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Impact of preculture temperature on peracetic acid-induced inactivation and sublethal injury of *L. monocytogenes* and subsequent growth potential of single cells

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ABSTRACT

The disinfectant peracetic acid (PAA) that is used in the food industry can cause sublethal injury in *L. monocytogenes*. The effect of preculture temperature on the inactivation and sublethal injury of *L. monocytogenes* cells due to PAA was evaluated by plating on non-selective and selective agar medium supplemented with 5 % (w/v) NaCl. *L. monocytogenes* cells were precultured at 30 °C, 20 °C or 4 °C, and the former was used as reference temperature. Preculture of cells at 20 °C or 4 °C and subsequent exposure to PAA at the respective growth temperatures caused higher injury compared to cells grown at 30 °C and exposed to PAA 20 °C and PAA 4 °C, respectively. Survival was also affected by the preculture temperature; 20 °C-grown cultures resulted in lower survival at PAA 20 °C. Nevertheless, preculture at 4 °C resulted in a similar number of surviving cells when exposed to PAA 4 °C compared to cells precultured at 30 °C and exposed to PAA at 4 °C. Flow cytometry was subsequently used to quantify outgrowth capacity of stressed and sublethal damaged populations following sorting of single cells in nutrient rich medium (Tryptone soy broth supplemented with yeast extract [TSBY]). PAA treatment affected the outgrowth of *L. monocytogenes* at single-cell level resulting in increased outgrowth-times reflecting higher single cell heterogeneity. To conclude, the response of *L. monocytogenes* when exposed to PAA depended on the preculture conditions, and the highly heterogeneous outgrowth potential of PAA-injured cells may affect their detection accuracy and pose a food safety risk.

1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes listeriosis, which is a rare but severe infectious disease with a high fatality rate (Gandhi and Chikindas, 2007). *L. monocytogenes* is able to grow at refrigeration temperatures, tolerate acidic and high osmolarity conditions as well as resists different sanitizers (Gandhi and Chikindas, 2007; Holah et al., 2002; Luque-Sastre et al., 2018). Due to its ubiquitous nature it can survive in food processing equipment and persists there for several months or years, posing a food contamination threat (Freitag et al., 2009; Halberg et al., 2014).

Disinfectants are used in food processing plants in order to efficiently control the presence and growth of pathogens such as *L. monocytogenes* and maintain hygiene on surfaces. Peracetic acid (PAA) is a commonly used disinfectant for food processing plant surfaces and is also used to decontaminate fruits and vegetables (Gonzalez et al., 2004; Shen et al., 2019; Zoellner et al., 2018). In previous studies, it was shown that PAA can induce high levels of sublethal injury in *L. monocytogenes* (Siderakou et al., 2022, 2021). In the food processing environment, inadequate disinfection and rinsing can leave residues of disinfectants at lower concentrations, and this may induce sublethal injury.

The application of any stress, including disinfection treatments, does not affect all the cells of a bacterial population equally. Different subpopulations that may occur include dead, healthy and moderately or severely injured cells (Wu, 2008). Consequently, the physiological status of the cell (i.e. unstressed, stressed, injured or severely injured) can influence its growth probability in a nutrient-rich environment, like food. Moreover, the stress increases the variability in the subsequent

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outgrowth of individual cells (Dupont and Augustin, 2009). Growth potential and variability in outgrowth of injured cells is of utmost importance for food safety, since they may remain undetected in testing procedures based on selective media and later resuscitate in foods during storage.

In order to better understand the response of *L. monocytogenes* and design appropriate strategies for the control of this versatile pathogenic bacterium in the food industry, information regarding the growth potential of injured *L. monocytogenes* cells after a stress is of relevance. The lag time duration is dependent on factors concerning the growth environment as well as factors related to the history of the cells which affect the cell's physiological state (Francois et al., 2007; Yue et al., 2019). The magnitude of the temperature deviation between consecutive conditions affects the lag times of the cells; small temperature shifts can result in decreased lag times (Mellefont and Ross, 2003). Additionally, the growth history of the cells can influence the subsequent stress response and may lead to adaptive changes (Koutsoumanis and Sofos, 2004; Lundén et al., 2003).

Considering the above, the effect of preculturing temperature on the survival and sublethal injury of PAA-treated *L. monocytogenes* was investigated. Furthermore, we assessed the impact of the disinfectant on the outgrowth of *L. monocytogenes* at single cell level.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Two strains of *L. monocytogenes* were used in this study, namely, ScottA (serotype 4b) and EGDe (serotype 1/2a). Strains were maintained in stock cultures at −80 °C in Tryptic Soy Broth (TSB; LAB M) supplemented with 0.6 % Yeast Extract (Y; Oxoid) and 20 % glycerol (Fluka). During the experiments both strains were maintained on slants of Tryptic Soy Agar (TSA, Oxoid) supplemented with 0.6 % w/v Yeast Extract (Y, LAB M) at 4 °C for up to three weeks.

For each strain, a single colony from a TSAY slant stock was transferred into 10 mL TSBY and incubated at 30 °C for 24 h. Subsequently, 0.1 mL of the 24 h culture was transferred in 10 mL fresh TSBY and incubated either at i) 30 °C for 18 h, ii) 20 °C for 28 h, or iii) 4 °C for 22 days. In all the aforementioned cases, incubation times of the cultures were selected based on preliminary experiments aiming to collect the cells in the mid-stationary phase of growth. Cells were harvested by centrifugation (2434*g* for 10 min at 4 °C), washed twice in Phosphate buffered saline (PBS) and finally diluted in 1 mL for EGDe or 10 mL PBS for ScottA so as to obtain a working culture of approximately 10^9 CFU/mL. PBS was prepared by dissolving 8.98 g Na_2HPO_4 (Merck), 2.72 g $NaH_2PO_4{\cdot}H_2O$ (Merck) and 8.5 g NaCl (Sigma-Aldrich) in 1 L deionized H_2O.

2.2. Treatment with in peracetic acid

The peracetic acid (PAA) stock solution of 75 ppm and the treatment working solution of 0.75 ppm were prepared on the day of the experiment by diluting the commercial product (peracetic acid 15 % pure, Applichem) in Microcosm Water (MW, Ultrapure water [Milli-Q]). Aliquots (10 mL) of PAA-treatment solution were kept either at 4 °C or 20 °C for 60 min prior to use for temperature equilibration. For each exposure assay, 0.1 mL of working culture of *L. monocytogenes* ScottA or EGDe was transferred into the PAA-working solution targeting a final concentration of approximately 10^7 CFU/mL. For the cultures grown at 4 °C, the exposure to PAA was performed at 4 °C for 90 min, while for the cultures grown at 20 °C, the exposure was performed for 90 min at 20 °C. Finally, for the cultures grown at 30 °C, the exposure was performed at 20 °C and 4 °C for 90 min, which was used as reference to assess the cell survival and injury, as these conditions were identified in a previous study to induce significant levels of injury (Siderakou et al., 2021).

2.3. Determination of L. monocytogenes population and sublethal injury

The CFU counts of *L. monocytogenes* population were measured during the exposure to PAA at 30 min time intervals. One mL of *L. monocytogenes* cell suspension was appropriately diluted in peptone physiological salt solution (PPS, Tritium Microbiologie) and plated onto selective and non-selective media. TSAY and TSAY supplemented with 5 % (w/v) NaCl were used to estimate the *L. monocytogenes* total (non-injured and injured) and non-injured population, respectively. The selective medium (TSAY-5 % NaCl) was used, as previously described by Siderakou et al. (2021) according to the maximum non-inhibitory concentration (MNIC) method. The MNIC of NaCl in TSAY for both ScottA and EGDe was determined to be 5 % (w/v) in preliminary experiments. Enumeration was carried out after 2 days at 37 °C for TSAY, and after 5 days at 37 °C for TSAY-5 % NaCl, respectively. The plate incubation times were determined as the maximum time needed for colonies to be formed on agar plates. The enumeration lower limit was 1.3 log CFU/mL. Sublethal injury was estimated according to Eq. (1), as the difference between cell numbers on TSAY and TSAY-5 % NaCl, which is the log ratio of non-selective to selective medium counts (Siderakou et al., 2021).

$$\text{Sublethal Injury } log \text{ ratio} = log\left(\frac{CFU_{TSAY}}{CFU_{TSAY\ 5\%NaCl}}\right) \quad (1)$$

2.4. Flow cytometry and single cell analysis

Flow cytometry experiments and cell sorting was performed with a BD FACSAria III™ cell sorter (BD Biosciences). Cells of *L. monocytogenes* were differentiated from electronic background noise exploiting a combination of forward scatter (FSC) and side scatter (SSC) using area and width parameters to obtain single cell events for sorting. Unstressed cells cultured in TSBY at 20 °C and stressed *L. monocytogenes* cells cultured at 20 °C and exposed to PAA at 20 °C for 30 min were used. Single cells were sorted in 384-wells plates (Greiner Bio-One, Austria) filled with 50 μL of TSBY per well. Single cell outgrowth in TSBY was measured in a SpectraMax 384 plus (Molecular Devices, USA) set at 37 °C and an optical density wavelength of 600 nm (OD_{600}). Optical density measurements were taken every 5 min for 72 h with 15 s shaking before each measurement. From the optical density data, the time-to-reach (TTR) of an outgrowing cell was quantified as the time to reach an OD_{600} increase of 0.2. Analysis of the results was done using software FlowJO V10 and Microsoft Excel.

Additionally, PAA-treated cells (cultured at 20 °C and treated with PAA at 20 °C for 30 min) were sorted in a range of concentrations (1; 10; 100; 1000; 10,000 cells) (i) in each well of 384-well plates containing TSBY and TSBY-5 % NaCl and (ii) onto TSAY and TSAY-5 % NaCl plates. The 384-well plates were incubated for 5 days in a sealed box with a wet cloth inside to prevent evaporation and agar plates were incubated at 37 °C. The TSAY and TSAY-5 % NaCl plates were incubated for 3 and 5 days at 37 °C, respectively. Growth was monitored qualitatively with (i) the appearance of turbidity in the nutrient broth in the well or (ii) the appearance of a colony on agar. Subsequently, the percentage of outgrowers was calculated with the MPN number technique according to Hurley and Roscoe (1983) using the excel calculator provided by Curiale (2000).

3. Results

3.1. Effect of preculture conditions on L. monocytogenes sublethal injury

The role of preculture temperature on the survival and sublethal-injury of *L. monocytogenes* after exposure to PAA was investigated. Two different preculture temperatures, namely 4 °C and 20 °C, were compared to that of 30 °C, an optimum and commonly used preculture temperature for *L. monocytogenes*. Specifically, the response of 20 °C-

grown *L. monocytogenes* cells subsequently exposed to PAA at 20 °C, was compared to that of 30 °C-grown and PAA-20 °C-exposed cells. Similarly, the response of 4 °C-grown *L. monocytogenes* cells subsequently exposed to PAA at 4 °C, was compared to that of 30 °C-grown and PAA-4 °C-exposed cells.

Preincubation of ScottA at 20 °C, followed by 60 and 90 min exposure to PAA at 20 °C, resulted in significantly lower number of surviving cells compared to the 30 °C-grown cultures (Fig. 1A). Similarly, for EGDe, surviving cells counts of 20 °C-grown cultures were significantly lower after a 60 min exposure to PAA at 20 °C than those of 30 °C-grown cultures (Fig. 1C). Also, the injured population of 20 °C-grown cultures was significantly higher when compared to those of the 30 °C-grown cultures after 30 min exposure to PAA at 20 °C for both strains (Fig. 1B and Fig. 1D). Overall, the decrease of the preculture temperature from 30 °C to 20 °C triggered a faster reduction in viability and a faster induction of injured cells upon PAA treatment at 20 °C.

On the other hand, PAA treatment at 4 °C was less effective than at 20 °C. During PAA treatment at 4 °C, the reduction of total number of surviving cells of 30 °C- and 4 °C-grown cultures was similar, and <1 log CFU/mL in 90 min for both strains (Fig. 2A and 2C). Regarding the injured population, differences were reported between the two strains. In EGDe, injury was significantly higher for cultures grown at 4 °C compared to the 30 °C-grown cultures after 30 and 60 min of exposure to PAA (Fig. 2D). In ScottA, significantly higher injury was reported for the 4 °C-grown cultures compared to the 30 °C-grown cultures after 30 min of exposure to PAA (Fig. 2B).

3.2. Flow cytometer and cell sorting on agar plates and in broth

Sorting cells at various concentrations on agar plates and in broth confirmed the observations from the standard plating technique. There was a difference in the percentage of cells recovering with and without the presence of NaCl after PAA treatment for 30 min at 20 °C, indicating the existence of two subpopulations due to injury. The number of intact cell survivors recovering on TSAY-5 % NaCl was approximately 2 logs smaller compared to the number of cells recovered on TSAY, and the EGDe strain was slightly more sensitive than the ScottA strain (Fig. 3).

3.3. Growth variability of PAA treated single cells after cell sorting

The outgrowth of PAA-treated ScottA cells was estimated for cultures grown at 20 °C and treated with PAA at 20 °C, which resulted in significant injury levels while survival was slightly affected. Non-treated and PAA-treated single cells were sorted in TSBY and their outgrowth kinetics were measured for up to 3 days. Untreated cells showed a similar response concerning the time after which growth was detected, and the time to reach an OD of 0.2 was estimated ranging from 15 to 20 h (Fig. 4A,C). In contrast, the majority of PAA-treated cells that were analyzed and sorted with the flow cytometer was considered to be injured (Fig. 3), showing notable diversity concerning the time of cell growth and a bimodal heterogeneity trend (Fig. 4B,C). Specifically, the time to reach an OD of 0.2 ranged from 18 to 61 h (Fig. 4C). These results highlighted that stress and injury, occurring from PAA, increased the variability in the outgrowth of cells.

4. Discussion

In this study we examined the potential effect of the preculture temperature on the survival and sublethal injury induced by PAA. We cultured the cells at the same temperature as applied during subsequent PAA treatment (4 °C and 20 °C) and compared their stress response to those cultures that were grown at the optimum temperature of 30 °C. We tried to approach a realistic scenario, using conditions relevant to food-processing, where cells can grow at the same temperature as the temperature from their subsequent exposure to disinfection stress and also in temperatures below optimum.

Our data suggested that the behavior of *L. monocytogenes*, when exposed to oxidative stress, depends on the preculture conditions. When treated with PAA at 20 °C, preculturing in the ambient temperature of 20 °C resulted in a lower number of survivors and higher levels of injury compared to cultures grown at 30 °C. On the other hand, PAA treatment at 4 °C and preculturing at 4 °C increased injury rather than affecting the survival compared to the cultures grown at 30 °C. The effect of precultural temperatures had been described by other studies suggesting that the conditions that cells encountered during their lifecycle can influence their response in subsequent environmental conditions (Chihib et al.,

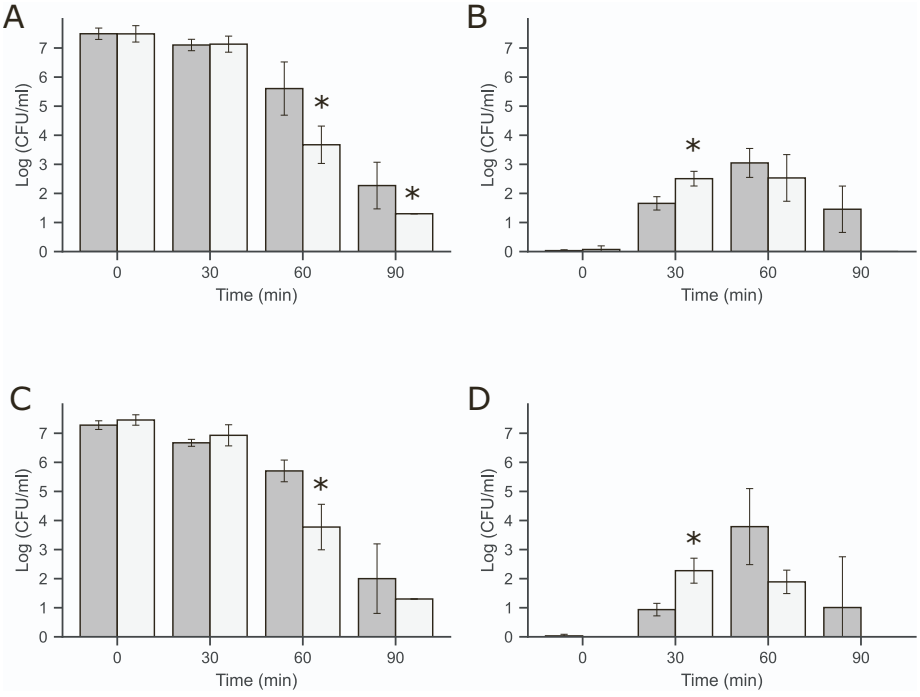

Fig. 1. Numbers (log CFU/ml) of total surviving (A, C) and log ratio of sublethally injured (B, D) *L. monocytogenes* ScottA (A, B) and EGDe (C, D) due to exposure to PAA at 20 °C *L. monocytogenes* cells were precultured at 30 °C for 18 h (dark gray bars) or 20 °C for 28 h (light gray bars). The population of sublethally injured cells was estimated as the log ratio of the total survivors over the intact ones. Asterisks indicate significant ($p < 0.05$) differences between populations grown at 30 °C and 20 °C.

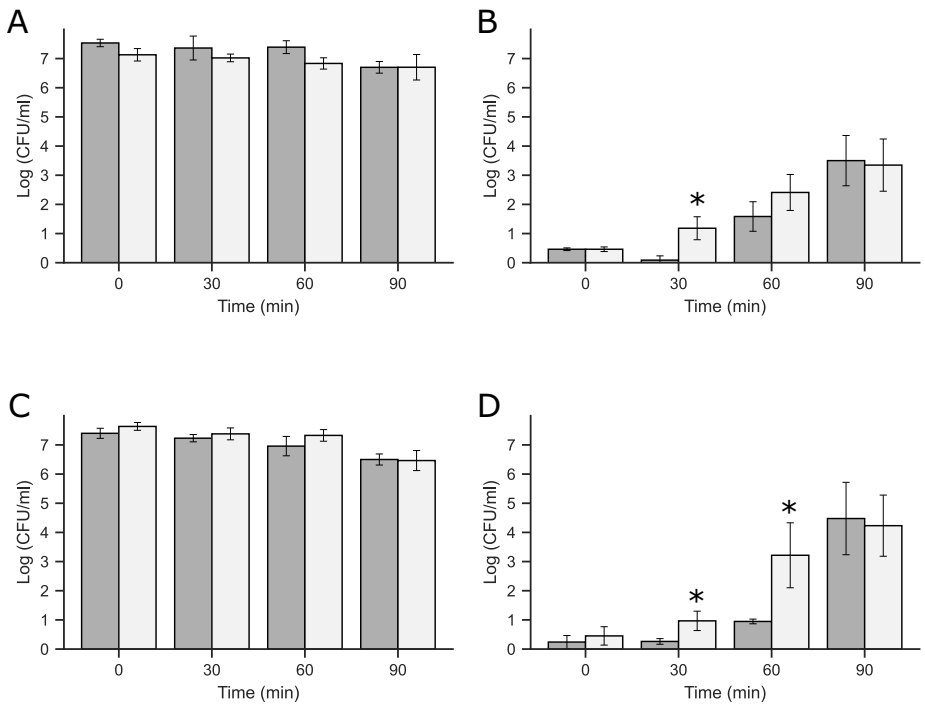

Fig. 2. Numbers (log CFU/ml) of total surviving (A, C) and log ratio of sublethally injured (B, D) *L. monocytogenes* ScottA (A, B) and EGDe (C, D) due to exposure to PAA at 4 °C. *L. monocytogenes* cells were precultured at 30 °C for 18 h (dark gray bars) or 4 °C for 22 days (light gray bars). The population of sublethally injured cells was estimated as the log ratio of the total survivors over the intact ones. Stars indicate significant ($p < 0.05$) differences between populations grown at 30 °C and 4 °C.

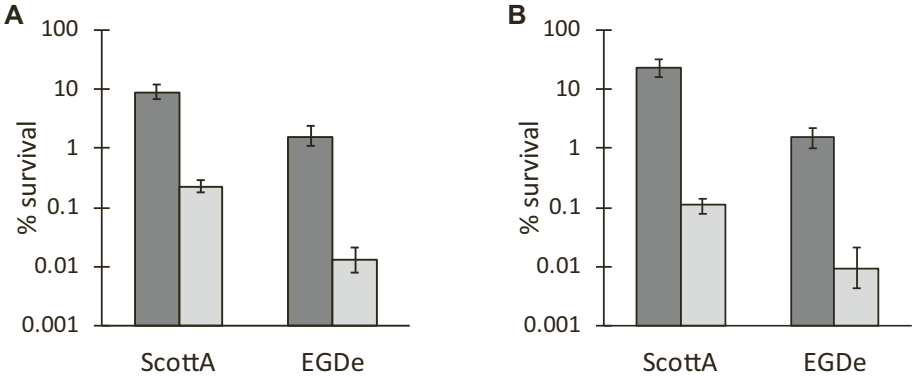

Fig. 3. Survival (%) of *L. monocytogenes* ScottA and EGDe treated with PAA (20 °C, 30 min, precultured at 20 °C) after cell sorting on (A) TSAY (dark gray bars) and TSAY-5 % (light gray bars) and (B) in TSBY (dark gray bars) and TSBY 5 % NaCl (light gray bars). Survival was estimated using the Most Probable Number method.

2011; Dykes, 2003; Mellefont and Ross, 2003; Yue et al., 2019).

L. monocytogenes, when grown in different temperatures, adjusts its membrane's fatty acid composition (Annous et al., 1997; Berry and Foegeding, 1997). Membrane modifications such as fluidity and permeability changes affect the pathogens' sensitivity to a subsequent stress exposure (Banerji et al., 2022; Li et al., 2002; Sibanda and Buys, 2022; Touche et al., 2023). The cellular membrane is the first barrier of the cell against the environmental stresses. PAA, like other peroxides act by oxidizing the cellular components. Peroxides have an affinity for membrane fatty acids which will eventually disrupt and penetrate the cell membrane. Inside the cytoplasm, PAA will cause a further disruption of more cellular components, such as enzymes and DNA (Zoellner et al., 2018).

L. monocytogenes, in the food processing environment, is subjected to numerous stresses that challenge its survival. Being a versatile bacterium, it can adapt to constant environmental changes and adverse conditions by inducing its stress response mechanisms. Under cold temperatures, *L. monocytogenes* adjusts its metabolism and promotes, among others, the expression of general stress response genes as well as the oxidative stress protection mechanism (Soni et al., 2011). Interestingly, De Abrew Abeysundara et al. (2019) showed that cold stress at 4 °C had a protective effect against oxidative stress induced by H_2O_2. We can speculate that PAA was still able to cause sublethal damage to cells grown at a low temperature but that the damage was not extensive enough to affect survival. Overall, different growth temperatures may induce cellular alterations in *L. monocytogenes* which can affect its susceptibility to sublethal damage. These findings indicate that PAA's efficiency regarding survival and sublethal injury may be dependent on the *L. monocytogenes* growth temperature and the temperature during disinfection.

Flow cytometry with cell sorting was used to estimate the heterogeneity in outgrowth of PAA-stressed cells on a single cell level. PAA did not affect all the cells of *L. monocytogenes* equally. The results on a population level (from standard plating technique as well as the MPN method) revealed two distinguished subpopulations, injured and intact. On a single cell level, by looking into the variation of the outgrowth

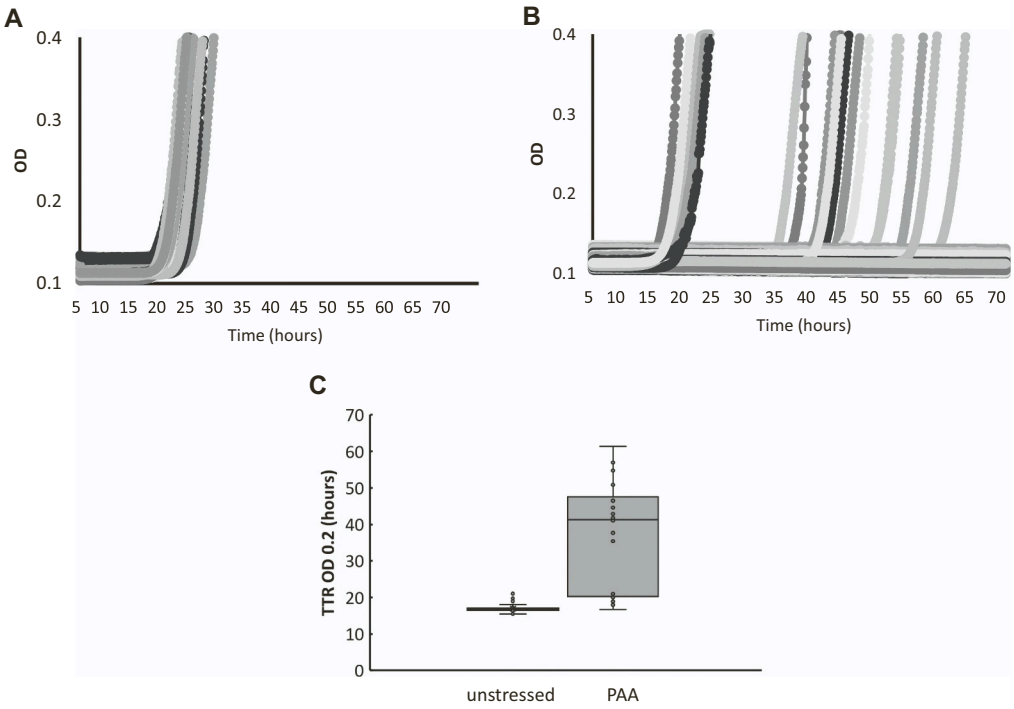

Fig. 4. Growth curves of *L. monocytogenes* ScottA (A, B) in TSBY starting from single cells. Cells were sorted with a flow cytometer and were either unstressed (A) or treated with PAA at 20 °C (B). Box plots show the variability in time of those well that reach an OD of 0.2 in 3 days of incubation (C).

times after the stress exposure, it can be suggested that even more physiological statuses can be present in the population. When a stress is imposed, the cells within the affected population can undergo different levels of damage that makes some cells more sensitive than others and these changes can result in different repair times (Koutsoumanis and Lianou, 2013; Ray, 1979) and the variability of lag times can increase when stress levels increase (Muñoz et al., 2010).

The duration of the lag phase can depend on the growth medium properties as well as the preincubation temperature of the cells. The lag phase can be shorter when changes between the pre-exposure and growth temperatures are smaller, because cells have been adapted to the incubation temperature (Francois et al., 2007; Yue et al., 2019). Pre-exposure to conditions that induce stress and injury can extend the variability and duration of the lag phase because the cells might require more time to repair the cellular damage and synthesize essential components for growth, such as proteins and nucleic acids (Dupont and Augustin, 2009; Wu, 2008).

The variability observed in the outgrowth of single cells can affect the pathogen's detection accuracy in foods. Especially slow growers can pose a higher risk in food-safety, because they may remain undetected. Moreover, food poisoning outbreaks can occur from a low number of cells which makes having insight in the variability of single cell response after stress of outmost importance. In line with this, Dupont and Augustin (2009), showed that, after an injury-inducing stress, the variability of the lag times of single cells was increased. History and cellular damage can affect the microorganism's resuscitation and may affect detection of *L. monocytogenes* in enrichment-based detection methods (Bannenberg et al., 2021)

Overall, this research highlights important aspects of the physiological state of the sublethal injury state. The response of *L. monocytogenes* and the level of injury induced by the oxidative stress of PAA depends on the preculture conditions. Understanding the effect of growth temperature on *L. monocytogenes'* response can contribute to a better understanding on how the pathogen persists in a continuously changing environment. Accounting for the variation and the extended duration of the growth times of the injured single cells, as well as their history, would help to optimize the safety strategies in the food industry. Evidence regarding the type of damage that PAA induces, as well as investigating the cellular modifications under different preculture temperatures in *L. monocytogenes* cells may contribute to a better understanding in the physiology of injured cells and their growth potential in foods and therefore it can be a subject of future investigation.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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