98	2.98	2.98

2.5. Assessment of L. monocytogenes sublethal injury by plate counting

Injured cells are non-culturable on selective media (BUSTA, 1976; Ray, 1979). A concentration of 5 % (w/v) NaCl was found to support only the growth of non-injured *L. monocytogenes*, being the maximum non inhibitory concentration (MNIC) (Siderakou et al., 2021). Thus, determination of sublethally injured population fractions was performed by subtracting the number of colonies on TSAYE supplemented with 5 % (w/v) NaCl (TSAYE+5 %NaCl; AppliChem) from those on TSAYE with 0.5 % (w/v) NaCl:

$$log\ \text{CFU injured cells} = log\ \text{CFU on TSAYE} - log\ \text{CFU on TSAYE} + 5\%\text{NaCl} \quad (2)$$

Percentage of sublethally injured cells was calculated by the following method:

$$\%\text{of injured cells} = \frac{100^{*}\ \text{CFU of injured cells}}{\text{CFU on TSAYE}} \text{at each time point} \quad (3)$$

0.1 ml of acid treated samples were serially diluted in Ringer's solution and spread-plated in duplicates on TSAYE and TSAYE+5%NaCl (Fig. 1). Enumeration of *L. monocytogenes* colonies was performed on TSAYE after incubation for 2 days at 37 °C and on TSAYE+5%NaCl after 5 days at 37 °C. This plate counting method has been proven efficient in detecting sublethal injury in *L. monocytogenes* induced by acetic acid and hydrochloric acid (Arvaniti et al., 2021). The limit of detection (LOD) was 1.0 log CFU/ml for all treatments.

2.6. Macroscopic observation of size colony variations (SCV)

Size colony variations (SCV) were identified via macroscopic colony observation after 2 and 5 days at 37 °C. Images were taken using a Scan 1200 HD automatic colony counter (Interscience).

2.7. Quantification of metabolically active, sublethally injured and dead sub-populations with fluorescence microscopy

In order to investigate, at population and single cell level, sublethal injury in *L. monocytogenes* and to describe the proportion of metabolically active, sublethally injured and dead cells during exposure to acid stress, plate counting was performed with the parallel use of fluorescence microscopy and CFDA/PI staining (Fig. 1). The same method was performed to examine induction of VBNC state with peroxy-acetic acid (Arvaniti et al., 2021). The comparative evaluation of results from plate counting versus fluorescence microscopy, after exposure to Ringer's solution adjusted with AA and HCl to pH 3.0, 2.7, and 2.5 at 20 °C for 5 h, revealed the extent of injury, at single cell level. Each experimental condition was evaluated in two independent biological replicates. The same clonal cells were stressed, for plate counting and fluorescence microscopy, at each biological replicate. Each sampling point was related to 4–6 images of different fields of view (FOV). Each field of view consisted of at least 80–100 cells.

2.8. Fluorescence microscopy

Metabolic activity and death were evaluated, using fluorescence microscopy and CFDA/PI staining, according to a previously used protocol (Arvaniti et al., 2021). Briefly, 100 µl of cells were stained in a final concentration of 10 µM 5(6)-CFDA (Sigma) solution in dimethyl sulfoxide (DMSO) and in 29.92 µM propidium iodide (PI) (LIVE/DEAD Baclight bacterial viability kit) solution in DMSO. Unstressed cells were used as positive control and ethanol killed cells (70 % ethanol for 30 min at room temperature) as negative. Evaluation of cell's staining status revealed 4 different population fractions: (i) metabolically active with membrane integrity - viable ($CFDA^{+}/PI^{-}$), (ii) membrane damaged - dead ($CFDA^{-}/PI^{+}$), (iii) metabolically active with damaged membranes - sublethally injured ($CFDA^{+}/PI^{+}$) and (iv) non-esterase active with membrane integrity cells ($CFDA^{-}/PI^{-}$) (Fig. 2). Images were taken using an inverted fluorescence microscope (Leica, DMi8) equipped with an oil immersion 100× phase contrast objective (NA = 1.25) and a DFC 7000T camera (Leica).

2.9. Image analysis

The images acquired via fluorescent microscopy have been processed and analyzed with an image analysis pipeline (consisting of a combination of image processing and machine learning techniques for metabolically active, sublethally injured and dead sub-populations quantification, respectively) at the single-cell level. As the basis for this analysis we used the workflow of BaSCA (Bacterial Single-Cell Analytics) methodology presented in (Balomenos et al., 2017, 2015; Balomenos and Manolakos, 2017) modified accordingly to process the particular type of images and acquisition process, copying with the specific microenvironment, i.e. presence of noisy background, artifacts, dead cells debris, etc. The pipeline modified for our purposes, allows high throughput estimation of single-cell properties, live/dead enumeration, and quantification. The developed methodology segments the cells on the images and quantifies their biophysical properties, and fluorescence intensities (green/red), revealing the living and the dead population fraction and provides data to assess dormancy. To achieve this goal a combination of image processing and machine learning techniques were employed consisting five stages: image preprocessing, bacterial colonies segmentation, single-cell segmentation and also cell tracking and lineage tree construction. More details can be found in earlier publications (Balomenos et al., 2017, 2015; Balomenos and Manolakos, 2017). After the cell segmentation process, the estimated single cell masks (outline of the detected cells) are used in order to quantify the corresponding

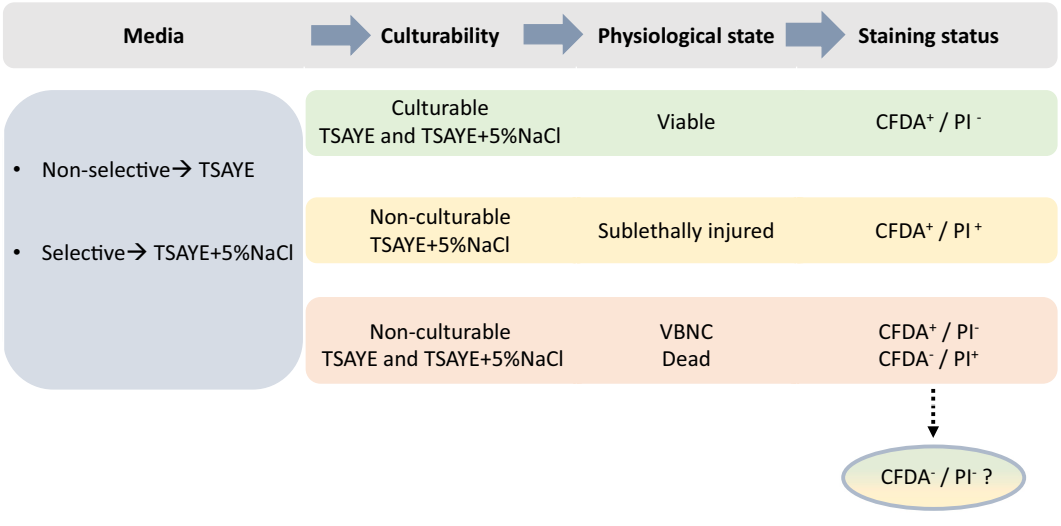

Fig. 2. Relationship between culturability, physiological state and staining status.

single-cell properties, and specifically the fluorescence of each cell and enumeration of the four possible subpopulations; i.e. $CFDA^{+}/PI^{-}$ (metabolically active with membrane integrity-viable), $CFDA^{-}/PI^{+}$ (membrane damaged-dead), $CFDA^{+}/PI^{+}$ (metabolically active with damaged membranes-sublethally injured), and $CFDA^{-}/PI^{-}$ (non-esterase active with membrane integrity). Furthermore, taking into account the dye leakage and its random distribution on the sample, along with the exposure and gain factors during image capture, we set an estimate of the threshold of dye intensity in order to bypass the background noise and consider the cell as true positive for each dye. To do so, we use the background intensities (outside of the cells mask) of each fluorescent dye image and compute their mean (mu) value and standard deviation (StD). Then the threshold is computed as: $Thres = mu + std$, which in a Gaussian distribution accounts for the 95.45 % of the values (here the values of the background). Next, we measure the pixel intensities inside each cell's area and the values above the aforementioned threshold are considered as dye markers inside this cell, characterizing the cell as dye positive ($CFDA^{+}$ and/or PI^{+}).

2.10. Relative gene transcription analysis

To assess the effect of acidic conditions on the relative transcription of the target genes, treated bacterial samples ($10^{9.3}$ CFU/ml) were collected every hour, for 5 h, during exposure to AA and HCl at pH 3.0, 2.7 and 2.5, at 20 °C. Samples were centrifuged (12,000 ×*g*; 1 min, 20 °C), the supernatant was discarded, and the pellet was resuspended in 200 μl of RNAlater Stabilization Solution (Ambion). Additionally, untreated control samples, in Ringer's solution, were collected at the same time points for the comparative quantification of gene transcription. Each experiment was performed in two independent biological replicates, RNA extraction was performed using Nucleospin® RNA kit (Macherey-Nagel). After extraction, RNA concentration and purity of the samples were measured using Nanophotometer (Implen GmbH).

Primers were designed through PrimerBlast (Ye et al., 2012). *clpP*, *mazE*, *mazF*, *relA*, *gadC*, *gadD*, *gadB*, *sigB*, *inlA* and *prfA* were selected due to their critical role in persistence and VBNC induction, stress response, virulence and pathogenicity (Table 3). Table 3 shows the primers sequences, respective amplicon sizes and the PCR efficiency of the primer pairs. The selection of best reference gene was conducted using NormFinder Excel plug-in v0.953 (Andersen et al., 2004). After evaluation of *IGS*, *rpoB* and *tpi* as candidate housekeeping genes; *tpi* was the gene with the most stable transcription profile and, therefore, it was used for normalization. Agarose gel electrophoresis (2.0 %) indicated amplification of a single PCR product of the expected size for our target genes.

cDNA synthesis was performed with the PrimeScript™ RT reagent Kit (Takara; RR047A), according to the manufacturer's instructions. Real-time qPCR was performed using KAPA SYBR qPCR Kit Master Mix (2×) for ABI Prism (Kapa Biosystems). Thermocycling conditions: initial denaturation at 95 °C for 20 s and then 40× (95 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s). Conditions for melting-curve analysis were 95 °C for 15 s and then 60 °C for 1 min and rise to 95 °C at 0.3 °C/s. Two RT reactions were performed for each sample; each cDNA was used for the evaluation of the transcription levels of the genes under study. qPCR reaction was carried out in a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Relative change in mRNA expression levels was determined using the mathematical model proposed by Pfaffl (Pfaffl, 2001):

$$\text{Fold of difference } \frac{E_{target}^{\Delta Ct \text{ target (control-sample)}}}{E_{ref}^{\Delta Ct \text{ ref (control-sample)}}} \quad (2)$$

Ct values were normalized with respect to reference genes and untreated control samples. Relative gene transcription data analysis was performed using Microsoft Excel 2010. The threshold cycle (CT) values of the target and reference genes obtained during the RT-qPCR

Table 3
Primers sequences, amplicon sizes and the PCR efficiency of the primer pairs.

Genes	Primer direction	Sequence (5′- > 3′)	Concentration (uM)	Amplicon size (bp)	PCR efficiency
Reference genes					
igs	Fwd	GGCCTATAGCTCAGCTGGTTA	0.5	135	1.899
	Rev	GCTGAGCTAAGGCCCCGTAAA	0.5		
rpoB	Fwd	CCGCGATGCGAAAACAAT	0.5	69	1.982
	Rev	CCWACAGAGATACGGTTATCRAATGC	0.5		
tpi	Fwd	AACACGGCATGACACCAATC	0.5	93	2.004
	Rev	CACGGATTTGACCACGTACC	0.5		
Persistence & VBNC state					
clpP	Fwd	ACTATCGGCATGGGTATGGC	0.5	165	2.037
	Rev	TAAAATGTGGCGAGCAGCGA	0.5		
mazE	Fwd	GGCTATGCAGAAATGGCGAC	0.5	130	1.994
	Rev	ATACGTCACCACGCTTCACC	0.5		
mazF	Fwd	AACGATTGACAAACAGCGCC	0.5	163	2.024
	Rev	ATACGTCACCACGCTTCACC	0.5		
relA	Fwd	CGCGAAATACCCAAGCGATG	0.5	160	1.975
	Rev	TTGCGATGGCGCAGGATATT	0.5		
Stress response					
gadC	Fwd	ATACAATCGGCGGCCTAACC	0.5	178	1.960
	Rev	CAGGCACTGACTTCACCCAT	0.5		
gadD	Fwd	CCAAACCGCCAAGCATACAC	0.5	174	2.033
	Rev	TGGCAGAAACGCTCGAGAAA	0.5		
gadB	Fwd	TTGCTTGGAGAAAACGTGCG	0.5	248	2.093
	Rev	ATCGTAGCGGCCTGTGTAAG	0.5		
sigB	Fwd	GCCGTAGAAGAGCTGACGAG	0.5	206	2.005
	Rev	CCATCATCCGTACCACCAACA	0.5		
Virulance & pathogenicity					
inlA	Fwd	AACTGTGACGCAGCCACTTA	0.5	156	2.059
	Rev	GTTTGGGCATCAAACCACCC	0.5		
prfA	Fwd	GCTATGTGCGATACCGCTTG	0.5	132	2.007
	Rev	CTCGGCTCTATTTGCGGTCA	0.5		

experiments were converted to $\log_2$ (fold of change) values.

2.11. Statistical analysis

To determine the statistically significant differences in the mean of *L. monocytogenes* population (log CFU/ml) at different time points the one-way analysis of variance was performed (ANOVA) and Tukey's honestly significant difference (HSD), using Statgraphics version 17.2.00. Differences were considered to be significant at the 95 % significance level.

Pearson correlation analysis was performed to compare the similarity in the transcription profiles of the two biological replicates along time. The coefficient correlation threshold (r) was 0.7 ($r > 0.7$ strong positive relationship). One Sample-*t*-test analysis was performed to investigate gene regulation. The $\log_2$ fold change ($\log_2$FC) value was examined as to whether it was statistically different from the selected threshold value (1 for up- and –1 for down-regulation). Statistically significant difference ($P < 0.05$) from the threshold value represented up- or down-regulation of gene transcription. The effect of time on gene transcription at each stress condition was evaluated with one-way analysis of variance (ANOVA). Statistical analysis was performed using Statgraphics (version 17.2.00).

3. Results

3.1. Induction of sublethal injury and VBNC state at single cell level

Evaluation of sublethal injury at population level, using plate counting, revealed that acetic acid treated cells showed statistically significant reduction in the total culturable (enumerated on TSAYE) as well as the non-injured population (enumerated on TSAYE+5%NaCl), after exposure to pH 3.0, 2.7 and 2.5 for 5 h at 20 °C. Incubation for 4 and 5 h at 20 °C in Ringer's solution at pH 3.0, adjusted with AA, resulted in statistically significant decrease of the total mean population ($P < 0.05$) (Fig. 3A). Increasing the stress intensity from pH 3.0 to pH 2.7 resulted in 1.94 log CFU/ml decrease after 5 h of exposure. At the same time, the population of non-injured cells decreased significantly by up to 2.28 log-units ($P < 0.05$) (Fig. 3C). Total culturable and non-injured population decreased up to 1.98 and 1.77 log units, respectively, after 3 h in Ringer's solution pH 2.5, adjusted with AA, at 20 °C. After 3 h of

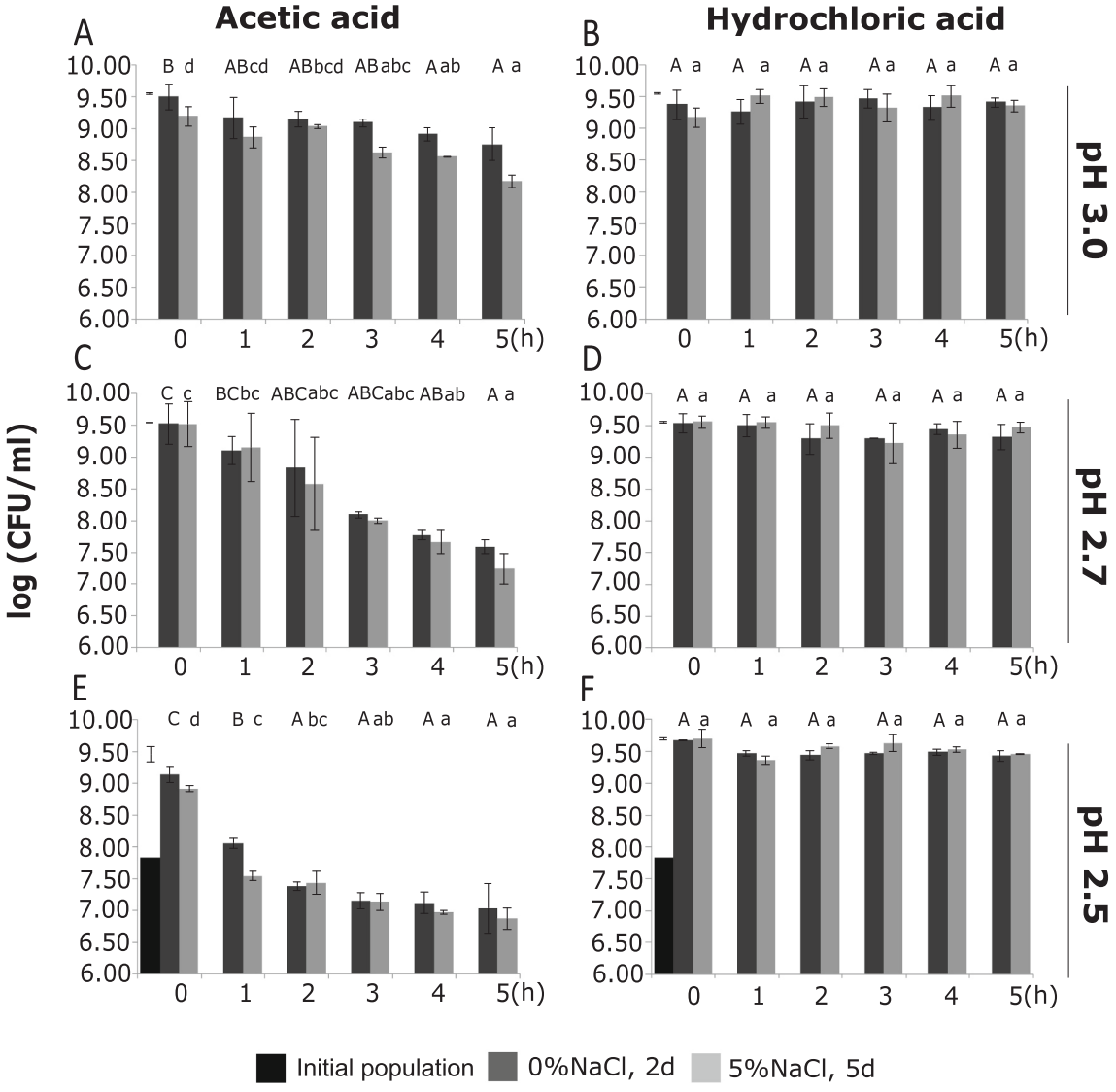

Fig. 3. Population (log CFU/ml) of *L. monocytogenes*, strain Scott A, after incubation for 0, 1, 2, 3, 4, 5 h at: AA (A) pH 3.0, (C) pH 2.7 and (E) pH 2.5 and HCl (B) pH 3.0, (D) pH 2.7 and (F) pH 2.5 at 20 °C. Total culturable (dark grey bars) and non-injured population (light grey bars) were enumerated after incubation for 2 days and 5 days at 37 °C, on TSAYE 0 % and 5 % NaCl, respectively. Bars represent mean values ± SD from one independent experiment. Statistically significant differences ($p < 0.05$, CI 95 %) are indicated with different uppercase (TSAYE) and lowercase (TSAYE-5%NaCl) letters, respectively.

exposure to AA pH 2.5 population levels (total and non-injured cells) remained stable at approximately 7.12 and 6.97 log CFU/ml (Fig. 3E).

In order to investigate sublethal injury in *L. monocytogenes* during exposure to acid stress at population and single cell level, plate counting was performed in parallel use to fluorescence microscopy using CFDA/PI staining. At single cell level, evaluation of metabolic activity and membrane integrity revealed 4 different population fractions: (i) metabolically active with membrane integrity - viable ($CFDA^+/PI^-$), (ii) membrane damaged - dead ($CFDA^-/PI^+$), (iii) metabolically active with damaged membranes - sublethally injured ($CFDA^+/PI^+$) and (iv) non-esterase active with membrane integrity ($CFDA^-/PI^-$). Quantification of the different sub-populations was performed to capture the proportion of cells in different physiological states at single-cell level. The fraction of $CFDA^+/PI^-$ (metabolically active with membrane integrity - viable) cells and the sub-population of double stained (metabolically active with damaged membranes - sublethally injured) cells is expected to recover on TSAYE (non-selective medium). On the other hand, sublethally injured cells ($CFDA^+/PI^+$) would not grow on TSAYE+5%NaCl (selective medium). $CFDA^-/PI^+$ cells are membrane damaged - dead (no metabolic activity and membrane damage) and as a result, they should not have recovery capacity on selective and non-selective media. Unstained cells - non-esterase active with membrane integrity ($CFDA^-/PI^-$) play a critical role in our experimental results, as they may represent viable, dormant, or sublethally injured cells, that have non-membranous injuries.

Sublethal injury of *L. monocytogenes* cells was evaluated comparatively (plate counting and fluorescence microscopy) after exposure to AA (pH 3.0, 2.7 and 2.5) at 20 °C. AA treated cells (total and non-injured) showed measurable logarithmic reduction at pH 2.7 and 2.5. At the same treatment conditions there was a significant percentage of $CFDA^-/PI^+$ cells, up to 70.54 % at pH 2.7 and 56.72 % at pH 2.5, after 5 h of exposure (Fig. 2D and F). Exposure to pH 3.0 adjusted with AA at 20 °C resulted in high percentages of metabolically active ($CFDA^+/PI^-$) cells (Fig. 2B). At the zero time of exposure (i.e., within 10 s after adding cells to the stress suspension), the percentage of viable cells ($CFDA^+/PI^-$) was 51.12 % and after 5 h of exposure decreased to 27.74 %. At the same time (5 h of exposure), the percentage of culturable cells on TSAYE was 4.53 %, while the percentage of double stained - sublethally injured

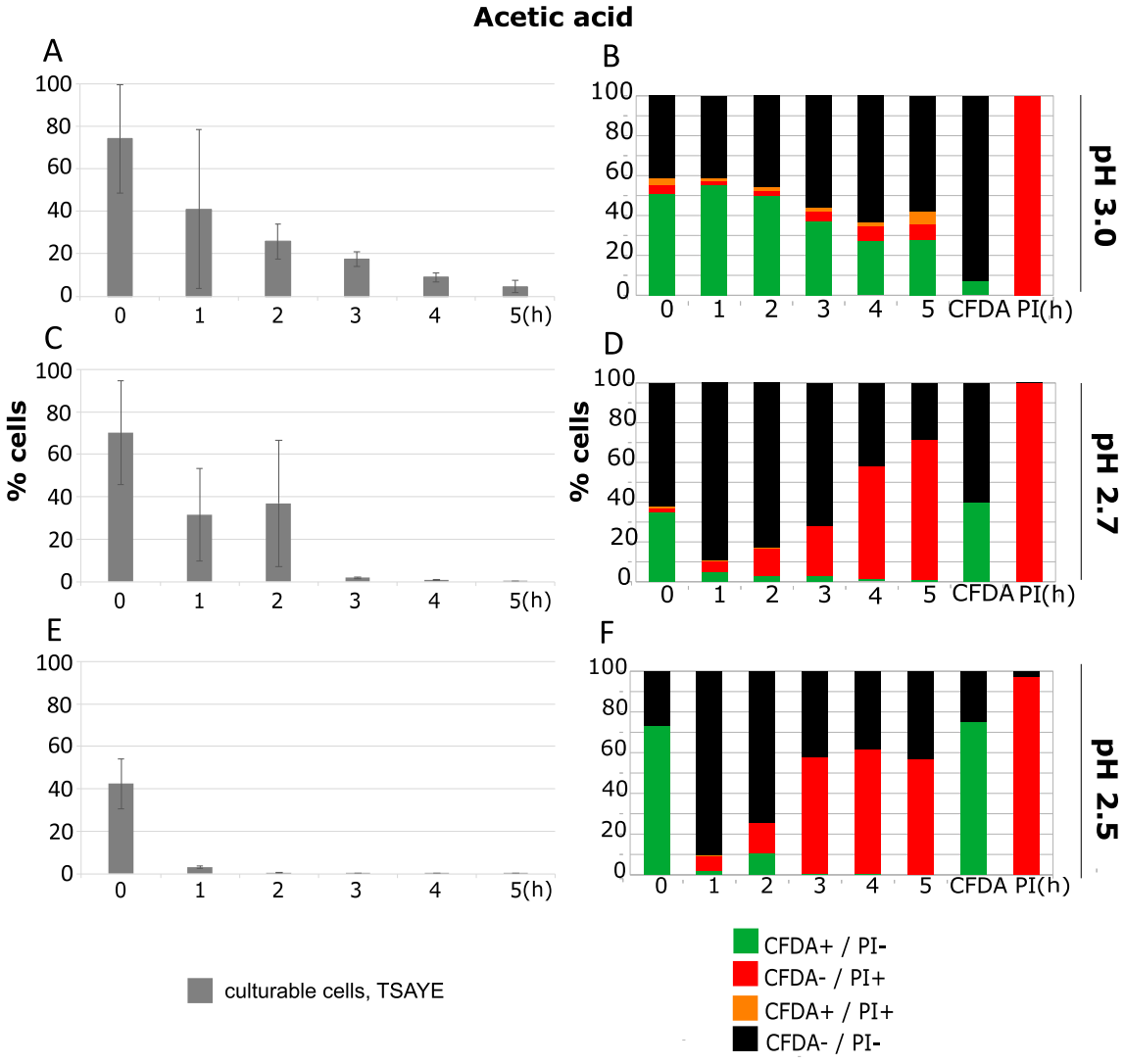

Fig. 4. Percentage of culturable *L. monocytogenes* cells, strain Scott A, on TSAYE (light grey bars) after incubation for 0, 1, 2, 3, 4, 5 h at: AA (A) pH 3.0, (C) pH 2.7 and (E) pH 2.5 at 20 °C. Each time point relates to several images of different fields of views (FOV). Cell enumeration results (%) exhibiting fluorescence ($CFDA^+/PI^-$ metabolically active with membrane integrity – viable; green, $CFDA^-/PI^+$ membrane damaged - dead; red, $CFDA^+/PI^+$ metabolically active with damaged membranes - sublethally injured; orange or $CFDA^-/PI^-$ non-esterase active with membrane integrity; black) considering several images of different fields of views (FOV) at each time point after treatment with AA pH 3.0 (B), pH 2.7 (D) and pH 2.5 (F). The experiment was performed in two independent biological replicates with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells 6.72 %. At pH 3.0, as the percentage of $CFDA^+/PI^-$ decreased over time, the percentage of $CFDA^-/PI^+$ and unstained cells increased. This pattern is clearer after 2 h of exposure in Ringer's solution at pH 3.0, adjusted with AA (Fig. 4B). Incubation for 4 and 5 h in Ringer's solution pH 2.7, adjusted with AA, resulted in 56.68 % and 70.54 % of $CFDA^-/PI^+$ cells (Fig. 4D). The percentage of viable cells ($CFDA^+/PI^-$) reduced from 34.70 %, at zero time of exposure to 0.84 %, after 5 h of exposure, while at the same time 28.34 % of unstained cells were enumerated. The percentage of culturable cells at the same time of exposure was 0.29 %. Exposure to pH 2.5, adjusted with AA, resulted in detectable percentages of metabolically active ($CFDA^+/PI^-$) cells after 1 and 2 h of exposure. Then, until the 5th h of exposure, the percentage of $CFDA^+/PI^-$ cells ranged from 0.64 to 0.21 %. In parallel, the percentage of $CFDA^-/PI^+$ increased from 7.29 % after 1 h of exposure to 56.72 % after 5 h of treatment. Sublethally injured cells were detected after 1, 4 and 5 h of treatment with AA pH 2.5 at 20 °C (Table 4). The percentage of metabolically active and membrane damaged cells ($CFDA^+/PI^+$) was up to 0.08 % after 1 h, 0.02 % after 3 h and 0.04 % after 5 h of exposure.

Adding together the percentages of $CFDA^+/PI^-$ (metabolically active with membrane integrity - viable), $CFDA^+/PI^+$ (metabolically active with damaged membranes - sublethally injured) and $CFDA^-/PI^-$ (non-esterase active with membrane integrity) cells showed that the enumerated percentages of culturable cells on TSAYE were lower than the potentially detected. Exposure to pH 3.0, adjusted with AA, for 4 and 5 h resulted in 8.87 % and 4.53 % of culturable cells, respectively, while the potentially culturable population on TSAYE, adding together the percentages of $CFDA^+/PI^-$, $CFDA^+/PI^+$ and $CFDA^-/PI^-$, was 92.84 % and 92.16 % after 4 and 5 h of exposure. Incubation for 4 and 5 h in Ringer's solution with pH 2.7, adjusted with AA at 20 °C resulted in 0.64 % and 0.29 % of culturable population on TSAYE compared to 43.32 % and 29.45 % that fluorescence microscopy results showed that could be identified. Finally, after 2 h of exposure to AA pH 2.5 at 20 °C the enumerated culturable population on TSAYE was 0.52 %, whereas the potentially culturable population was calculated up to 85.10 %. These, results indicate that the unstained fraction of cells remained undetected and thus non-culturable on TSAYE.

HCl treated cells (total and non-injured population) remained culturable after 5 h of exposure at 20 °C. Comparisons of the mean population of *L. monocytogenes* after treatment with HCl pH 3.0, 2.7 and 2.5 revealed that the total and the non-injured population remained at the same level throughout stress exposure (Fig. 3).

At single cell level fluorescence microscopy revealed significant percentages of double stained ($CFDA^+/PI^+$) cells after HCl treatment, indicating sublethal injury, which was not detected at population level, using plate counting (Table 5). After 1 h of exposure to Ringer's solution pH 3.0, adjusted with HCl, 0.17 % of cells were double stained ($CFDA^+/PI^+$), (Fig. 5B). The percentage of viable and membrane damaged - sublethally injured cells ($CFDA^+/PI^+$) increased over time and reached up to 4.60 % after 5 h of exposure. At time zero of exposure (within 10 s) $CFDA^-/PI^+$ (membrane damaged - dead) cells were up to 1.96 %, while after 5 h of exposure this percentage decreased to 0.65 %. Increasing the stress intensity from pH 3.0 to pH 2.7 led to an increase in the percentage of double stained cells (Fig. 5D), from 7.52 % after 1 h of exposure to HCl pH 2.7 to 12.26 % after 5 h of exposure. $CFDA^-/PI^+$ cells were up to 1.09 % at time zero of exposure and increased to 2.90 % after 1 h of exposure. After 2, 3 and 4 h of exposure to pH 2.7, adjusted with HCl, the percentage of $CFDA^-/PI^-$ (non-esterase active and membrane intact cells) decreased from 54.73 %, at time zero, to 41.49 %, 40.47 % and 36.03 %, respectively (Fig. 5D). After 2 and 3 h treatment with HCl at pH 2.5, the percentage of sublethally injured cells reached up to 20.18 % and 24.59 %, respectively, while after 5 h, 13.72 % of cells were $CFDA^+/PI^-$ (viable), 0.99 % were stained as $CFDA^-/PI^+$ (dead), 1.99 % were $CFDA^+/PI^+$ (sublethally injured) and 83.30 % of cells were $CFDA^-/PI^-$ (non-esterase active and membrane intact) (Fig. 5F).

Incubation for 2 h in Ringer's solution of pH 3.0 and 2.5, adjusted with HCl, resulted in a stochastic change in cells' morphology from rod-shaped to spherical (Fig. 6). At pH 3.0, the differently shaped cells were $CFDA^+/PI^-$ (Fig. 6A), while at pH 2.5 some of them appeared as $CFDA^-/PI^-$ (Fig. 6B), potentially indicating a different physiological state in the dormancy continuum. Finally, evaluation of the potentially culturable population, including the proportion of unstained cells, compared to the enumerated on TSAYE after HCl treatment did not reveal non-culturable population fractions.

3.2. Size colony variations

As recently reported (Arvaniti et al., 2021), evaluation of sublethal injury after treatment with acetic acid and hydrochloric acid did not show colony morphology variants in the total culturable population (CFU on TSAYE). In that study, the stress exposed population level was 10^7 CFU/ml. Phenotype heterogeneity was evaluated through visual inspection after treatment with acid stress following 2 days of incubation at 37 °C. When $10^{9.5}$ CFU/ml were exposed to Ringer's solution pH 2.7, adjusted with AA, at 20 °C, we observed two sub-populations on TSAYE after 1 h of exposure, one with the phenotype of untreated cells and one resembling SCVs (Fig. 7). The induction of SCVs seemed to be time of exposure dependent. SCVs appeared after 1 h of treatment with AA pH 2.7 at 20 °C and were present until the 4th h of exposure, indicating the existence of a slow growing potentially persistent subpopulation.

3.3. Effect of acid stress on the transcriptional profile of the selected genes

Regarding the effect of AA on the relative transcription of the target genes, specific combinations of acid/pH conditions were identified as showing statistically significant ($P < 0.05$) down-regulation of six genes: *clpP* – AA/pH 3.0 after 4 h, *clpP* - AA/pH 2.7 after 0, 2, 4 and 5 h of exposure and *inlA* - AA/pH 2.7 after 2 h. The condition AA/pH 2.7 after 5 h of exposure, showed significant ($P < 0.05$) up-regulation of *relA*. Pearson correlation revealed that after exposure to AA pH 3.0 at 20 °C, only *sigB* showed strong positive correlation ($r > 0.7$) between the two biological replicates along time, while after exposure to AA pH 2.7 at 20 °C, *clpP*, *mazE*, *relA*, *gadC* and *sigB* also showed strong positive correlation ($r > 0.7$). Exposure to Ringer's solution pH 2.5, adjusted with

Table 4
Population log (CFU/ml) and percentage (%) of *L. monocytogenes* sublethally injured cells after incubation for 5 h to Ringer's solution pH 3.0, 2.7 and 2.5, adjusted with AA, at 20 °C.

	Exposure time (h)	Sublethal injury log (CFU/ml)			Sublethal injury %		
AA		pH 3.0	pH 2.7	pH 2.5	pH 3.0	pH 2.7	pH 2.5
20 °C	0	0.30	*	0.22	0.000000066	*	0.000000122
	1	0.30	*	0.51	0.000000141	*	0.000002887
	2	0.11	0.25	*	0.000000091	0.000000640	*
	3	0.47	0.10	0.02	0.000000239	0.000001010	0.000007478
	4	0.36	0.11	0.15	0.000000274	0.000002381	0.00001065
	5	0.56	0.35	0.16	0.000000674	0.000006667	0.000014154

*No sublethally injured cells.

Table 5
Population log (CFU/ml) and percentage (%) of *L. monocytogenes* sublethally injured cells after incubation for 5 h to Ringer's solution pH 3.0, 2.7 and 2.5, adjusted with HCl, at 20 °C.

	Exposure time (h)	Sublethal injury log (CFU/ml)			Sublethal injury %		
HCl		pH 3.0	pH 2.7	pH 2.5	pH 3.0	pH 2.7	pH 2.5
20 °C	0	0.21	*	*	0.000000075	*	*
	1	*	*	0.11	*	*	0.000000044
	2	*	*	*	*	*	*
	3	0.14	0.08	*	0.000000054	0.000000068	*
	4	*	0.08	*	*	0.000000049	*
	5	0.05	*	*	0.000000044	*	*

*No sublethally injured cells.

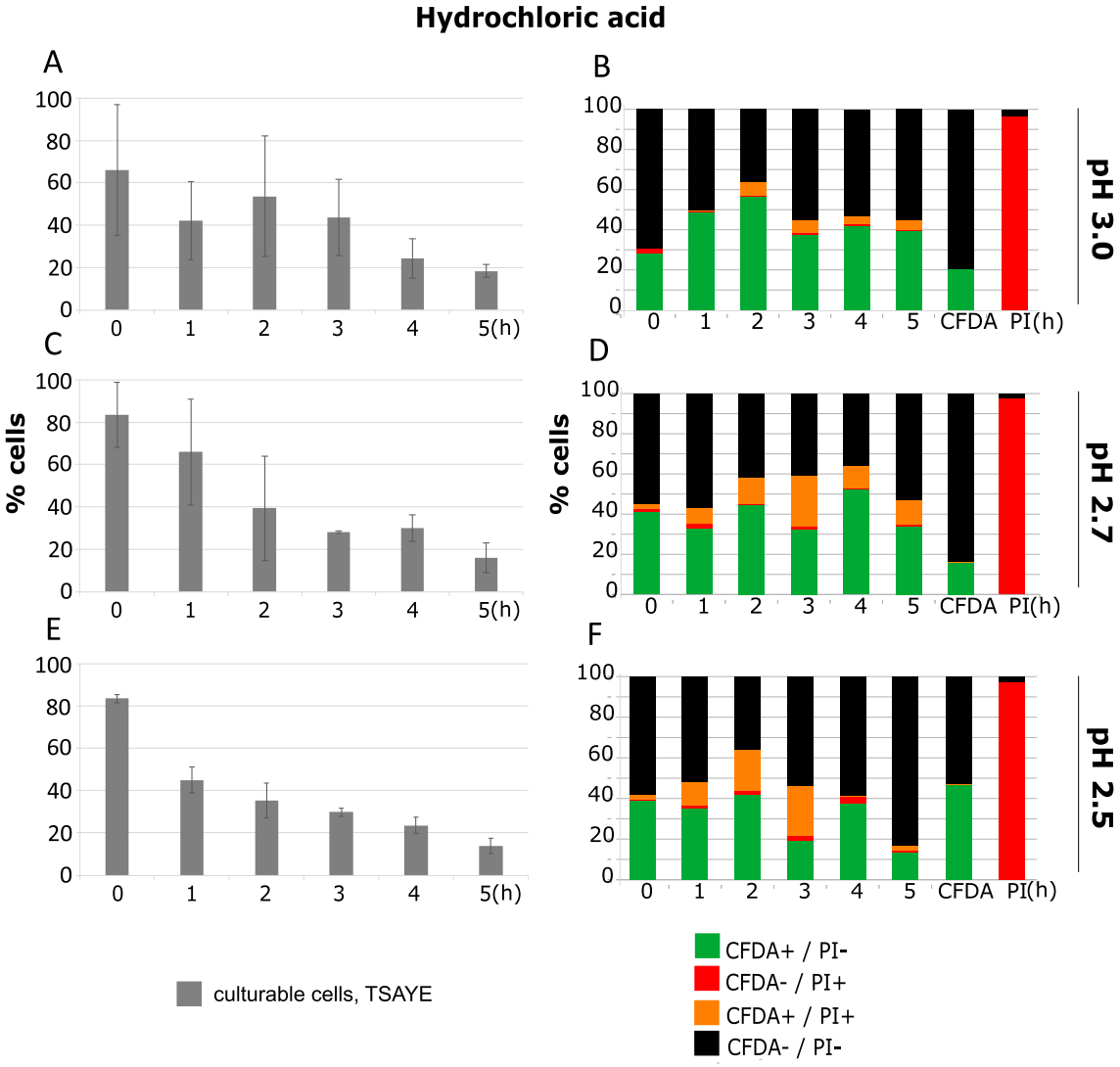

Fig. 5. Percentage of culturable *L. monocytogenes* cells, strain Scott A, on TSAYE (light grey bars) after incubation for 0, 1, 2, 3, 4, 5 h at: HCl (A) pH 3.0, (C) pH 2.7 and (E) pH 2.5 at 20 °C. Each time point relates to several images of different fields of views (FOV). Cell enumeration results (%) exhibiting fluorescence ($CFDA^+/PI^-$ metabolically active with membrane integrity – viable; green, $CFDA^-/PI^+$ membrane damaged - dead; red, $CFDA^+/PI^+$ metabolically active with damaged membranes - sublethally injured; orange or $CFDA^-/PI^-$ non-esterase active with membrane integrity; black) considering several images of different fields of views (FOV) at each time point after treatment with HCl pH 3.0 (B), pH 2.7 (D) and pH 2.5 (F). The experiment was performed in two independent biological replicates with similar results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

AA, did not show any statistically significant up- or down-regulated cases nor strong correlation.

A pattern of gene up-regulation was observed during treatment with AA pH 2.7 and HCl pH 3.0 at the 3rd h of exposure (Fig. 8). One-way analysis of variance (ANOVA), considering the impact of incubation time on gene regulation, revealed that the transcription levels of *clpP*, *mazE*, *mazF*, *relA* and *gadB* were significantly differentiated (up-regulated) after exposure for 3 h to AA pH 2.7 at 20 °C compared to the 2nd h of exposure ($P < 0.05$). Statistically significant differences on gene transcription (up-regulation) were also observed for *clpP*, *gadC*, *gadD*,

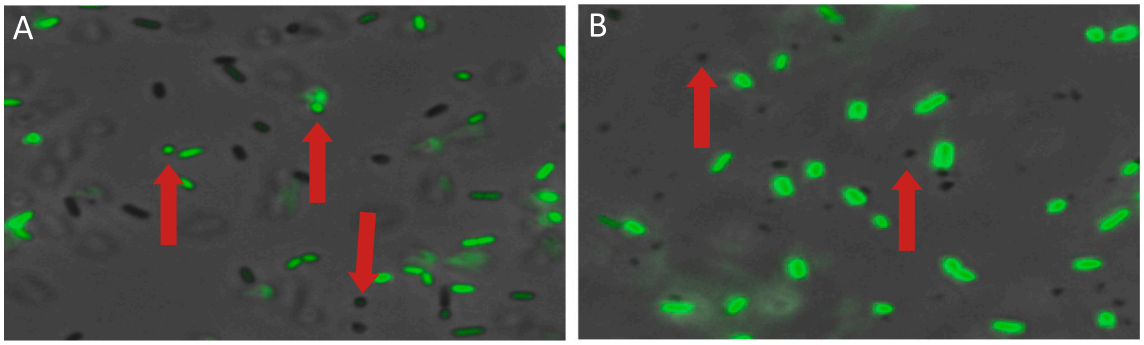

Fig. 6. Double stained cells (CFDA/PI staining) exhibiting fluorescence ($CFDA^+/PI^-$ or $CFDA^-/PI^-$) after incubation for 2 h at 20 °C in Ringer, pH (A) 3.0 (B) 2.5, adjusted with HCl. Arrows show differently shaped cells.

gadB and *sigB* after 3 h of exposure to HCl pH 3.0 at 20 °C.

4. Discussion

Standardized microbiological methods and guidelines have been established so that food industries and the authorities can examine food products for the presence of *L. monocytogenes*. However, bacterial heterogeneity, injury and VBNC state-limitations may lead to potentially undetected hazards, causing foodborne outbreaks or be the reason for insufficient evidence to enable source attribution (Arvaniti and Skandamis, 2022). Laboratory media used in food diagnostics may contain selective compounds, to which, sublethally injured cells are sensitive, leading to underestimation of a product's actual microbial level (Jasson et al., 2007; Restaino et al., 2001). Studies focusing on the heterogeneity in single-cell outgrowth of *Listeria monocytogenes* have shown that heat-stressed cells present higher single-cell heterogeneity in half Fraser enrichment broth compared to non-selective BHI (Bannenberg et al., 2021b) and variability in lag duration of different strains (Bannenberg et al., 2021a), affecting the successful detection of the pathogen using ISO 11290–1:2017. Sublethally injured, persisters and cells in the VBNC state are able to evade detection. Conventional culture-based detection methods may underestimate the real microbial load of a product or present false negative results, due to the presence of sublethally injured and dormant cells (Lee et al., 2014). Thus, decisions based only on conventional culture-based techniques may falsely conclude about "hazard-free" products (Arvaniti and Skandamis, 2022). As a result, there is a need for alternative single cell detection methods to evaluate sublethal injury and dormancy phenomena. Furthermore, deciphering the activation of the dormancy continuum associated molecular mechanism will help us identify gene expression biomarkers related to dormancy, non-culturable states.

Defining cell viability is really challenging especially in terms of individual cell (Barer, 2012). On the other hand, cell death may be easier defined as the point where the extent of injury is beyond the ability of a cell to repair and as a result there is no-growth (Bogosian and Bourneuf, 2001). In our experimental design, in order to distinguish the existing sub-populations (viable, sublethally injured, VBNC, dead) we used fluorescence microscopy with a mixture of CFDA and PI staining. Fluorescence techniques are widely used for the characterization of persistence and the VBNC state to define the physiological status of individual cells (Arvaniti and Skandamis, 2022). Wideman et al. concluded that Live/Dead BacLight™ staining and CFDA-DVC staining are the most accurate assays for the detection of the VBNC state in *L. monocytogenes* (Wideman et al., 2021). As with most techniques fluorescence microscopy shows limitations delineated by three principal issues, namely, photobleaching of fluorescent probes, their toxicity and phototoxicity, and a limited spatial resolution of images (Dobrucki, 2013). To answer the question of whether a cell remains viable the use of lipophilic, uncharged and non-fluorescent fluorogenic substrates, such as CFDA, can be used to detect esterase activity. Esterases are present in all living organisms, and these enzymes can be used to provide information on the metabolic state of bacterial cells. The enzyme-substrate reaction could be considered as energy independent. However, dead or dying cells with damaged membranes rapidly leak the dye, even if they retain some residual esterase activity (Joux and Lebaron, 2000). Poor dye uptake and active dye extrusion are related to fluorogenic esterases (Bunthof et al., 1999; Molenaar et al., 1992). On the other hand, membrane integrity can be used as an indicator of cell damage or cell death. In our experimental design PI was used as an indicator of membrane integrity. PI binds to DNA of any base composition and may insert from either the major or the minor groove (Neidle and Berman, 1983) with little or no sequence preference (Nelson and Tinoco, 1984). CFDA/PI staining enabled us to detect the different physiological states that co-exist in the total population (i) metabolically active with membrane integrity - viable ($CFDA^+/PI^-$), (ii) membrane damaged - dead ($CFDA^-/PI^+$), (iii) metabolically active with damaged membranes - sublethally injured ($CFDA^+/PI^+$) and (iv) non-esterase active with membrane integrity ($CFDA^-/PI^-$).

Each of our biological replicates was paired with a positive (untreated cells stained with CFDA) and a negative (70 % ethanol killed cells stained with PI) control (cells stained from the same bacterial clone with the stressed cells). A percentage of unstained cells was quantified in positive controls. We hypothesize that the detectable unstained subpopulation could not adjust rapidly to the change of solution and pH, i.e., from TSBYE, pH 5.1 ± 0.01, to Ringer's solution, pH 7.5 ± 0.5, and thus, did not show any fluorescence (Arvaniti et al., 2021). As Breeuwer et al. reported, CFDA is a pH-sensitive fluorescent probe (Breeuwer and Abee, 2000), that permeates the cell membrane and then is converted by cytosolic esterases to the highly fluorescent carboxyfluorescein (CF), which because it is negatively charged, remains inside the cell (Graber et al., 1986). Bunthof et al. showed that *Lactococcus lactis* cells extruded the probe rapidly upon the addition of lactose (Bunthof et al., 2000). This may be another explanation for the existence of unstained cells in our positive control, as the activated cultures were incubated for 18 h at 30 °C in TSBYE, which contains 2.5 g/liter glucose (Arvaniti et al., 2021). However, in most of the treatment conditions the percentage of unstained cells decreased over time, indicating that the role of $CFDA^-/PI^-$ is critical for the interpretation of our results. On the other hand, ethanol-treated cells were well stained with PI in all of the examined cases, indicating that there is no doubt about the ability of propidium iodide to successfully enter and stain membrane damaged cells.

Acidity is an inherent property of many foods, including fruit, honey and fermented food products (Pérez-Díaz et al., 2017). It may also be a hurdle to the growth of spoilage and pathogenic bacteria, that greatly improves food shelf-life and safety (Leistner and Gorris, 1995). Fernando Pérez-Rodríguez et al. in an external scientific report for EFSA provided extent search on the frequency of use of organic acids, among them acetate, as preservatives in commercial RTE placed on the EU

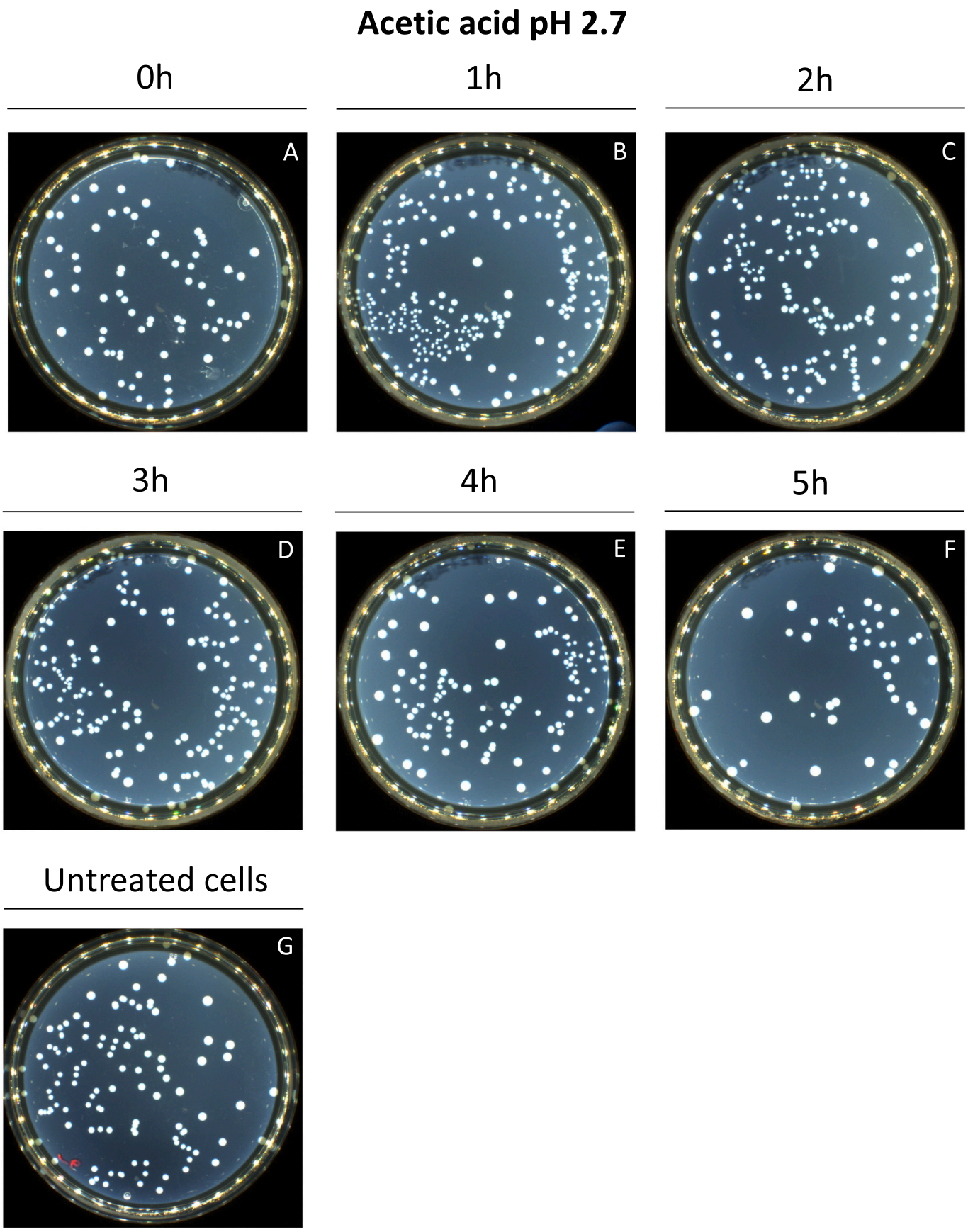

Fig. 7. Size colony variations after exposure of *L. monocytogenes* to AA pH 2.7 for (A) 0 h, (B) 1 h, (C) 2 h, (D) 3 h, (E) 4 h and (F) 5 h at 20 °C. Total population on TSAYE showed alterations in colony morphotype. Based on macroscopic colony observation, two sub-populations were observed (untreated cell's colony phenotype (G) and small colony variations-SCV) indicating phenotype heterogeneity.

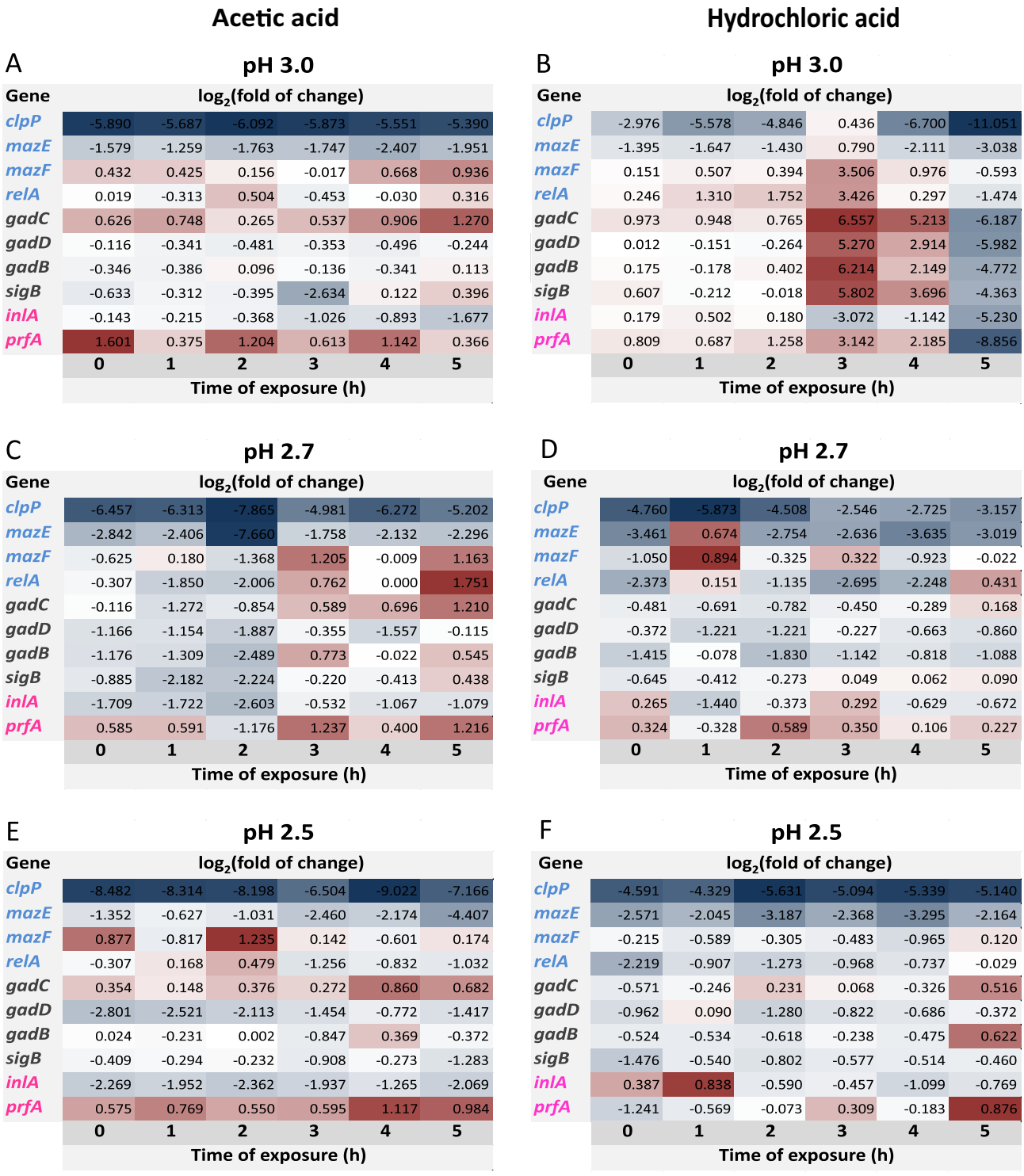

Fig. 8. Effect of acid stress on the relative gene transcription of *clpP*, *mazE*, *mazF*, *relA*, *gadC*, *gadD*, *gadB*, *sigB*, *inlA* and *prfA* during exposure of *L. monocytogenes*, Scott A strain, to Ringer, pH 3.0, 2.7 and 2.5 adjusted with AA (A, C, E) and HCl (B, D, E) for 5 h at 20 °C. Ct values were normalized to tpi transcription levels (reference gene) and to reference samples (control/untreated cells) and then were converted to $\log_2$ (fold change). Values of $\log_2$ (fold change) represent the means of two biological and two technical replicate.

market during the last 10 years. Their search was carried out in the Mintel-Global New Products Database and they reported that the presence of acetate/diacetate in cooked meat products (sliced, pâte and sausages) was 10.83 %, in smoked and marinated fish products 6.84 % and in soft and semi-soft cheese 1.28 % during the period of 2006 to 2016 (Pérez-Rodríguez et al., 2017). According to FDA, examples of acidified foods include beans, cucumbers, cabbage, artichokes, cauliflower, puddings, peppers, tropical fruits, and fish, singly or in any combination. They have a water activity (aw) >0.85 and have a finished equilibrium pH of 4.6 or below. These foods may be called "pickles" or "pickled", such as pickled vegetables and eggs. Except from the inherent acidity of foods or the addition of organic acids as food preservatives, pathogens may also colonize acidic niches such as the stomach (pH ≈ 1–2), where gastric cells produce HCl, the vaginal epithelium (pH ≈ 4),

or the colon (pH ≈ 5.7) (Fallingborg, 1999; O'Hanlon et al., 2019).

In our study, treatment with acetic acid and hydrochloric acid adjusted to pH 3.0, 2.7 and 2.5 at 20 °C for 5 h was performed. Acid stress induces different degrees of bacterial injury. The expression of sublethal injury can be manifested by the loss of characteristic growth capabilities of cells (BUSTA, 1976; Hurst, 1977; Wesche et al., 2009). Metabolic injury has been defined as the inability of bacterial cells to grow on defined minimal media, which is often temporary, as cells retain their resuscitation capacity (Gilbert, 1984; Hurst, 1984). The inability of bacteria to proliferate or survive on media containing selective agents is called structural injury. Structural injury may also be temporary if metabolic repair from injury occurs. In this case, sufficient recovery time under appropriate environmental conditions is needed. To cope up with extreme acidic environment, pathogens rely on pH homeostatic or membrane proton efflux system. Drop of cellular pH leads physiological changes to the cytoplasmic and periplasmic components, that is, low pH induced unfolding of enzymes and other vital proteins, DNA damage and membrane destruction (Panwar et al., 2021). Weak organic acids exert a more deleterious effect than strong inorganic acids. Strong acids dissociate completely in aqueous solutions. Cell membranes are semipermeable to protons, which can only pass the membranes with the use of energy dependent systems, such as H^+ adenosine triphosphatases (ATPases), Na^+/H^+ antiporters or electron transport systems (Humphery-Smith and Hecker, 2006). On the other hand, weak acids, protonated at low pH (depending on the pKa of their acidic groups) and therefore uncharged and more lipophilic, tend to passively penetrate cell membranes (Ita and Hutkins, 1991). At low pH, the undissociated form of a weak acid, like acetic acid, permeates the cell membrane and dissociates intracellularly, acidifying the pH_i and collapsing transmembrane proton gradients (Hirshfield et al., 2003; Lund et al., 2020; Mira and Teixeira, 2013).

Indeed, AA treated cells showed significant population reduction (total and non-injured), while exposure to pH 2.7 and 2.5 resulted in an increase in the percentage of $CFDA^-/PI^+$ (membrane damaged – dead) cells after 2 h. The role of unstained cells in AA treated samples is critical for the interpretation of our results, as they are non-membrane injured and non-esterase active bacteria. A possible explanation is that they represent sublethally injured cells, non – membrane damaged thus PI^-, in a transient physiological stage between injury and death, or dormant cells with very slow metabolic activity, thus $CFDA^-$. Injury is a potentially transient, reversible state, resulting from cumulative cellular damage (Bogosian and Bourneuf, 2001). In the first case (transient stage of injury and death), unstained cells should recover in non-selective media (TSAYE) only together with $CFDA^+/PI^-$ (metabolically active with membrane integrity – viable) and double stained (metabolically active with damaged membranes - sublethally injured) cells. Evaluation of the potentially culturable population on TSAYE, adding together the percentage of $CFDA^+/PI^-$, $CFDA^+/PI^+$ and $CFDA^-/PI^-$ cells in comparison with the percentage of the enumerated culturable population on TSAYE, revealed that unstained cells are a non-culturable population fraction. It is conceivable that unstained cells represent non-esterase active cells with membrane integrity and thus may be dormant with very slow metabolic activity or within a transient state of injury. Another possible interpretation of the role of unstained cells in AA treated samples may be that cells get shocked after exposure to AA due to intracellular acidification, while retaining their membrane integrity ($CFDA^-/PI^-$). However, this fraction could not recover on TSAYE. In detail, after 4 and 5 h of exposure to AA pH 3.0 the percentage of culturable population was 8.87 % and 4.53 %, while the percentage of unstained cells at the same time of exposure was 63.39 % and 57.68 %. Increasing stress intensity from pH 3.0 to pH 2.7 resulted in 0.64 % of culturable cells after 4 h of treatment. The percentage of unstained cells at the same exposure conditions was 41.66 %. At the same conditions (AA pH 2.7), after 1 h of exposure macroscopic observation revealed variations in the morphology of total population colonies (CFU on TSAYE). Clement Vulin et al. described nonstable SCVs as small size colonies, which are not a consequence of mutation, and their alteration in colony morphotype is not retained upon sub-cultivation (Vulin et al., 2018). According to our results, SCVs were observed until the 4th h of exposure to Ringer's solution, adjusted with AA, at 20 °C. Leimer et al. have also reported that acidic conditions favours the induction of non-stable SCVs and non-replicating persisters (Leimer et al., 2016). Finally, treatment with AA pH 2.5 for 2 h resulted in 0.52 % culturable cells on TSAYE, while the percentage of $CFDA^-/PI^-$ cells was 74.45 %. Unstained cell sub-populations remained non-culturable, leading to an underestimation of the enumerated microbial level. Increasing exposure time to 3, 4 and 5 h resulted in 0.25 %, 0.18 % and 0.12 % of culturable cells, while at the same conditions membrane damaged – dead, $CFDA^-/PI^+$, cells were up to 57.64 %, 61.38 % and 56.72 %. This indicates that a sub-population of non-culturable cells, that is not included in the dead fraction of cells, exists.

Molecular analysis showed that after 5 h of exposure to Ringer's solution pH 2.7, adjusted with AA, at 20 °C, *relA* was significantly up-regulated. Also, transcription levels of *clpP*, *mazE*, *mazF*, *relA* and *gadB* were statistically differentiated (up-regulated) after exposure for 3 h to AA pH 2.7 at 20 °C compared to the 2nd h of exposure. Thus, exposure to pH 2.7 with AA for 5 h at 20 °C induced persistence in *L. monocytogenes*, Scott A strain. This fraction of cells remained non-culturable, representing a potential increased risk, under the prism of resuscitation. Bacterial stringent response is the mechanism underlying bacterial persistence (Maisonneuve and Gerdes, 2014). RelA is responsible for the accumulation of ppGpp (Maisonneuve and Gerdes, 2014). Shyp et al. showed that there is a positive allosteric feedback during stringent response by the ppGpp alarmone (Shyp et al., 2012). As a result, rapid increases of ppGpp sustain and amplify intrinsic (i.e., transcription and translation rates) and extrinsic noise (fluctuations of [PolyP] and thereby in Lon activity), that could activate toxins and contribute to formation of persisters (Maisonneuve and Gerdes, 2014).

Fluorescence microscopy coupled with CFDA/PI staining revealed fractions of sublethally injured HCl treated cells, that were not detected using plate counting in selective and non-selective media. This is explained by the fact that population heterogeneity cannot be detected with population scale methods, as the numerically superior population dominates individual cells with different physiological state, which in case of sublethally injured or VBNC cells constitutes a small fraction of the total population (Arvaniti and Skandamis, 2022). Furthermore, a stochastic change in cell's shape, from rod-shaped to spherical, was detected after exposure to pH 3.0 and 2.5, adjusted with HCl, for 2 h at 20 °C. Lipoidal cell membrane damage and decreased fluidity are consequences of low pH, that may lead to morphological changes of cells (Guan and Liu, 2020; Streit et al., 2008). Membrane status regulates viability of cells under stress conditions; the maintenance of a characteristic cell shape is an actively physical property of bacterial cells with selective value. Cell shape affects nutrients diffusion because a spherical shape would have the lowest surface-to-volume ratio compared to non-spherical shapes (Yulo and Hendrickson, 2019). The formation and maintenance of rod shape is conferred to Mre proteins, MreB, MreC and MreD (Wachi et al., 1989). Pichoff et al. reported that the loss or perturbation of MreBCD may lead rod-shaped bacteria to have spherical morphology (Pichoff and Lutkenhaus, 2007). Interestingly, the differently shaped cells exhibited green fluorescence ($CFDA^+/PI^-$) after exposure to pH 3.0, adjusted with HCl, for 2 h. At the same time of exposure increasing stress intensity from pH 3.0 to pH 2.5 resulted in unstained and differently shaped cells. These results indicate that exposure to pH 3.0 and 2.5, adjusted with HCl, for 2 h induced a small fraction of cells in a potentially different physiological state within the dormancy continuum. Morphological changes are related to the VBNC state (Li et al., 2014), as a strategy to minimize energy requirements (Biosca et al., 1996; Gray and Postgate, 1976). Cell rounding is also found in many species in the VBNC state (Adams et al., 2003; Cook and Bolster, 2007). However, as Pinto et al. reported morphological changes alone cannot be used as a critical parameter to judge VBNC induction

(Pinto et al., 2015).

5. Conclusions

Evaluation of sublethal injury at single cell level, induced by AA, revealed fractions of non-culturable sub-populations in dormancy states (i.e., persistence). On the other hand, treatment with HCl resulted in sublethally injured sub-populations that were not detected using selective and non-selective plate counting. Furthermore, a stochastic change in cell shape suggests that a small fraction of cells likely entered a different physiological state in the dormancy continuum. The results of our study provide a deeper insight into the sublethal injury state, especially regarding its physiological and molecular characteristics. Also, they provide the food science community with quantitative data that can contribute to a more accurate prediction of sublethal injury and as a result improve quantitative risk assessment analyses.

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Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2022.109983.

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